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Doping in Sports

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Doping in Sports

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Preface

Although the definition of doping has been modified over the years, its meaning may be pharmacologically understood as attempts to enhance performance (mainly strength and endurance) in sport by illegal administration of pharmaceuticals or application of prohibited methods (e.g. blood transfusions). Regardless of its individual motivation (e.g. unbounded ambitions, collective chauvinism or excessive financial interest), the doping phenomenon has been increasing in relevance for many years. However, any attempt to describe it from a scientific perspective faces the problem that systematic pharmacological principles are less important than the possible uncovering of its administration in doping control analyses.

Over time, we have seen that some of the early and most potent stimulating agents (e.g. amphetamine) have almost disappeared because relevant dosages are easily detected in doping controls. Instead, alternative and less efficient drugs (e.g. caffeine, modafinil) were used until they appeared on the relevant lists of prohibited substances. In some cases, even untested and unapproved drugs (bromantane, carphedone) were administered to circumvent positive doping controls.

Similarly, the 'progress' of doping with anabolic compounds was pharmacologically characterised by a loss of efficacy, which is notably paralleled by performance deterioration in highly 'doping susceptible' disciplines (compare world records in shot put). Originally, both injectable and orally administered steroids with high myotrophic potential (stanozolol, nandrolone, metandienone) were abused, resulting in significant gain in muscle mass and performance. Their replacement by lower levels of endogenous steroids could still combine reasonable effects with a moderate risk of discovery. Following further analytical improvements to differentiate endogenous and synthetic steroids (carbon isotope mass spectrometry), the application of mimetics and prohormones became popular. The latter (e.g. androstenedione) were temporarily legally available as 'nutrition supplements' and were thus abused in large amounts, although the significance of intended pharmacological effects was not proven. The BALCO affair (Bay Area Laboratory Co-operative), an intentional systematic development of new pharmaceutical analogues of anabolic steroids (tetrahydrogestrinone, THG) for doping

purposes, was certainly intriguing but due to the great effort required and the high risks involved it probably does not represent a general tendency.

Relevant detection time windows differ significantly and have to be seen in relation to the duration of possible performance-enhancing effects. Application of amphetamines, for instance, to stimulate sympathetic and central nervous systems is associated with high therapeutic substance concentrations during the performance and can be easily identified "in-competition". In contrast, effects of anabolic substances or enhancement of oxygen transport capacity may last longer than the presence of the respective doping agents in the body. Unannounced out-of-competition tests were therefore introduced to specifically search for anabolic substances.

Analytically, the detectability in urine, the main specimen in doping control, and the corresponding detection time windows of relevant compounds are mainly governed by their pharmacokinetics. Detailed knowledge of the biotransformation and excretion kinetics of prohibited compounds is therefore essential in doping control. Quite often, pharmacologically irrelevant terminal metabolites are examined in great detail to enable a long-term detection of steroid abuse.

Recent advances in the development of doping strategies are not restricted to the development of new compounds. Forms of administration are also optimised to avoid the detection of administered substances. Anabolic steroids which were classically administered by intramuscular injection of their esters or taken orally became available as sublingual or buccal tablets and in particular as transdermal gels, enabling an efficient application of low dosages with good bioavailability and moderate detection windows.

Some new developments occurred in the 1990s when cheaper and safer recombinant peptide hormones became available. Erythropoietin (EPO), growth hormone (hGH) and insulin-like growth factor (IGF-1) pose outstanding analytical challenges because of their potentially endogenous nature and their pulsatile biosynthesis. Quantitative analyses are not eligible as proof of administration, and alternative procedures to differentiate the complexity of isoforms (hGH) or glycosylation (EPO) became necessary.

This development from classic – yet highly potent – compounds to new replacement strategies reflects a major challenge in doping control: old compounds and methods are still state-of-the-art and their control needs to be maintained while new analytical procedures must be permanently included. The time lapse between the clinical trials of a new drug with a misuse potential and the introduction of the drug might be used to develop a possible detection strategy. Detection methods for specific androgen receptor modulators (SARMs) have already been developed and the substances are included on the prohibited list even before any preparation is registered.

In addition to potentially performance-enhancing substances, masking agents have been prohibited because compounds influencing their analytical detectability were used. A fascinating facet of the BALCO affair was the documented production of "the cream", a transdermal testosterone preparation with an in-built fraction of the masking agent epitestosterone to prevent an adverse analytical finding.

Recently, genetic aspects and techniques have gained importance in doping analysis, for instance to understand inter-individual variations (pharmacogenomics of testosterone glucuronidation) or as a diagnostic tool (reporter gene biomarkers). Moreover, the possible abuse of developments in gene therapeutic treatment has revealed a new potential for manipulation (gene doping). The first attempts to detect this are in progress.

The development of doping analysis in human sports has been closely related to the abuse and detection of illegal compounds in animal sports (particularly horse racing), while aspects like the availability of substances, species-related biochemical particularities and specific regulation of the acceptance of medications define their speciality. Similarly, the application of inappropriate dosages of anabolic compounds in bodybuilding and their illegal use in food-producing animals are not fully comparable to situations in sport, but permit useful insights into biotransformation, pathobiochemistry and the appearance of side effects and attempts to treat them.

Finally, doping cannot be properly understood without some knowledge of its legal implications. The abuse of certain compounds is restricted by a trade-off between potential gain (honour, social, money) and risk (costs, sanctions or legal penalties). The classification of potentially harmful doping agents as scheduled compounds, their control and limitation of their availability are therefore also as important as analytical means.

A comprehensive overview of the health risks of doping practices and their side effects would exceed the scope of this volume. However, we chose to include some aspects which have not yet been covered extensively in the literature: the side effects of anabolic-androgenic steroids from a forensic point of view and the risks of steroid abuse observed from a cardiologist's standpoint.

As early as 1980, the International Association of Athletics Federations (IAAF) initiated an accreditation programme for doping control laboratories, which was later taken over by the International Olympic Committee (IOC) and the World Anti-Doping Agency (WADA). Major concepts of quality assessment in analytical chemistry (e.g. identification criteria in mass spectrometry) originated in this process. The anchoring of quality control in the concepts of the International Organization for Standardization (ISO) provides the documentation of adequate competence. The fact that analytical results are periodically the subject of public contention reflects the high awareness of doping and strong financial interests rather than scientific insufficiencies.

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History of Doping and Doping Control

Rudhard Klaus Müller

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Keywords History of doping • Definition of doping • Anti-doping regulations • Doping control

1 The Expression "Doping"

Although attempts to enhance athletic performance are probably much older, the word "doping" was first mentioned in 1889 in an English dictionary. It described originally a mixed remedy containing opium, which was used to "dope" horses.

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"Dope" was a spirit prepared from the residues of grapes, which Zulu warriors used as a "stimulant" at fights and religious procedures and which also reportedly was called "doop" in Afrikaans or Dutch.

Later, the meaning of "dope" was extended in a broader sense to other beverages with stimulating properties. The expression was introduced into English Turf Sport about 1900 for illegal drugging of racehorses.

2 Early Attempts of Doping

According to reports of Philostratos and Galen, various remedies were used to enhance athletic performance as early as the end of the third century BC (Burstin 1963). Chinese physicians recommended the use of Ma Huang (an extract from the plant Ephedra) to increase performance over 5,000 years ago, when this drug was usually used to suppress coughing and to stimulate circulation (Abourashed et al. 2003).

The Indian physician Sutruta recommended the eating of testicles to enforce virility around 300 B.C., and the Huns consumed testicles before battles – obviously with the same aim (Chinery 1983). Hallucinogenic mushrooms were taken in the third century BC to enhance performance during Olympic competitions, which were held between 776 BC and 393 AD (Burstin 1963; Prokop 1970, 1972; Hanley 1983).

Critically considered, the materials available at that time may probably be categorized between nutrients or nutritional supplements (like eggs, meat, blood) and real "drug-like" substances with objectively expected activity (like bull testicles, or alcoholic beverages with their nevertheless two-edged effect on performance).

This antique "doping" was strictly prohibited by the rules of the classic Olympic Games, just as today. The sanctions were however much more severe in the old Greek Olympics as well as in horse doping: Prokop (2002) mentions that even death penalties were given. When Emperor Theodosius abolished the ancient Games in the year 395 AD, the reasons he gave were that they had become "a hotbed of cheating, affronts to human dignity and doping" (Dirix and Sturbois 1998).

3 Doping and its Emerging Prohibition

While there are some reports about doping – including related attempts and their prohibition – we do not yet have any knowledge about their role in a large gap of time from the Greek Olympics through the Middle Ages to our modern times.

The use of the strong stimulant cocaine (coca leaves) in parallel to caffeine (coffee, guarana, cola nuts and mate tea) is reported from Latin America. Incas

were reportedly able to run the distance from Cuzco to Quito (1750 km) in 5 days under the influence of those stimulants – almost incredible even with cocaine (Wadler and Hainline 1989).

Later, strychnine, caffeine, cocaine and alcohol were often used by cyclists and other endurance athletes in single as well as combined administrations. Among the preparations promising to provide power was "Mariani wine", first produced and patented 1863 by Angelo Mariani, and made from Bordeaux wine and coca extracts. Among high-ranking consumers (e.g. Thomas Edison, Henrik Ibsen, Jules Verne), Pope Leo XIII conferred a gold medal on Vin Mariani. This beverage became later forbidden under the "Opium" (or Narcotics) Law in Germany in 1920 (Eckart 2003).

Increasing use of mineral drugs/poisons such as arsenic, and developing knowledge about vegetable drugs, might have led to their use for this "paramedical" purpose also. The purification, definition and structural elucidation of alkaloids and other active ingredients of plants, overlapping with the first synthetic organic pharmaceuticals, were the final preconditions for the beginning of the "modern era" of doping in the nineteenth century.

Numerous individual doping cases were reported between the late nineteenth and the second half of the twentieth century, when official testing of human athletes was initiated. In parallel, the first definitions and regulations against doping in sport appeared. The International Athletic Federation (IAAF) was the first to ban the use of stimulating substances in sport, but this remained inefficient until testing possibilities were available.

Although certainly less important, the opposite of normal doping has also played some role in history: "doping to lose" or "negative doping" – attempts to impede the performance of athletes, as well as horses (initially called "anti-doping") or other competing animals, by clandestine administration of sedatives etc.

Contemporary allegations to explain adverse analytical findings in urine samples as consequences of the introduction of doping agents by others can be considered as an extrapolation of these early attempts at sabotage.

A series of cases are reported by Ludwig Prokop (Prokop 1957, 1970, 1972, 2002), although in general briefly and not always with original sources. Following his documentation, the first doping case was detected at a British Channel swimming event in 1865 (Pini 1964). The death of the cyclist Linton in the Paris–Bordeaux race was correlated to an overdose of caffeine. (This might have possibly been a simplification with regard to the very low toxicity of caffeine.)

Six-day cycling races (established in 1879) and professional boxing as well as horse and dog racing obviously fostered the increase of doping attempts with alcohol, cocaine, caffeine, heroin, nitroglycerol and strychnine. The marathon winner at the St. Louis Olympics in 1904, Thomas Hicks, received – besides the consumption of raw eggs and brandy – injections of strychnine during the run.

Various mixtures were tried to increase the stimulation of these substances. Such as:

- Alcohol, caffeine and nitroglycerol,
- Cocaine and heroin,
- Alcohol and cocaine

(Dorando Pietri, London 1908).

In 1908 Belgian football teams tried to enhance performance by breathing pure oxygen, but they obviously abandoned this attempt soon.

It is hard to envisage how these first cases might have been discovered – by direct observation, by confession or otherwise.

The first application of chemical analysis in connection with a series of unexpected results in horse racing occurred in Austria at the beginning of the twentieth century. Ludwig Prokop reports that the Austrian Jockey Club – following suspicious events during horse races – invited the Russian chemist Bukowski to come from St. Petersburg to Vienna. He was able to detect alkaloids in the horse saliva. While Bukowski kept his method secret, Professor Siegmund Frenkel elaborated his own method at Vienna University. In 1910/1911, 218 analyses were performed, leading to several sanctions being imposed on the coaches after positive findings in the horse saliva samples.

While restrictions for the use of pharmaceuticals in sports have reportedly been introduced since 1920, and the IAAF prohibited doping (use of stimulating agents) in 1928, official testing of humans was not yet performed. Therefore, the restrictions remained ineffective due to the lack of testing possibilities.

In 1966 the international federations UCI (cycling) and FIFA (football) introduced doping tests in their World Championships. The International Olympic Committee (IOC) instituted its own Medical Commission and set up its first List of Prohibited Substances in 1967. It introduced the first tests at the Summer Olympic Games in 1968 in Mexico.

The tests performed during competitions proved to be ineffective in controlling long-acting agents such as anabolic agents due to the possibility of withdrawing them prior to competitions, while the performance enhancement lasted long enough after the excretion of the agent itself. This led to the conclusion that sample collections were also necessary outside the competition. Despite some resistance they were introduced and now form the backbone of doping control.

The spectacular stanozolol misuse of the runner Ben Johnson at the 1988 Summer Olympic Games in Seoul caused a shock among the sports community and led to an increased readiness to support effective doping control measures. In parallel, the number of potential doping agents began to increase continuously with the development of synthetic organic drugs, which still continues.

Nevertheless – and notwithstanding the overwhelming number of pharmaceuticals on the worldwide market – the development of new pharmacological agents has been slowed down considerably by the very strict requirements of toxicological testing prior to registration. Because the agents under development are known about much earlier than their final registration and introduction to the market, this contradicts the

popular assumption in the media that doping control and analysis would always remain far behind the progress of doping strategies.

3.1 Stimulants

Early doping agents were mainly stimulants (cocaine, caffeine, strychnine, etc.). The introduction of synthetic phenylethylamine derivatives (mainly amphetamine [Benzedrine] and methamphetamine [Pervitin]) as strong-acting stimulants led to their increased use in sports (as well as in the military for improved vigilance). Amphetamines and analeptics (central, respiratory, and psychotonic) as well as several alkaloids (e.g. ephedrine, strychnine), narcotics and some hormones therefore became prohibited as the first classes of doping agents.

Following L. Prokop (2002), cases of misuse of amphetamines occurred first between 1950 and 1970. Amphetamine was first synthesised in 1887 (Edeleanu 1887) simultaneously with the isolation of ephedrine. Gordon Alles synthesised it again in 1927 in Los Angeles while searching for derivatives of ephedrine, and coined the term amphetamine. Since 1932, Smith, Kline and French sold inhalation remedies containing amphetamine, called “Benzedrine”, and after 1936 also Benzedrine tablets. Amphetamines were reportedly used for the treatment of child hyperactivity and as stimulants during the Second World War.

In a cycling competition in 1955, five samples out of 25 tested positive for amphetamine (Venerando 1963). In 1956, a cyclist needed psychiatric treatment after amphetamine misuse. During several high-level cycling competitions (e.g. World Amateur Championship, Professional Cycling World Championship Zürich, Austria Tour) numerous cyclists (e.g. from Austria, The Netherlands, and Poland) tested positive and had to be sanctioned. It was reported that several athletes carried amphetamines in their clothing.

As a psychological aspect of doping and its motivation, placebo doping effects were objectively shown by Ludwig Prokop as early as 1957: he included more than 100 athletes and control persons in a study and showed significant psychological and physiological effects after the administration of placebo remedies.

3.2 Anabolic Agents

Anabolic steroids came into use in sports first as agents supporting recovery after massive stress and exhaustion. They were developed after the isolation and structural elucidation of the mother compound testosterone, the principal male sexual hormone, in the 1930s. Testosterone was first isolated as a crystallised pure substance by E. Lacqueur and coworkers in 1935 (Freeman et al. 2001). A. Butenandt elucidated the structure. His chemical synthesis, published almost simultaneously with that of L. Ruzicka and A. Weltstein, was honoured by the

Nobel Prize in 1939. Soon after, numerous synthetic derivatives were synthesised and used as pharmaceuticals in parallel to the natural hormone.

The activity of a hormone governing (male) sexual functions had been revealed much earlier, when A.A. Berthold linked testicular action to circulating blood constituents. Interestingly, an experiment of C.-E. Brown-Sequard in Paris, attempting to prove the activity of a testicle extract (dog, guinea pig) in 1889 was considered ridiculous by his colleagues after his statement of a marked restoration of his feeling of wellbeing and vigour. This led to him and his successors abandoning research for several decades, until it was initiated anew in 1927 in Chicago by the group of F.C. Koch.

Among the various actions and side effects of anabolic steroids, their potential for enhancing aggressive behaviour (still under discussion due to the difficulty of gaining objective proof of causality; Müller and Müller-Platz 2001) has gained importance less in sport than in forensic aspects – having been claimed as an explanation and excuse of violent behaviour and violent crimes. Testosterone was given to soldiers during World War II to increase their aggression, and Adolf Hitler used testosterone just after it had been synthesised (Spitzer 2005b).

The structural elucidation of testosterone was followed by the synthesis of numerous derivatives as well as by the revelation of the complete "family" of steroid hormones (precursors, metabolites and also antipodes of the main androgenic hormone testosterone).

Soon after the 1950s, the class of anabolic androgenic steroid hormones became the leading group in the statistics of doping cases and later also the leading group in adverse analytical findings, after the methodology enabled laboratories to detect them (see also Duchaine 1989; Fahey and Fritz 1991; US FDA 2008; Wright and Cowart 1990; Yates and Wolff 1993; Fainaru and Williams 2003). Special problems such as the need to distinguish exogenous intake of the endogenous compounds from their normal, physiological presence in the body and in urine led to the concept of the testosterone/epitestosterone ratio (T/E) of Manfred Donike (Donike et al. 1983).

Clinical tests (e.g. ketoconazol administration, the "steroid profile") and later the application of isotope ratio mass spectrometry, IRMS (Ayotte 2009) have been included as additional means to suspect, to detect or to prove the misuse of testosterone and other – mainly endogenous – anabolic steroids.

When pursuing directed hints led to the detection of clenbuterol in 1992 (Beckett 1992; Maltin et al., 1987a, b), and to the suspicion of its obvious misuse as a replacement for anabolic steroids, a definition problem became apparent. While the examples for the agents belonging to the respective prohibited classes was followed by the expression ". . . and related substances", an argument arose over the meaning of the term "related". Clenbuterol – already known for its misuse in animal nutrition for enhanced muscle growth – was undoubtedly an anabolic, but certainly no steroid. Hence some experts suggested that clenbuterol – due to its side effect – had to be considered as a prohibited agent, whereas others claimed that it was not related due to its nonsteroidal chemical constitution.

3.3 Fatalities with Presumptive Correlation to Doping

Lethal cases contributed to the growing public awareness of this problem, such as the death of the Danish cyclist Knut Enemark Jensen in the Summer Olympic Games in 1960 in Rome and of the British cyclist Tommy Simpson in1967 at Mont Ventoux (Houlihan 1999) during the Tour de France (Blickensdörfer 1972). Fatal cases in other sports at that time were also related to doping, e.g. the weightlifter Billi Bello in 1963 (with heroin) and Dick Howard, a 400-m hurdler in 1960.

But it has to be emphasised that such a correlation of sudden death in sport to doping at that time was very probably a suspicion rather than a justified diagnosis according to forensic standards. Even the detection of doping in an actual case or beforehand cannot really prove that doping was the cause of death, and this can be relevant even vice versa (Bux et al. 2008). An example of erroneous suspicion of earlier doping occurred in the early 1990s after the sudden death of the American athlete Florence Griffith-Joyner. The autopsy revealed a natural cause of death (intracranial bleeding by rupture of an obviously connatal brain stem cavernoma). Primarily, the media assumed her death was the lethal consequence of doping during her active career. On the other hand, a natural cause of death does not necessarily exclude any earlier use of doping agents. Only nowadays are such rare fatal cases of death in active periods of high performance sport thoroughly investigated (Bux et al. 2008; Goldmann 1984; Kohler et al. 2008; Lüderwald et al. 2008; Raschka 2008).

When single urine analyses immediately pre or also post mortem provide no unequivocal conclusions, hair analyses have provided very helpful additional information about possible long-term doping (Sachs and Möller 1989; Sachs and Kintz 2000; Lüderwald et al. 2008).

4 Development of General Anti-Doping Regulations

In 1928 the International Amateur Athletic Federation became the first International Sport Federation to ban the use of stimulating substances (WADA 2009a). The IOC claims to have contemplated doping problems at the Olympic Games since its IOC Sessions in 1937/1938 in Warsaw and Cairo (Dirix and Sturbois 1998). It stated in 1938: “The practise of doping is to be condemned utterly, and any person accepting or offering to supply dope should not be allowed to enter amateur meetings or the Olympic Games.” Alerts were given by Drs. Ludwig Prokop and Albert Dirix in 1952 and 1956, “regarding obvious signs of the reckless use of medicinal substances, while some athletes exhibited symptoms which were worrying to say the least”.

At the IOC Session in Athens in 1961, a Medical Commission was created, and at the Session in Madrid in 1965 Prince Alexandre de Merode (Belgium) presented a report on doping problems in the light of the Tokyo Games in 1964 (Dirix 1986). This report can be considered as the starting point for the anti-doping efforts of the

IOC and its Medical Commission. Beginning with the first anti-doping legislation in France in 1963 and several international congresses, anti-doping laws and regulations were issued in a series of countries and by International Federations (IFs) in sport. Since then, UCI and FIFA introduced doping tests in 1966, and several other international federations followed in subsequent years.

The first doping tests at the Olympics were taken during the Winter Games in Grenoble and the Summer Games in Mexico in 1968, when the first disqualifications occurred. In parallel to the first regulations, anti-doping commissions were established, e.g. France 1959; Austria 1962; Council of Europe and Italy 1963; IOC (Medical Commission) 1961/1967.

The approach of the Mexico City Games brought the problem of doping into particularly sharp focus and prompted further debate at top level within the IOC. At the 66th Session of the IOC in Teheran from 6 to 9 May 1967, the problems associated with drug testing, the list of products and the methods used for doping and gender testing for the 1968 Games were expounded by the retiring chair of the IOC Medical Commission, Sir Arthur Porritt. Prince Alexandre de Merode (Belgium) was appointed Chairman of the IOC Medical Commission on May 9, 1967, and held the chairmanship until his death in 2003.

The Medical Commission of the IOC also elaborated scientifically based requirements for doping analyses and for the qualification and equipment of anti-doping laboratories, which only after IOC accreditation are exclusively entitled to perform analyses for international competitions including the Olympic Games.

On 3rd September 1968, from the Château de Vidy in Lausanne, IOC President Avery Brundage issued in a press release a circular letter to all IFs, NOCs and IOC Members on the IOC initiative towards an anti-doping campaign (Dirix and Sturbois 1998). This document contained the following general statements about the standpoint of the IOC towards doping:

> “. . . The use of dope has always been prohibited by Olympic Rules and by those of most amateur sport organisations. . . .
>
> . . . It was never intended that the IOC itself should take responsibility for testing seven or eight thousand competitors . . .
>
> . . . The International Olympic Committee has its rules, it has defined dope, and it should see that provisions are made by the Organising Committee for testing. But the actual testing is left in the hands of others. This is a responsibility that the IOC is not prepared to take. The responsibility of the IOC is to have intelligent regulations to see that the adequate facilities are provided, and that correct methods are used.”

Under the supervision of the IOC Medical Commission, doping tests were first carried out during the Winter Olympic Games in Grenoble and during the Summer Games in Mexico City in 1968. Most IFs introduced doping tests in the 1970s. In 1986, the IOC inaugurated the International Olympic Charter against Doping in Sport, and in 1989 the Anti-Doping Convention of the Council of Europe was finalised. The Appendix summarises the historical period of changing doping definitions and regulations up to that time (Council of Europe 1989; Dirix 1984, 1992, 1986; Todd and Todd 2001).

5 Doping Analysis and Accreditation of Anti-Doping Laboratories

Although the first attempts at detecting doping practices reach back for about a century, the analytical possibilities with regard to sensitivity and certainty of results remained rather restricted until the 1970s. While the first attempts had probably started with test-tube chemistry, the recommendations for analyses during the 1972 Olympic Games in Munich comprised thin-layer chomatography and gas chromatography (Beckett et al. 1967; Beckett and Cowan 1979; Clasing et al. 1974; IOC 1972; Donike and Kaiser 1971; Donike et al. 1974; Merode 1999; Hemmersbach 2008; Kim et al. 1999). Immunoassays had been used in some laboratories for pre-testing (screening), but positive results were considered as preliminary and had to be confirmed by chromatography (Brooks et al. 1975, 1979).

Quickly following the technical development of new analytical principles, mass spectrometry (MS) and its combination with gas chromatography became the standard instrumentation for the detection and quantitation of the majority of doping agents. This principle and similar "hyphenated techniques" combine the high separation power of a chromatographic technique with the very high information capacity and sensitivity of the mass spectrometer (Catlin 1987, 1999; Donike et al. 1974, 1976, 1983, in his booklet "Dopingkontrollen" 1978–1996; Hemmersbach and de la Torre 1996; Maurer 2006; Müller et al. 2003; Pfleger et al. 2000; Westwood et al. 1999).

GC–MS (the combination of gas chromatography with unit mass resolution mass spectrometry) has been extended in its possibilities by the introduction of high resolution mass spectrometry (HRMS), tandem mass spectrometry (MS–MS) and isotope ratio mass spectrometry (IRMS) as well as by the combination of MS with liquid chromatography (LC) instead of GC during the 1990s (Aguilera et al. 1996; Ayotte 2006; Becchi et al. 1994; Horning and Donike 1994; Horning et al. 1996; Mareck et al. 2008; Pozo et al. 2008; Shackleton et al. 1997; Thevis and Schänzer 2005, 2007; Thevis et al. 2005; WADA 2008a, b, c, d.

The increasing complexity of the analyses and the growing importance of the analytical results with regard to the consequences (sanctions) of doping offences in the course of the establishment of strict regulations led to the adoption by the IAAF at first of restrictive rules for analytical procedures and quality testing for laboratories performing drug tests. The IAAF Council accepted the rules for accreditation (as part of a continuous accreditation programme) in Dakar in April 1979. In 1981, a joint communiqué of the IAAF Medical Commission and the IOC's Sub-Commission for Doping and Biochemistry in Sport combined the intention and initiative and accredited the first anti-doping laboratories worldwide: Cologne (Germany), Kreischa (Germany/GDR), Leningrad (USSR), London (GB), Magglingen (Switzerland) and Montreal (Canada). Up to 2008, the number of accredited anti-doping laboratories (WADA 2008c), now accredited by WADA according to the International Standard for Laboratories (WADA 2008b), and based in addition on the International Standard ISO 17025, increased to 34.

The challenges to the laboratories for the analysis of prohibited doping agents and methods grew considerably during the 1990s due to the misuse of new substances and methods, mainly peptide hormones like erythropoietin (EPO), human growth hormone (hGH) and others. Most newer doping agents of lower molecular size (below 1000 Da) can be included into the normal scheme of GC–MS or LC–MS procedures without great difficulty, and have sometimes been detected even if completely unused and more or less unknown, and have afterwards been explicitly included in the Prohibited List, e.g. clenbuterol 1992 (Maltin et al. 1987a, b); bromantane 1994 (Badyshtov et al. 1995; Bumat et al. 1997; Grekhova et al. 1995; Kudrin et al. 1995); carphedone or phenylpiracetam 1996 (Müller et al. 1999; Kim et al. 1999); RSR13 (Breidbach and Catlin 2001); hydroxyethyl starch (HES) (Thevis et al. 2000; Deventer et al. 2006); tetrahydrogestrinone (THG) 2004 (Catlin et al. 2004; Fainaru and Williams 2003; Jasuja et al. 2005).

For the peptide and glycoproteine hormones (with much larger molecules between 10^3 and 10^5 Da and with many different chemical properties), immunoassays – partly combined with electrochemical separation – as well as an "indirect approach" have been introduced. The direct approach identifies the hormone itself (and has to distinguish the exogenous product from the physiological one), while the indirect principle quantifies and evaluates parameters which are physiologically correlated with the hormone (such as erythropoietin haemoglobin or hematocrit, ferritin, soluble transferrin receptor, reticulocytes) (Catlin et al. 2002; Gore et al. 2003; Lasne 2001; Lasne and de Ceaurriz 2000; Lasne et al. 2002; Parisotto et al. 2000, 2001).

The question of whether blood sampling would either be desirable or would become necessary for the detection of doping agents (and methods) in general or in particular for peptide hormones was intensively discussed in the 1990s due to the legal impact: blood sampling was partly considered legally unacceptable, while a sample category whose sampling could be refused would remain ineffective in doping control (Donike et al. 1996). At present, blood sampling is becoming more and more usual in connection with the storage of health tests and individual "blood profiles" of athletes, and can be used if necessary in addition to the traditional urine sample if the nature of agents (or methods) so requires. This will very probably be necessary in connection with approaches to detect autologous blood doping, and perhaps also for other factors enhancing oxygen transport capacities.

Hair as a potential sample material for doping detection has also caused discussion: many doping agents are incorporated into growing hair and remain stable for long periods, so that a longer individual history of the incorporation of agents can be gained by hair analysis. The very sensitive hyphenated MS-techniques permit detection even in a few milligrams of available hair material. On the other hand, a single administration of low dose is hardly detectable (and with some delay, due to the slow growth of the hair out of the follicle), so that a negative hair analysis cannot exclude a unique doping offence, whereas it can elucidate retrospectively a repeated consumption (Müller and Thieme 2000; Sachs and Kintz 2000).

The inclusion of gene doping in the Prohibited List (since 2003) created a new challenge for doping control and analysis (Müller 2001). While methods for the detection of genetic constitution and changes as well as methods for genetic manipulation are already very far developed experimentally, their transfer into medical treatment – and therefore also the possibility to misuse potentially performance-enhancing manipulations in sport – is not yet practicable. In spite of this, research projects on the elaboration of detection methods – whether directed to the detection of genetic changes themselves or of the indirect markers related to them – are under way. The results will decide whether the necessary completely new techniques can be introduced in the laboratories accredited for doping control, or how far cooperation with specialised (e.g. forensic DNA) laboratories will be indicated.

6 Doping and the Cold War

Between about 1970 and 1990, sport in general and doping in sport undoubtedly played their roles not only for the personal ambition of athletes, coaches, officials and even some physicians, but also were misused for nationalism, for competition among ideologies and political systems and as a welcome tool to balance other national inferiorities. While in democratic states doping was applied individually or organised by single teams, sport associations or institutions (partly made easier due to the still weak or differing rules and insufficient control), in a series of mainly Eastern European states doping was organised systematically by governments and parties in their struggle for acknowledgement or recognition against the background of their rather inferior economic and scientific performances. Although this was characteristic for all satellite states of the Soviet Union, the German Democratic Republic (East Germany) led this strategy based on its intention to obtain international recognition as a second German state following the division of Germany by the Allies at the end of the Second World War. As a matter of fact, the astonishing results in high performance sports by this comparatively small country at this time (certainly not only, but in considerable part, as a result of the secret systematic doping strategy by "supporting agents" – "Unterstützende Mittel, UM") were very much acknowledged throughout the world, creating an image which disregarded the political suppression by and the growing economical inferiority of this regime (Berendonk 1992; Franke 1997; Spitzer 1989; Spitzer et al. 1999; Teichler 2003; Spitzer 2005a).

This was a very complex problem ranging from scientifically based practices to secret research, irresponsible applications disregarding acute and long-term side effects, from obvious coercion or deception of athletes to the readiness of others to reach higher performances even with the risk to health, to misuse of laboratory results for the avoidance of adverse findings at international competitions, etc.

Anabolic androgenic steroids – registered pharmaceuticals (testosterone itself, its various esters and derivatives including the legendary dehydrochloromethyltestosterone named Oral-Turinabol®) as well as synthetic substances developed in

secret research projects – were certainly the most important doping substances during these decades. The risks were increased when the synthetic agents were applied without the rigorous toxicological testing that is compulsory for the official registration of new pharmacological agents.

More effective test methods have led to a remarkable drop in the level of top results in some sports, which obviously could not be attained in a natural way. Some track and field results of this time – obviously obtained under the action of those agents – are still waiting to be equalled again, very probably for this reason.

The consequences of the widespread use of doping in sports in the GDR were a series of court trials against former sport officials responsible, and claims after the German reunification in 1990 of numerous athletes alleging late health problems. Attempts to elucidate and solve as far as possible the malpractices encountered considerable difficulties of proof due to the time interval and the secrecy of the whole strategy in the 1970s and 1980s. Notwithstanding the undisputable fact of state-supported doping in countries behind the "Iron Curtain" and of the associated doping-related sports results, doping has undoubtedly been applied also in many other countries, although more as an individual misbehaviour than as a strategic practice. Nevertheless, the general standpoint both of officials and of the public has slowly developed from acceptance through a negative image to total prohibition; this is still continuing.

7 Developments from the 1990s Onward

The Anti-Doping Convention of the Council of Europe (1989) was the first step on the side of states towards international harmonisation in the fight against doping, including obligations of governments and their dialogue with international and national federations and associations in sport. A Monitoring Group was established as the forum of the member states, seconded by the Sport Division under the Directorate General in the Council of Europe. This Convention was open for the membership of states inside and outside Europe from the beginning, and the actual membership has grown to 47 in 2008.

For the various aspects of the common activities, Advisory Groups of experts have been established for legal issues, education, science and for the common database. The Monitoring Group as a kind of "parliament" or General Assembly for this Anti-Doping Convention convenes usually twice a year in Strasbourg, whereas the Advisory Groups meet according to actual necessities.

Starting in the 1980s, rumours came up alleging the misuse of peptide hormones – mainly erythropoietin (EPO) and human growth hormone (hGH) – in sports. The lack of detection methods for proving the external origin of those hormones (whose clinical detection and quantitation has been possible for a long time) hampered confirmation of the suspicions for about a decade.

After the elucidation of the effects of blood transfusions and of the correlation between oxygen partial pressure and haemoglobin formation, Paul Carrot (1869–1957) and Catherine Deflandre assumed in 1906 that erythropoiesis would be governed by a humoral factor. After contradictions and final confirmation, Allan Jacob Erslev was able to show the existence of this factor, erythropoietin, in 1953. Eugene Goldwasser and Leon Orris Jacobson detected its production by the kidney in 1957 and first isolated the hormone in 1977 from human urine. The identification of the human EPO gene by Fu-Kuen Lin followed at Amgen in 1983. The first cloning and expression of recombinant human EPO gene was obtained in 1984 by Sylvia Lec-Huang in New York, followed soon after by the manipulation of mammalian cells (Fisher 2003).

Following the spectacular events at the Tour de France in the second half of the 1990s, in 1998 the IOC proposed the idea of an international Anti-doping Agency. First discussed at a World Conference in Lausanne in February 1999, the International Olympic Committee, the Council of Europe and the Monitoring Group to its Anti-Doping Convention, as well as several representatives of Governments, played an active role in supporting the foundation of the World Anti-Doping Agency, WADA, in December 1999. After having first established an office in Lausanne, WADA moved to Montreal as a political decision with the intention of emphasising the independence of this new institution from the IOC and from the international federations. This independence is guaranteed by a foundation based on Swiss Law, and mainly by the constitution of the governing bodies by equal numbers of representatives from sport (Olympic Movement, i.e. IOC and International Federations) and governments (delegates of states and international bodies such as the Council of Europe). The financial support of WADA is also shared between those two partners, sport (Olympic Movement) and Governments. Europe pays almost half (47.5%) of the governmental contribution, while the remaining 52.5% is contributed by the other four continents.

In the following years, the foundation and establishment of WADA created an entirely new situation. The first big success was the elaboration of the World Anti-Doping Code (WADC) in a huge project and with extraordinarily broad discussion until 2003, when this document was adopted at the World Conference in Copenhagen. It replaced the Medical Code of the IOC and the Olympic Movement Anti-Doping Code of 1999 and became the first anti-doping regulation with worldwide acceptance – after resentment and intensive discussion by all international federations.

Associated with the WADC are the International Standards (level 2 document) for the Prohibited List (WADA 2008a), for the Laboratories, for Testing and for Therapeutic Use Exemptions (TUEs). A third level of documents comprises Recommendations or Guidelines for related activities like education/prevention, result management, etc.

The need to harmonise the consequences of doping offences internationally and among the various federations and associations led to an international court of arbitration, CAS (Court of Arbitration in Sport, Lausanne), which is now considered the supreme authority in disputed cases (McLaren 2001).

The responsibility for the Prohibited List was taken over by WADA in 2003. The annual review is opened for discussion inside WADA and with its stakeholders. The WADA List Committee usually issues a draft with the suggested changes, and after extensive discussions with stakeholders the final version is accepted by the WADA Executive Committee at the end of September and becomes effective on 1 January the following year.

The inclusion of gene doping as a prohibited method was the most significant change since then, notwithstanding the inclusion of new substances, withdrawal of single compounds (most importantly caffeine), the establishment of a paralleling Monitoring Programme for permitted agents with some potential for doping, changes in the structure of the list and in the status of different classes and substances, and clarification of the processing of adverse findings.

A problem of definition – the widening of the lists of examples of prohibited classes by adding the expression "... and other substances with similar chemical structure or similar biological activity" could not be eliminated despite intensive discussions (Müller et al. 2000). The difficulty of obtaining complete lists of relevant agents and the possibility of misuse of "gaps" by synthesising designer compounds (such as was attempted with THG, tetrahydrogestrinone: Catlin et al. 2004; Fainaru and Williams 2003) are impeding "closed" lists so far.

When it remained a legal problem for governments formally to join a body based on a national law like WADA (a foundation under Swiss Law) and to agree about obligations derived from such an international document like the World Anti-Doping Code, after some discussions an idea originating from the Anti-Doping Convention of the Council of Europe (COE) and its Monitoring. Group led to the elaboration of the global UNESCO Anti-Doping Convention (UNESCO 2005). While the theoretical possibility to extend the (open) Convention of the COE to all other states has not found support, this UNESCO Convention was elaborated in several conferences and adopted in 2006. After ratification by the adopted minimum of 30 states, it came into effect in 2007. A General Assembly will be the future governing body, besides a permanent bureau at the UNESCO Headquarter in Paris.

The World Anti-Doping Code was revised by a third Anti-Doping World Conference in Madrid in November 2007; the new version came to effect on January 1, 2009 (WADA 2009b).

Similarly, and subsequent to the formation of those international bodies, National Anti-Doping Organisations (NADOs) as well as Regional Anti-Doping Organisations (RADOs) have been established in a series of states and regions, mostly combining the efforts of sport organisations with governmental and public participation in the fight against doping. This again has led to an international group ANADO (Association of National Anti-Doping Organisations), which aims to the worldwide harmonisation of doping control measures.

For the Anti-Doping Laboratories, the World Association of Anti-Doping Scientists (WAADS) provides the forum for exchange of knowledge and problems as well as the possibility to communicate with one voice to the other bodies bearing responsibility in this common activity.

Appendix 1 Historical Definitions of Doping

1933 Beckmanns Sport Lexikon

Doping, the use of stimulating (performance enhancing) agents, which shall push the atlete beyond his/her normal limits of performance.

Used for this purpose are:

Adrenalin, extracts of testes, caffeine, digitalis, strychnine, camphor, nicotine, cocaine, colanine, heroin, morphine, arsenic, phosphorous, calcium, alcohol etc.

The application of doping agents is rejected for reasons of sports ethics and health and is sanctioned in many sport disciplines by dismissal and sanctions.

1952 Association of German Sport Physicians (Deutscher Sportärztebund)

The intake of any pharmaceutical – regardless its activity – with the intention to enhance performance during a competition is considered as doping.

1952 Keysers Sportlexikon

Doping: Administration of stimulating agents for the enhancement of performance in sport. Application before or during the competition causes disqualification.

1956 Lexikon des Sportes (Dictionary of Sport)

Doping: Attempts by artificial stimulants of any kind, to enhance the performance of the body beyond the natural limits.

1963 Council of Europe (Madrid)

If a necessary medical treatment carried out by any means, which by its nature is capable to enhance physical performance beyond the normal level, this is considered as doping and excludes the capability to compete.

1963 Council of Europe, Committee of General (Out-of-school) Education

Doping is the administration or the use of xenobiotic substances in each form and on each way or of physiological substances in abnormal form or by abnormal ways to healthy persons with the only aim of artificial and unfair enhancement of performance for competition. In addition, various psychological measures for performance enhancement of the athlete have to be considered as doping.

1965 Belgian Law

It is the intention of this Law that doping is considered as the use of substances or the application of methods for the artificial enhancement of performance of an athlete, who participates in a competition or prepares for a competition, if the use can be harmful for his physical or mental integrity.

The Anti-Doping Committee recommends that the Legislative should prepare such a list of substances and methods, including an adequate declaration of the prohibited doses of these substances.

1970 German Sports League (Deutscher Sportbund)

Doping is the attempt to obtain an increase of performance of athletes for competition by nonphysiological substances. Doping substances as defined by these Guidelines are phenylethylamine derivatives (strong central stimulants or "Weckamines", ephedrines, adrenaline derivatives), narcotics, analeptics (camphor- and strychnine derivatives), sedatives, psychopharmaceuticals and alcohol.

1971 Medical Commission of the International Olympic Committee:

All substances, also if applied for therapeutic purposes, which influence performance by their composition or dosage, are doping agents, including in particular

1. Sympathomimetic amines (e.g. amphetamines, ephedrines, etc.)
2. Central stimulants (e.g. strychnine, analeptics, etc.)
3. Narcotic analgesics (e.g. morphine, methadone, etc.)

1971 German Track and Field Association (Deutscher Leichtathletik-Verband, DLV)

"Each athlete is prohibited to take part in a competition, if he/she is under the influence of pharmaceuticals on the prohibited list. The proof of doping is obtained by qualitative detection. Time, dosage and potential of the agent are irrelevant."

1976 International Olympic Committee

A definition of doping is not introduced.

Doping comprises the application of the substances on the following list. This list contains 76 different agents.

1. Psychomotoric stimulants: amphetamines and derivatives. Sympathomimetic amines: ephedrine and derivatives. Central nervous stimulating substances: analeptics, strychnine
2. Narcotics and analgesics: morphine and derivatives
3. Vasodilatators: nitrites
4. Anabolic steroides

1977 German Association of Sport Physicians (Deutscher Sportärztebund)

1. Doping is the attempt at unphysiological enhancement of performance of an athlete by application (intake, injection or administration) of a doping substance by the athlete or an assisting person (e.g. team leader, coach, physician, nurse or physiotherapist) prior to a competition and, for the anabolic hormones, also during training.
2. Doping substances according to these guidelines are in particular: Phenylethylamine derivatives ("Weckamines"), ephedrines, adrenaline derivatives, narcotics, analeptics (camphor and strychnine derivatives) and anabolic hormones. In specific sports, additional substances can be prohibited as doping agents (e.g. alcohol, sedatives, psychopharmaceuticals).

1978 German Association of Sport Physicians
Doping is the use of substances from the prohibited classes of agents:

(a) Psychomotoric stimulants
(b) Sympathomimetic amines
(c) Various stimulants of the Central Nervous System
(d) Narcotis and analgesics
(e) Anabolic steroids

1986 German Association of Sport Physicians
Doping is the use of substances belonging to the prohibited classes of agents, and the application of non-permitted measures like blood doping.

Five classes of substances are defined as doping agents:

1. Psychomotoric substances (stimulants)
2. Narcotics
3. Anabolic steroids
4. Beta-receptor blockers
5. Diuretics

1988 International Olympic Committee
Doping is the use of substances from the prohibited classes of agents and the use of prohibited methods.

List of prohibited classes of substances and methods

I. Prohibited classes of substances
   A. Stimulants
   B. Narcotics
   C. Anabolic steroids
   D. Beta-receptor blockers
   E. Diuretics
II. Prohibited methods
   A. Blood doping
   B. Pharmacological, chemical and physical manipulation
III. Substances, permitted with certain restrictions
   A. Alcohol
   B. Local aneasthetics
   C. Corticosteroids

1989 Anti-Doping Convention of the Council of Europe
According to this Convention are defined

"Doping in sport" the administration to or the use of pharmacological doping agents or of doping methods by athletes.

(a) Pharmacological doping agents or doping methods according to paragraph 2 are those doping agents or doping methods which have been prohibited by the respective international sport organisations and are included in

lists, which according to article II.1b. are confirmed by the Monitoring Group,
(b) "Athletes" are those persons who participate regularly in organised sporting activities.

Until the date when a list with the prohibited doping agents and methods has been confirmed by the Monitoring Group according to Article II.I.b., the list of agents and methods attached to this Convention is valid as List of Agents and Methods.

Doping in this sense means each attempt to enhance performance by means, which normally are not administrated to the organism, whereby the intention of stimulation is essential and the manner of administration is irrelevant.

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Biochemical and Physiological Aspects of Endogenous Androgens

Andrew T. Kicman

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Abstract This review attempts to give a synopsis of the major aspects concerning the biochemistry of endogenous androgens, supplemented with several facets of physiology, particularly with respect to testosterone. Testosterone continues to be the most common adverse finding declared by World Anti-Doping Agency accredited laboratories, such samples having an augmented testosterone to epitestosterone ratio. Knowledge regarding the precursors and metabolism of endogenous testosterone is therefore fundamental to understanding many of the issues concerning doping with testosterone and its prohormones, including the detection of their administration. Further, adverse findings for nandrolone are frequent, but

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this steroid and 19-norandrostenedione are also produced endogenously, an appealing hypothesis being that they are minor by-products of the aromatization of androgens. At sports tribunals pertaining to adverse analytical findings of natural androgen administration, experts often raise issues that concern some aspect of steroid biochemistry and physiology. Salient topics included within this review are the origins and interconversion of endogenous androgens, the biosynthesis of testosterone and epitestosterone, the mechanism of aromatization, the molecular biology of the androgen receptor, the hypothalamic–pituitary–testicular axis, disturbances to this axis by anabolic steroid administration, the transport (binding) of androgens in blood, and briefly the metabolism and excretion of androgens.

Keywords Androgen • Biochemistry • Physiology • Production • Metabolism • Aromatization • Testosterone • Nandrolone • Epitestosterone

1 General Introduction

This review of endogenous androgens attempts to give a synopsis of the major aspects concerning the biochemistry of endogenous androgens, supplemented with relevant facets of physiology, particularly with respect to testosterone. Given the breadth of the field, this brief review cannot address all features of androgen biochemistry, but singled out for special attention are certain topics that have particular relevance to anti-doping in sport. The biological action of testosterone in different tissues, directly and by its aromatization or 5α-reduction, is not discussed herein but is covered most comprehensively elsewhere (Nieschlag and Behre 2004). The author regrets that his article cannot be exhaustive in terms of references but hopes that a sufficient number of key articles have been cited to allow the reader to pursue topics of interest. The detection of administration of natural androgens for doping control has been described in many publications, e.g. Kicman and Gower (2003); Kicman et al. (2009b); Mareck et al. (2008); Schänzer (2004); including the current Handbook. It is intended that this review act as a complement to these articles by providing relevant information on endogenous androgens regarding their biosynthesis, secretion, mechanism of action, regulation, transport in blood, metabolism and excretion.

2 Structure and Activity

The naturally occurring androgens are C_{19} steroids, some of which are illustrated in Fig. 1.

The androgenic (masculinizing) activity of androgens has been assessed by in vivo responses, such as growth of the capon's comb or the growth of accessory sex organs of male rat (Brooks 1975) and much more recently by several different

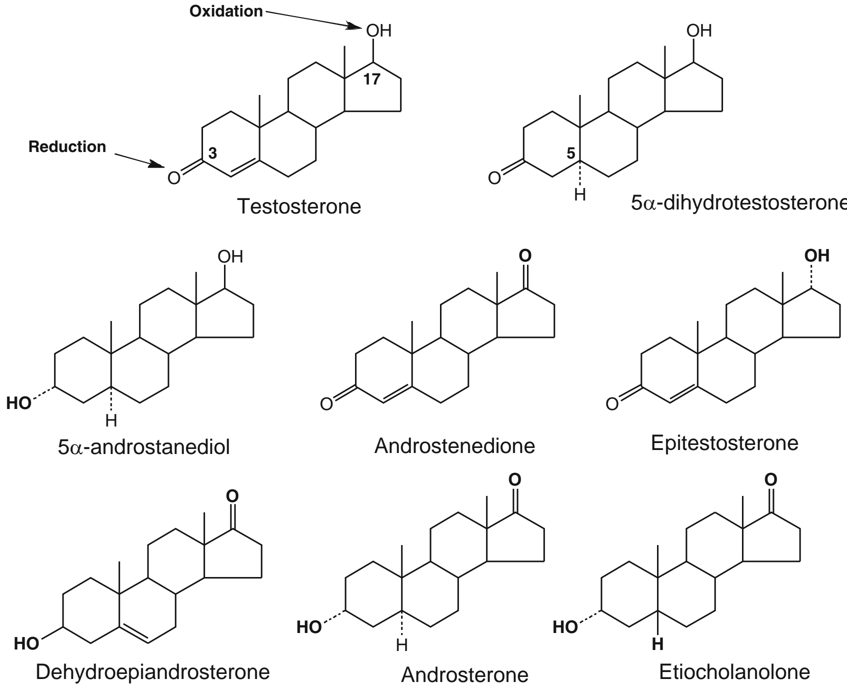


Fig. 1 Structures of endogenous androgens. The groups in *bold text* highlight the changes compared to testosterone and DHT. Testosterone and 5α-dihydrotestosterone (DHT) are displayed in the top row. Oxidation of the 17β-hydroxyl group of these androgens or reduction of the 3-oxo group results in a loss of activity (middle row) as does conversion of both groups (bottom row). Epitestosterone is a 17α-epimer of testosterone and has no androgenic activity, and neither do the 5β-reduced androgens, such as etiocholanolone

types of in vitro bioassay (Roy et al. 2008). From these assays, conclusions can be drawn about the structure–activity relationship of endogenous androgens. The most biologically active androgens are 5α-dihydrotestosterone (DHT) and testosterone, both of which have a 17β-hydroxyl group and a 3-oxo group, and, indeed, DHT binds with greater affinity to the androgen receptor, as discussed elsewhere (Kicman 2008). The reduction of the 3-oxo group results in some loss of activity and the oxidation of the 17β-hydroxyl group results in considerable loss of activity. The metabolism of DHT to 5α-androstanediol (5α-androstane-3α,17β-diol), as occurs readily in the skin, results in some loss of activity, the relative binding to the androgen receptor (of the rat) being about a fifth that of DHT (Chang et al. 1983). Oxidation of the 17β-hydroxyl group of testosterone to androstenedione results in a considerable loss of androgenic activity, as may be expected of an androgen that binds weakly in an androgen receptor competitive binding assay compared to that of DHT (androstenedione $K_d = 648 \pm 21$ nM, DHT $K_d = 10 \pm 0.4$ nM), though it does appear to retain a direct genomic effect (Jasuja et al. 2005). The 17-oxo steroids, dehydroepiandrosterone (DHEA) and androsterone, also

possess a 3β- and 3α-hydroxyl group respectively and have even less biological activity than androstenedione. Indeed, recent studies, using in vitro assays incorporating endogenous androgen receptors, have demonstrated that DHEA has negligible androgenic activity (Chen et al. 2005), exerting a minimal effect on "normal" human prostate cells as opposed to its activity in prostate cancer cell lines such LNCaP (Arnold et al. 2008). Much of the androgenic activity of DHEA and androstenedione in vivo can be attributed to the peripheral conversion of these steroids to testosterone and DHT following their secretion (see next section). DHEA can act also bind to the estrogen receptors α and β (Chen et al. 2005), and as an adjunct, 5-androstenediol (androst-5-ene-3β,17β-diol), formed from DHEA in the steroid biosynthetic pathway, can activate both androgen and estrogen target genes (Gingras et al. 1999; Miyamoto et al. 1998).

Androgens with the orientation of the hydrogen atom attached to C-5 in the 5β-configuration have an A-ring that is approximately perpendicular to the laminar (plane with thickness) of the rest of the steroid molecule, e.g. 5β-dihydrotestosterone, etiocholanolone. 5β-Metabolites are not known to have any androgenic activity either but they still retain another biological activity associated with androgens, in that they can stimulate the production of haeme in the bone marrow and liver (Besa and Bullock 1981). Epitestosterone is an epimer of testosterone, having a 17α-hydroxyl group, and this steroid is considered to have weak anti-androgenic activity (Starka 2003; Starka et al. 1989).

3 Origin and Interconversion of Secreted Androgens

In eugonadal men, circulating testosterone is almost exclusively of testicular origin, more than 95% being directly secreted, with a small amount being derived from peripheral metabolism of weaker androgens, namely androstenedione (Luke and Coffey 1994; O'Malley and Strott 1999). The biosynthesis of testosterone occurs in the 500 million or so Leydig cells, which constitute only a few percent of the total testicular volume (Rommerts 2004).

The blood production rate of testosterone (total testosterone that enters the plasma/serum from all sources) in the eugonadal male is approximately 3–7 mg/day, as calculated by infusions of a radioactive tracer. More recent investigations, using stable isotope-labelled analogues of testosterone for infusion and mass spectrometry (MS) for detection, have shown general agreement with this range. Vierhapper et al. (1997) using gas-chromatography–MS reported a rate of 147 ± 31 μg h^{-1}, which is equivalent to 3.7 ± 2.2 mg/day (assuming a constant rate of production). Wang et al. (2004), using liquid chromatography tandem–MS, reported the rate as 7.22 ± 1.15 mg/day in young Asian men and 9.11 ± 1.11 mg/day in young white men (no statistically significant difference) and a significantly lower value in middle-aged men of 3.88 ± 0.27 mg/day. The reference interval for serum concentration of testosterone in eugonadal men is usually quoted around 3–10 ng mL^{-1} (10–35 nmol L^{-1}).

In healthy women, the production rate of testosterone is much less than in men, being in the order of 0.1–0.4 mg per day (Burger 2002); the serum concentration of testosterone is usually quoted as being <1 ng mL^{-1} (<3.5 nmol L^{-1}). As a consequence of the relatively small amount of testosterone produced in the female, the secretion of weaker androgens by the ovary and adrenal cortex have much greater relevance than in the male, in particular their peripheral conversion making a major contribution to the production of testosterone (as discussed later in this section).

A review on androgen secretion in the normal female by Longcope (1986), despite being published over 20 years ago, contains most of the relevant data and research citations to date with respect to androgens in venous blood of the ovary and adrenal, the arteriovenous concentration gradients across these glands being important indicators of their relative contribution to endogenous production. In addition, the results of investigation by Moltz et al. (1984) are of relevance. Androstenedione and DHEA (but not DHEA sulphate) are the major androgens secreted into the ovarian vein. Secretion arises from the theca cells of the follicle, the corpus luteum and the stromal cells, under LH control, and will vary somewhat during the menstrual cycle. The secretion of androstenedione and testosterone are highest with maximum secretion of estrogen during the menstrual cycle, just prior to or at the time of ovulation, and then gradually fall during the luteal phase, the ovary not secreting significant amounts of testosterone during the early follicular phase (Lloyd et al. 1971; McNatty et al. 1976; Moltz et al. 1984).

The major androgens secreted by the adrenal cortex are androstenedione, DHEA and DHEA sulphate (Moltz et al. 1984; Nieschlag et al. 1973), ACTH being the primary secretagogue of both cortisol and the adrenal androgens (de Peretti and Forest 1976). DHEA secretion follows a diurnal rhythm similar to that of cortisol whereas DHEA sulphate does not (Brooks 1984). 11β-Hydroxyandrostenedione is secreted exclusively by the adrenals because the expression of 11β-hydroxylase is unique to that gland, CYP11B1 being present in the mitochondria of the zona fasciculata/reticularis (Payne and Hales 2004). The 11β-hydroxylated metabolites of this steroid, such as 11-ketoandrosterone, are also unique, so they can be used as endogenous reference C_{19} steroids that are unaffected in ^{12}C:^{13}C isotope content by testosterone administration, when carbon isotope ratio mass spectrometry is applied for doping control purposes (Cawley et al. 2005).

The findings by Vande Wiele et al. (1963), over 45 years ago, demonstrated that there is peripheral conversion between DHA sulphate and DHEA, that DHEA is peripherally converted to androstenedione, and that there is peripheral interconversion between androstenedione and testosterone. The interconversion of these steroids has been further discussed elsewhere (Allolio and Arlt 2004; Brooks 1984; Gower 1984a), with the degree of interconversion between DHEA and its sulphate being a recent subject of discussion (Siiteri 2005). The peripheral conversion of androstenedione to testosterone is 12–14% in both sexes but this contributes to about half of the circulating testosterone in women (Bardin and Lipsett 1967; Horton and Tait 1966) and thus probably accounts for much of the in vivo activity ascribed to androstenedione. As an adjunct, it follows that oral administration of androstenedione significantly raises

circulating testosterone in women (Bassindale et al. 2004; Brown et al. 2004; Kicman et al. 2003; Leder et al. 2002), and prohormones of androstenedione (and isomers of androstenediol) were widely available as ‘dietary supplements’ until the USA Anabolic Steroid Control Act was passed in 2004. With respect to the sources of the remaining half of the endogenous testosterone produced in healthy women, judging by the literature, the relative contributions of the adrenal and ovary remain somewhat unresolved. There is some indication that testosterone is constantly secreted by the adrenal cortex, as supported by analysis of venous effluent from healthy volunteers (Moltz et al. 1984). Even so, the adrenal synthesis of testosterone appears to be minimal, and its contribution should not be confused with the numerous reports concerning testosterone secreting adrenal adenomas. A confounding factor in accurately measuring the adrenal secretion of testosterone is the analytical sensitivity and the specificity required but, in the future, LC-tandem MS should help to overcome these difficulties. Other factors are the possibility that sampling procedure may alter the rate of secretion and the obvious difficulties faced in organising studies involving the catheterisation of ovarian and adrenal veins of healthy individuals.

The more potent androgen, DHT, is also secreted by the testis (Hammond et al. 1977; Pazzagli et al. 1974), although approximately half of the DHT produced in men is from the peripheral conversion of testosterone (Saez et al. 1972). The production rate has been reported as being 17–28 $\mu g\ h^{-1}$ (Toscano and Horton 1987; Vierhapper et al. 1997), which is about a tenth that of testosterone. However, in women, DHT can be considered to be more significant, as the production rate is one third that of testosterone, the major peripheral precursor being androstenedione (Mahoudeau et al. 1971). DHT is converted to 5α-androstanediol but this can be also converted back to DHT.

Epitestosterone, the epimer of testosterone, is of relevance to drug testing in sport as the urinary testosterone to epitestosterone ratio is augmented following testosterone administration. It was first reported to be isolated from urine in 1964 by Brooks and Giuliani and also Korenman et al. (1964). A number of studies support the testis as being a major source of epitestosterone in eugonadal men. In men, testosterone-induced suppression of LH decreases the urinary excretion rate of epitestosterone glucuronide (Anderson et al. 1997; Dehennin and Matsumoto 1993; Kicman et al. 1999; Palonek et al. 1995), e.g. administration of 200 mg testosterone enanthate weekly i.m. for 16 weeks decreases urinary epitestosterone glucuronide to $<10\%$ of pretreatment values (Anderson et al. 1997). Conversely, hCG stimulation results in an increase in urinary excretion of epitestosterone glucuronide (Cowan et al. 1991; Wilson and Lipsett 1966). The concentration of plasma epitestosterone increases during puberty (Lapcik et al. 1995), a comparison between peripheral and spermatic venous plasma in men (24–36 years) showing that testicular epitestosterone secretion represents approximately half of the total epitestosterone production (Dehennin 1993), based on a urinary production rate of about 220 μg/24 h (Wilson and Lipsett 1966). In the female, a source of epitestosterone is the ovary, this steroid having been identified in follicular fluid (Dehennin et al. 1987). Adrenal stimulation can cause an increase in epitestosterone production, as evidenced by an increase in plasma epitestosterone concentration and the

urinary excretion rate of epitestosterone (free steroid plus glucuronide conjugate) in the hypogonadal male but this appears to be countered in the eugonadal male by a concomitant decrease in testicular steroidogenesis, probably as a result of induced hypercortisolaemia (Kicman et al. 1999). Further investigations are required to prove whether the adrenal cortex can synthesize epitestosterone itself or whether it exclusively secretes a precursor steroid(s), which is then peripherally converted.

4 Biosynthesis of Androgens: Steroidogenic Enzymes

4.1 Introduction

Androgens are synthesized from cholesterol, which is in turn supplied from three different sources. Cholesterol is synthesized from acetyl coenzyme A by enzymes in the cellular microsomes and cytosol. Cholesterol is also supplied by the hydrolysis of esterified cholesterol stored within the steroidogenic cells. Another source of cholesterol is from the uptake of plasma low density lipoprotein. Lipoprotein is internalized by a specific receptor-mediated endocytosis and the cholesterol released by lysosomal action.

The steroidogenic enzymes in the pathway from cholesterol to testosterone have been reviewed recently and in depth by Payne and Hales (2004). These enzymes can be classified into two major categories of proteins, (a) the cytochrome P450 haeme-containing proteins and (b) the hydroxysteroid dehydrogenases, as briefly discussed here:

(a) Cytochrome P450 enzymes. The nomenclature for the cytochrome P450 superfamily of enzymes has been established, based on the genes that encode them. The nomenclature employs the root term "CYP" in the human and "Cyp" for other species, followed by a number to designate the family, then a letter to denote the subfamily (when two or more exist) and finally a number to denote the individual member of the subfamily, which specifies the individual gene. Italics are used when referring to the gene, e.g. *CYP11A1* encodes the protein CYP11A. The cytochrome P450 superfamily of enzymes are sometimes called mixed function oxidases or monoxygenases, where molecular oxygen is split with one atom being incorporated into the substrate and the other is reduced to water. This incorporation of oxygen commonly results in hydroxylation, such as with steroids, but many other catalysed reactions can occur with other substrates, e.g. N-oxidation, sulfoxidation, epoxidation, dealkylation. The general reaction for hydroxylation is:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

(b) Hydroxysteroid dehydrogenases. These are oxidoreductases requiring coenzymes for their function, the cofactors being nicotinamide adenine dinucleotide (NAD(H)) and its phosphate (NADP(H)). It is the oxidised form of these

cofactors that function in catalysis as hydrogen acceptor and the reduced form as a hydrogen donor:

$$NAD^{+} + 2H^{+} \rightleftharpoons NADH + H^{+}$$

The directional preference of the hydroxysteroid dehydrogenases is governed by the relative affinities of the enzymes for the cofactors and the gradients of these cofactors in subcellular compartments (Mizrachi and Auchus 2008).

4.2 Biosynthesis of Testosterone

The steroid biosynthetic pathway from cholesterol is given in Fig. 2, and details of the enzymes involved in Table 1, together with which tissues these proteins are specifically expressed in, and their subcellular location. Each of the P450 enzymes is the product of a single gene whereas there are two isoforms for 3β-hydroxysteroid dehydrogenase/isomerase in the human and several isozymes of the 17β-hydroxysteroid dehydrogenase, each being also a product of a distinct gene. The preferred route for formation of androgens is the Δ^5 pathway from pregnenolone to DHEA to androstenedione, which is readily converted to testosterone in the testis (Fig. 2). This conversion also occurs in the ovary but, relatively, the

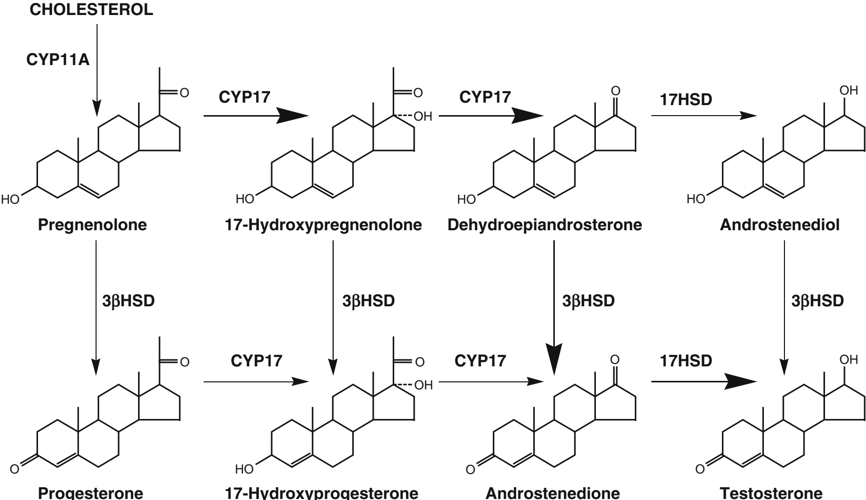


Fig. 2 The steroid biosynthetic pathway to testosterone and the enzymes involved. The *larger arrows* represent the major route in the human.
Note: in the testis, the chief isoforms of 3β-hydroxysteroid dehydrogenase/isomerase and 17β-hydroxysteroid dehydrogenase are 3βHSDII and 17βHSD3 respectively. 17βHSD3 is not expressed in the ovary but 17βHSD5 may account for the biosynthesis of testosterone in normal and abnormal states of ovarian function. 17βHSD5 can be expressed in human adrenal tumour cells but if actual synthesis of testosterone in the adrenal cortex of healthy adults does occur it appears to be minimal

Table 1 Enzymes Involved in the biosynthesis of androgens[a]

Human gene	Protein name	Action	Examples of synonyms	Tissue-specific expression	Subcellular location[b]
CYP450s					
CYP11A1	CYP11A	Cleaves the cholesterol side-chain between C20 and C22	P450scc, cholesterol side chain cleavage, cholesterol desmolase	Testis, ovary, adrenal cortex, placenta	Mitochondria
CYP17	CYP17	17α-hydroxylation of C_{21} steroids followed by cleavage of the C17–20 bond to produce the androgens (C_{19} steroids)	P450c17, 17α-hydroxylase/17, 20-lyase	Testis (Leydig cells only), ovary (thecal cells), adrenal cortex	Microsomes
Hydroxysteroid dehydrogenases					
HSD3B1	3β-HSDI	Converts 5-ene-3β-hydroxylated steroids to 4-ene-3-oxo steroids	3βHSD/Δ^5-Δ^4 isomerase, 3β-hydroxy-Δ^5 steroid dehydrogenase	Placenta, skin	Mitochondria and microsomes
HSD3B2	3β-HSDII	Converts 5-ene-3β-hydroxylated steroids to 4-ene-3-oxo steroids	3βHSD/Δ^5-Δ^4 isomerase, 3β-hydroxy-Δ^5 steroid dehydrogenase	Testis, ovary, adrenal cortex	Mitochondria and microsomes
HSD17B3	17HSD3	Converts androstenedione to testosterone	17βHSD3	Testis (Leydig cells)	Microsomes
HSD17B5	17HSD5	Converts androstenedione to testosterone	17βHSD5	Ovary	Microsomes

[a]Much of this table was extracted from the review by Payne and Hales (2004). The presence of 17βHSD5 in the ovary has been recently reported by Goto et al. (2006)

[b]Despite microsomes being artefactual vesicles created by cell homogenization, the subcellular location of enzymes bound to the smooth endoplasmic reticulum are generally referred to as microsomes or the microsomal fraction

amount of testosterone secreted is much smaller than from the testis. The end-point of androgen biosynthesis in the adrenal cortex appears to be with the formation of androstenedione and if testosterone is synthesised there, it is in a minimal amount.

Another biosynthetic precursor of testosterone is androstenediol (androst-5-ene-3β,17β-diol) but the biosynthetic route via this steroid is considered to be minor. Even so, it is worth noting that androstenediol appears to be rarely targeted as an analyte in studies on biosynthesis but the analysis for this steroid may become easier with the availability of LC-MS/MS.

4.2.1 CYP11A: Cholesterol Side-Chain Cleavage

The testis, ovary, adrenal glands and the placenta were considered to be unique in their ability to cleave the cholesterol side chain in the mitochondria to produce the C21 steroid pregnenolone. In recent years, however, evidence has been obtained for the expression of cholesterol side-chain cleavage enzyme CYP11A in the nervous system and cardiac tissue, as well as the presence of other CYP450s allowing the de novo synthesis of some steroid hormones (Payne and Hales 2004). The mechanism of cleavage of cholesterol by CYP11A involves two hydroxylation steps, first on C22 and then on C20 to yield 20,22R-dihydroxycholesterol that is then cleaved to yield the C21 steroid pregnenolone and the 6-carbon isocaproic aldehyde, which is then oxidised to isocaproic acid. The synthesized pregnenolone is then transferred to the smooth endoplasmic reticulum where it is the precursor for further steroid synthesis. The formation of testosterone from pregnenolone is via two main pathways. The first route is via a series of intermediates, each with a double bond between C-5 and C-6 and a 3β-hydroxy group; this is commonly referred to as the Δ^5 pathway (Δ is the Greek symbol for capital delta and together with the superscripted number denotes the presence of the double bond in the steroid structure). The second route is via a series of intermediates with a double bond between C-4 and C-5 and a 3-oxo group; this is called the Δ^4 pathway. In vitro experiments on human testicular tissue indicate that both the Δ^4 and Δ^5 pathways are important but the latter pathway is the major route of synthesis of testosterone in the human (Fig. 2).

4.2.2 CYP17: Pregnenolone/Progesterone Side-Chain Cleavage

CYP17 catalyses 17α-hydroxylation and cleavage of the C17–20 bond. In this reaction, 17α-hydroxypregnenolone and 17α-hydroxyprogesterone are formed as intermediates in the Δ^5 and Δ^4 pathways respectively, followed by cleavage of the side chain of these C21 steroids to form the C_{19} steroids DHEA and androstenedione. The distinction between the two activities is functional and not genetic or structural (Miller 1988). The activity of human CYP17 favours the Δ^5 pathway, the conversion of pregnenolone to DHEA being preferential to that of progesterone to androstenedione. The evidence for this favoured route is from clinical observations and biochemical studies concerning the adrenal glands and subsequently it has also been demonstrated that this is also the preferred route in the testis (Fluck et al. 2003).

4.2.3 3β-Hydroxysteroid Dehydrogenase/Isomerase (3βHSD)

3β-Hydroxysteroid dehydrogenase/isomerase exists as two isoforms in the human. The testis, ovary and adrenal gland express 3βHSD II whereas in skin, placenta, and breast tissue 3βHSD I is expressed (Payne and Hales 2004). 3βHSD catalyses the conversion of the Δ^5-3β-hydroxysteroids to the Δ^4-3-ketosteroids. Two sequential reactions occur in this conversion. Firstly, the dehydrogenation of the 3β-hydroxy group requiring the coenzyme NAD+ yields the Δ^5-3-keto intermediate and NADH. The NADH then activates the isomerization of the double bond, flipping it from the Δ^5 position to the Δ^4 (Thomas et al. 2003).

4.2.4 17β-Hydroxysteroid Dehydrogenase

Up to ten 17βHSDs have been discovered in the human, all being encoded by separate genes. The characteristics of these enzymes have been reviewed by Luu-The (2001), Moeller and Adamski (2006), and Payne and Hales (2004). Their substrate specificity is broader than was expected, and the role of some of these enzymes in vivo are probably not in steroid metabolism but in basic metabolic pathways (Moeller and Adamski 2006).

17βHSD3 is the key enzyme in the synthesis of testosterone, being expressed within the adult Leydig cells of the testis (Payne and Hales 2004). 17βHSD3 reduces androstenedione to testosterone, with NADPH as the preferred cofactor. The human ovary lacks expression of 17βHSD3 (Zhang et al. 1996) but another isozyme 17βHSD5 appears to be predominantly expressed there (Qin and Rosenfield 2000), which may account for the biosynthesis of testosterone in normal and abnormal states of ovarian function (Luu-The 2001; Nelson et al. 2001; Qin et al. 2006). 17βHSD5 has been also shown to be expressed in human adrenal tumour cells (Qin and Rosenfield 2005) but if actual synthesis of testosterone in the adrenal cortex of healthy adults does occur then it is minimal. Using the reverse transcription-polymerase chain reaction, 17βHSD5 and to a much lesser extent 17βHSD3 transcripts have been detected in the fetal adrenal cortex (first trimester), as was testosterone as “validated” by GC–MS/MS, although it should be emphasised that only one ion transition was presented as evidence in the supplement accompanying the report by Goto et al. (2006).

4.3 5α-Dihydrotestosterone

The irreversible conversion of testosterone into DHT is catalysed by the 5α-reductase, Table 2 giving details of the isozymes types 1 and 2 (Russell and Wilson 1994). In the human, the type 1 enzyme dominates in nongenital skin and scalp, the isozyme being induced at or during puberty and continuing thereafter (Thigpen et al. 1993; Zouboulis et al. 2007), but the type 2 predominates in beard

hair follicles (Zouboulis et al. 2007). The type 2 isozyme is the predominant, if not exclusive, isozyme in the prostate, epidydymis, seminal vesicles and genital skin, and both isozymes are present in the liver (Thigpen et al. 1993; Zouboulis et al. 2007). Testosterone is thus considered a prohormone in sexual tissue and skin, being readily converted to DHT, which binds with greater affinity to the androgen receptor. Intracellular metabolism of weaker androgens DHEA and androstenedione is also important as these steroids can be converted to testosterone (by 3βHSD and 17βHSD) and then be reduced to DHT, such as occurs in the skin (Zouboulis et al. 2007; Zouboulis and Degitz 2004) and prostate gland (Suzuki et al. 2007).

By contrast, in the human skeletal muscle (collected less than 12 h post-mortem), 5α-reductase activity (either type 1 or 2) is not detectable (Thigpen et al. 1993; Zouboulis et al. 2007) so testosterone itself is chiefly binding to the androgen receptor. A number of animal studies, mainly in the rat, also reported that intracellular DHT is low in skeletal muscle (Bartsch et al. 1980; Gloyna and Wilson 1969; Krieg et al. 1976; Massa and Martini 1974), and that its presence is further diminished because of the high activity in this tissue (and cardiac tissue as well) of the enzyme 3α-hydroxysteroid-dehydrogenase, the enzyme that converts DHT irreversibly to 5α-androstanediol (Massa and Martini 1974; Smith et al. 1980).

4.4 Aromatization and Nandrolone

A question that is of special relevance to drug testing in human sport is whether nandrolone (19-nortestosterone) is formed as a minor product of aromatization of endogenous androgens?

Aromatase (CYP19) (Table 2) is responsible for the conversion of androstenedione to estrone and likewise testosterone to estradiol. The classical pathway involves successive three oxidation steps, the first two occurring on C-19 angular methyl group to form a gem-diol, followed by dehydration, and (what was not well understood) a final oxidation step leading to cleavage of the bond between C-10 and C-19 to afford aromatization of the A-ring and formic acid (Fig. 3). Various schemes have been put forward for the "mysterious" third oxidation step, as reviewed by Cole and Robinson (1990), but recently the necessity of dehydration of the 19-gem-diol prior to the final catalytic step has been called into question by the findings of Hackett et al. (2005) using density field calculations. 1β-Hydrogen atom abstraction from substrates in the presence of the 2,3-enol (formed from enolisation of the 3-keto functional group) shows a remarkable low energy barrier. Ab initio molecular dynamics confirmed a dehydrogenase action of aromatase in the final step, involving 1β-hydrogen abstraction followed by gem-diol deprotonation, as depicted in Fig. 4.

Estrogen production increases during pregnancy and because plasma nandrolone can be detected during gestation (Reznik et al. 1987), as can urinary 19-norandrosterone (a diagnostic metabolite of 19-norandrogens) often in concentrations exceeding 5 ng mL^{-1} (de Boer et al. 1993; Mareck-Engelke et al. 1998; Mareck-Engelke et al.

Table 2 Enzymes engaged in the 5α-reduction and aromatization of testosterone

Human gene	Protein name	Action	Examples of synonyms	Tissue-specific expression	Subcellular location (see also note below)
		5α-REDUCTION			
SRD5A1	3-oxo-5α-steroid 4-dehydrogenase 1	Converts testosterone to 5α-dihydrotestosterone	Steroid-5α-reductase 1; Type I 5α-reductase	Permanently expressed in skin from time of puberty, liver	Microsomes, nucleus
SRD5A2	3-oxo-5α-steroid 4-dehydrogenase 2	Converts testosterone to 5α-dihydrotestosterone	Steroid-5α-reductase; Type II 5α-reductase	Male accessory reproductive glands, liver	Microsomes, nucleus
		Aromatization			
CYP19A1	CYP19A1	Aromatization of androgens; 19-norandrogens as a by-product?	Aromatase, P450arom, estrogen syntase	Ovary (granulosa cells), testis (Leydig cells), placenta, adipose tissue, bone	Microsome

Testosterone is considered to be a prohormone in androgenic target tissue (male accessory reproductive glands and skin), being converted by 5α-reductase (SRD5A1 and SRD5A2) to 5α-dihydrotestosterone, this metabolite binding with greater affinity to the androgen receptor compared to testosterone. 19-Nortestosterone (nandrolone) and 19-norandrostenedione are potential by-products of the aromatization of testosterone and androstenedione respectively

Fig. 3 Classical aromatization pathway. R is a 17-oxo group in androstenedione and a 17β-OH group in testosterone, these androgens being aromatized to estrone and estradiol respectively

Fig. 4 A proposed mechanism of the final aromatization step, which can be summarized by 1β-hydrogen atom abstraction followed by gem-diol deprotonation. Figure adapted from Hackett et al. (2005)

2002; Van Eenoo et al. 1999), it has been assumed that nandrolone is a by-product of the aromatization process (as could be 19-norandrostenedione, which can be then reduced to nandrolone by 17βHSD). Moreover, hCG stimulation of testicular steroidogenesis causes an increase in the urinary excretion rate of 19-norandrosterone and a concomitant rise in plasma estradiol (Reznik et al. 2001). Could an alternative reaction path be initiated by hydrogen atom abstraction from the gem-diol, rather than the 1β-hydrogen leading to C10–C19 homolysis? In vitro experiments are required to

investigate whether demethylation without aromatization can occur, together with ab initio molecular dynamics to help elucidate the mechanism.

In the anti-doping context, it is worth emphasising that the natural urinary excretion of 19-norandrosterone is very small (Nandrolone Review 2000; Nandrolone Progress Report 2003; Ayotte 2006). Findings from a recent study have demonstrated that only a few samples collected from a large cohort of non-pregnant women had a 19-norandrosterone concentration naturally greater than 1 ng mL^{-1} but, nevertheless, none exceeded the reporting threshold specified by the World Anti-Doping Agency (Walker et al. 2009).

4.5 Epitestosterone

A suggested biosynthetic precursor of epitestosterone is from the oxidation of epiandrostenediol (androst-5-ene-3β,17α-diol) by 3βHSD. This proposal is based on the identification of epiandrostenediol in human testis tissue (Ruokonen et al. 1972) and the significant correlation between concentrations of epitestosterone and androst-5-ene-3β,17α-diol in spermatic vein plasma (Dehennin 1993), which indirectly supports that this androstenediol epimer is a substrate for 3βHSD within testicular tissue.

The peripheral metabolism of testosterone to epitestosterone is negligible (Donike et al. 1983; Dray and Ledru 1966) and its conversion from androstenedione (androst-4-ene-3,17-dione) or DHEA appears to be minimal (Brooks and Giuliani 1964; Wilson and Lipsett 1966). With oral administration of hundreds of milligrams of androstenedione, an increase in the urinary excretion rate of epitestosterone (Catlin et al. 2002) was reported and subsequently the conversion of androstenedione to epitestosterone by 17αHSD activity has been demonstrated in human embryonic kidney cells using HPLC as the analytical probe (Bellemare et al. 2005). The enzyme also catalyses the conversion of three other 17-ketosteroids but the catalytic efficiency of 3βHSD and 17αHSD for DHEA and androstenedione respectively suggests that the main pathway is from conversion of DHEA to androstenedione and then reduction to epitestosterone and the minor pathway is through the reduction of DHEA to epiandrostenediol and then oxidation to epitestosterone (Bellemare et al. 2005). As an adjunct, it has also been proposed that epiandrostenediol can be formed as a by-product of the synthesis of a 16-androstene, 5,16-androstadien-3 β-ol (androsta-5,16-dien-3β-ol) from pregnenolone (Weusten et al. 1989). The putative pathways for formation of epitestosterone are given in Fig. 5.

5 Mechanisms of Androgen Action

The mechanisms of androgen action, including those concerning xenobiotic anabolic steroids, have been reviewed in detail elsewhere (Kicman 2008). The effects of androgens are modulated at the cellular level by the steroid-converting enzymes

Fig. 5 Proposed synthetic pathways to epitestosterone. Note: 5,16-androstadien-3β-ol = androsta-5,16-dien-3β-ol, under the revised nomenclature of steroids of 1989 (IUPAC/IUB); the former name is used in papers describing the putative pathways

within the particular target tissue and most probably at the molecular level due to differences in the distribution of androgen receptor coregulators.

With respect to modulation at the cellular level, in reproductive target tissues and skin, testosterone can be considered to be a prohormone, being readily converted by 5α-reductase to the more potent androgen DHT, whereas testosterone itself is chiefly binding to the androgen receptor in skeletal muscle, as discussed in Sect. 4.3. In other tissues, such as adipose tissue and parts of the brain, testosterone is converted by aromatase to the estrogen, estradiol. In bone, the mechanism of action of the anabolism of androgens has not been entirely elucidated but both a direct effect of testosterone and a mediated effect by aromatization to estradiol are important (Orwoll 1996; Zitzmann and Nieschlag 2004). Aromatase expression and activity is significant in human skeletal muscle (Larionov et al. 2003) but whether

the conversion of androgens to estrogens within this tissue is physiologically important for mediating some of the myotrophic effect of androgens is yet to be determined.

The molecular biology of the androgen receptor has been reviewed by Klocker et al. (2004). The modulation of the effects of androgens may also occur at the molecular level due to differences in the distribution of androgen receptor coregulators in various tissues. The androgen receptor belongs to the nuclear receptor superfamily (Mangelsdorf et al. 1995), consisting of a DNA binding domain, a ligand binding domain and at least two transcriptional activation domains, AF-1 and AF-2. In contrast to other steroid receptors, most androgen receptor transcriptional activity is mediated through the N-terminal AF-1 domain. In target tissues, i.e. the cells that contain androgen receptors, androgens bind to the receptor ligand-binding domain, causing dissociation of the receptor from its protein chaperones, the resultant conformational (allosteric) change making the receptor active (Fig. 6). Following dissociation from the chaperone complex, the activated androgen receptor is translocated from the cytoplasm into the nucleus. Activated receptors interact as homodimers with the androgen response element on the chromatin, the effect of the two receptors binding being cooperative (greater affinity and stability). This attachment to the DNA in turn triggers the formation of a transcription complex,

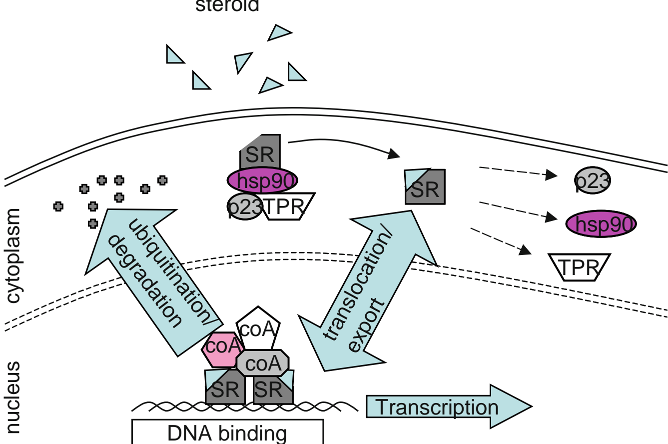


Fig. 6 Regulation of steroid receptor action. In the absence of hormone, the steroid receptor exists as an inactive oligomeric complex with the molecular chaperone heat shock protein Hsp90 and p23, and co-chaperones utilizing tetratricopeptide repeat (TPR) motifs. After hormone binding, the receptor–Hsp90 complex disassociates and the activated receptor is translocated into the nucleus. Activated receptors interact as homodimers with the steroid response element on the chromatin, triggering the formation of a transcription complex, a cluster of coregulators resulting in gene activation, transcription of the gene, protein translation, and a resultant alteration in cell function, growth or differentiation. This figure is redrawn by the author but was based on part of the figure in the article by Weigel and Moore (2007). Figure reproduced from Kicman (2008), with permission of the Nature Publishing Group

a cluster of coregulators (also called comodulators) that fit around the receptors like "pieces in a jigsaw puzzle". Coregulators can be either positive or negatively regulatory proteins, referred to as coactivators or corepressors, respectively (Perissi and Rosenfeld 2005). Co-activator and corepressor complexes are required for nuclear-receptor-mediated transcriptional regulation, generally liganded receptors recruiting coactivators resulting in gene activation, transcription of the gene, translation and a resultant alteration in cell function, growth or differentiation. This is a developing field and the comparative importance of many of these coregulators is yet to be established for any particular cell type, let alone their relative in vivo importance in examining tissue differences in androgen action. An appealing hypothesis is that modulation of the androgen receptor can occur as a result of anabolic steroids and non-steroidal selective androgen receptor modulators ("SARMs") inducing specific conformational changes of the androgen receptor complex, which then affects subsequent interaction with various coregulators in different tissues.

6 The Hypothalamic–Pituitary–Testicular Axis

6.1 Control of Testicular Steroidogenesis

Testicular function is controlled by feedback loops involving the hypothalamus and the anterior pituitary. The anterior pituitary secretes the two gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH stimulates steroidogenesis in the Leydig cells of the testis, resulting in the synthesis and release of testosterone. The testosterone within the testis is at much higher concentration than in the peripheral circulation, and it is this intra-testicular concentration together with FSH that is important in spermatogenesis, which occurs in the epithelium of the seminiferous tubules. As an adjunct, the Leydig cells appear to be able to produce a small amount of testosterone independent of LH stimulation, as proven following hypophysectomy in the rat (Turner et al. 1985). In addition, there is also indirect proof through the fact that with exogenous testosterone administration to eugonadal men, a low rate of testicular steroidogenesis remains because the urinary excretion of epitestosterone remains detectable (Anderson et al. 1997; Dehennin and Matsumoto 1993), with concentrations several-fold higher than in hypogonadal men (Kicman et al. 1999). This independent production of testosterone and also the intracellular metabolism of testosterone to DHT is of particular interest in the field of male hormonal contraception, as it is thought that these low intra-testicular concentrations of androgens may help to support spermatogenesis; to achieve azoospermia, the administration of a progestogen is thus of considerable importance as it has a direct inhibitory effect on Leydig cell steroidogenesis (Walton et al. 2006), as well as its main action in suppressing gonadotropin secretion (Anderson and Baird 2002).

LH secretion is tightly regulated, being stimulated by gonadotropin-releasing hormone (GnRH) and inhibited by the sex steroids testosterone and estradiol in the male (estradiol also exerts a negative feedback during the female menstrual cycle but with the rising tide of estradiol in the late follicular phase of the menstrual cycle a positive feedback action also occurs leading to a massive preovulatory surge in LH and FSH). GnRH is a single decapeptide hormone that is secreted episodically by GnRH neurones in the hypothalamus, as elegantly reviewed by Cone et al. (2003). Its release is governed by a complex neuronal network that integrates multiple external and internal factors to control fertility, with steroid hormones playing a pivotal role. GnRH is released into a capillary bed directly connected to another capillary bed in the anterior pituitary by small blood vessels (a portal system), this particular vascular link being referred to as the hypophyseal–portal circulation. The GnRH binds to its receptors in the anterior pituitary causing the pulsatile release of both LH and FSH (Van Vugt et al. 1985). In turn, testosterone and estradiol are secreted in contemporaneous pulses into the gonadal veins of the testis (Winters and Troen 1985).

The importance of the pulsatile pattern of release of GnRH, as opposed to steady state infusion which leads to down-regulation of GnRH receptors, was first established by studying anaesthetized rhesus monkeys with hypothalamic lesions (Belchetz et al. 1978). Shortly afterwards, it was demonstrated that human idiopathic hypogonadotrophic hypogonadism, a disorder characterized by low plasma gonadotropins, can be treated by episodic administrations of GnRH using a portable infusion pump (Hoffman and Crowley 1982). There is a log-linear relationship between the amount of GnRH and the amplitude of the ensuing LH pulse (Spratt et al. 1986).

Circulating testosterone exerts a negative feedback to diminish LH secretion, both directly and by aromatization to estradiol, thus maintaining a relative constant concentration of androgens in the blood of the male, taking into account the small diurnal variation. In eugonadal men about 80% of the total daily production of estradiol is derived from the peripheral conversion of testosterone, with only 10–20% being directly secreted by the testis (Baird et al. 1969). Early studies indicated that this peripheral aromatization of testosterone may play an important role in the feedback of gonadotropin release. Infusions of estradiol at its production rate lower the plasma LH concentration (Brooks et al. 1974), whereas the estrogen antagonist clomiphene citrate raises circulating concentrations of both gonadotropins (Winters et al. 1979), feedback being apparent at the hypothalamic and pituitary level (Santen 1975; Winters and Troen 1985). Historically, many studies were performed to elucidate the regulation of gonadotropin secretion and the interested reader is referred to the review by Plant (1986). Subsequently, it was suggested that the restraint by testosterone is largely conditional on its aromatization within the hypothalamo-pituitary compartment (Schnorr et al. 2001) or that at least some of the restraint of LH secretion is mediated by the aromatization of testosterone at the pituitary level (Bagatell et al. 1994), whereas other investigations indicate that peripheral aromatization is sufficient for feedback, without central aromatization being a prerequisite (Raven et al. 2006; Rochira et al. 2006). Evidence has also been provided of an

estrogen-independent effect of testosterone (Boyar et al. 1978; Gooren et al. 1987; Hayes et al. 2001; Urban et al. 1988; Veldhuis et al. 1992).

A major problem in assessing the mechanisms of endogenous gonadotropin feedback mechanisms is that gonadotropin secretion reflects the integrated response of both the hypothalamus and the pituitary. In an attempt to overcome this problem, a few investigations also studied the effects of various drug interventions in men deficient in GnRH due to idiopathic hypogonadotrophic hypogonadism (IHH), the volunteers continuing to receive their pulsatile GnRH therapy throughout the study period, thus representing a hypothalamic clamp where any alteration in gonadotropin secretion can only reflect a pituitary site of action (Bagatell et al. 1994; Finkelstein et al. 1991a,b; Sheckter et al. 1989). This approach was employed by Pitteloud et al. (2008a) and the response of LH secretion was compared to that in "normal" (eugonadal) men, giving valuable insight into the mechanisms feedback of sex steroids at the levels of the hypothalamus and pituitary. The drug these investigators chose for administration was ketoconazole, a cytochrome P450 inhibitor, which achieved chemical castration and inhibition of aromatase, followed by the co-administration of doses of testosterone or estradiol to restore serum concentrations critically to basal values, rather than supraphysiological levels, in both normal and the GnRH deficient men (also, dexamethasone was administered as ketoconazole inhibits the production of cortisol). Apart from serum concentrations of LH and LH pulse amplitude being measured, most importantly, an assay for the free α subunit of LH was performed to assess changes in GnRH pulse frequency, which is superior to LH as a marker of GnRH release at fast pulse rates. Testosterone administration to eugonadal men slowed the pulse frequency of GnRH even though estradiol levels were suppressed, indicating that testosterone feedback can occur directly at the hypothalamus without the requirement of aromatization. By contrast, testosterone administration to the IHH men failed to diminish either the mean concentrations of serum LH or LH-amplitude, and as the GnRH therapy was maintained at a constant dose and frequency, this lack of response indicates that testosterone's negative feedback at the pituitary is mediated by aromatization, a finding consistent with previous reports. A response to estrogen replacement was elicited, confirming this feedback pathway. Estradiol replacement in the eugonadal men lowered the serum LH concentrations by lowering the pulse frequency without any effect on pulse amplitude, indicating that the dominant site of estradiol feedback is on the hypothalamus. Pitteloud et al. concluded that the model of sex steroid feedback suggests (1) testosterone and estradiol have independent effects on LH secretion; (2) that the inhibition of LH by testosterone requires aromatization for its pituitary but not its hypothalamic effects; (3) estradiol has dual sites of feedback but its predominant effect is on the hypothalamus (Fig. 7).

At one time it was thought that DHT might be also important in the modulation of LH secretion. The importance of the 5α-reduction of testosterone to DHT for the inhibitory control of LH secretion was deduced from a form of pseudohermaphroditism. The affected individuals have a hereditary deficiency of the 5α-reductase system and thus have subnormal serum concentrations of DHT. The majority of these patients have mildly elevated serum LH and FSH values

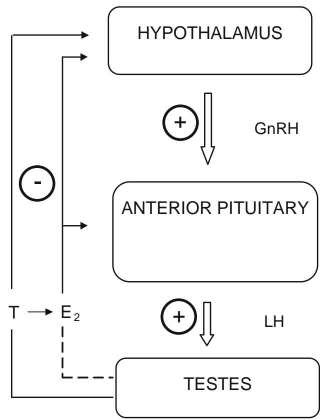


Fig. 7 The hypothalamic–pituitary–testicular axis showing the principal negative feedback pathways, based on current knowledge

although serum testosterone and estrogen concentrations are elevated or normal (Imperato-McGinley et al. 1979, 1990; Martini 1982). Later, there was evidence to contradict the importance of DHT in feedback mechanisms in eugonadal men, in that the administration of the 5α-reductase inhibitor, finasteride, caused no discernable effect on serum LH (or FSH) concentrations compared to another group given a placebo despite the approximately 80% decrease of the serum concentration of DHT in the treatment group (Rittmaster et al. 1992). Further, although DHT administered in supraphysiological doses to eugonadal men can suppress LH secretion (Ando et al. 1978; Coutts et al. 1997; Keenan et al. 1987; Kicman et al. 1995; Kuhn et al. 1984; Stewart-Bentley et al. 1974), more physiologically appropriate doses have little or no effect (Sherins and Loriaux 1973; Wang et al. 1998).

Inhibin B is important in the modulation of FSH secretion. The inhibins are a family of glycoprotein hormones that are produced by the gonads in both sexes. They consist of an α and β subunit, and there are two forms of the β subunit, giving rise to the dimeric forms A and B. In the case of the male, the Sertoli cells of the testis produce and secrete inhibin B, providing the principal negative feedback regulating FSH secretion (Boepple et al. 2008), with an added but modest feedback effect by estradiol (Pitteloud et al. 2008b). This modulation of FSH release by inhibin B accounts for the differential release of FSH compared to that of LH.

6.2 Disturbances to the Hypothalamic–Pituitary–Testicular Axis

6.2.1 Physical Exercise

The effects of physical exercise on the endocrine system have been extensively researched but concise accounts of the findings with regard to circulating testosterone may be found within the reviews by Christiansen (2004) and Friedl (2005).

Results of comparative studies indicate that male athletes at rest have a lower free and total testosterone concentration, being about 60–85% of age-matched untrained men. Nonetheless, the circulating concentration is still within the reference interval ("normal range") (Christiansen 2004), and reproductive dysfunction is uncommon in male athletes. Short-term exercise, whether maximal or submaximal (5–30 min), normally results in an acute increase in circulating testosterone, probably due to a transitory decrease in hepatic clearance as a result of reduced blood flow through the liver. By contrast, a decline in circulating testosterone is associated with prolonged exercise, the size of the (temporary) decline increasing with the duration of continued exercise, possibly due to alterations in hepatic and extra-hepatic (particularly skeletal muscle) metabolism of testosterone. LH pulsatile frequency and amplitude are generally unaffected by training, as discussed by Christiansen (2004) and Maimoun et al. (2003), supporting the hypotheses that the alteration in circulating testosterone is by peripheral effects. Even so, the LH production rate, the product of pulse frequency and secretory burst mass, has been shown to be marginally blunted overnight following resistance exercise, indicating also a central effect, although it should be noted that singularly neither frequency or the burst mass was significantly changed in that investigation (Nindl et al. 2001).

6.2.2 Exogenous Androgens

The administration of anabolic steroids suppresses gonadotropin secretion by the negative feedback loop of the hypothalamic–pituitary–gonadal axis in both men and women and can result in infertility, testicular atrophy, disturbances of the menstrual cycle and secondary amenorrhea (Graham and Kennedy 1990).

The inhibitory effect on LH secretion following testosterone administration is dose-related, feedback being exerted by the parent steroid as well as by its conversion to estradiol (as described in the previous section). Continuous infusion of testosterone at twice the mean production rate in men, i.e. 15 mg/day, suppressed plasma LH by $39 \pm 13\%$ (Loriaux et al. 1977), thus indicating that modest amounts of testosterone used surreptitiously by some athletes should cause a partial suppression in LH and testicular steroidogenesis. As part of a study investigating discriminating parameters for the detection of transdermal application of testosterone formulated as a hydroalcoholic gel, Geyer et al. (2007) measured serum testosterone and LH concentrations. The dose administered was a 100 mg daily to eugonadal men, which is the recommended maximum dose to be administered in the treatment of hypogonadal men as a replacement therapy. Continuous administration over 6 weeks resulting in approximately two- to threefold increase in serum testosterone in the majority (6 out of the 9) of the male volunteers, and a distinct decrease in the serum LH concentrations in the same volunteers to less than 50% of the pre-treatment value, and in some samples to 0.1 IU L^{-1}, substantially less than the reference range for the assay U (1.7–8.6 IU L^{-1}). Intermittent administration of the gel also resulted in a decrease serum LH concentrations in the majority of volunteers.

Supraphysiological doses of DHT in eugonadal men cause suppression of LH secretion (Ando et al. 1978; Coutts et al. 1997; Keenan et al. 1987; Kicman et al. 1995 Kuhn et al. 1984; Stewart-Bentley et al. 1974) and consequently there is a decrease in testicular steroidogenesis, reflected by a decrease in the urinary excretion of testosterone, its 5β-metabolites, 5β-androstanediol and etiocholanolone, and epitestosterone (Coutts et al. 1997; Donike et al. 1995; Kicman et al. 1995; Southan et al. 1992).

Several investigations have demonstrated that xenobiotic anabolic steroids will suppress LH secretion, for example fluoxymesterone administered in oral doses of 40–50 mg daily over 3–4 days markedly diminishes plasma LH (Swerdloff and Odell 1968; Vigersky et al. 1976) as does methandienone administered as 15 mg per day over 2 months (Holma and Adlercreutz 1976). The degree of depression of gonadotropin concentrations varies, not least depending on the dose and duration, ranging from no change to profound suppression, as discussed in the review by Wright (1980). Androgens which lack the C-19 angular methyl group, such as nandrolone, also have strong progestational activity, and these will increase the suppression of gonadotropin secretion. When anabolic steroids are used in large amounts, and/or in combinations such as the stacking regimens that bodybuilders/power athletes follow, then there is a profound disturbance of the HPG axis (Alen and Hakkinen 1987; Martikainen et al. 1986). Recovery is usually within a few months of cessation of administration but there are a few case reports of persistent hypogonadism (Jarow and Lipshultz 1990; Menon 2003; van Breda et al. 2003), these relying on the veracity of the patients with respect to when they say they withdrew from using anabolic steroids.

7 Transport: Binding of Androgens in Blood

Steroids circulate in the blood, mainly bound to the proteins, with only a small proportion circulating as unbound hormones, sometimes referred to as "free steroid" (Dunn et al. 1981). The major binding proteins for androgens are albumin and sex-hormone binding globulin, both being produced by the liver. Human serum albumin has a molecular weight of approximately 66,000 Da, consisting of a single polypeptide chain of 585 amino acid residues (Kragh-Hansen 1981). SHBG is a homodimeric glycoprotein with a molecular weight of 85,600 Da of which 14% is carbohydrate, with some microheterogeneity due to variability in sugar content (Petra 1991).

Circulating albumin has a vast capacity for binding a wide variety of compounds, both endogenous and exogenous, not least because of its high serum concentration (0.6–0.8 mmol L^{-1}). Human serum albumin has anionic properties with over 200 negative charges per molecule at pH 7.4 (Silverman et al. 1986). Despite this, albumin binds (possibly preferentially) negatively as well as positively charged molecules, as well as electrostatic neutral compounds, there appearing to be at least six binding regions on the molecule, with one or two primary sites and a

number of sites with lower affinity (Kragh-Hansen 1981). Endogenous steroids (non-conjugated) are neutral compounds and bind to albumin with low affinity, the association (binding) constant varies somewhat but is in the region of $\sim 10^4 M^{-1}$, as comprehensively listed by Dunn et al. 1981) (the interested reader is referred to the data in the last paragraph of the Materials and Methods section of that paper). More recently, Amundsen and Siren (2007) assign association constants to steroids not previously reported, these being epitestosterone, androstenedione and the xenobiotic anabolic steroids methyltestosterone and fluoxymesterone. The low affinity of neutral steroids with albumin is partially due to the inability of steroids to form ionic bonds but as albumin is present in a high concentration, a large proportion of circulating steroids is nonetheless bound to this protein, i.e. albumin has a low affinity but a high capacity for steroids. Indeed, even in pregnancy, a physiological state characterised by high circulating steroid concentrations, such is the enormous capacity of albumin for binding steroids that 99% of its available binding sites remain unoccupied (Dunn et al. 1981).

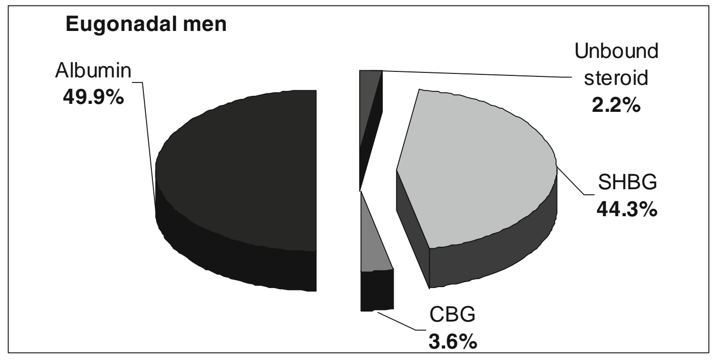
The more specific steroid-binding proteins, sex-hormone binding globulin (SHBG) and corticosteroid-binding globulin (CBG; transcortin) circulate in much lower concentrations (in the nmol L^{-1} range) compared to albumin but they have relatively a much higher binding affinity with certain steroids, i.e. these binding globulins have a high affinity but low capacity. SHBG has a high binding affinity with sex steroids and it is also referred to as "testosterone-binding globulin" or "testosterone-estrogen binding globulin". Each monomer of this homodimeric glycoprotein has a binding site so each SHBG has the capacity to bind two steroid ligands (Avvakumov et al. 2001). Its binding affinity with testosterone and estradiol and other steroids possessing a 17β-hydroxylated substituent is around 10^9 M^{-1}. In contrast, the binding affinity with the 17α-hydroxylated steroid, epitestosterone, is likely to be similar to that of other non-17β-hydroxylated steroids, such as androstenedione ($K_a = 2.9 \times 10^6 M^{-1}$). CBG has the highest affinity for corticosteroids but also binds androgens with a much lower binding affinity, e.g. testosterone ($K_a = 10^6 M^{-1}$) has about one twentieth the binding constant of cortisol.

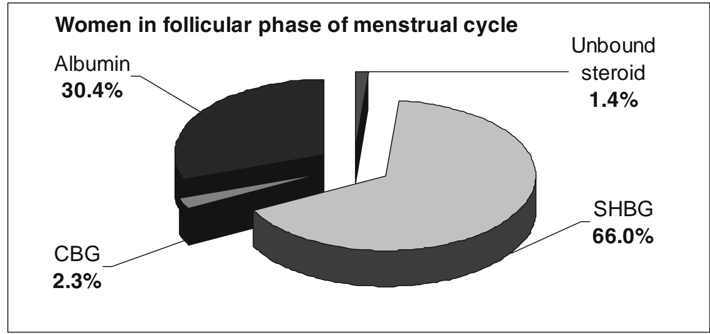
Dunn et al. (1981) reported, using computer simulation, that in eugonadal men, testosterone occupies 36% of the binding sites of SHBG, and taking into account binding with androstenediol, DHT, DHEA and other steroids, this leaves about 44% of the binding sites unoccupied. In women, who have much lower amounts of circulating androgen, 82% of the binding sites are unoccupied. Increasing the testosterone concentration will result in increasing binding until the sites of SHBG are saturated. With respect to the strength of binding of different 17β-hydroxylated steroids, the association constant of DHT with SHBG ($K_a = 5.5 \times 10^9 M^{-1}$) is approximately three times greater than that of testosterone ($K_a = 1.6 \times 10^9 M^{-1}$), which in turn is about twice that of estradiol ($K_a = \sim 10^9 M^{-1}$). As the energy of binding of SHBG with 17β-hydroxylated steroids is far higher compared with these steroids to albumin, at equilibrium the proportion of unbound ligand compared to the protein-bound fraction is relatively much smaller with SHBG than with albumin. As an adjunct, results from a study by Saartok et al. (1984) has indicated that the introduction of a 17α-methyl group into testosterone, or removal

of its 19-methyl group, has a negative influence on binding with SHBG, whereas 1α-methyl substitution on DHT increased the affinity by approximately fourfold compared to DHT itself (note: the investigation utilised human serum and not isolated SHBG).

Unbound steroids are available for transport across cell membranes and kinetic studies have shown that albumin-bound steroids may supplement the free steroid pool due to the rapid equilibrium between binding and dissociation (Mendel 1990; van den Beld et al. 2000), the steroids dissociating from the albumin in the capillary space to then enter tissues (Manni et al. 1985; Pardridge 1981). The fraction of 17β-hydroxylated steroids tightly bound to SHBG is widely considered to be unavailable for diffusing out of the bloodstream, despite some speculation to the contrary, and thus the SHBG-bound fraction is accepted as being biologically inactive. There is some evidence, however, of a mechanism independent of steroid nuclear receptors, where SHBG can bind to receptors on the cell surface. The importance of this non-genomic pathway, and others, at physiological concentrations of androgens is not currently known (Kicman 2008). Leaving aside any possible non-genomic effects, as only unbound hormones and albumin-bound hormones are generally considered bioavailable, SHBG is therefore the most important circulating protein for controlling the amount of steroids available to responsive cells. The plasma concentration of SHBG in eugonadal women is twice that compared to eugonadal men (and consequently the unbound proportion of testosterone is lower, often being quoted as being about 1% compared to approximately 2% in men; Fig. 8). Some caution must be exercised, nonetheless, with respect to the proportion of unbound testosterone quoted for the female, as often the free concentration is calculated using either equilibrium dialysis approach or an algorithm, both of which depend on accurate measurement of total testosterone. Recently, the accuracy of immunoassay in the measurement of low concentrations of serum total testosterone, such as found in women, has been called into question and newer methods based on mass spectrometry are helping to address this issue (Kane et al. 2007; Matsumoto and Bremner 2004; Swerdloff and Wang 2008). Furthermore, approaches based on the algorithm approach require, as input, the concentration of SHBG (as well as total testosterone and albumin, and the association constants of testosterone with SHBG and also with albumin) but Miller et al. (2004) found approximately twofold higher values using an immunoradiometric assay compared to a radioimmunoassay. This disparity may be due to differences in antibody recognition of various forms of SHBG, including glycosylated variants.

Administration of testosterone causes a fall in plasma SHBG concentration whereas estrogen administration stimulates synthesis of SHBG concentration. An in vitro study has demonstrated that as testosterone binds more strongly to SHBG relative to estradiol, a fall in SHBG concentration results in a rise in the ratio of unbound testosterone/estradiol and, conversely, a rise in SHBG concentration results in a fall in the testosterone/estradiol ratio (Burke and Anderson 1972). It was thus proposed that SHBG may play an important role as a "biological amplifier" for sex steroid action, where androgens and estrogens promote their own action by altering the SHBG level accordingly. The conclusions drawn from





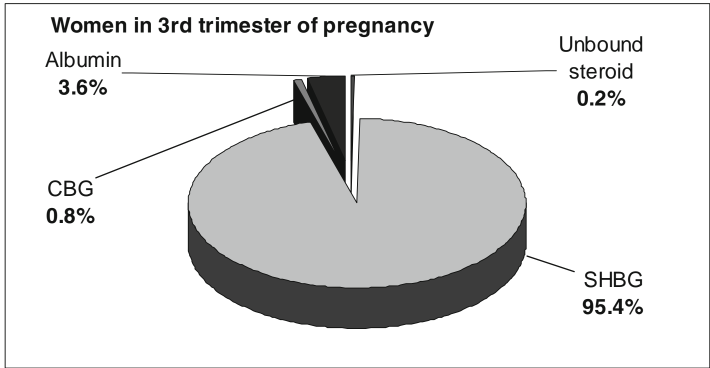


Fig. 8 The proportion of testosterone bound to plasma proteins in the eugonadal male, the menstruating female and the pregnant female (third trimester), based on the data presented by Dunn et al. (1981). The illustration provides a useful impression of the relative proportions but it would be inappropriate to place great weight on the absolute accuracy of the numbers quoted, for reasons discussed within the text

this in vitro study, however, are unlikely to apply in vivo in eugonadal men, as the HPT axis will respond to a fall in non-SHBG bound testosterone with an increase in LH and testosterone, assuming that non-SHBG bound testosterone is driving the feedback inhibition (de Ronde et al. 2005a, b). Of course, with chronic testosterone

administration for performance enhancement in sport, this adjustment back to the steady state cannot occur and therefore amplification would be expected.

During pregnancy, presumably under the influence of the rapidly increasing estrogen concentration, there is a large increase in SHBG concentration, rising five- to tenfold by week 30 (Moore and Bulbrook 1988) and, as SHBG-bound steroids are protected from metabolism, this results in a large increase in the total testosterone concentration but, as logic dictates, a fall in the unbound testosterone concentration. Conversely, with the loss of estrogen during the menopause, there is a decrease in SHBG concentration, which results in an increase in free testosterone (Morley and Perry 2003). The decrease in levels of SHBG associated with hypothyroidism and increased levels with thyroid hormone excess may be also due to changes in estrogen formation (Griffin and Wilson 2003).

The disappearance of human SHBG has been investigated after its administration to the Rhesus monkey (Longcope et al. 1992) and the rabbit (Cousin et al. 1998), and regardless of the discrepancy in metabolism between these two species, there was a biphasic serum profile with an initial half-life (alpha) of approximately 3 h and a terminal half-life of over 30 h. The prolonged half-life of SHBG is compatible with its relative stability and thus drug-induced changes in the circulating concentration of SHBG in the human would be expected to be also relatively slow, with a gradual change in the clearance of sex steroids. Modest to severe falls in SHBG concentration have been reported during chronic self-administration of high doses of anabolic steroids by male bodybuilders and power athletes (Alen and Hakkinen 1987; Bonetti et al. 2008), with as much as a 90% reduction occurring based on measurement by competitive immunoassay (Ruokonen et al. 1985). Conversely, use of oral contraceptives that contain estrogens causes an increase in SHBG and CBG concentration; after only one cycle of use the SHBG concentration can double (Wiegratz et al. 2003). Progestogen administration can result in inhibition of SHBG synthesis and, accordingly, the type of progestogen accompanying the estrogen in combined oral contraceptives will affect the degree of rise in SHBG (Moore and Bulbrook 1988). Of interest is how changes in SHBG synthesis may affect the urinary testosterone/epitestosterone (T/E) ratio, for example when a woman switches from use of a combined oral contraceptive to a progestogen-only contraceptive. A fall in circulating SHBG should increase the amount of bioavailable testosterone for urinary excretion but clearance of epitestosterone is likely to remain largely unaffected due to its probable low binding affinity. Theoretically, the urinary T/E ratio should increase as a result, even though the urinary excretion of both these steroids would be expected to be low due to suppression of ovarian steroidogenesis.

8 Catabolism and Excretion of Endogenous Androgens

The main site of androgen metabolism is in the liver, which is particularly rich in steroid catabolic enzymes. Extrahepatic androgen catabolism can also be significant, e.g. 5α-androstanediol and its glucuronide conjugate are derived from extra-splanchnic metabolism of DHT, mainly in the skin (Toscano 1986).

Fig. 9 Metabolism of testosterone, with figures attached to the arrows indicating the approximate proportion of testosterone which is metabolized by that route (Brooks 1975). All steroids are excreted predominantly as conjugates but only the glucuronide conjugate of testosterone is shown

Testosterone is subjected to extensive phase 1 metabolism (Fig. 9), involving oxidation of the 17β-hydroxyl group to androstenedione, the reduction of the A-ring to yield the 5α- and 5β-androstanediones and then the action of the 3α- and 3β-hydroxysteroid dehydrogenases to form the tetrahydro-17-oxosteroid metabolites, androsterone and etiocholanolone, as major metabolites, and to a much lesser extent epiandrosterone. The isomeric 5α- and 5β-androstane-3α, 17β-androstanediols are also minor metabolites of testosterone and a very small proportion is converted to estradiol (Brooks 1975). Apart from oxidoreductive reactions at positions 3, 4 and 17, hydroxylation by the action of CYP enzymes may possible occur, especially following testosterone administration, as discussed by Rendic (1993).

Androgens in urine are almost entirely conjugated. Phase II reactions, concerning the conjugation with UDP glucuronic acid (UDPGA) to yield glucuronides and with 3′-phosphoadenosine-5′-phosphosulphate (PAPS) to yield sulphates, has been reviewed extensively (e.g. Gower 1984b). The quantitative determination of urinary steroid glucuronide conjugates (in men) and mono- and disulphates (in men and women) show that the large majority of the androgen metabolites are excreted in the urine mainly as glucuronide conjugates, a notable

exception being the 3β-hydroxylated steroids, which are predominantly excreted as sulphates, DHEA sulphate being particularly abundant (Janne 1970; Setchell et al. 1976). The conjugation of androgens to glucuronic acid or sulphate is chiefly performed by enzyme systems in the liver, although these systems are also present in other tissues such as kidney and intestine. This conjugation makes the steroid metabolites even more water soluble. A small fraction of androgen metabolites is excreted via the bile into the small intestine, although the sulphate conjugates in particular may be reabsorbed there as part of the enterohepatic circulation. The fact that sulphate conjugates are also tightly bound to albumin in the blood may explain why they are not so rapidly excreted compared to steroid glucuronide conjugates.

Typical concentrations of urinary androgens, based on a database of ~10,000 samples and analysis by GC–MS following glucuronidase hydrolysis, show that testosterone is approximately 40 ng mL^{-1} (Aguilera et al. 1999), 5β- and 5α-androstanediol (median values) are 121 and 68 ng mL^{-1} (Aguilera et al. 1999), and androsterone and etiocholanolone are 1,000 to 4,000 ng mL^{-1} (Aguilera et al. 2000). The supporting science to this data has not been published but the data originates from the anti-doping laboratory at UCLA, and these sorts of measured concentrations are consistent with the experience of other WADA-accredited laboratories, such as the one in London. Mareck et al. (2008) have reviewed the factors influencing the androgen profile in doping control analysis.

It has been well recognized that the clinical assay used for urinary 17-oxo steroids is a poor index of androgenic status, since proportionately about two thirds originates from adrenal steroid metabolism (Bethune 1975; Grant and Beastall 1983). The adrenal steroids secreted that are metabolized to urinary androsterone and etiocholanolone glucuronides are androstenedione, DHEA and DHEA sulphate. Moreover, ACTH secretion is increased in times of physiological stress, as can occur (moderately) with athletes in competition and with sustained arduous training, which results in increased adrenal androgen output and thus the urinary excretion of androsterone and etiocholanolone should be raised. This adrenal contribution will attenuate the sensitivity of the gas chromatography-combustion-isotope ratio mass spectrometry test for testosterone administration based on targeting androsterone and etiocholanolone rather than urinary testosterone itself.

Epitestosterone is an important analyte for detecting testosterone administration by athletes. Currently, as only urine samples are collected for analysis and because testosterone is also endogenously produced, a test for the detection of testosterone administration is based on the ratio of urinary testosterone to epitestosterone (T/E) in urine, which is independent of the urinary dilution (Donike et al. 1983). The circulating concentration and production rate of testosterone in men is about 15–30 times greater than that of epitestosterone (Handelsman et al. 1996; Kicman et al. 1999; Wilson and Lipsett 1966), albeit based on limited sample numbers. Due to differences in extent of metabolism, about 1% of testosterone is excreted into urine as testosterone glucuronide compared with about 30% of epitestosterone glucuronide, resulting in similar urinary concentrations of these steroid glucuronides and thus the T/E ratio approximates to 1. The ratio also approximates unity in healthy women. When testosterone is administered the ratio is augmented due to

the increased excretion of urinary testosterone, and a laboratory reporting threshold of 4 has been chosen by the WADA.

Genetic polymorphism can affect the excretion of steroids and this has implications for doping control. The deletion polymorphism of UGT2B17 is associated with significantly lower glucuronidation rates of testosterone, which diminishes the excretion of testosterone glucuronide but does not seem to affect epitestosterone glucuronide (Jakobsson et al. 2006). The UGT2B17 del/del genotype appears to be much more common in Asian than Caucasian men, and this inter-ethnic variation in metabolism has implications for the detection of testosterone administration test based on an absolute T/E reporting threshold (Bowers 2008; Kicman and Cowan 2009a; Schulze et al. 2008). Further, Borts and Bowers found that concentrations of both glucuronide and sulphate conjugates of testosterone and epitestosterone were lower in a Chinese population compared with a Caucasian mixed group (Borts and Bowers 2000).

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Phase-II Metabolism of Androgens and Its Relevance for Doping Control Analysis

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Abstract Phase-II metabolism has a major contribution to androgen metabolism, converting the highly non-polar compounds to a more easily excreted form prior to their excretion in urine. In the human body the main phase-II metabolic reactions are glucuronidation and sulphonation. These reactions are catalysed by enzymes, which are categorised into families and further subfamilies based on their function and similarities of their amino-acid sequences. Due to inter-individual variation of the metabolising enzymes and their activities, the metabolic patterns of prohibited substances should be estimated for efficient doping control. In addition to target analytes the phase-II reactions have an effect on the selection of sample preparation procedure, chromatographic technique and ionisation method of the analysis routine. For method development and identification purposes adequate reference material is required, and to replace the laborious *in vivo* excretion studies, *in vitro* methodologies have been implemented to produce intact phase-II metabolites of androgens.

Keywords Androgen • Phase-II • Glucuronidation • sulphonation • *In vitro* assays

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1 Phase-II Metabolism of Androgens

Due to their highly non-polar character, androgens are extensively modified by several metabolic reactions prior to their excretion in urine. These metabolic pathways are divided into phase-I and phase-II reactions, and transform the steroid substrate into a less toxic, less active and more polar form, typically terminating the pharmacological activity of the androgen. Phase-I reactions (i.e. functionalisation) involve oxidation, hydrolysis and reduction (Gibson and Skett 1994), which introduce new functional groups for the subsequent phase-II reactions (i.e. conjugation). For the endogenous androgens and for exogenous anabolic androgenic steroids (AAS) the main phase-II reactions are conjugation with glucuronic acid (glucuronidation) or with a sulfo-moiety (sulphonation), glucuronidation being the most predominant pathway in human metabolism. In general, conjugation plays a remarkable role in the metabolism of androgens, as the unconjugated fraction of androgens has been reported to contain only less than 3% of the total amount of androgens excreted in urine (Dehennin and Matsumoto 1993).

1.1 Glucuronide Conjugation

In human, conjugation with glucuronic acid is the major conjugation reaction of androgens (Mulder et al. 1990). Glucuronidation is a bimolecular nucleophilic substitution reaction (S_N2), which is catalysed by uridine diphosphoglucuronosyltransferases (UGTs; E.C. 2.4.1.17) and uses uridine-5′-diphosphoglucuronic acid (UDPGA) as the co-substrate. The reaction leads to the attachment of the highly polar glucuronic acid moiety to the steroid structure with the immediate inversion of the configuration to yield a β-glycosidic bond (Fig. 1). In most cases the activity of the xenobiotics or endobiotics is terminated by glucuronidation, but exceptionally higher toxicity has been reported for the D-ring glucuronide conjugates of 17β-hydroxyestrogens, testosterone and dihydrotestosterone in comparison to the corresponding parent compounds (Vore and Slikker 1985).

Testosterone + Uridine 5′-diphosphoglucuronic acid (UDPGA) —UGT→ Testosterone glucuronide + UDP + H_2O

Fig. 1 A UGT-catalysed glucuronide conjugation between testosterone and uridine-5′-diphosphoglucuronic acid (UDPGA)

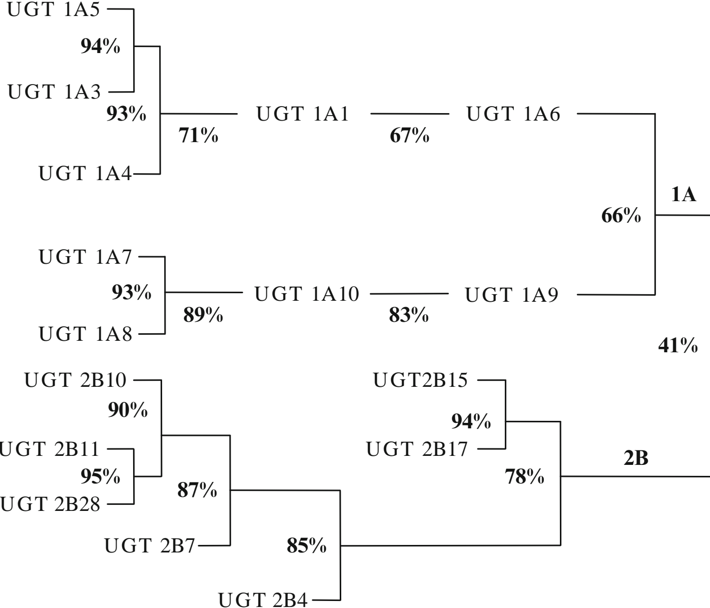

Fig. 2 Sequence similarity of human UGT isoenzymes

UGTs are a family of membrane-bound enzymes of the endoplasmic reticulum. According to recent updates the human genome encodes at least 19 UGTs (Mackenzie et al. 2005), which range from 529 to 534 amino acids in size (Tukey and Strassburg 2000). These expressed proteins have been divided into two families (UGT1 and UGT2) on the basis of their sequence similarity (Fig. 2), which is higher than 38% within a single family (Tukey and Strassburg 2000, 2001). According to the even higher sequence homology, the enzyme families are categorised into further subfamilies (Burchell et al. 1991; Mackenzie et al. 1990, 1997, 2005).

The most important enzymes involved in glucuronidation of androgens are members of subfamilies UGT1A and UGT2B (Barbier and Bélanger 2003; Hum et al. 1999; Turgeon et al. 2001). The main site of glucuronidation is the liver (Table 1), although extra-hepatic glucuronidation has been observed in kidney, intestine, lung, and prostate. In the human body the members of the subfamily UGT2A are expressed in the nasal epithelium (Lazard et al. 1991), and despite the observed glucuronidation activity towards androgens, their relevance in the androgen biotransformation is minor in comparison to UGT1A and UGT2B enzymes (Sten et al. 2009).

The superfamily of UGTs is a diverse group of isoenzymes capable of glucuronidating a wide variety of substrates and different functional groups, e.g. –OH, –COOH, $–NH_2$, –SH and C–C (Radominska-Pandya et al. 1999). With androgens, however, the site of conjugation is typically a hydroxyl group, although interesting evidence of the regio- and stereoselectivity of UGTs has been shown, especially in the activities of UGT2B enzymes (Jin et al. 1997; Kuuranne et al. 2003; Sten et al. 2009).

Table 1 Examples of human UGT isoenzymes, of their tissue distribution and of their reported androgen substrates (A: androsterone, 5α-A: 5α-androstane-3α,17β-diol, DHT: dihydrotestosterone, E: epitestosterone, Etio: etiocholanolone, T: testosterone)

Isoform	Major sites of distribution	Examples of androgen substrates	References
UGT1A1	Liver, bile duct, stomach, colon	None	
UGT1A3	Liver, bile duct, stomach, colon	A	Gall et al. (1999)
UGT1A4	Liver, bile duct, colon	5α-A	Green and Tephly (1996)
UGT1A5	Not defined	None	
UGT1A6	Liver, bile duct, stomach, colon, brain	None	
UGT1A7	Oesophagus, stomach	None	
UGT1A8	GI-tract	DHT, E, T	Cheng et al. (1999)
UGT1A9	Liver, colon, kidney	None	
UGT1A10	GI-tract	A, DHT	Cheng et al. (1999); Tukey and Strassburg (2000)
UGT2A1	Nasal epithelium	E, T	Sten et al. (2009)
UGT2B4	Liver, prostate, testis, mammary gland, lung, kidney	5α-A, A	Hum et al. (1999); Turgeon et al. (2001)
UGT2B7	Liver, mammary gland, lung, kidney	A, E, T and several other hydroxysteroids	Gall et al. (1999); Turgeon et al. (2001)
UGT2B10	Liver, prostate, testis, mammary gland, lung, kidney	None	
UGT2B11	Liver, prostate, testis, mammary gland, lung, kidney	5α-A	Jin et al. (1997)
UGT2B15	Liver, prostate, testis, mammary gland, lung, kidney	5α-A, DHT, T	Green and Tephly (1996); Hum et al. (1999); Turgeon et al. (2001)
UGT2B17	Liver, prostate, testis, mammary gland, lung, kidney	5α-A, DHT, A, E, Etio, T	Beaulieu et al. (1996); Hum et al. (1999)
UGT2B28	Liver, mammary gland	A, T	Lévesque et al. (2001)

1.2 Sulphonation

In addition to glucuronide conjugation, sulphonation (often called also as sulfate conjugation) also plays a major role in the modulation of pharmacological activity of a wide variety of endogenous compounds and xenobiotics in the human body. The sulfonation (Fig. 3) is catalysed by sulphotransferase enzymes, which transfer the sulfo moiety (SO_3) from a co-substrate, 3′-phosphoadenosine-5′-phosphosulfate (PAPS), to the specific substrate (Mulder and Jakoby 1990). In mammals the sulfotransferases (SULTs) are divided into two classes, namely membrane-bound forms, which are not active in the biotransformation of xenobiotics, and soluble forms, typically cytosolic sulfotransferases which are involved in the sulphonation of xenobiotics and small compounds (Glatt et al. 2001; Kauffman 2004; Chapman et al. 2004). The nomenclature of SULTs is often confusing due to an extensive overlap of substrate specificity of the enzymes. All cytosolic SULTs are members of a single superfamily and according to the literature there are currently 13 known isoforms of human SULT enzymes (Lindsay et al. 2008) categorised into families SULT1, SULT2, SULT4 and SULT6 (Table 2). Within each family the identity of amino-acid sequence is higher than 45%, whereas the similarity is above 60% within each subfamily. Although gluronidation is the main conjugation pathway of androgens in human, steroids possessing a 3β-hydroxyl function are an exception and are sulfonated to exceptionally high extent, dehydroepiandrosterone (DHEA) being a model compound of endogenous androgens (Gower et al. 1995). Catalysing activity towards androgens has been reported with SULT1E1 (Coughtrie 2002), 2A1 (Shimada et al. 2001), and 2B1a and 2B1b (Her et al. 1998). Unlike with UGTs, with SULTs each enzyme displays a unique tissue distribution, SULT1A1 being the main hepatic isoenzyme (Barker et al. 1994).

Table 2 Examples of human SULT isoenzymes, examples of their tissue distribution and of their reported androgen substrates

Isoform	Major sites of distribution	Examples of androgen substrates	References
SULT1A1	Adult liver	None	
SULT1A2	Not defined	None	
SULT1A3	GI tract	None	
SULT1B1	Adult liver and GI tract	None	
SULT1C2	Fetal kidney, lung and GI tract	None	
SULT1C4	Fetal kidney and lung	No substrate known	
SULT1E1	Fetal kidney, lung and GI tract, adult liver and endometrium	DHEA	Coughtrie (2002)
SULT2A1	Fetal and adult, adenal gland and liver	DHEA, epiandrosterone, androsterone, testosterone	Nowell et al. (2000) Aksoy et al. (1993)
SULT2B1a	Adrenal glands, liver and small intenstine	None to high extent	Lindsay et al. (2008)
SULT2B1b	Liver, GI tract, spleen, thymus, lung, prostate, ovary and adrenal gland	DHEA and other 3β-Hydroxysteroids	Coughtrie (2002)
SULT4A1	Brain	No substrate known	

The table is modified from Lindsay et al. (2008)

Fig. 3 A SULT-catalysed sulphonation between testosterone and 3′-phosphoadenosine 5′-phosphosulfate (PAPS)

1.3 Genetic Polymorphism

Inter-individual or inter-ethnic differences observed in metabolic pathways may be caused by genetic variation, e.g. polymorphism, which may alter the expression or the activity of a specific metabolic enzyme. For the UGT isoenzymes polymorphism has been reported at least for seven genes, namely *UGT1A1*, *1A6*, *1A7*, *2B4*, *2B7*, *2B15*, and *2B17* (Miners et al. 2002; Jakobsson et al. 2006). Due to the significant role of UGTs in the elimination of xenobiotics, genetic polymorphism of UGTs is of toxicological and physiological importance, e.g. in the occurrence of exceptionally slow or rapid metabolic behaviour of an individual, and in evaluation of therapeutic doses or drug interactions. For the SULT family there are also reports of the common single nucleotide polymorphism, which is associated with the variation in enzyme activity and thermal stability (Pachouri et al. 2006). Inter-ethnic variation has been reported especially for SULT1A1, although instead of androgen metabolism the polymorphism plays a role in the risk of lung cancer from cigarette smoking (Lindsay et al. 2008).

From the practical doping control point of view, the most significant issue of genetic polymorphism is connected to the steroid profiling and to the detection of administration of exogenous testosterone (T) in particular. The current trigger for further investigations is when the ratio of urinary glucuronide conjugated T to epitestosterone (E) exceeds the value $T/E = 4$. The ratio is dramatically affected if an individual is a homozygotic carrier of a common genetic deletion of *UGT2B17*, the frequency of which is much higher in Asian than, e.g., in Caucasian populations (Jakobsson et al. 2006, 2008). As the glucuronidation of T is reduced, the ratio may remain below the threshold even in the case of testosterone administration. On the other hand, there are also individuals showing "naturally elevated" T/E ratios, i.e. values above the threshold without an exogenous application of testosterone or its precursors. To exclude the effect of genotype on the test of the doping control result, there is a desire to shift the methodology towards subject-based testing, monitoring the status of an individual against his/her own steroid profile instead of the large population, as described in detail in Ayotte (2009).

2 Relevance of Phase-II Metabolism in Doping Control Analysis

The relevance of metabolic reactions in doping control originates from the desired targeted screening of the most prominent metabolites, which should take into account the potential inter-individual variation and the time of the administration. Phase-I metabolism of androgens plays a significant role in these structural modifications and strongly determines the applied analytical approaches, such as the selection of separation and ionisation methods. The development of instrumentation providing interfacing of liquid chromatographic (LC) separation to mass spectrometric (MS) detection, especially via electrospray ionisation (ESI), has opened up broad possibilities for the direct analysis of thermolabile, non-volatile, bulky and polar compounds, such as glucuronide-conjugated or sulfonated androgens (Bowers and Sanaullah 1996; Borts and Bowers 2000; Kuuranne et al. 2000; Hintikka et al. 2008). Although this combination could make the simultaneous detection of the total steroid fraction possible it is not totally free from interferences, such as matrix effects. Nowadays the routine screening procedure of endogenous and exogenous steroids is typically carried out in the hydrolysed urine sample and the analytical method is selected from the basis of chromatographic and mass spectrometric properties of individual analytes.

Phase-II metabolic routes, however, have a particular effect on selection of the appropriate sample preparation procedure, as the hydrolytic properties of glucuronide-conjugated and sulfonated androgens are significantly different. Information on the metabolic fate of an analyte is of great importance with issues, e.g. (1) decision of the general approach (chemical or enzymatic hydrolysis), (2) activity of the enzyme (β-glucuronidase only or with sulfatase activity) and (3) required hydrolysis conditions (amount of the enzyme, hydrolysis temperature, duration of the incubation and pH). Many sulfonated steroids are not efficiently hydrolysed in enzymatic processes, but application of more efficient methods, such as solvolysis, may lead to degradation of certain analytes (Gomes et al. 2009). As a consequence, no universal analysis method for steroid conjugates is available and the recent methods rely mainly on the enzymatic hydrolysis of target compounds (e.g. with *E. coli*) and focus thus on the analysis of glucuronide-conjugated fraction of steroids (Schänzer and Donike 1993). Intact phase-II metabolites, nevertheless, may be needed in the monitoring of the hydrolysis stage of sample cleanup.

3 *In Vitro* Production of Phase-II Metabolites

For method development and identification purposes of a new banned substance, relevant reference compounds are needed in doping control analysis. Traditionally the metabolic behaviour and the resulting target compounds of a new steroid have been examined in excretion studies in human subjects. This approach, however, requires a heavy administrative and medical workload with adequate ethical

approvals. Also other practical problems may be encountered with the isolation and purification of the metabolites, which represent the distribution of a single or few subjects. Severe problems are also faced with pipeline products or compounds from illicit producers, when there is no sales permission or legal pharmaceutical product available for administration. To ensure a fast response to analytical challenges, the development and set-up of new analytical methods or implementation of new compounds in present procedures should be prompt.

Animal models, e.g. monkey, are used to characterise pharmacokinetic and toxicological properties of androgens (Barbier et al. 2003), as well as tissue slices or crude enzyme preparations obtained from animal or human metabolic organs (Kuuranne et al. 2002, 2008a). The enzyme preparations can be used, e.g., in the small scale production (typically less than 1 mg amounts) of reference material by selecting the co-substrates, enzyme preparations, other additives and incubation conditions (Table 3) to mimic only phase-I or phase-II metabolic reactions separately, or to combine all the potential pathways in one assay (Kuuranne et al. 2002, 2008a,b). Sulphonation has been described as "high affinity–low capacity" phase-II reaction *in vivo* and the predominant reaction at low substrate concentration, whereas glucuronidation prevails at high substrate concentration (Lindsay et al. 2008). There are also differences in the set-up of *in vitro* assays, as the product inhibition restricts easily the activity of SULTs (Chapman et al. 2004). But in general terms, the *in vitro* metabolic assays of androgens are relatively straightforward to perform. As human liver enzymatic preparations are nowadays commercially available, easy and safe to handle and store, and the method does not require specific instrumentation or hardware, it may be easily applied to an operating doping control laboratory.

Recent sophisticated cloning and expression techniques of, e.g., recombinant human UGT isoenzymes (Kurkela et al. 2003) enable deeper characterization of the affinity, specificity and kinetic properties of a specific enzyme towards a certain substrate. For example, the members of the UGT2B subfamily are well known for their activity towards hydroxysteroids and they have been reported to exhibit both regio- and stereoselective glucuronidation of androgens (Jin et al. 1997; Sten et al. 2009). Gaining wider experience on the substrate specificity or overlap of enzyme

Table 3 Examples of the *in vitro* assay screening conditions for androgens

Component	Phase-I assay	Phase-II assay	
		Glucuronidation	Sulphonation
50 mM phosphate buffer + 5 mM $MgCl_2$	100 µl	100 µl	100 µl
Substrate	50 µM	50 µM	10 µM
D-Saccharic acid 1,4- lactone		5 mM	
NADPH	5 mM		
UDPGA		5 mM	
PAPS			20 µM
Enzyme, S9 fraction	0.5 mg ml^{-1}		0.5 mg ml^{-1}
Enzyme, microsomal fraction		0.5 mg ml^{-1}	

NADPH: nicotinamide adenine dinucleotide phosphate; PAPS: 3′-phosphoadenosine-5′-phosphosulfate; UDPGA: uridine-5′-diphosphoglucuronic acid

subfamilies provides information on the factors affecting the inter-individual variation, such as genetic polymorphism, and may thus also assist in interpretation of the results in doping control.

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Detecting the Administration of Endogenous Anabolic Androgenic Steroids

Christiane Ayotte

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Abstract The detection of the administration of an androgen such as testosterone that could be present normally in human bodily fluids is based upon the methodical evaluation of key parameters of the urinary profile of steroids, precisely measured

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by GC/MS. Over the years, the markers of utilization were identified, the reference ranges of diagnostic metabolites and ratios were established in volunteers and in populations of athletes, and their stability in individual subjects was studied. The direct confirmation comes from the measurement of δ^{13}C values reflecting their synthetic origin, ruling out a potential physiological anomaly. Several factors may alter the individual GC/MS steroid profile besides the administration of a testosterone-related steroid, the nonexhaustive list ranging from the microbial degradation of the specimen, the utilization of inhibitors of 5α-reductase or other anabolic steroids, masking agents such as probenecid, to inebriating alcohol drinking. The limitation of the testing strategy comes from the potentially elevated rate of false negatives, since only the values exceeding those of the reference populations are picked up by the GC/MS screening analyses performed by the laboratories on blind samples, excluding individual particularities and subtle doping. Since the ranges of normal values are often described from samples collected in Western countries, extrapolating data to all athletes appears inefficient. Furthermore, with short half-life and topical formulations, the alterations of the steroid profile are less pronounced and disappear rapidly. GC/C/IRMS analyses are too delicate and fastidious to be considered for screening routine samples. An approach based upon the individual athlete's steroid profiling is necessary to pick up variations that would trigger further IRMS analysis and investigations.

Keywords Testosterone • detection • steroid profiles • GC/MS • GC/C/IRMS

1 Introduction

1.1 Testosterone

For the past 20 years, the two anabolic androgenic steroids most frequently reported by laboratories in adverse findings are testosterone and 19-nortestosterone (IOC Medical Commission, personal communications 1983–1999; WADA 2008). Testosterone is available in a wide range of forms and dosages. In North America, for example, excluding the veterinary formulations, it is contained in gels (topical: *Androgel, Testim* 2.5 or 5 mg per packet), films (transdermal: *Androderm* 2.5 or 5 mg per 24 h) and suspensions (intra-muscular injections). Converted to 17β-esters, it is available for oral use (undecanoate – *Andriol* 40 mg), and dissolved in oil for intra-muscular injections (propionate – *Testex*, enanthate – *Delatestryl*, cypionate – *depo-testosterone*: 59 to 200 mg mL^{-1}). Testosterone is also offered in combination with estradiol (Health Canada 2008; FDA 2008). Elsewhere in the world, other forms and preparations can be found.

Testosterone is prohibited in sport. The first marker of its misuse, as proposed in the early 1980s, is a ratio of urinary glucuroconjugated testosterone to epitestosterone (*T*/*E* value) exceeding the range of values normally measured in human (Donike et al. 1983).

1.2 Historical Background

In the early 1980s, when the Medical Commission of the International Olympic Committee first introduced testosterone (17β-hydroxyandrost-4-en-3-one) on its list of banned substances, the sole measurement of a urinary *T*/*E* value above 6 was thought to reflect its administration (epitestosterone is not a metabolite of exogenous testosterone and, in some instances, its excretion is even diminished by negative feedback). In 1992, the definition of what constituted a positive finding was modified to take into account the natural conditions reported to have produced false positive results (Namba et al. 1988; Raynaud et al. 1992). A ratio higher than 6 was considered to reflect the administration of testosterone *unless there is evidence that this ratio is due to a physiological or pathological condition*. Further tests, such as comparison of the athlete's other test results or vaguely defined endocrinological investigations, were required for borderline, gray-zone values that could not take one readily out be readily attributed to doping (IOC Medical Commission, List of banned substances and methods of doping May 1992). Acceptable physiological and pathological conditions and endocrinological investigations were matters of dispute before the tribunals. In absence of clear guidelines, several often opposed interpretations coexisted, other tests, some extreme, were proposed requiring the virtual sequestration of the athlete and the administration of ketoconazole in order to differentiate the normally elevated value from the one due to doping (Kicman et al. 1993). The need for complementary markers was universally stressed.

Meanwhile, as other testosterone-related steroids were misused, more frequently so when hormonal supplements containing precursors could be purchased legally, and reports were made of the growing utilization of new formulations of testosterone delivering much lower dosages, it became apparent that looking only at high *T*/*E* values would not suffice. The concept of urinary steroid profiling was applied to this field of testing; the reference ranges and individual stability of other parameters were determined. Corroborative evidences are now collected through the routine measurement of the concentrations and relative ratios of testosterone, epitestosterone, final and inactive metabolites, androsterone (Andro: 5α-androstan-3α-ol-17-one), etiocholanolone (Etio: 3α-hydroxy-5β-androstan-17-one), androstanediols (5α-diol: 5α-androstan-3α,17β-diol and 5β-diol: 5β-androstan-3α, 17β-diol) which, as a minimum, constitute the steroid profile.

At the end of the 1990s, just when a top-level sprinter and his expert endocrinologist convinced his national federation that the increased urinary testosterone

concentration and *T*/*E* value of his reported positive sample were most certainly due to alleged physiological conditions such as the influence of permitted supplements on his adrenal system combined with “stress factors, sleep deprivation, prolonged sexual interaction and alcohol”, the utilization of the isotope ratio mass spectrometry (IRMS) became the essential complementary tool, offering a direct method for the detection of metabolites of synthetic origin. Currently, the World Anti-Doping Agency requests that all samples presenting abnormal steroid profiles such as *T*/*E* values above 4 or abnormal levels of metabolites be investigated, preferably by a further GC/C/IRMS of the sample (WADA 2008).

2 General Approach to the Measurement of Urinary Steroids

2.1 From Immunoassays to Mass Spectrometry

The radioimmunoassays employed in the 1970s for the detection of 19-norsteroids, 17-methylsteroids and testosterone (Brooks et al. 1975; Rogozkin et al. 1979; Chaĭkovskiĭ et al. 1983; Bílek et al. 1987) were definitely abandoned in the 1980s when low-cost, user-friendly, software-operated instruments combining high resolution gas chromatography and quadrupole filter mass spectrometry (GC/MS) became accessible, allowing the automated analysis of batches of samples with high sensitivity and specificity.

The metabolites of steroids are mostly excreted in the conjugated, principally glucuroconjugated, form and are amenable to GC/MS analysis following, however, rather extensive sample preparation. With relatively few modifications brought to the methods proposed in the early 1980s for urinary steroid analysis (Shackleton and Whitney 1980; Shackleton 1986; Vestergaard 1980), the steroids are most frequently isolated from the urine matrix by solid phase extraction, the glucuronides are hydrolysed enzymatically, and converted into pertrimethylsilylated derivatives (TMS-enols (ketone), TMS-ethers (hydroxyl)) (Donike 1980). Their GC/MS detection in the low ng mL^{-1} range is carried out by monitoring specific ions (SIM mode). Their GC/MS detection in the low ng/mL range is carried out by monitoring specific ions (SIM mode) of relevant metabolites, often molecular ions or fragments corresponding to the loss of a methyl group. General comprehensive screening methods allow the sensitive detection of the characteristic metabolites of prohibited anabolic androgenic steroids, the presence of which is solely due to doping, and the precise measurement of the steroid profile of endogenous steroids (Ayotte et al. 1996). While, recently, liquid chromatography and tandem mass spectrometry (LC/MS/MS) with electrospray ionization (ESI) has been proposed for the direct analysis of free and conjugated metabolites of purely exogenous steroids, steroid profiles are still being acquired by GC/MS. Confirmatory assays by LC/MS/MS or LC/MS-TOF of testosterone and epitestosterone conjugates or hydrazone derivatives have been suggested in order to permit their detection and identification when present at low levels (Borts and Bowers 2000; Saudan et al. 2006; Danaceau et al. 2008).

2.2 Sample Preparation for GC/MS Analyses

In order to allow comparison of steroid profiles often essential to detect anomalies related to doping, test results must be routinely obtained in a manner that ensures accuracy. Screening analyses are normally conducted on a single aliquot of the urine samples, grouped in batches also containing a quality control sample of known composition. Confirmatory analyses of a parameter exceeding the ranges of values normally measured in humans must provide a clear identification of the analyte, and its quantification in triplicate is required (WADA 2004). The assays include appropriate internal standards. Of crucial importance, the enzymatic hydrolysis of the steroid glucuronides can be carried out on an extract purified by solid phase extraction (SPE, most often on C18 cartridges) (Shackleton and Whitney 1980) but also directly on the urine. In the latter case, extra care must be taken to ensure complete deconjugation of key steroids that are not hydrolysed at the same rate and to exclude undesirable side-activities (Vestergaard 1978; Graef et al. 1977). Purified β-glucuronidase from *E. coli* was shown to maintain the integrity of the steroid profile while chemical hydrolysis or methanolysis were not found to be suitable for that application. Among other unsuitable preparations, some crude mixtures of *Helix pomatia* are known to possess unwanted enzymatic activities causing the alteration of the urinary steroids, for example the conversion of the 3β-hydroxy-5-ene steroids to 3-oxo-4-ene steroids by the action of 3β-HSDH:NAD oxidoreductase and 3-oxo-5-4-en-isomerase and the oxidation of 3-hydroxysteroids (Massé et al. 1989; Vanluchene et al. 1982; Messeri et al. 1984; Leyssens et al. 1998; Graef et al. 1975). Extensive reviews of factors altering the urinary steroid profiles are available (Geyer et al. 1996; Mareck et al. 2008).

2.3 Stability of Urinary Steroid Conjugates

Testosterone glucuronide is known to be stable in urine for at least 1 year when the specimen is stored below –20°C (Venturelli et al. 1995; Robards and Towers 1990), while one study reported the formation of free testosterone and free dihydrotestosterone in urine samples kept at 18 and 37°C (Kjeld et al. 1977). Testosterone and epitestosterone conjugates are stable for months in sterile urine kept at 4°C and –20°C (Jiménez et al. 2006), while the addition of a bacteriostatic agent was required to preserve their integrity in urine samples kept at 4°C and 37°C for a week (Saudan et al. 2006). Spiked in sterile urine, authentic standards of androsterone, etiocholanolone, testosterone, epitestosterone sulfates and glucuronides are stable even upon incubation at 37°C; dehydroepiandrosterone-3β-sulfate, like other sulfoconjugated steroids, is less stable showing partial deconjugation. Since samples are not collected under sterile conditions, skin bacteria and intestinal microflora (staphylococci, enterobacteria, *E. coli*, etc.) are often present. Delays in delivering the samples to the laboratory and improper refrigeration during

transportation and storage may offer good conditions for microbial growth; the effects on the urinary steroids are easily detectable and consist mainly in the hydrolysis of the steroid conjugates and in oxidoreductive reactions leading to the abnormal presence of steroids in the free form and the accumulation of 5α- and 5β-androstan-3,17-dione, the β-isomers being more rapidly altered. Signs of microbial degradation should obviously be absent to ensure the accurate measure of the steroid profile (Ayotte et al. 1996, 1997; Hemmersbach et al. 1997).

3 Testosterone and Epitestosterone

3.1 Reference Ranges

The concentrations of excreted metabolites can be compared once adjusted for a specific gravity of 1,020[1] (Donike et al. 1993). In the North American athletic population, the normalized concentration of testosterone and epitestosterone glucuroconjugates does not exceed 130 ng mL^{-1} in the vast majority of male samples, while levels lower than 30–40 ng mL^{-1}, often below the limits of quantification, are measured in females (Fig. 1); similar results were reported for Midland Europeans (Ayotte 2008; Donike 1993; Geyer et al. 1997; Lévesque and Ayotte 1999a, b and references cited herein). The *T*/*E* value is often given as the direct ratio of the always measurable peak area (*m*/*z* 432 for the pertrimethylsilylated derivatives) when the steroids cannot be reliably quantified considering the uncertainty of the measure (WADA 2004).

As shown from the analysis of the same principally North-American population, *T*/*E* values are most frequently close to 1 and the 99% cut-off values of both male and female populations are 4.6 and 4.3, respectively (Ayotte 2008)[2].

The *T*/*E* values follow a bimodal distribution that parallels the pattern of glucuroconjugated testosterone (Figs. 1 and 2). The presence of a minor population of ratios at values below 1 due to lower concentrations of testosterone glucuronide was reported; it was shown to be more representative of Asians (Ayotte 2008; Ayotte et al. 1996; Baenziger and Bowers 1994; Catlin et al. 1997; de la Torre et al. 1997). This observation was explained recently when that pattern of testosterone excretion was shown to strongly correlated with UGT2B17 (uridine diphosphoglucuronosyl transferase) deletion polymorphism, more frequently present in Asians (Jakobsson et al. 2006).

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[1] $\text{Concentration}(1.020) = \text{Concentration measured} \times \frac{1.020 - 1}{\text{Specific gravity (sample)} - 1}$

[2] It was already known in the 1960s that epitestosterone and testosterone were excreted in similar amounts (e.g. De Nicola et al. 1966).

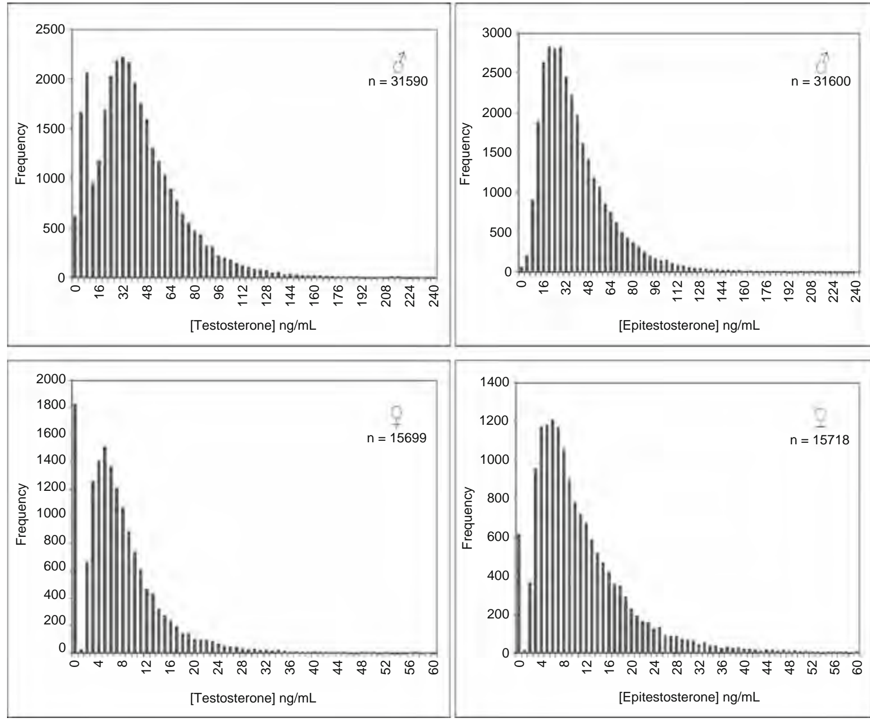

Fig. 1 Distribution of urinary testosterone (*left*) and epitestosterone (*right*) (enzymatically hydrolyzed glucuronide) in male athletes (*upper panels*) and female athletes (*lower panels*). Data obtained from routine sample analysis in Montréal Laboratory on a mostly North-American athletic population and volunteers (Ayotte 2008). Legends translated from French. Reproduced with permission of *Revue francophone des laboratoires*

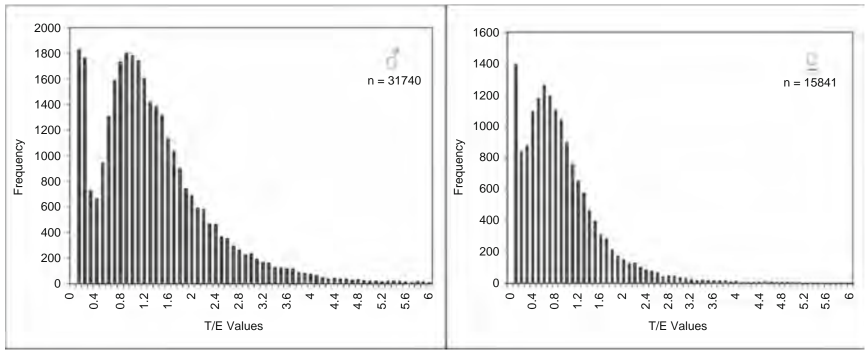

Fig. 2 Distribution of *T*/*E* values measured in male ($n = 31{,}740$, *left*) and female ($n = 15{,}841$, *right*) athlete samples (Ayotte 2008)

3.2 Stability of Individual Parameters

The comparison of the different parameters of the steroid profile measured from previous or subsequent test results, considering the population reference ranges and the expected individual stability, supports a doping violation when the value is found to be a statistical outlier to the athlete's norm. The application of steroid profiling or *longitudinal studies* to doping controls was proposed in the early 1990s (Donike 1993).

Several groups evaluated the stability of parameters of the steroid profile in volunteers and athletes. The ratios of 5α- to 5β-metabolites (e.g., androsterone to etiocholanolone) are the most stable parameters of the individual steroid profile (Geyer et al. 1997; Mareck-Engelke et al. 1993). With regards to the *T*/*E* values, in males, individual values were found to normally fluctuate by less than 30% (coefficient of variation, cv %) from the mean, whether located at 6 or 1. The *T*/*E* values of female volunteers and athletes show more variation: totally random fluctuations of 10–64% from the mean were recorded throughout the menstrual cycle of female volunteers, while the ratios of female athlete's samples can vary to the same extent when followed over years (Ayotte 2008; Mareck-Engelke et al. 1995, 1996a,b, 1997).

Obviously disrupted profiles of *T*/*E* values and concentrations of testosterone glucuronide, both increased to abnormal values, reflects the administration of testosterone, and for a decade, anti-doping scientists evaluated cases following the report of an elevated *T*/*E* value in a given sample (Ayotte 1997; Geyer et al. 1997; Garle et al. 1996; Baenziger and Bowers 1994; Catlin et al. 1997). Typical individual variation of *T*/*E* values in male athletes and in female athletes and volunteers are shown in Figs. 3–5 respectively; disrupted athletes' profiles supporting the misuse of testosterone-related steroids are also presented (Ayotte 2008).

The remarkable stability of some parameters of the individual urinary steroid profile has provided the first evidence for manipulation of the sample collection during doping controls. Samples supposed to belong to different athletes were tested and found to possess the same *T*/*E*, androsterone to etiocholanolone, androsterone to testosterone and 5α- to 5β-diol ratios, a highly improbable situation. Further DNA investigations revealed that the samples belonged to one person, but not to any of the three athletes concerned (Thevis et al. 2007).

4 Alteration of the Urinary Steroid Profile

4.1 Testosterone

The impacts on the more global urinary steroid profile were studied following the oral or intra-muscular administration of testosterone in single or repeated doses, and potential complementary parameters were evaluated (Dehennin and Masumoto 1993; Garle et al. 1996; Palonek and Garle 1993). In relation to the *T*/*E* values more specifically, it can be concluded that there are indeed modifications although not for all subjects in a way that would cause a threshold fixed at 6 or 4 to be exceeded, and not during the same period. Importantly, it was shown that with very

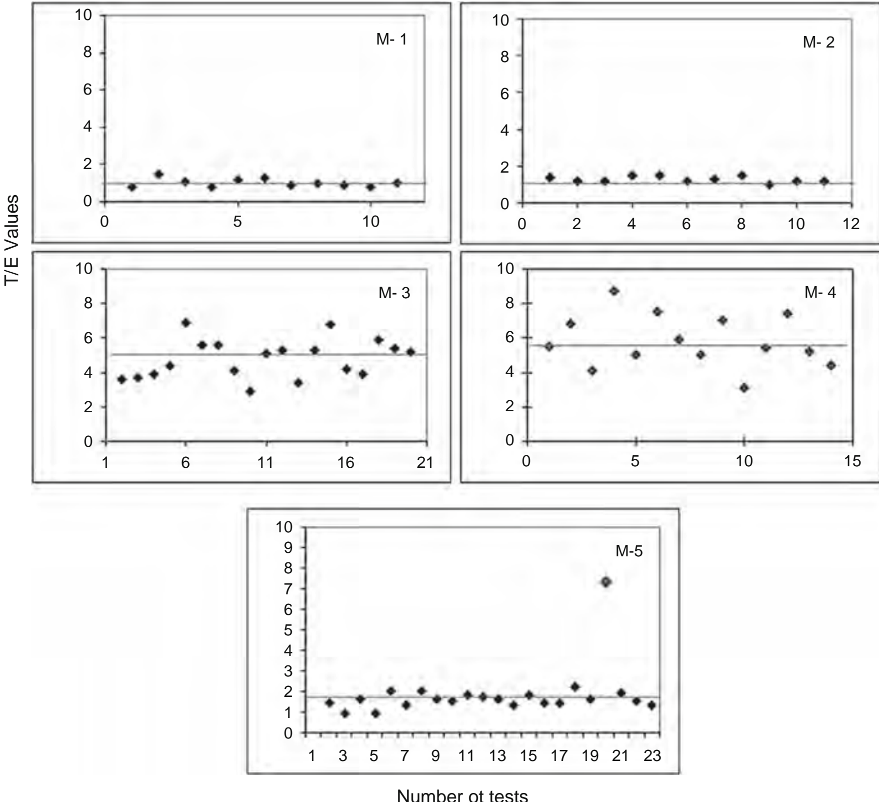

Fig. 3 Longitudinal studies of *T*/*E* values in male athletes' samples collected and analyzed over years: M-1 (mean: 1,03, coefficient of variation: 22%), M-2 (mean: 1,3, coefficient of variation: 13%), M-3 (mean: 4.8, coefficient of variation: 23%), M-4 (mean: 5,8, coefficient of variation: 26%), M-5 (mean of basal values: 1,5, coefficient of variation: 22%)

low basal *T*/*E* values, the increase following the i.m. administration of testosterone would probably not be picked up by the GC/MS screening assay, since only a few specimens had values exceeding the population-based threshold (Shackleton et al. 1997a,b). The expression of UGT2B17 deletion polymorphism, more frequent in certain populations, produces *T*/*E* values which are clearly lower than 1 and will remain within the population-based reference ranges, i.e. under 4, following the administration of the endogenous anabolic androgenic steroid (Jakobsson et al. 2006; Shackleton et al. 1997a,b).

The administration of oral preparations results in increased values that can be detected, however for less than 24 h even when taken daily for 21 days, while daily or intermittent usage of a testosterone-containing gel (100 mg per day) did not systematically alter the *T*/*E* values in a way that would be revealed by the GC/MS analysis as exceeding the population-based reference range (Wright et al. 1993; Geyer et al. 2007). For the latter, other ratios such as 5α-diol to epitestosterone were found to be informative. Although suggested as evidence of testosterone administration, the

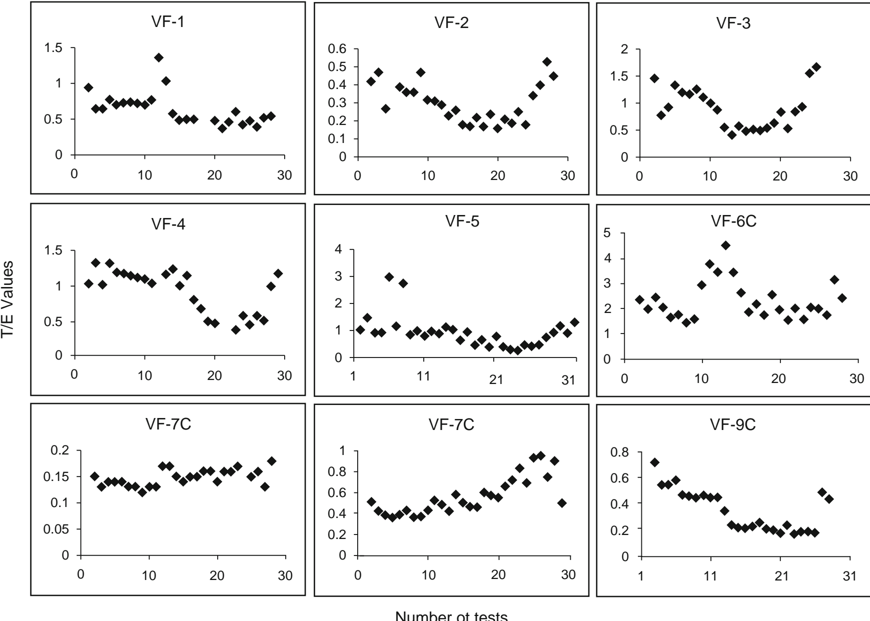

Fig. 4 Random variation of *T*/*E* values in nine healthy female volunteers over an entire month of the menstrual cycle (including volunteers utilizing birth control medications). Mean *T*/*E* values ranging from 0.2 to 3.4 with individual coefficients of variation of up to 60% (Ayotte 2008, reproduced with permission of Revue francophone des laboratoires)

suppression of epitestosterone and luteinizing hormone (LH) is not systematic and no other universal complementary urinary parameter could be picked up by the GC/MS analysis that would allow clear differentiation from normally elevated *T*/*E*s.

In the majority of cases though, when the values postadministration are compared to the subject's basal ones, the abnormal variation can be picked up, again suggesting the more sensitive individual profiling approach.

4.2 Dihydrotestosterone, Androstenedione and Dehydroepiandrosterone

It was reported that in the early 1980s East German scientists, informed that a test would soon be adopted to detect the use of testosterone, looked for alternatives such as dihydrotestosterone (DHT, 17β-hydroxy-5α-androstan-3-one), biosynthetic precursors androstenedione (androst-4-ene-3,17-dione) or dehydroepiandrosterone (DHEA, 3β-hydroxyandrost-5-en-17-one) and developed short-acting preparations and nasal sprays containing testosterone or androstenedione (Franke and Berendonk 1997). Fifteen years later, a first hormonal supplement, euphemistically described as dietary, DHEA, became commercially available in the USA for oral self-administration. It was

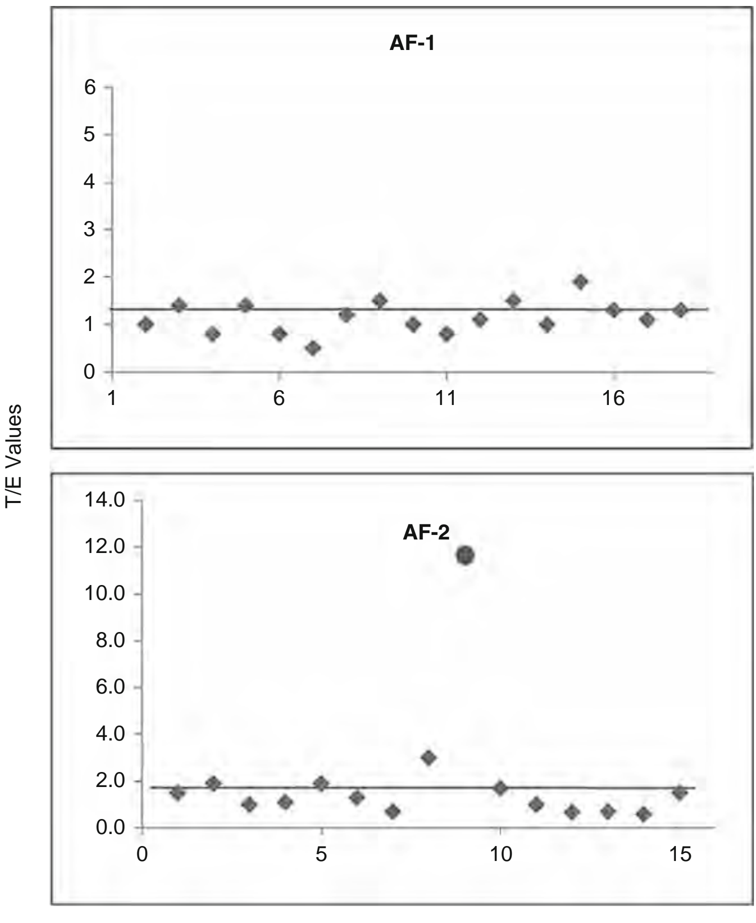

Fig. 5 Longitudinal studies of two female athletes *T/E* values collected over time: AF-1 showing normal *T/E* values (mean: 1.9) and variation (29%). AF-2 showing a characteristic disruption attributed to misuse of testosterone-related steroids (mean of negative values: 1.4; outlier value circled)

quickly followed by androstenedione. Since January 2005, the distribution of all pro-hormones and *designer* steroids has stopped at least officially, with the exception of DHEA that can still legally be purchased in some countries such as the USA.

The sole evaluation of the *T/E* values does not allow the detection of the administration of all testosterone-related steroids. Obviously, DHT, testosterone 5α-reduced metabolite, produces an increased excretion of the related 5α-metabolites; it does not alter the *T/E* values but enhances instead the ratio of 5α- to 5β-metabolites e.g., androsterone to etiocholanolone, and 5α-androstanediol to 5β-androstanediol. Several adverse analytical findings were simultaneously reported in the early 1990s and that has apparently stopped its use as a doping

agent (Donike et al. 1995). That episode, however, stressed the importance of monitoring parameters of the steroid profile other than the *T*/*E* value and the concentrations of testosterone and epitestosterone.

The oral administration of DHEA and androstenedione, biological precursors of testosterone, will not necessarily result in the increased excretion of testosterone or epitestosterone glucuronide. As a matter of fact, the ratios are not always altered and when they are, the effects are very short; strong inter-individual variations were observed (Ayotte et al. 2001; Bosy et al. 1998; Bowers 1999; Lévesque and Ayotte 1999a, b; Van Eenoo et al. 1998). With both steroids, the excretion of androsterone and etiocholanolone is drastically increased during the following few hours, however at different rates and varying with time, particularly when DHEA is involved. While clearly abnormal levels of DHEA glucuronide can be measured during a few hours, the presence of characteristic metabolites such as 6α-hydroxyandrostenedione, 6β-hydroxyepiandrosterone (sulfoconjuguated, persistent) and the abnormal ratio of the sulfates of 7β-hydroxydehydroepiandrosterone to 16α-hydroxyandrosterone could have a diagnostic value, at least during the first few days (Lévesque and Ayotte 1999b), while 3α,5-cyclo-5α-androstan-6β-ol-17-one seems detectable for slightly longer periods (Cawley et al. 2004).

In all those cases, when performed, the complementary IRMS analysis was found particularly useful and the δ^{13}C values of urinary metabolites reflected their exogenous origin even when their levels or the *T*/*E* values were within the population reference ranges.

5 Measurement of the δ^{13}C Values of Urinary Steroid Metabolites

5.1 *Principle of the GC/C/IRMS Analysis*

Many articles review the principle of GC/C/IRMS analysis of organic substances and its application in diversified fields (for example Brenna et al. 1997; Meier-Augenstein 1999).

Precise carbon isotope analysis measures minute difference in the isotopic abundance of ^{13}C; it is achieved by the utilization of the GC/C/IRMS instrument, a combination of gas chromatography (GC) separation/on-line combustion (C)/isotope ratio mass spectrometry (IRMS). The analytes separated and eluted from the gas chromatograph are converted to CO_2 by passing through a copper oxide combustion tube. The mass spectrometer is set for measuring simultaneously ions at *m*/*z* 44 ($^{12}C^{16}O_2$), 45 ($^{13}C^{16}O_2 = {}^{12}C^{17}O^{16}O$) and 46 ($^{12}C^{18}O^{16}O$); the latter ion is utilized to calculate and subtract the contribution of ^{17}O containing isotopomer to *m*/*z* 45. Water formed during the combustion process is removed by a cryogenic trap or a semi-permeable membrane of *Nafion* in order to prevent the formation of interfering HCO_2^+. Hence, the ratio of ions (*R*) at *m*/*z* 45–44 reflects the abundance of ^{13}C to ^{12}C of the analyte; it is not given as such but against an international reference and expressed in the standard δ-notation per mil (‰), according to the following well-known equation:

$$\delta^{13}C_{analyte} = (R_{analyte}/R_{reference}\text{-}1) \times 1000(‰).$$

The international reference was initially ^{13}C rich carbonate, *Pee Dee Belemnite* (PDB). Its ^{13}C at% is 1,1112 and $\delta^{13}C$ value is 0.00‰, consequently, all $\delta^{13}C$ values measured against that of PDB will be negative. PDB is now replaced by CO_2 calibrated against it (e.g. NBS 19).

5.2 IRMS for Determining the Origin of Organic Compounds

IRMS is the best way to determine the origin of organic substances, including the urinary metabolites of endogenous anabolic androgenic steroids, and many examples can be found in the literature.

CO_2 is fixed in plants by different pathways, namely the C_3 (3-phosphoglycerate or Calvin cycle), C_4 (oxaloacetate or Hatch–Slack) and CAM (Crassulean acid metabolism), and therefore since plants discriminate differently against ^{13}C, the carbon isotopic signature of their organic compounds will reflect their origin. Sugar beet, wheat, barley and rice are C_3-plants, while sugar cane, corn and millet are C_4-plants; the $\delta^{13}C$ values of their biomolecules are typical and range respectively from -22 to -35 and from -10 to -18. Hence, depending on its origin, the $\delta^{13}C$ value of an organic compound can vary by more than $10^0/_{00}$. Plant sterols extracted from soy, diosgenin, a steroid saponin from wild yam (*Discorea*) or derived precursor sterols serve as starting material for the synthesis of commercial steroids and pro-hormones such as androstenedione and DHEA that will possess the carbon isotopic signature reflecting their single vegetal origin characteristic of C_3-plants (Ueki et al. 2002).

Different $\delta^{13}C$ values were measured in exhaled CO_2 from people living in Europe and USA, reflecting the C_4-plant rich diet of the latter, but, in spite of the different diets, human urinary steroids originating from normal metabolic processes in general show carbon isotopic profiles differing from those originating from commercial synthetic steroids.

5.3 $\delta^{13}C$ Values of Urinary Steroid Metabolites

A new approach to the detection of the misuse of testosterone in sport was proposed in 1990 (Southan et al. 1990) and by the end of that decade, several groups confirmed the potential of the GC/C/IRMS analysis of selected urinary metabolites to detect their exogenous origin and, therefore, prove with direct evidence the administration of testosterone or testosterone-related steroids (Aguilera et al. 1996; Becchi et al. 1994; Shackleton et al. 1997a, b; Horning et al. 1997). The diagnostic metabolites selected then were most frequently the pair 5α- / 5β-diols and androsterone etiocholanolone isolated from the urine following deconjugation along with the internal reference steroids, cholesterol or pregnanediol. Steroids were most often analyzed as acetylated derivatives. Endogenous $\delta^{13}C$ values were described as well as those measured in samples

collected further to the administration of potentially endogenous steroids (testosterone, DHEA and epitestosterone) and often differences of more than 3–6‰ were found (Aguilera et al. 1999, 2002; Horning et al. 1997; Shackleton et al. 1997a, b). It was first proposed to consider absolute δ^{13}C values of acetatylated androstanediols lower than −29.0‰ as a proof of their synthetic origin and to utilize a threshold of 1.1 for the ratio of the δ^{13}C values of the two metabolites to the reference steroid (Shackleton et al. 1997a, b).

Nowadays, the GC/C/IRMS analyses are routinely integrated into the detection and confirmation of the administration of endogenous anabolic androgenic steroids as well as epitestosterone that could be utilized as a masking agent of the *T*/*E* value. Technically, careful and extensive sample preparation is required and must be shown to exclude isotopic fractionation in order to obtain valid results, while the resolution and sensitivity of the instrument are not comparable to contemporary GC/MS. The peaks corresponding to the analytes, metabolites or reference steroids must be resolved, of good shape and free from interference. Typical GC/C/IRMS chromatograms of the principal analytes are shown in Fig. 6 (Ayotte, unpublished results).

To compensate for individual and analytical variations, in every sample the difference between the values of each diagnostic metabolite and other unaffected urinary steroids chosen as internal reference is determined and compared to corresponding values in untreated subjects (reference ranges). The difference will not be the same for each analyte and must be greater than the normal variation (biological fractionation) to support the exogenous origin of the metabolite. The difference of δ^{13}C values to reference of androsterone and etiocholanolone, although easier to analyze, may however be less pronounced with small doses, both being final metabolites of several androgens and therefore considered to be weak markers of administration when compared to the primary metabolites, i.e. testosterone itself, 5α-androstan-3α,17β-diol, 5β-androstan-3α,17β-diol, and DHEA, which are definitely best markers. The endogenous reference steroids most frequently chosen are pregnanediol (pgdiol), 16(5α)-androsten-3α-ol, 11-ketoetiocholanolone, and 11-hydroxyandrosterone (Geyer et al. 2007; Maître et al. 2004; Saudan et al. 2004).

6 External Factors Influencing the GC/MS Steroid Profile

6.1 Anabolic Agents and Inhibitors of Reductase

The administration of other synthetic anabolic androgenic steroids can alter the excretion of the metabolites measured in a normal urinary steroid profile, although none are produced directly by the exogenous compound (Donike et al. 1990). The discovery of a designer steroid in an athlete's sample originated from the recording of abnormally low levels of endogenous steroid metabolites during routine screening tests. Further investigations revealed the presence of 13-ethyl-17-hydroxy-18,19-dinor-17α-pregn-4-en-3-one, norbolethone, not commercially available, therefore pointing to a secret source (Catlin et al. 2002).

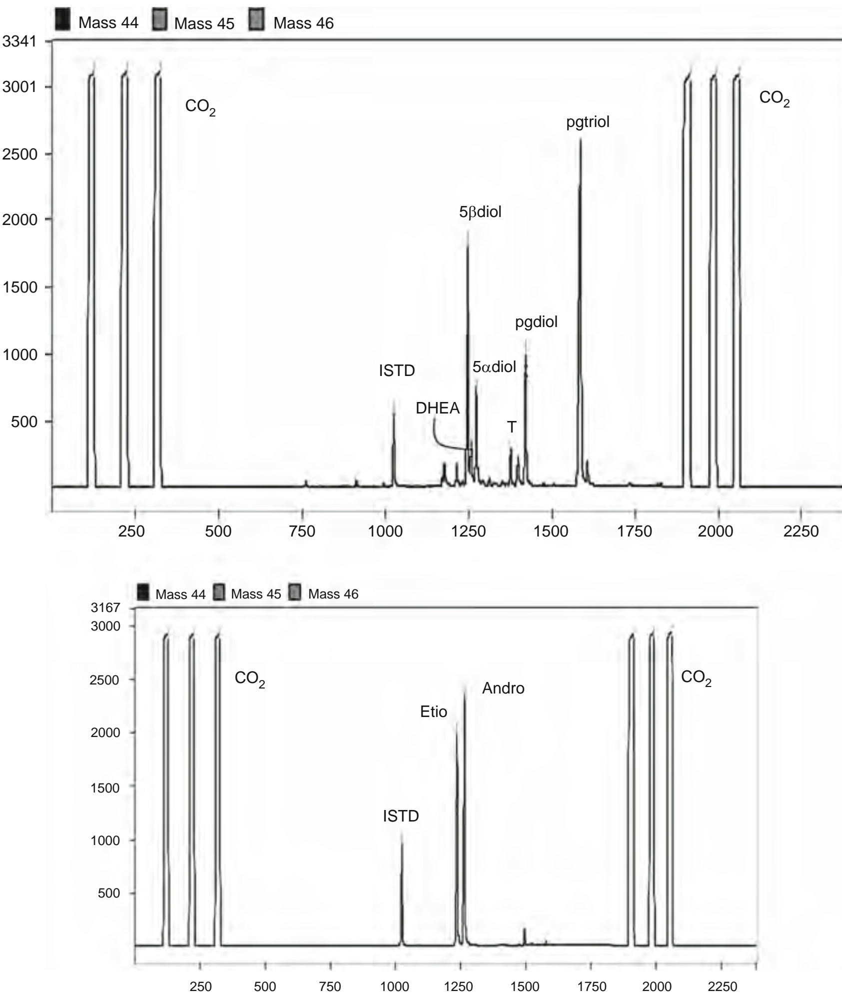

Fig. 6 Chromatograms of GC/C/IRMS analyses of isolated and HPLC purified urinary metabolites and reference steroids (hydrolyzed glucuronides, underivatized)

Finasteride, an inhibitor of 5α-reductase has a marked effect on the steroid profile, altering the ratios of 5α- to 5β-metabolites (the ratio of androsterone to etiocholanolone ranges typically from 1 to 0.2) (Thevis et al. 2007).

6.2 Ethanol

The determination of the origin of urinary metabolites is essential in the most complex although very rare cases where the excretion of testosterone and *T*/*E*

Table 1 Summary of GC/MS, GC/C/IRMS and ethanol measurement results in four cases where findings were attributed to alcohol drinking (Ayotte 2008, unpublished results)

Gender	*T*/*E*	[T] (ng mL^{-1})	Andro/T	Ethanol (mmol L^{-1})	IRMS results (origin[a])
F	8.8[b]	72	6.4	7	Endogenous
M	12[c]	52	62.3	7	Endogenous
M	5	850	5.3	29	Endogenous
M	3.1	95	8.1	14	Endogenous

[a]Deduced origin of the urinary metabolites, androsterone, etiocholanolone, 5α-diol, 5β-diol and testosterone based upon their δ^{13}C values which did not differ from the endogenous reference compounds and were found to be normal
[b]Athlete's norm: 1.3 ($n = 2$)
[c]Athlete's norm: 5.8 ($n = 4$)

values is drastically increased to clearly abnormal levels by the ingestion of large, inebriating quantities of alcohol (Karila et al. 1996; Falk et al. 1988; Mareck-Engelke et al. 1995). In several cases involving male and female athletes investigated recently, the parameters of the steroid profiles were clearly consistent with the administration of testosterone with abnormally high levels of testosterone and 5β-androstanediol glucuronides, resulting in high *T*/*E* and low androsterone to testosterone values. The δ^{13}C values of all the metabolites reflected conclusively their endogenous origin and the presence of ethanol in all those specimens confirmed the "physiological condition." Typical results produced by heavy alcohol drinking are summarized in Table 1.

6.3 Supplements

Several supplements are advertised as having the potential to increase the natural secretion of testosterone but those claims remain mostly unsupported. For example, after weeks of treatment, *Tribulus terrestris* had no influence on serum testosterone, androstenedione and LH and on the *T*/*E* values (Neychev and Mitev 2005; Rogerson et al. 2007).

7 From Population-Based to Subject-Based Testing Approach

The accurate quantification of all the parameters of the steroid profile is essential to the detection of testosterone-related anabolic androgenic steroids. On the other hand, the extensive sample purification of a relatively high volume of urine required for analytes present at low levels, and the high technical skills necessary to obtain accurate results from the GC/C/IRMS analysis has limited its successful implementation in all laboratories. Nonetheless, the limits of a method strictly based upon comparison with population-based reference ranges are manifest. With this

approach, only the samples in which the parameters of the steroid profile exceed the upper limit of the statistical distribution of values measured in the reference population are further investigated by a GC/C/IRMS analysis or a comparison to the athlete's other test results. Considering that *T*/*E* values slightly greater than 4 are present normally in 1–2% of male athletes' samples, precious resources are wasted by preparing and analyzing systematically those samples with GC/C/IRMS while a quick review of previous test results would provide a clear answer. Worse, the samples in which the doping-increased values do not exceed those measured in the reference population are simply missed.

More efficient detection of the misuse of testosterone-related steroids requires the shift to a subject-based approach (Sottas et al. 2007, 2008). Only the comparison of individual values would permit more systematic spotting of the effects of a testosterone-related steroid. The identity of the athlete is blind to the laboratory and, consequently, only the testing authorities can verify the individual stability of the steroid profile. With the proper statistical tools and a rapid exchange of information, the IRMS analysis could be done on a sample which upon first screening had not revealed values exceeding the population-based threshold. The World Anti-Doping Agency has taken steps to implement such subject-based testing by developing an "athlete's passport" where blood parameters and urinary steroid profiles will be kept and evaluated.

Endogenous "natural" anabolic androgenic steroids are misused by athletes and sophisticated testing methods are utilized for their detection, in vain however outside a subject-based approach.

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Synthetic Anabolic Agents: Steroids and Nonsteroidal Selective Androgen Receptor Modulators

Mario Thevis and Wilhelm Schänzer

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Abstract The central role of testosterone in the development of male characteristics, as well as its beneficial effects on physical performance and muscle growth, has led to the search for synthetic alternatives with improved pharmacological profiles. Hundreds of steroidal analogs have been prepared with a superior oral bioavailability, which should also possess reduced undesirable effects. However, only a few entered the pharmaceutical market due to severe toxicological incidences that were mainly attributed to the lack of tissue selectivity. Prominent representatives of anabolic-androgenic steroids (AAS) are for instance methyltestosterone, metandienone and stanozolol, which are discussed as model compounds with regard to general pharmacological aspects of synthetic AAS. Recently, nonsteroidal alternatives to AAS have been developed that selectively activate the

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androgen receptor in either muscle tissue or bones. These so-called selective androgen receptor modulators (SARMs) are currently undergoing late clinical trials (IIb) and will be prohibited by the World Anti-Doping Agency from January 2008. Their entirely synthetic structures are barely related to steroids, but particular functional groups allow for the tissue-selective activation or inhibition of androgen receptors and, thus, the stimulation of muscle growth without the risk of severe undesirable effects commonly observed in steroid replacement therapies. Hence, these compounds possess a high potential for misuse in sports and will be the subject of future doping control assays.

Keywords Doping • Mass spectrometry • Sport • SARMs • Steroids

1 Introduction

Traditional endocrinology commonly aims to correct hormone deficiencies by replacing the missing hormone. Modern chemistry, biochemistry and biotechnology have enabled the synthesis of many hormones with structures identical to their respective natural analogs. Those drugs have been employed for the treatment of numerous diseases, e.g. recombinant human insulin, recombinant human erythropoietin, or synthetic testosterone for diabetes mellitus, anemia, or hypogonadism, respectively. However, the substitution of a deficient hormone by its synthetic counterpart may not always be the best option, for various reasons such as disadvantageous pharmacokinetic profile, reduced bioavailability, or redundant biological actions. Hence, after testosterone (Scheme 1, **1**) was identified as the

Scheme 1 Chemical structures of testosterone (**1**, mol wt = 288), methyltestosterone (**3**, mol wt = 302), metandienone (**2**, mol wt = 300) and stanozolol (**4**, mol wt = 328)

active principle responsible for the development of male characteristics (Ruzicka and Wettstein 1935), the search for more potent and tissue-selective analogs was started, which yielded thousands of steroidal compounds within the last 80 years. Comprehensive studies conducted in particular in the 1950s and 1960s (Maisel 1965) were primarily based on the so-called *levator ani*-test (Eisenberg and Gordan 1950) (later referred to as the Hershberger assay (Hershberger et al. 1953)) that provided detailed information about the androgenic and anabolic properties of synthesized drug candidates. Major goals were for instance the treatment and management of debilitating diseases such as AIDS, osteoporosis, and hypogonadism, and fertility control (Kerr and Congeni 2007). The reasons for looking for drugs other than testosterone itself were its comparably low oral bioavailability (caused by a severe first-pass metabolism) as well as its metabolic conversion into 5α-dihydrotestosterone (DHT), which represents a bioactive steroid that significantly amplifies the androgenic activity of testosterone. Thus, synthetic analogs were desired that are (a) orally active, and (b) no substrate for human 5α-reductases and aromatase (Mohler et al. 2005). While the majority of the synthesized products had steroidal nuclei, recent developments focused on nonsteroidal and tissue-selective drugs, which were termed selective androgen receptor modulators (SARMs). These drug candidates possess the ability to activate the androgen receptor but have shown considerably reduced incidences of undesirable effects in proof-of-concept studies. In the following, pharmacological aspects of and detection strategies for selected AAS as well as SARMs are presented and discussed.

2 Pharmacological Aspects and Mode of Action of AAS

Shortly after the discovery and characterization of testosterone, its limited oral bioavailability became evident and alternatives for therapeutic purposes were the subject of numerous studies. Several options were considered such as different parenteral applications (intramuscular injection, intravenous or transdermal administration, but also buccal or sublingual routes) and chemical derivatization (Gooren and Bunck 2004).

One of the first synthetic analogs to testosterone prepared by the Noble laureate Ruzicka was 17α-methyltestosterone (Ruzicka et al. 1935). The alkylation of testosterone at C-17 decelerated the metabolic degradation and yielded a comparably stable drug (Scheme 1, **2**), the conversion and excretion of which was studied in detail by several groups (Emmens and Parkes 1939; Quincey and Gray 1967; Sandberg and Slaunwhite 1956; Schänzer et al. 1992). The presence of the 17α-methyl residue inhibited the oxidation of steroids to 17-keto analogs and, thus, enhanced their bioavailability. Further reduction of the metabolic clearance rate was accomplished by modifications of the steroidal A-ring structure. The introduction of a double bond between C-1 and C-2 yielded metandienone (Scheme 1, **3**) (Meystre et al. 1956; Vischer et al. 1955), and the condensation of the A-ring to a pyrazole structure gave rise to stanozolol (Scheme 1, **4**) (Clinton

et al. 1961). Both derivatives demonstrated a markedly increased plasma half-life and oral bioavailability (Arnold et al. 1963; Beyler et al. 1961) due to significantly slowed rates of metabolic transformation (Kochakian 1976; Schänzer 1996). An additional major goal of synthetic AAS was the separation of androgenic and anabolic activities of new steroids as commonly evaluated by the Hershberger assay. Myotrophic activity was assessed by the increase of weight of the *levator ani* muscle of young castrated male rats or by means of nitrogen balance studies (Arnold et al. 1963; Kochakian 1950). The index for androgenicity was the growth of the ventral prostate gland. Numerous studies were conducted and comprehensively summarized (Potts et al. 1976), which demonstrated the advantageous properties of methyltestosterone (MT) (Dorfman and Kincl 1963), metandienone (MD) (Ercoli et al. 1962) and stanozolol (ST) (Potts et al. 1960) as compared to orally administered testosterone with regard to oral bioavailability and myotrophic activity.

2.1 Mode of Action of AAS

The effect of AAS on target tissues is mediated mainly through identical processes that are responsible also for the efficacy of testosterone. These are discussed in detail by Kicman (2009).

2.2 Metabolism of AAS

Principal metabolic reactions of synthetic anabolic steroids are closely related to those reported for testosterone. Major pathways are based on enzymes such as cytochrome P450, 17β-hydroxysteroid dehydrogenase, 3α-/3β-hydroxysteroid dehydrogenase and 5α-reductases, which are primarily responsible for the conversion of testosterone into active or inactive metabolic products (Rommerts 2004). These systems as well as additional metabolizing enzymes also affect most synthetic AAS and yield typical metabolic products that are commonly used as target analytes in doping controls as presented in Table 1 (Schänzer 1996). Using three representatives MT, MD and ST, typical and characteristic phase-I metabolites are presented in the following, and a generalized overview of most common metabolic pathways involving the A/B-ring of 3-oxo-steroids and the D-ring of 17-hydroxysteroids is depicted in Scheme 2. The reduction of the 3-oxo-residue and the adjacent double bond between C-4 and C-5 of the A-ring commonly yields three out of four possible combinations as depicted in Scheme 2a. In contrast, 3-oxo-1,4-diene steroids are preferably transferred to 3α,5β-oriented metabolites (Scheme 2b). The D-ring of 17-hydroxylated steroids is frequently subjected to oxidation and reduction reactions yielding 17-oxo-steroids if no 17-alkylation is

Table 1 Main target substances in human urine for the detection of anabolic steroid misuse

Anabolic steroid	Main screened substance(s)
1-Androstenedione	1-Androstenedione
	3α-Hydroxy-5α-androstan-17-one
	3α-Hydoxy-5α-androst-1-en-17-one
	17β-Hydroxy-5α-androst-1-en-3-one
	5α-Androst-1-ene-3α,17β-diol
1-Androstenediol	See 1-Androstendione
Bolasterone	7α,17α-Dimethyl-5β-androstane-3α,17β-diol
Boldenone	Boldenone
	5β-Androst-1-en-17β-ol-3-one
	5β-Androst-1-en-3α-ol-17-one
Boldione	See Boldenone
Calusterone	7β,17α-Dimethyl-5β-androstane-3α,17β-diol
	17β-Hydroxy-7β,17α-dimethyl-androst-4-en-3-one
	7β,17α-Dimethyl-5α-androstane-3α,17β-diol
Clostebol	4-Chloro-androst-4-en-3α-ol-17-one
	4-Chloro-5α-androstan-3α-ol-17-one
	4-Chloro-5β-androstan-3α-ol-17-one
	4-Chloro-androst-4-ene-3α,17β-diol
Danazol	2α-Hydroxymethylethisterone
Dehydrochlormethyltestosterone	6β-Hydroxy-4-chloro-1,2-dehydro-17α-methyltestosterone
	4ξ-Chloro-3α,6β,17β-trishydroxy-17α-methyl-5β-androst-1-en-16-one
Dehydro-17-methylclostebol	4-Chloro-17α-methyl-androst-4-ene-3ξ,17β-diol
Desoxymethyltestosterone	Desoxymethyltestosterone (17α-methyl-5α-androst-2-en-17β-ol)
Drostanolone	2α-methyl-5α-androstan-3α-ol-17-one
Ethylestrenol	17α-Ethyl-5β-estrane-3α,17β-diol
Fluoxymesterone	9α-Fluoro-18-nor-17,17-dimethyl-androst-4,13-dien-11β-ol-3-one
	9α-Fluoro-17α-methyl-androst-4-ene-3α,6β,11β,17β-tetra-ol
Formebolone	2-Hydroxymethyl-17α-methyl-androsta-1,4-diene-11α,17β-diol-3-one
Furazabol	16β-Hydroxyfurazabol
4-Hydroxytestosterone	4-Hydroxy-androst-4-ene-3,17-dione
Mestanolone	17α-Methyl-5α-androstane-3α,17β-diol
	17β -Methyl-5α-androstane-3α,17α-diol
	18-Nor-17,17-dimethyl-5α-androst-13-en-3α-ol
Mesterolone	1α-Methyl-5α-androstan-3α-ol-17-one
Metandienone	17-Epimetandienone
	6β-Hydroxymetandienone
	17α-Methyl-5β-androstane-3α,17β-diol
	17β-Methyl-5β-androst-1-ene-3α,17α-diol
	18-Nor-17,17-dimethyl-5β-androsta-1,13-dien-3α-ol
	18-Nor-17β-hydroxymethyl,17α-methyl-androst-1,4,13-trien-3-one
Metenolone	1-Methylene-5α-androstan-3α-ol-17-one
Methandriol	17α-Methyl-5β-androstane-3α,17β-diol
Methasterone	Methasterone (2α,17α-dimethyldihydrotestosterone)
	2α,17α-dimethyl-5α-androstane-3α,17β-diol
6α-Methyl-androstendione	3α-hydroxy-6α-methyl-5β-androstan-17-one
Methyldienolone	Methyldienolone

(*continued*)

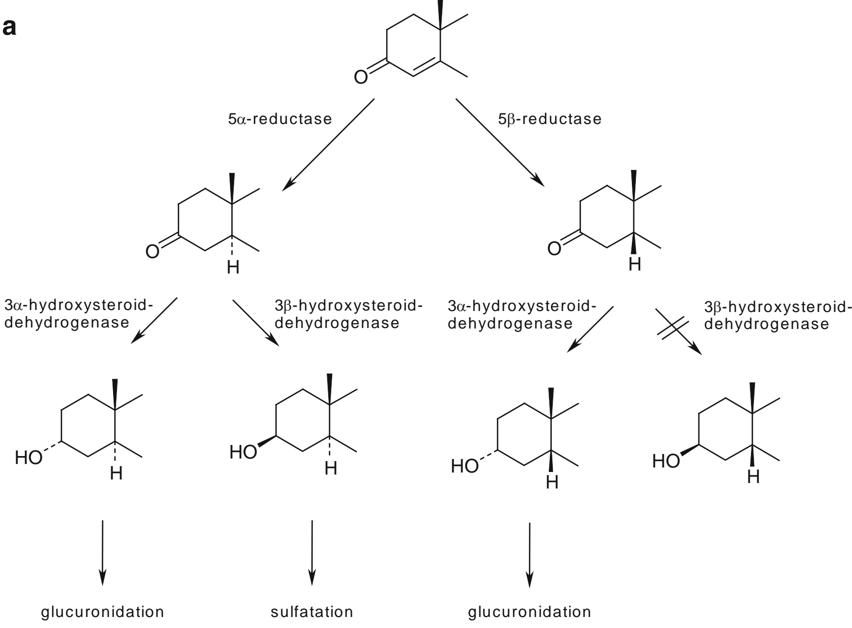
Table 1 (continued)

Anabolic steroid	Main screened substance(s)
17-Methylnortestosterone	17α-Methyl-5β-estrane-3α,17β-diol
	17α-Methyl-5α-estrane-3α,17β-diol
18-Methyl-19-nortestosterone	13β-ethyl-3α-hydroxy-5α-gonan-17-one
	13β-ethyl-3α-hydroxy-5β-gonan-17-one
Methyltestosterone	17α-Methyl-5α-androstane-3α,17β-diol
	17α-Methyl-5β-androstane-3α,17β-diol
Methyl-1-testosterone	Methyl-1-testosterone
	17α-Methyl-5α-androst-1-en-3α,17β-diol
	17β-Methyl-5α-androst-1-en-3α,17α-diol
	17α-Methyl-5α-androstane-3α,17β-diol
Methyltrienolone	Methyltrienolone
Mibolerone	7α,17α-Dimethyl-5β-estrane-3α,17β-diol
Nandrolone	5α-Estran-3α-ol-17-one
	5β-Estran-3α-ol-17-one
19-Norandrostenediol	see Nandrolone
19-Norandrostenedione	see Nandrolone
Norbolethone	13β,17α-Diethyl-3α,17β-dihydroxy-5β-gonane
Norclostebol	4-Chloro-4-estren-3α,17β-diol
Norethandrolone	17α-Ethyl-5β-estrane-3α,17β-diol
Oxabolone	4-Hydroxynorandrostendione
Oxandrolone	17-Epioxandrolone
Oxymesterone	Oxymesterone
Oxymetholone	17α-Methyl-5α-androstane-3α,17β-diol
Prostanozol	Prostanozol
	17-Oxo-5α-androstano[3,2-c]pyrazole
Quinbolone	see Boldenone
Stanozolol	3′-Hydroxystanozolol
	3′-Hydroxy-17-epistanozolol
	16β-Hydroxystanozolol
	4β-Hydroxystanozolol
Stenbolone	3α-Hydroxy-2-methyl-5α-androst-1-ene-17-one
1-Testosterone	See 1-Androstendione
Tetrahydrogestrinone	Tetrahydrogestrione
Trenbolone	Trenbolone
	17-Epitrenbolone

present. In addition, hydroxylation at C-16 is observed that results also in 16-oxo-metabolites (Scheme 2c).

2.2.1 Methyltestosterone and Metandienone

MT was one of the first synthetic analogs to testosterone with oral bioavailability. Studies on its metabolism yielded a few major products (Scheme 3a) described as 17α-methyl-5α-androstane-3α,17β-diol (**5**) and 17α-methyl-5β-androstane-3α, 17β-diol (**6**) (Rongone and Segaloff 1962; Segaloff et al. 1965) (the counterparts to androsterone and etiocholanolone as derived from testosterone). Moreover,



Scheme 2 Common metabolic pathways of 3-oxo-steroids with (**a**) 4-ene structure and (**b**) 1,4-diene nucleus

corresponding analogs resulting from 17-epimerization (Scheme 2a, **7** and **8**) were determined (Schänzer et al. 1992), which usually represent less than 5% of the total amount of renally excreted metabolic products of MT. All of these phase-I metabolites (**5–8**) were conjugated to glucuronic acid yielding respective phase-II metabolites before urinary excretion.

Despite closely related structures, MD provided several metabolites which were found in addition to the only common one 17α-methyl-5β-androstane-3α,17β-diol

Scheme 2 (continued) In addition, typical metabolic reactions occurring at the D-ring of 17-hydroxysteroids are illustrated (**c**)

Scheme 3 Major unconjugated metabolites of selected AAS: (**a**) methyltestosterone (**2**) yields 17α-methyl-5α-androstane-3α,17β-diol (**5**, mol wt = 306), 17α-methyl-5β-androstane-3α,17β-diol (**6**, mol wt = 306), 17β-methyl-5α-androstane-3α,17α-diol (**7**, mol wt = 306) and 17β-methyl-5β-androstane-3α,17α-diol (**8**, mol wt = 306); (**b**) metandienone (**3**) yields 6β-hydroxymetandienone (**9**, mol wt = 316), 18-nor-17,17-dimethylandrosta-1,4,13-trien-3-one (**10**, mol wt = 282), 17-epimetandienone (**11**, mol wt = 300), 17β-hydroxy-17α-methyl-5β-androst-1-en-3-one (**12**, mol wt = 302), 17β-methyl-5β-androst-1-en-3α,17α-diol (**13**, mol wt = 304), 18-nor-17,17-dimethyl-5β-androsta-1,13-dien-3α-ol (**14**, mol wt = 284), 17α-methyl-5β-androst-1-en-3α,17β-diol (**15**, mol wt = 304), 18-nor-17β-hydroxymethyl-17α-methyl-5β-androsta-1,13-dien-3-one (**16**, mol wt = 298), and 17α-methyl-5β-androstane-3α,17β-diol (**6**, mol wt = 306); (**c**) stanozolol (**4**) yields 3′-hydroxystanozolol (**17**, mol wt = 344), 3’-hydroxy-17-epistanozolol (**18**, mol wt = 344),16β-hydroxystanozolol (**19**, mol wt = 344), and 4β-hydroxystanozolol (**20**, mol wt = 344)

Scheme 3 (continued)

(**6**) as outlined in Scheme 3b. MD was further converted to its 6-hydroxylated analog (**9**) (Rongone and Segaloff 1963), 17-epimerized (**11**) (Macdonald et al. 1971; Schänzer et al. 1992), and the combined reduction, epimerization and/or elimination of water yielded the most common target analytes of MD in doping controls referred to as epimetendiol (17β-methyl-5β-androst-1-en-3α,17α-diol, **13**) and 18-nor-epimetendiol (18-nor-17,17-dimethyl-5β-androsta-1,13-dien-3α-ol, **14**) (Masse et al. 1991; Schänzer et al. 1991). More recently, a new long-term

metabolite named 18-nor-17β-hydroxymethyl,17α-methyl-5β-androsta-1,13-dien-3-one (**16**) was detected (Schänzer et al. 2006), which is observed up to 21 days after single administration of MD. Compounds **9** and **11** were found unconjugated in administration studies; however, the existence of a labile conjugate of **9** to an unknown ligand was reported (Dürbeck and Büker 1980; Schänzer 1996), and 17-epimerization (**11**) was demonstrated to result from 17β-sulfates rearranging to 17-epimeric products from 17α-methyl AAS (Schänzer et al. 1992). All other phase-I metabolic products referred to in Scheme 3b were determined as conjugates of glucuronic acid.

2.2.2 Stanozolol

ST differs in its metabolic reactions considerably from MT and MD due to its pronounced structural disparity resulting from the pyrazol residue condensed to the A-ring of the steroidal core (Scheme 1, **4**). Major phase-I metabolites observed and identified in human urine were 3′-hydroxystanozolol (**17**), 3′-hydroxy-17-epistanozolol (**18**), 16β-hydroxystanozolol (**19**), and 4β-hydroxystanozolol (**20**) (Masse et al. 1989; Schänzer et al. 1996a; Schänzer et al. 1990) as illustrated in Scheme 3c. In addition, seven further metabolic products were detected and characterized, which are not considered in this chapter due to their comparatively low abundance in urine specimens. The metabolites **17**, **19** and **20** were conjugated to glucuronic acid before renal elimination, while metabolite **18** was excreted without conjugation to its corresponding glucuronide or sulfate.

2.3 Undesirable Effects of AAS

AAS possess a series of undesirable effects, a major reason for their rather limited clinical utility. Numerous effects associated with steroid replacement therapies and, thus, use and misuse of AAS have been studied and reported over more than 60 years, as comprehensively reviewed recently (Casavant et al. 2007; Kerr and Congeni 2007). The most critical issues have been the lack of prospective studies, which have rarely been conducted, primarily for ethical reasons. Moreover, the virtually unlimited varieties of AAS and assumed supraphysiological dosages applied in case of steroid misuse have complicated the evaluation of observations. However, numerous case reports, retrospective (postmortem) analyses, and animal models revealed at least five groups of side effects, categorized into cardiovascular, endocrine, hepatic, psychiatric, and musculoskeletal consequences.

2.3.1 Cardiovascular Issues

Cardiovascular consequences attributed to AAS misuse include several serious health issues such as systemic hypertension, increased left ventricular posterior

wall thickness (cardiac hypertrophy), impaired diastolic function, and atherosclerosis, all of which might contribute to the significant number of sudden death cases observed amongst AAS abusers.

The risk of atherosclerosis was demonstrated to correlate with decreased serum levels of high-density lipoprotein and concomitantly increased values for low-density lipoprotein. These changes were found to be significantly higher in studies involving 17-alkylated steroids instead of testosterone formulations (Hall 2005). Moreover, increased platelet reactivity was observed and considered relevant for various myocardial infarctions and strokes (Ferenchick et al. 1992; Ferenchick 1991). The occurrence of cardiac hypertrophy and impaired relaxation combined with an altered diastolic filling was reported in numerous (fatal) cases of AAS misuse. The latter phenomenon is assumed to result from an increased incorporation of collagen into myocardial structures and the presence of necrotic areas (Kindermann 2006; Luke et al. 1990; Thiblin et al. 2000).

2.3.2 Endocrine Issues

The endocrine system is also considerably affected by the administration of AAS, in particular with regard to the biosynthesis of testosterone and the regulatory hormones belonging to the hypothalamic–pituitary–gonadal axis. Long-term abuse of AAS in men has been associated with testicular atrophy, as a decrease in the gonadotrophins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) was observed. Consequently, azoospermia and infertility were reported, which are usually reversible but, depending on the amount and duration of AAS misuse, persist for months after cessation. In addition, the susceptibility of steroidal hormones to aromatases results in an increased availability of estrogens, which causes feminization of men in terms of voice pitch and, more seriously, irreversible gynecomastia.

2.3.3 Hepatic Issues

The most prominent hepatic consequences correlated with AAS misuse are liver toxicity, in particular associated with 17-alkylated steroids such as MT, MD and ST (Hall 2005; Ishak and Zimmerman 1987) causing benign hepatic adenomas or hepatocellular carcinoma. In addition, structural lesions and blood-filled cavities (peliosis hepatis) have been reported (Hickson et al. 1989).

2.3.4 Psychiatric Issues

Various studies stressed the fact that the misuse of AAS is correlated with major changes of mood, aggressive behavior and also depression (Pope and Katz 1994; Su et al. 1993), which vary between individuals and are related to the dosage of AAS. Upon cessation, these effects are short-lived. A potential for physical

dependence has not been demonstrated but the addictive potential, however, cannot be ruled out.

2.3.5 Musculoskeletal Issues

The administration of AAS was demonstrated to cause structurally disordered alignments of collagen fibrils in tendons. The resulting instability combined with increased muscle strength entails a higher risk of ruptures and failure during physical activity (Miles et al. 1992).

3 Pharmacological Aspects and Mode of Action of SARMs

The clinical benefits of steroid replacement therapies such as the management of debilitating diseases and osteoporosis have been evident in the past, for instance with the significant increase of AAS prescription for the treatment of AIDS in the late 1980s. However, the severe undesirable effects outlined above have resulted in a significantly reduced use of steroidal agents lately, and the search for nonsteroidal SARMs was successful for the first time in 1998 with the discovery of an analog to bicalutamide, an androgen receptor antagonist (Scheme 4, **21**) (Dalton et al. 1998). Subsequently, various SARM drug candidates were prepared on the basis of chemically diverse cores such as propionanilides, bicyclic hydantoins, and 2-quinolinones as illustrated in Scheme 4. Lead drug candidates have advanced to phase-I and phase-II clinical trials (Bhasin et al. 2006; Chen et al. 2005b; Gao and Dalton 2007a; Thevis and Schänzer 2007a) and recent proof-of-concept studies have demonstrated the enormous selectivity to muscle or bone tissue and significantly reduced side effect profiles (vide supra). These advantageous pharmacological properties are attributed primarily to the fact that SARMs activate the androgen receptor but are not substrates for 5α-reductases and aromatases (Chen et al. 2005b; Gao and Dalton 2007b; Mohler et al. 2005). Consequently, the metabolic amplification of androgenic or estrogenic functions of testosterone and AAS in tissues such as the prostate is eliminated and, thus, various undesirable effects are excluded.

Recent receptor binding studies provided additional insights into potential mechanisms underlying the tissue-selectivity of SARMs (Bhasin et al. 2006). Ligand binding to the androgen receptor (AR) induces conformational changes in the ligand-binding domain corresponding to the particular structure of the ligand. As a consequence, the altered surface topology is suggested to enable interactions with different cofactors, which contribute to a tissue-specific gene regulation. The concerted action with androgen response elements, co-regulators or transcription factors is proposed to allow for tissue-selective stimulation or inhibition of the AR.

Steroid binding to the AR is well documented, and major anchors are the 3-keto and 17-hydroxy functions of testosterone, which interact with the amino acid residues Arg 752, Asn 705 and Thr 877, respectively (Poujol et al. 2000; Sack

et al. 2001; Waller et al. 1996). Moreover, hydrophobicity as additionally provided by spacious alkyl substituents at C-6, C-7, C-11, C-12 or C-17 contributes to increased receptor binding affinities. Hence, structures of SARMs lead drug candidates were optimized with respect to these findings (Kim et al. 2005; Marhefka et al. 2004), and functional groups were designed to mimic the features of the natural AR ligands testosterone and dihydrotestosterone (Bohl et al. 2004; Ostrowski et al. 2006; Yin et al. 2003). The most advanced propionanilide-derived SARM (GTx, Inc., Scheme 4, **22**) bears a nitro residue and a chiral hydroxyl function, which mimic the 3-keto- and 17β-OH-group of DHT, respectively, establishing interactions with the earlier identified amino acid residues Gln 711, Arg 752, and Asn 705 (Bohl et al. 2004). Analogously, receptor binding studies with a bicyclic modulator (BMS-564929, Scheme 4, **24**) were recently published where ligand–receptor interactions were also observed at amino acids Arg 752 and Asn 705 (Ostrowski et al. 2006).

3.1 Metabolism of SARMs

Due to the comparatively young history of nonsteroidal SARMs, metabolism studies have been published only for a few lead drug candidates employing in vitro and in vivo approaches. In rats, compound **22** (Scheme 4) underwent extensive hydrolysis at the amide bond yielding 3-(4-fluorophenoxy)-2-hydroxy-2-methyl propanoic acid (**26**) and the corresponding 4-nitro-3-(trifluoromethyl) aniline (**27**), and the latter was further metabolized by acetylation and/or

Scheme 4 Chemical structures of selected SARMs: bicalutamide (**21**, mol wt = 430), propionanilides (**22**, mol wt = 402, and **23**, mol wt = 441), bicyclic hydantoin BMS-564929 (**24**, mol wt = 305), and 2-quinolinone LGD 2226 (**25**, mol wt = 392)

hydroxylation and sulfonation. Moreover, ring hydroxylation with and without reduction of the nitro residue (**29** and **30**) and subsequent conjugation to glucuronides and sulfates was reported (Wu et al. 2006). A brief summary is illustrated in Scheme 5a. Employing human, rat and dog liver enzyme preparations, the metabolism of compound **23** (Scheme 4) was studied in detail (Gao et al. 2006). In analogy to compound **22**, amide bond cleavage was observed yielding the metabolic product **27** and **31** (Scheme 5b). In addition, due to the presence of the *N*-acetyl function, **23** was extensively hydrolyzed yielding deacetylated counterparts to the parent drug (**32**) and other metabolic products (**33** and **34**). Another study using human liver microsomes further demonstrated the formation of an *O*-dephenylated metabolite (**35**) resulting from both candidates, **22** and **23**, and formation of glucuronic acid and sulfate conjugates (Kuuranne et al. 2008). Recent in vivo experiments with rats and dogs substantiated the findings of the earlier in vitro studies (Perera et al. 2006) and further revealed the presence of a methylated analog to **23**. However, the site of alkylation has not yet been conclusively determined. A selection of metabolic products of **23** is summarized in Scheme 5b.

Metabolism studies regarding other SARMs such as BMS-564929 (Scheme 4, 2**4**) or LGD-2226 (Scheme 4, 2**5**) have not yet been published. However, their oral bioavailability was demonstrated (Miner et al. 2007; Ostrowski et al. 2006) and will entail investigations of pharmacokinetic, pharmacodynamic and metabolic profiles.

3.2 Clinical Applications

The clinical utility of SARMs is manifold and includes numerous indications and health conditions (Cadilla and Turnbull 2006; Gao and Dalton 2007a). Major targets that have been mentioned are the treatment of hypogonadism, androgen deficiency in the aging male, prevention and treatment of muscle wasting and osteoporosis (Miner et al. 2007), and benign prostatic hyperplasia as well as male contraception (Chen et al. 2005a). These prospects are based on results obtained from preclinical and clinical studies, and a recent investigation has demonstrated the enormous potential associated with SARMs. A double-blind, randomized, placebo-controlled clinical trial with a drug candidate termed ostarine with 120 subjects (60 elderly men and 60 postmenopausal women) showed a dose-dependent increase in total lean body mass without a prescribed diet or exercise regimen. Within a three-month treatment period, an increase of 1.4 kg of muscle mass compared to placebo was observed, and functional performance measured by a stair climb test was significantly improved (GTx 2006).

3.3 Undesirable Effects of SARMs

The major advantage of SARMs compared to AAS is their considerably reduced pattern of side effects. Currently, no long-term studies are available, but first

Scheme 5 Major unconjugated metabolites of selected SARMs: (**a**) **22** yields 3-(4-fluorophenoxy)-2-hydroxy-2-methyl propanoic acid (**26**, mol wt=206), 4-nitro-3-(trifluoromethyl)aniline (**27**, mol wt =214), 4-amino-**22** (**28**, mol wt=372), hydroxylated 4-amino-**22** (**29**, mol wt = 388), and hydroxy-**22** (**30**, mol wt=418); (**b**) (**23**) yields 3-(4-acetylamino-phenoxy)-2-hydroxy-2-methyl propanoic acid (**31**, mol wt=253), 4-nitro-3-(trifluoromethyl)aniline (**27**, mol wt=214), deacetylated compound **23** (**32**, mol wt=399), 3-(4-amino-phenoxy)-2-hydroxy-2-methyl propanoic acid (**33**, mol wt=211), deacetylated 4-amino-**23** (**34**, mol wt=369), and the *O*-dephenylation product (4-nitro-3-(trifluoromethyl))-2,3-dihydroxy-2-methyl-propionanilide (**35**, mol wt=308)

proof-of-concept studies with ostarine have demonstrated favorable profiles concerning cardiovascular and endocrine aspects (GTx 2006). Commonly observed effects on serum PSA (prostate-specific antigen), sebum production (skin and hair), or serum LH were not reported. However, the treatment with ostarine resulted in a dose-dependent decrease in both LDL and HDL cholesterol levels, with the average LDL/HDL ratio for all doses tested remaining in the low cardiovascular risk category.

4 Detection Strategies for AAS and SARMs

Traditionally, anabolic androgenic steroids have been comprehensively detected in urine samples using gas chromatographic–mass spectrometric (GC–MS) methods since they complemented and finally replaced radioimmunoassay-based techniques (Brooks et al. 1975) in the early 1970s. Screening and confirmation methods are commonly performed, employing benchtop GC–MS systems (Schänzer 1998; Schänzer and Donike 1996; Ayotte et al. 1996), and numerous adverse analytical findings have arisen from the identification of AAS or respective metabolic products (Kicman and Gower 2003; Saugy et al. 2000). In addition, the use of high resolution instruments (such as magnetic sector analyzers) as well as tandem mass spectrometers have provided powerful, fast, and reliable tools for detecting AAS, especially those requiring utmost specificity and sensitivity such as the long-term metabolites of MD or the major metabolic product of nandrolone termed 19-norandrosterone. However, marginal chromatographic properties of various analytes, also after derivatization, and enormous improvements in interfacing liquid chromatography to tandem mass spectrometry (LC–MS/MS) encouraged the development of alternative approaches utilizing the novel technology of LC–MS/MS to complement the existing analytical methods for AAS. In particular thermo-labile compounds and those with an exceptional polarity, e.g., trenbolone and related substances, were transferred from traditional GC–MS to LC–MS/MS procedures (Thevis and Schänzer 2007b).

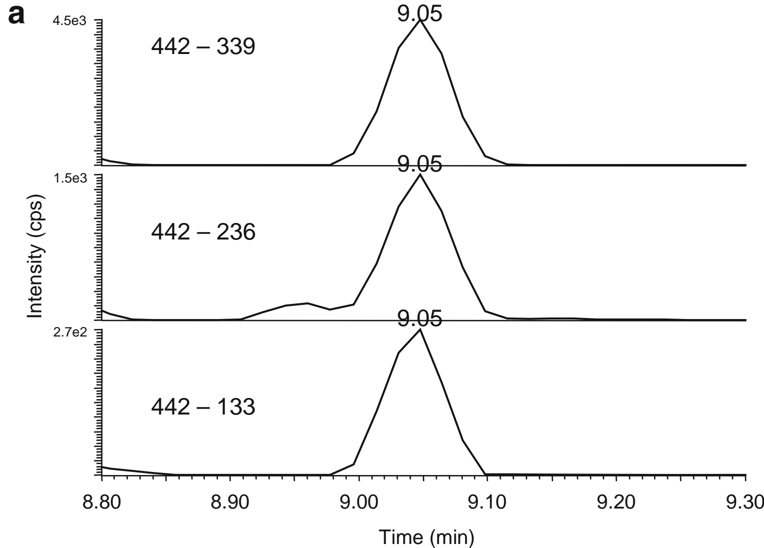
4.1 Sample Preparation and Analysis of AAS

Regardless whether urine specimens are prepared for GC–MS or LC–MS/MS analysis, samples are commonly adjusted to a pH of 5.0–5.2 and enzymatically hydrolyzed using β-glucuronidase. Subsequently, unconjugated steroids and respective phase-I metabolites are purified by means of liquid–liquid extraction (LLE) using diethyl or *tert*-butyl methyl ether (Donike et al. 1984). The organic layer is separated and evaporated to dryness, and dry residues are either subjected to derivatization (for GC–MS) or reconstituted in LC solvents. The derivatization of analytes, usually based on trimethylsilylation of hydroxyl residues and keto

functions (Donike and Zimmermann 1980), tremendously improves gas chromatographic and mass spectrometric properties of most analytes, which provides highly informative mass spectra and excellent detection limits.

4.1.1 GC–MS Analyses

Screening for AAS and metabolic products by GC–MS has been accomplished by GC–MS systems that contain conventional single quadrupole mass analyzers (Bowers 1998; Donike and Zimmermann 1980; Donike et al. 1984; Hatton and Catlin 1987; Kicman and Gower 2003; Saugy et al. 2000). The use of selected ion monitoring (SIM) of the most abundant, characteristic and/or specific fragment ions of target compounds has enabled the sensitive qualitative and quantitative detection of numerous AAS, some of which require detection limits as low as 1–2 ng mL^{-1} (Schänzer 1998; Schänzer and Donike 1996; Ayotte et al. 1996). Usually, up to four ions are recorded per analyte to ensure its identity and reduce the frequency of unnecessary repetitions of sample preparation and analysis. The option of quadrupole MS was complemented by so-called high sensitivity instruments, which were introduced in 1993 (Horning and Donike 1994) with high resolution magnetic sector mass spectrometers and became mandatory in sports drug testing in 1996 (Bowers 1997). It has considerably enhanced detection and identification capabilities in doping controls, especially with regard to long-term excreted metabolites (Schänzer et al. 1996a, b). Primarily due to the high financial burden, alternatives were sought and found in tandem mass spectrometers commonly composed by ion trap devices. The gain in sensitivity as accomplished by MS/MS(/MS) experiments has also allowed the detection of the specified analytes 3′-hydroxystanozolol (**17**), 17α-methyl-5β-androstane-3α,17β-diol (**6**), 19-norandrosterone, and clenbuterol at required detection limits (Marcos et al. 2002; Mateus-Avois et al. 2005; Munoz-Guerra et al. 1997). Those compounds have been particular targets in sports drug testing due to their frequent misuse; hence, long-term traceability has been of great interest and research focused on improved retrospective is an ongoing pursuit. A recent example for “old drugs and new targets” was found in 18-nor-17β-hydroxymethyl-17α-methyl-5β-androsta-1,13-dien-3-one (**16**). The EI-mass spectrum of the trimethylsilylated metabolite of MD and respective extracted ion chromatograms are illustrated in Fig. 1. The data were obtained from a urine specimen tested positive after administration of MD. The utility of this comparably new long-term target in sports drug testing (Schänzer et al. 2006) has become evident with an increase of adverse analytical findings associated with the misuse of MD of more than 300% in the Cologne doping control laboratory in 2006 (Fusshöller et al. 2007). Out of approximately 11,000 urine samples, 68 (0.6%) tested positive for compound **16** proving the misuse of MD. In comparison to the years 2003–2005, where the total number of doping control urine specimens also ranged between 10,000 and 12,000 per year, only 12–15 adverse analytical findings were reported. Moreover, in 2006 a total of 126 doping violations with MD were determined, 54% of which were identified utilizing the long-term metabolite **16**.



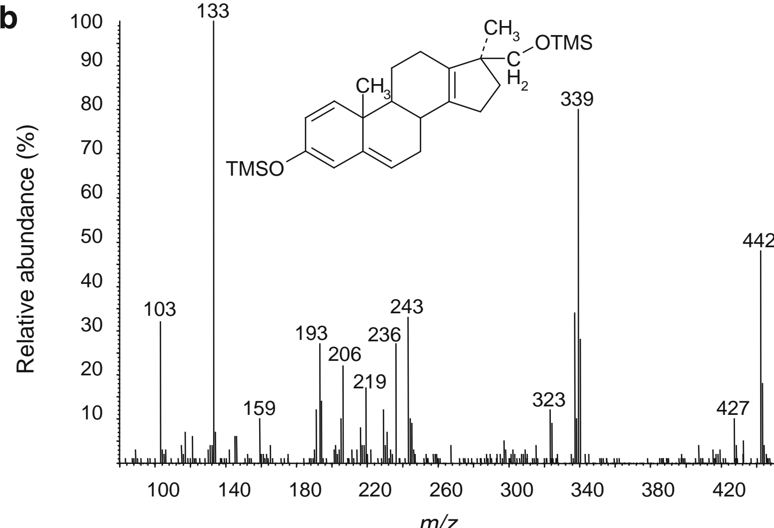


Fig. 1 (**a**) Extracted ion chromatograms of *m/z* 339, 236 and 133 resulting from an GC–MS/MS analysis indicating the presence of the long-term metabolite 18-nor-17β-hydroxymethyl-17α-methyl-5β-androsta-1,13-dien-3-one (**16**) in a urine specimen; (**b**) EI-mass spectrum of **16** after trimethylsilylation (mol wt = 442)

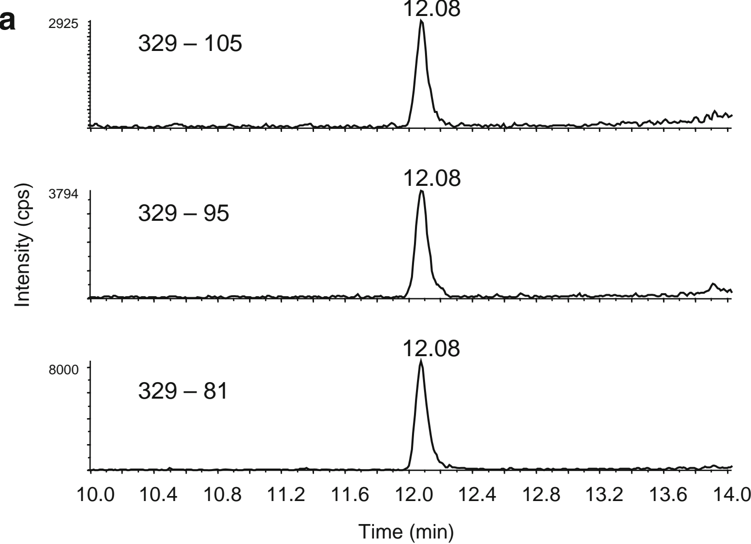
4.1.2 LC–MS(/MS) Analyses

Because various AAS (e.g., trenbolone, gestrinone, stanozolol, etc.) possess marginal gas chromatographic properties even after derivatization, LC–MS(/MS)-based approaches were established that allow for a comprehensive determination of these compounds according to the minimum required performance limits requested by WADA (WADA 2004). The most eligible substances are those possessing a (cross-)conjugated electron system such as MD or trenbolone and analogs, or a high proton affinity due to heteroatoms incorporated in the steroid core as found in the case of ST. Consequently, several methods enabling the detection of these drugs and metabolic products in urine specimens have been presented (Deventer et al. 2006; Leinonen et al. 2004; Mareck et al. 2004; Mazzarino and Botre 2006; Pozo et al. 2007a), some of which focused particularly on stanozolol (Thevis et al. 2005b, 2006a), androstadienolones (Thevis and Schänzer 2005) or drugs designed for doping practices only (Catlin et al. 2004; Thevis et al. 2005a). In particular the case of tetrahydrogestrinone (THG) stressed the need for complementary methods allowing the sensitive detection of drugs and substances with rather limited GC properties, a major reason for the successful camouflage of THG in doping controls for many years. In addition, attempts to cover more AAS using LC–MS(/MS) approaches were published recently, either utilizing the ability of the analytes to generate adduct ions (Pozo et al. 2007b) or employing first-generation product ions (e.g., derived from the elimination of water) as precursor ions in MS/MS experiments (Trout et al. 2007) or derivatization (Borges et al. 2007). In addition, new ionization techniques such as atmospheric pressure photo ionization (APPI) (Leinonen et al. 2002) as well as reactive desorption electrospray ionization (DESI) have been described (Huang et al. 2007), potentially allowing the determination of a broader spectrum of analytes using LC–MS/(MS) systems.

All of these strategies are based on tandem mass spectrometry which provides the specificity and sensitivity to unambiguously identify prohibited drugs in doping control samples. In Fig. 2, the extracted ion chromatograms of a urine specimen fortified with 0.2 ng mL^{-1} of stanozolol is shown in combination with the product ion mass spectrum derived from the protonated precursor ion at *m*/*z* 329. The specificity of the ion transitions (329–81, 329–95, and 329–105) enabled the unequivocal detection and identification according to commonly accepted guidelines as established by WADA.

4.2 Sample Preparation and Analysis of SARMs

The structural diversity of SARMs is a key factor in sample preparation and analysis of this new class of emerging drugs. As outlined in Scheme 4, polar functions are present in all representatives of SARMs, but leading to positive (2-quinolinones) and negative (arylpropionamides, bicyclic hydantoins) charges. Moreover, the metabolic fate of these compounds is yet to be fully elucidated;



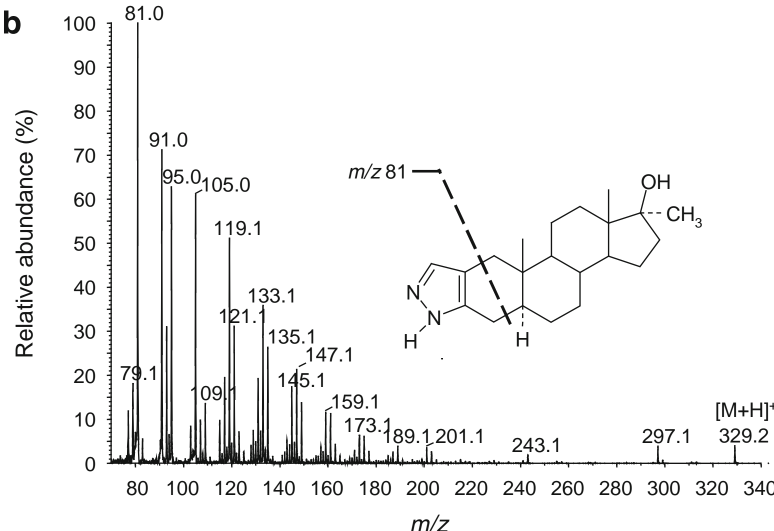
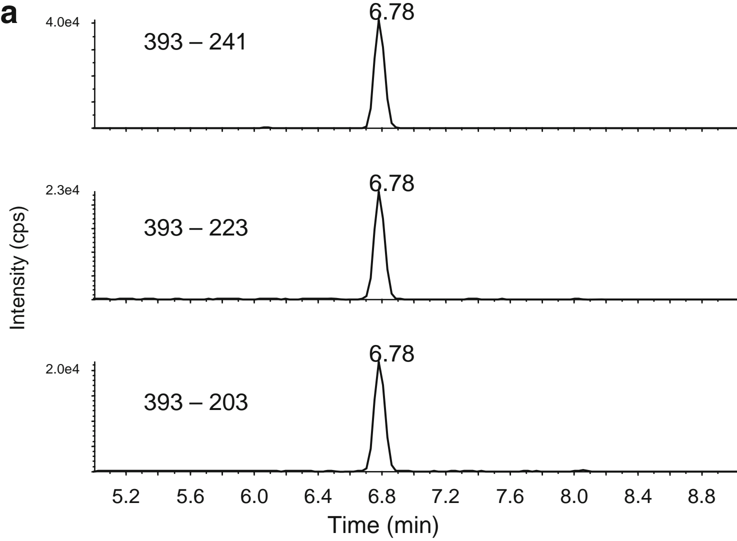


Fig. 2 (**a**) Extracted ion chromatograms 329–81, 329–95, and 329–105 obtained from a urine sample fortified with 0.2 ng mL^{-1} of stanozolol; (**b**) ESI-product ion spectrum derived from the protonated molecule of stanozolol $[M+H]^{+}=329$

hence, SPE and LLE were chosen for initial studies on the detection of SARMs in doping control analysis. The arylpropionamides **22** and **23** were described as *metabolically stable* (Marhefka et al. 2004), and first reports on in vitro as well as in vivo metabolism studies demonstrated a limited conversion of these into

degradation or conjugation products (vide supra). A simple SPE of spiked urine specimens followed by LC-ESI-MS/MS analysis enabled the detection of 1 ng mL^{-1} of **22** and **23**, and the simultaneous measurement of precursor ion scans on most common product ions derived from the arylpropionamide core complemented the



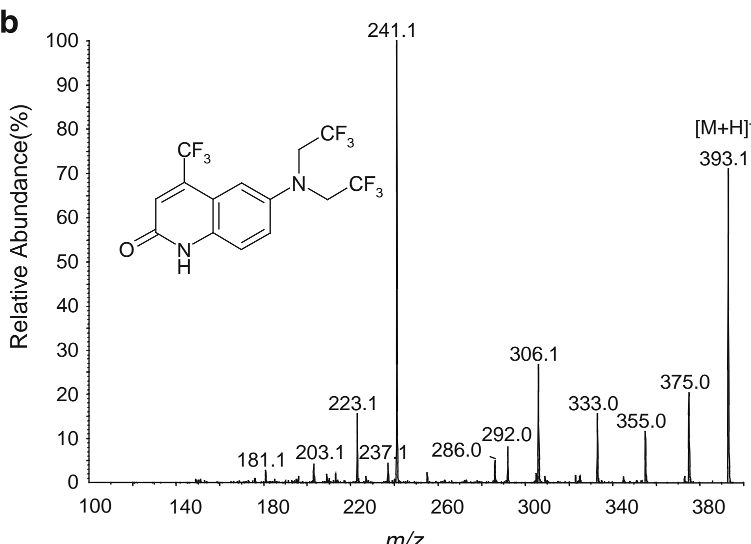


Fig. 3 (**a**) Extracted ion chromatograms 393–241, 393–223, and 393–203 obtained from a urine sample fortified with 1 ng mL^{-1} of LGD-2226 (**25**); (**b**) ESI-product ion spectrum derived from the protonated molecule of **25** $[M+H]^{+}=393$

assay to allow a comprehensive screening for related drugs as well as their metabolites (Thevis et al. 2006b). The bicyclic hydantoin BMS 564929 (Scheme 4, **24**) was also extracted from spiked urine samples using SPE, but due to its poorly acidic nature and limited proton affinity, methanol adduct formation under negative ESI conditions was employed to allow detection limits of 5–20 ng mL^{-1} (Thevis et al. 2007b). In contrast, 2-quinolinone-derived SARMs such as LGD-2226 (Scheme 4, **25**) were isolated from urine specimens by means of common LLE, and LC–MS/MS using positive ESI enabled the detection of 0.2 ng mL^{-1} (Thevis et al. 2007a). A typical extracted ion chromatogram of characteristic ion transitions derived from a urine specimen enriched with 1 ng mL^{-1} of LGD-2226 and its product ion mass spectrum are depicted in Fig. 3.

5 Conclusions

The anabolic steroids were referred to as the Gremlins of Sport (Todd 1987), and in the light of the developments of doping controls, this fact seems to have remained true. Attempts to enhanceme performance surreptitiously by means of anabolic agents are frequently detected among elite athletes, and neither concerns about health, ethical issues nor sanctions have built barriers high enough to prevent athletes from also administering designer drugs without clinical approval. Anabolic agents will play a key role in sports drug testing in the future, especially because of a continuously increasing number of new compounds and drugs entering the market. However, sophisticated strategies based on mass spectrometry but also complementary techniques such as bioassays (Nielen et al. 2006) will help to reduce extensive misuse.

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Nandrolone: A Multi-Faceted Doping Agent

Peter Hemmersbach and Joachim Große

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Abstract Nandrolone or nortestosterone, an anabolic-androgenic steroid, has been prohibited by doping control regulations for more than 30 years. Although its main metabolism in the human body was already known at that time, and detection of its misuse by gas or liquid chromatographic separation with mass spectrometric

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detection is straightforward, many interesting aspects regarding this doping agent have appeared since.

Over the years, nandrolone preparations have kept their position among the prohibited substances that are most frequently detected in WADA-accredited laboratories. Their forms of application range from injectable fatty acid esters to orally administered nandrolone prohormones. The long detection window for nandrolone ester preparations and the appearance of orally available nandrolone precursors have changed the pattern of misuse.

At the same time, more refined analytical methods with lowered detection limits led to new insights into the pharmacology of nandrolone and revelation of its natural production in the body.

Possible contamination of nutritional supplements with nandrolone precursors, interference of nandrolone metabolism by other drugs and rarely occurring critical changes during storage of urine samples have to be taken into consideration when interpreting an analytical finding.

A set of strict identification criteria, including a threshold limit, is applied to judge correctly an analytical finding of nandrolone metabolites. The possible influence of interfering drugs, urine storage or natural production is taken into account by applying appropriate rules and regulations.

Keywords Doping control • Nandrolone • Nortestosterone • Prohormones • Norandrosterone

1 Introduction

Although no reliable data are available from that time, nandrolone (19-nortestosterone, 17β-hydroxy-estr-4-en-3-one) was presumably one of the substances of choice when athletes began using anabolic-androgenic steroids as performance-enhancing drugs in the 1960s. The expectation that the desired anabolic effect, i.e. increase of muscle mass due to better nitrogen retention and stimulated protein formation, combined with highly intense training would lead to an overall improved performance, particularly in sports dependent on muscle strength, became certain at the beginning of the 1970s. Realising the danger for fairness and ethics in sports, the Medical Commission of the International Olympic Committee (IOC) decided in 1974 to ban the misuse of anabolic-androgenic steroids. After some deficiencies in the detection strategy for nandrolone were eliminated at the beginning of the 1980s, the fight between the doping analysts and the nandrolone users who were trying to avoid being tested positive was characterised by the refinement of analytical methods with lowered detection limits, leading finally to new insights into the pharmacology of nandrolone on the one hand and adjustments regarding the use of specific preparations and their route of administration on the other.

Nandrolone is thus a good example of a doping agent that reflects how doping control and doping analysis have developed over the years. It can exemplify many aspects of how the misuse of doping agents has changed and how the acquirement of new knowledge has adapted the fight against doping. Examples ranging from different - in some cases subtle - application patterns to the inadvertent intake of contaminated dietary supplements, and from the ingestion of meat from treated animals, to the natural formation of nandrolone in the body, are facets of this doping agent.

Nandrolone preparations are almost entirely converted in the body to two main phase I metabolites and excreted as conjugates of norandrosterone (NA, 3α-hydroxy-5α-estran-17-one) and noretiocholanolone (NE, 3α-hydroxy-5β-estran-17-one). Findings of these metabolites are, however, not unambiguously linked to the intentional administration of nandrolone. They may have other origins, such as natural production, interference through other drugs, contamination in nutritional supplements and possible changes during storage. Improvements in analytical techniques and increasingly lower detection limits have led to knowledge about the natural production of nandrolone in the body in concentrations that hitherto were undetectable. Consequently, a threshold value has been set for NA in a doping context. Concentrations in urine that are lower than 2 ng mL^{-1} are not to be considered as an adverse analytical finding.

2 Nandrolone: Prohibited List and Adverse Finding Statistics

The 2009 Prohibited List issued by the World Anti-Doping Agency (WADA) specifies the substances and methods which are prohibited, either at all times or only in competition, for all athletes falling under the jurisdiction of the World Anti-Doping Code (World Anti Doping Agency 2008b). Nandrolone is an example of a prohibited substance from this list, and belongs to the class of anabolic agents, subgroup exogenous anabolic-androgenic steroids. Nandrolone can be regarded as an "old" doping agent that was extensively used decades ago in various forms of application, and it is still used to enhance performance or is even unintentionally ingested (see Sects. 5 and 6). The WADA statistics for 2004 showed that anabolic-androgenic steroids comprised 36% of all adverse analytical findings reported by accredited laboratories and, of these, 28.5% were associated with nandrolone metabolites (World Anti Doping Agency 2005). These figures had been reasonably stable for the previous 15 years. However, in recent years the absolute numbers of nandrolone adverse findings and the relative percentage have continuously decreased (see Fig. 1). This moderate decline may be explained by improvements in anti-doping strategies, even though great regional differences still remain, and in particular by increasingly efficient testing procedures, notably unannounced out-of-competition tests, having apparently deterred more athletes from using anabolic steroids. The disproportionately large drop of nandrolone findings compared to those of metandienone and stanozolol may be due to a downturn of propagation and

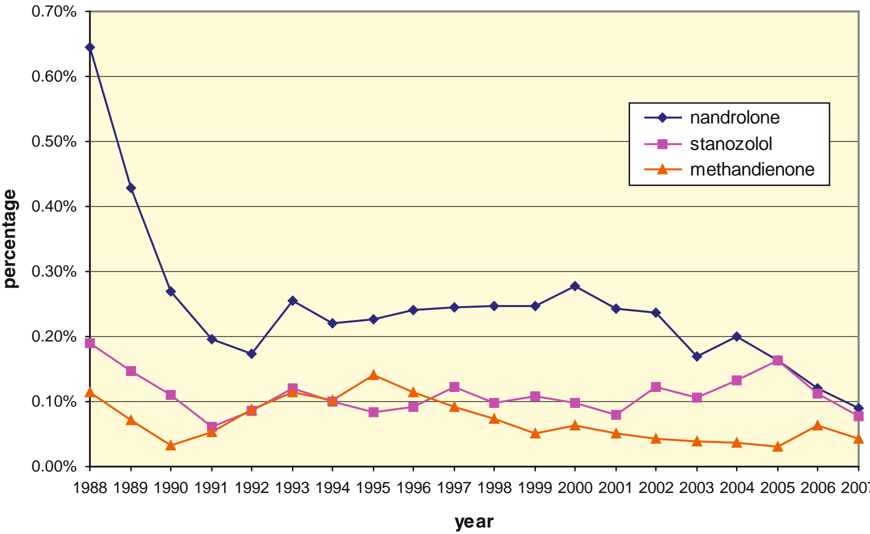

Fig. 1 A sample adverse analytical findings of specific anabolic-androgenic steroids reported by accredited anti-doping laboratories

distribution of nandrolone prohormones after the Anabolic Steroid Control Act 2004 came into force in 2005 (The Senate of the United States 2004). Furthermore, it can be assumed that a few of the nandrolone findings in the years before 2004 might have been caused by the then unknown and very rare in situ formation of nandrolone metabolites in urine from endogenous precursors (see Sect. 9).

In 2007, the total number of doping tests worldwide amounted to 224,898, and 4,402 of these samples resulted in 4,850 adverse findings, 203 of which were nandrolone metabolites. Nandrolone is therefore still one of the most frequently detected doping agents worldwide, only surpassed in absolute numbers by testosterone atypical findings (1,607), amphetamine (430) and cannabis (576) (World Anti Doping Agency 2008a).

3 Nandrolone and Related AAS

3.1 Urinary Excretion and Analysis

The anabolic-androgenic steroid, nandrolone, was synthesised in the early 1950s (Birch 1950; Wilds and Nelson 1953). As shown in Fig. 2, nandrolone is structurally very like testosterone. Only the methyl group in the 19 position is missing; consequently, nandrolone is also called nortestosterone or 19-nortestosterone. The metabolism of nandrolone was studied soon after (Engel et al. 1958) and its

Testosterone Nandrolone (19-Nortestosterone)

Fig. 2 Structural formulas for testosterone and nandrolone (19-nortestosterone)

phase I metabolism largely follows the metabolism of testosterone, with oxidation of the hydroxyl group in the 17 position (catalysed by 17β-hydroxysteroid dehydrogenase) forming a 17-oxo-steroid and complete reduction of the A-ring, i.e. the double bond in the 4 position (5α- and 5β-reductase, respectively) and the 3-keto function are hydrogenated. As a result of this, the two main phase metabolites, NA (5α-isomer) and NE (5β-isomer) are formed (see Fig. 3). Both metabolites are mainly excreted as glucuronic acid conjugates. The structure of the main phase I metabolites was confirmed with the help of synthesis in 1960 (Counsell 1961; Kupfer et al. 1960) and is described in a number of publications (Bagchus et al. 2005; Baume et al. 2004; Schänzer 1996). NA and NE have also proved to be the main phase I metabolites of nandrolone-related steroids like norandrostenedione (estr-4-ene-3,17-dione) and norandrostenediol (estr-4-ene-3β,17β-diol) (Kintz et al. 1999a; Schrader et al. 2006). 3β-Hydroxy-isomers (3β-hydroxy-5α/β-estran-17-one, i.e. norepiandrosterone and norepietiocholanolone) are also excreted, but only as sulphates (Torrado et al. 2008b). The metabolism of nandrolone and nandrolone-related steroids is illustrated in Fig. 3.

After some early approaches with the help of radioimmunoassay techniques (Dugal et al. 1977; Brooks et al. 1979) the analysis of the glucuronidated metabolites, NA and NE, has been favoured to detect doping with nandrolone or its precursors. Numerous publications, mainly based on gas chromatographic separation with mass spectrometric detection (GC–MS), have described possible analytical methods for these metabolites. Generally, the NA and NE conjugates are hydrolysed followed by an extraction and derivatisation step before the MS detection is performed (Bjorkhem and Ek 1982; Masse et al. 1985; Schänzer and Donike 1993). A few efforts have been made to determine and characterise intact glucuronidated nandrolone metabolites by GC–MS and liquid chromatography coupled to mass spectrometry (LC–MS) (Thevis et al. 2001a; Thevis et al. 2001b; Hintikka et al. 2008a; Hintikka et al. 2008b).

Urine has been the preferential matrix for doping analysis, but nandrolone metabolites have been detected in plasma (Schrader et al. 2006; Torrado et al. 2008a) and hair (Gambelunghe et al. 2007; Kintz et al. 1999b; Segura et al. 2000).

To obtain a complete picture of the urinary metabolites, the sulphate fraction of the relevant metabolites should also be taken into account. Recent publications have described analytical procedures for the direct detection of sulphate conjugates

Fig. 3 Phase I metabolism and urinary excretion of nandrolone and the nandrolone-related steroids

by LC–MS (Strahm et al. 2007). Studies of the excretion of nandrolone precursors have indicated that the NA sulphate even outweighs the glucuronide at a late excretion state (Strahm et al. 2009). Sulphate metabolites may be taken into consideration to increase the retrospection in the detection of 19-norsteroids after oral administration (Torrado et al. 2008b).

3.2 Criteria for Issuing an Adverse Analytical Finding

The criteria for reporting adverse analytical findings of NA and/or NE, which indicates the administration of preparations containing nandrolone or nandrolone precursors, are laid down as a Technical Document under WADA's International Standards for Laboratories (World Anti Doping Agency 2009). Any analytical finding of NA and/or NE in the initial testing will be forwarded to a confirmation analysis with a second aliquot of the same urine sample.

The final analytical result is then checked against each point in the set of criteria. These criteria include the following points with a reference to the section where the respective backgrounds are discussed:

(a) In addition to meeting the identification criteria, the laboratory must demonstrate that the concentration of NA in the urine sample significantly exceeds a threshold value of 2 ng mL^{-1}. This threshold is corrected if the urine specific gravity exceeds 1.020, according to the formula

$$\text{Threshold} = (\text{Specific gravity of the } \textit{Sample} - 1)/(1.020 - 1) * 2\ \text{ng}\,\text{mL}^{-1}$$

In order to take into account measurement uncertainty a decision limit (DL) has been defined by adding a guard band of 0.4 ng/ml to the adjusted threshold (see Sects. 8, 8.2–8.4).

In the case of a urine sample from a female athlete, the laboratory must demonstrate

(b) That the presence of low levels of 19-norandrosterone is not due to pregnancy (see Sect. 8.1).
(c) Whether it is reasonable that the 19-norandrosterone was excreted in the amount measured consequent to the intake of norethisterone by verifying that the major isomer of glucuroconjugated tetrahydronorethisterone is present (see Sect. 7.1).

Additionally, for urine samples from males and females

(d) That the sample meets the criteria of "stability". In urine samples from males and females with an NA concentration below 10 ng mL^{-1} and exhibiting all of the indicated features of "unstable" urine (comparable levels of NA and NE where NA/NE < androsterone/etiocholanolone (A/E), shall be submitted to a stability test before reporting an adverse analytical finding (see Sect. 9). An IRMS-analysis shall prove the exogenous origin of NA.

If the results of the confirmation analysis fulfil identification criteria and the issues indicated under (a)–(d), an adverse analytical finding will be reported. All the questions surrounding adverse nandrolone findings have been discussed in a detailed report by a UK working group (Callicott and Kicman 2003), and an excellent review on the significance of NA in the urine samples of athletes has been published (Ayotte 2006).

4 Clinical Use and Abuse

Anabolic-androgenic steroids have been of interest for several fields of clinical applications for more than half a century. The attraction of nandrolone preparations compared to the body's own anabolic-androgenic steroid, testosterone, is mainly due to differences in their biochemical activity and transformation in the body, which reduce the possible side effects of AAS use.

Studies of the binding characteristics to the androgen receptor have shown (Bergink et al. 1985) that nandrolone has a higher affinity than testosterone,

whereas the situation for the respective dihydro metabolites after 5α-reductase-catalysed biotransformation is just the opposite. It is likely that testosterone and/or nandrolone exert their effect on muscle cells in their native molecular forms (Celotti and Cesi 1992), whereas in the androgenic tissue, with much 5α-reductase available, the reduced forms dominate. This therefore explains why nandrolone shows increased anabolic-androgenic differentiation (Kicman and Gower 2003), even though only one type of androgen receptor exists (Mooradian et al. 1987).

Another important difference between testosterone and nandrolone biotransformation is related to its ability to be aromatised into an oestrogen. Unlike testosterone, nandrolone is not a substrate for the cytochrome P450 aromatase enzyme catalysed transformation to an oestrogen (Behre et al. 2001), although nandrolone-related substances may undergo aromatisation via a different pathway in the liver (Kuhl and Wiegratz 2007).

These and other characteristics of nandrolone have promoted both clinical use and doping abuse since its synthesis more than 50 years ago.

In clinical contexts, nandrolone is used for pathological conditions characterised by negative nitrogen balance, catabolic states like major burns, cancer (Puccio and Nathanson 1997) and AIDS (Mulligan et al. 2005; Sattler et al. 2002; Storer et al. 2005), and in trials for the treatment of osteoporosis (Geusens 1995), chronic kidney failure and aplastic anaemia (Gardner 1985). Nandrolone has, moreover, been the subject of much research into the development of contraceptives for men (Nieschlag et al. 2003; Rajalakshmi 2005; Schürmeyer et al. 1984). Even ophthalmological preparations containing nandrolone sodium sulphate to support cornea healing are available and may interfere with doping analysis (Avois et al. 2007).

However, while the application of nandrolone preparations for clinical use has decreased in recent decades, their misuse as anabolic agents aimed at their potential performance-enhancing properties has increased.

In particular in the bodybuilding scene, the abuse of anabolic-androgenic steroids is characterised by the combination of several different preparations, generally in time intervals or "cycles". Doses used in a doping context are often 5–10 times higher than those employed in clinical use (Llewellyn 2009; Sinner and Bachmann 2004).

A new form of application arose in the mid-1990s. Nandrolone-related steroids like norandrostenediol and norandrostenedione were sold at first as oral, later also as sublingual and transdermal preparations without any association with medical treatment (see Sect. 6).

5 Different Forms of Nandrolone Application: Impact on Metabolism and Excretion

When injected, nandrolone is usually administered as an ester derivative, either as the shorter acting phenyl propionate (e.g. Durabolin) or more widely as the longer acting decanoate (e.g. Deca-Durabolin, see Fig. 4). Pharmacokinetic studies of

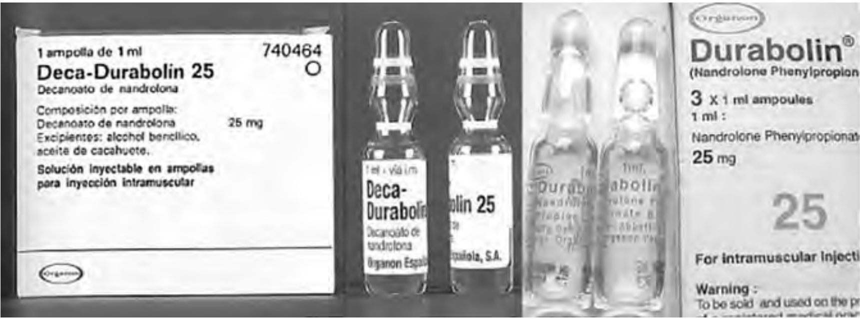

Fig. 4 Nandrolone decanoate and nandrolone phenylpropionate as the active substances in preparations for injection (Sinner and Bachmann 2004)

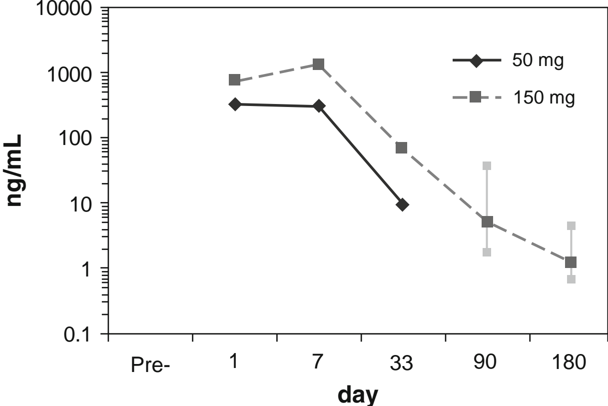

Fig. 5 Concentration of norandrosterone in urine following injection of, respectively, 50 and 150 mg of nandrolone decanoate (semi-logarithmic scale) (from Bagchus et al. 2005)

nandrolone following injection of nandrolone esters have been performed in serum in a number of connections, and a suitable study (Bagchus et al. 2005) describes the concentration relationships in both serum and urine with data linked to various dosages. Following single injections of, respectively, 50, 100 and 150 mg nandrolone decanoate, the levels in serum and urine were monitored over time. As Fig. 5 shows, the metabolite, NA, is traceable in urine in concentrations higher than the threshold value of 2 ng mL^{-1} for 6 months after injection of a single dose of 150 mg nandrolone decanoate. Thus, the metabolites, NA and NE, can be traced for a very long time following administration of depot preparations when nandrolone occurs as fat-soluble, long-chained ester derivatives.

Traceability over time depends mainly on the pharmaceutical formulation and the route of administration. Following intake of the nandrolone-related steroids available as tablets or capsules for oral intake, the metabolites are excreted in

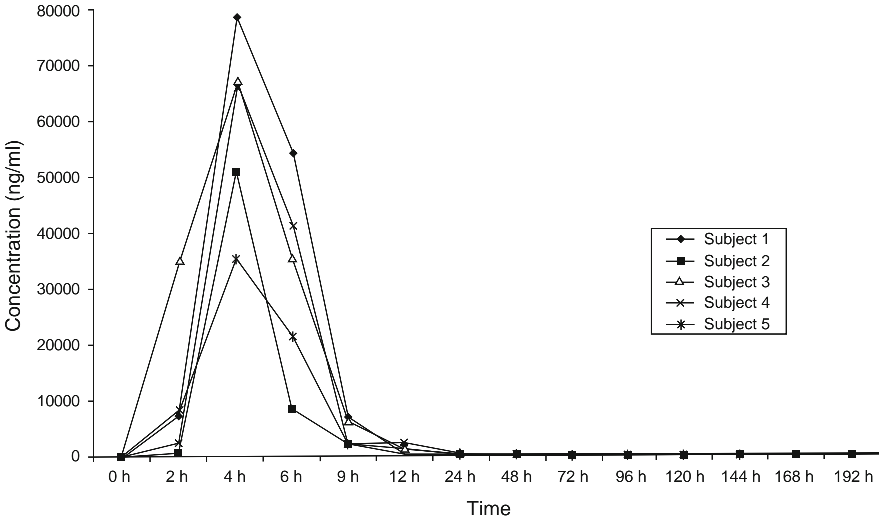

Fig. 6 Concentration of norandrosterone in urine in five test persons following intake of 5 mg of 4-norandrostenedione (from De Cock et al. 2001)

extremely high concentrations in the initial hours. Owing to fast phase I metabolism, they are rapidly excreted in urine and are only traceable for a few days (2–6) after their intake, in any case after one dosage (see Fig. 6 from De Cock et al. 2001). Several comparative studies investigating metabolism and excretion of 19-norsteroids after oral (Torrado et al. 2008b), sublingual (Schrader et al. 2006) and transdermal (Thieme et al. 2003) administration revealed that the bioavailability of the physiologically active species increases in this direction. The detectability which is dependent upon the excretion half-life of the terminal metabolites, NA-glucuronide and NE-glucuronide, behaves in the same manner.

6 Nutritional Supplements and Doping

In this section, the term nutritional supplement covers everything from preparations containing supplements of vitamins and minerals to so-called ergogenous supplements marketed for their performance-promoting effect.

6.1 Prohormones

As mentioned earlier, the nandrolone-related steroids, norandrostenediol and norandrostenedione, exist as oral preparations, and they became available as

"nutritional supplements" in the mid-1990s. The reason for this was that legislation regulating dietary supplements and herbal preparations in the USA, "The Dietary Supplement Health and Education Act" (U.S. Food and Drug Administration 1994), was enacted in 1994 (Millman and Ross 2003). The concept of dietary supplement was extended to apply to everything not covered by the stringent drug or steroid legislation; moreover, regulation of the sale of dietary supplements and herbal preparations was greatly relaxed. As long as the preparations were not marketed as having a therapeutic effect and no health hazard was documented, restrictions on their sale could not be implemented. The manufacturers were entrusted with the quality control of dietary supplements. Following a great deal of effort and many rounds, the legislation in the USA was made more stringent in 2004 (The Senate of the United States 2004).

Norandrostenediol and norandrostenedione are often referred to as prohormones or precursors. A closer look at their molecular structures reveals that many different isomers were on the market, including norandrost-4-ene-3,17-dione (estr-4-ene-3,17-dione), norandrost-5-ene-3,17-dione (estr-5-ene-3,17-dione), norandrost-4-ene-3,17-diol (estr-4-ene-3β,17β-diol, bolandiol), and norandrost-5-ene-3,17-diol (estr-5-ene-3β,17β-diol). These compounds are chemically and pharmacologically closely related to nandrolone. The terms precursor or prohormone allude to the fact that the substance is converted to the active hormone inside the body (Schrader et al. 2006). As already shown in Fig. 3, norandrostenediol and norandrostenedione have the same main metabolites as nandrolone, NA and NE (Uralets and Gillette 1999). Based on a single urine sample, it will be impossible to say anything about the origin of the metabolites. The prohormones were designed in such a way as to circumvent the stringent steroid legislation in the USA, and the products could thus be sold as dietary supplements without restrictions. Even though they were banned in Europe and many other parts of the world, these preparations could be easily obtained in a rapidly growing Internet market. Fig. 7 illustrates an example of the Internet sale of nandrolone-related steroids as a "dietary supplement".

Although those prohormones have never been investigated with the aim of introducing a registered pharmaceutical preparation, in recent years several studies have been undertaken to investigate their effects (Ziegenfuss et al. 2002) and anabolic-androgenic properties. Studies on the bioactivity of previously not-studied steroids also support the classification of these substances as prohibited. It has recently been shown that if the most popular nandrolone prohormone, norandrostenedione, is administered subcutaneously in mice it highly selectively stimulates the growth of skeletal muscle, but has only weak androgenic properties (Diel et al. 2008).

All the nandrolone prohormones are placed on WADA's Prohibited List (World Anti Doping Agency 2008b), even though little has been done to investigate their performance-promoting effect (Burke et al. 2000). In a study of norandrostenedione and norandrostenediol in resistance-trained men, 344 mg/day of norsteroid supplementation had no effect on strength or body composition (van Gammeren et al. 2002).

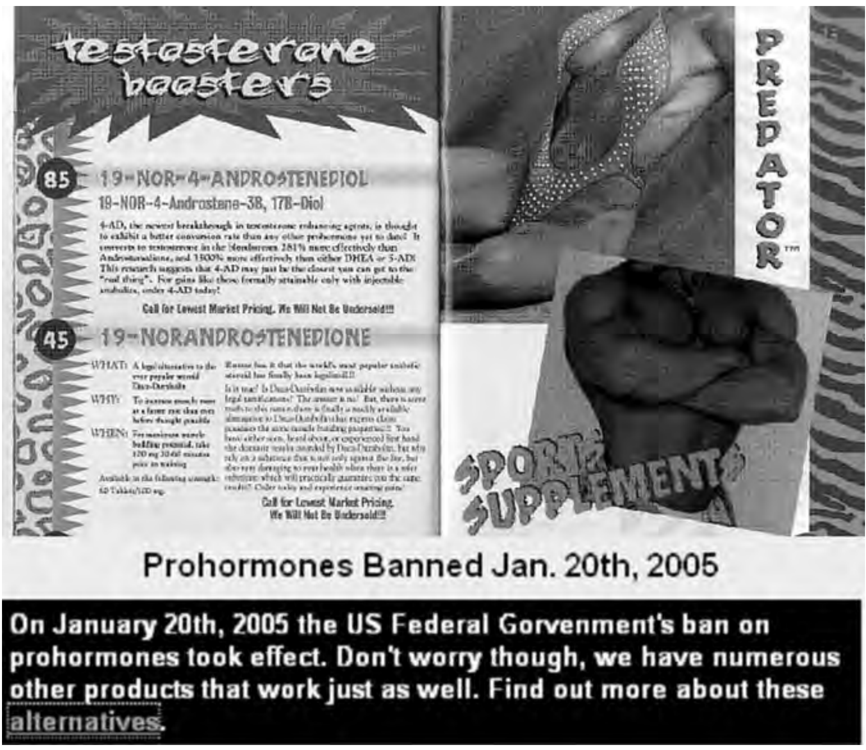

Fig. 7 Example of the Internet sale of nandrolone-related steroids as a nutritional supplement (before 2005). The lower part shows the information presented on web-sites, where nandrolone prohormones were advertised before (Bodybuilding.com 2009)

6.2 Contaminated Nutritional Supplements

Another consequence of the sale of prohormones is the problem of contaminated dietary supplements. Inadequate or erroneous labelling of preparations means that a certain risk is linked with the intake of dietary supplements and herbal preparations. Several reports have revealed prohormones in preparations even when nothing in the declaration of contents suggests that they are included. Lack of quality control at the production stage of the preparations and/or product manipulation may be reasons for this. Even extremely low concentrations in dietary supplements, trace amounts, may lead to a positive doping test (Ayotte et al. 2001; Catlin et al. 2000; Geyer et al. 2000). A comprehensive study performed by the IOC in 2000 and 2001, where 634 dietary supplements from 13 countries were investigated, revealed that 14.8% of them contained prohormones that were not listed in the declaration of contents, but that could have led to a positive doping test (Geyer et al. 2004). Obviously, the problem with contaminated nutritional supplements has decreased since the US legislation has restricted the over-the-counter availability of nandrolone prohormones. However, it

is still discussed in numerous publications (de Hon and Coumans 2007; Parr et al. 2004; Tseng et al. 2005; Maughan 2005; Stepan et al. 2008).

In order to protect the athletes, many national anti-doping agencies and national Olympic Committees have developed programmes to assess the risk of ingesting a contaminated nutritional supplement (Australian Sports Anti-Doping Authority 2008; Olympiatoppen Norway 2004; United States Anti-Doping Agency 2003).

These programmes point out that athletes must be aware that there is a real danger that a dietary supplement may give rise to a positive doping test. Athletes themselves are responsible for what they take as dietary supplements. When a positive doping test is obtained, it is up to the athlete to prove that the intake was neither intentional nor careless. This proof may be difficult to acquire, and there is every reason to undertake a risk assessment before taking dietary supplements. In any case, if an athlete purchases nutritional supplements from companies which offer prohibited substances in their sales catalogues, he or she runs a very big risk.

7 Interferences

7.1 Administration of Progestins Derived from Norethisterone

As a result of an improved detection technique for nandrolone metabolites based on the formation of trimethylsilyl-enol-ethers prior to GC–MS analysis and its application to a large number of doping control samples, including some from females using contraceptives, a hitherto unknown peculiarity in the metabolism of the progestin norethisterone and its prodrugs (Schindler et al. 2003) (see Fig. 8) was discovered in the second half of the 1980s (de Boer et al. 1988). In addition to the major urinary metabolites formed by A-ring reduction (5β-tetrahydronorethisterone and to a lesser extent 5α-tetrahydronorethisterone), the GC–MS analysis revealed the typical signals of NA (and less NE) in low amounts. It could be demonstrated that the appearance of these typical nandrolone metabolites was the result of a metabolic reaction and not due to contamination of the parent drug or artificial formation in the analytical process. In a recent study the amount of 19-norandrostenedione as impurity in pharmaceutical preparations of norethisterone was found to be lower than 0.05% (Walker et al. 2009c). The pattern of both metabolic routes shows a distinct difference in the proportion of the 5α- and 5β-metabolites: while the 5β-isomer tends to be favoured in the case of the ethynylated compounds (5α- and 5β-tetrahydronorethisterone), in the de-ethynylated metabolites the 5α-isomer (NA) clearly dominates over the 5β one (NE). This indicates that nandrolone or norandrostenedione are formed as intermediate products by de-ethynylation of norethisterone prior to the A-ring reduction (see Fig. 9). After this metabolic pathway had been elucidated, the Medical Commission of the International Olympic Committee initially, in February 1987, prohibited the use of norethisterone, but revoked this ruling only half a year later in response to appeals from the United States Olympic Committee and the Canadian Sports Council. The main arguments

OH O **Norethisterone**

OH **Lynestrenol**

OH O **Norethynodrel**

OH HO **Ethynodiol**

Fig. 8 Norethisterone and its prodrugs

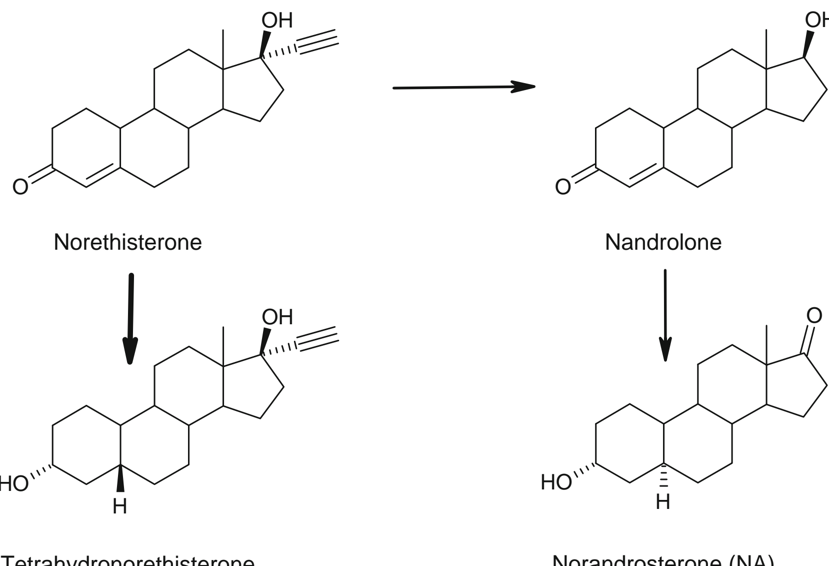

Fig. 9 Scheme of norethisterone metabolism: the A-ring reduction of norethisterone gives mainly the 5β-isomer of tetrahydronorethisterone, whereas after de-ethynylation to nandrolone to a minor degree the 5α-isomer norandrosterone (NA) is excreted into urine. A more detailed investigation of the norethisterone metabolism has recently been published (Walker et al. 2009b)

for this final decision were that an anabolic effect after norethisterone administration was assumed to be negligible and attempts to mask a simultaneous misuse of nandrolone could be discovered by monitoring the metabolic profile. This appeared reasonable if contraceptives commonly containing 0.35–1 mg of norethisterone (pure or as acetate) were used, resulting in levels of NA of about 2 ng mL^{-1} and less. However, it had been shown (Clausnitzer and Große 1988) that with higher dosages of administered progestin yielding urinary NA concentrations of 10 ng mL^{-1} and even higher, the medical use (5–15 mg and more daily in the treatment of menstrual dysfunction) could not be clearly differentiated from the fraudulent intention of veiling the late elimination phase of NA after injection of a nandrolone preparation. The actual impact of a scenario like this obviously remained insignificant, although at that time when unannounced out-of-competition testing was far from being reality, the potential of such masking could not be neglected. A recent large-scale investigation, where single untimed urines were collected from 1202 female volunteers, 38 of whom were taking norethisterone containing contraceptives, determined NA concentrations ranging from 0.5 to 4.1 ng/ml (Walker et al. 2009a).

7.2 5α-Reductase Inhibitor Administration

A different interference with the excretion of nandrolone metabolites may occur when the 5α-reductase inhibitors, finasteride or dutasteride, are administered (Thevis et al. 2007). 5α-Reductase inhibitors are mainly used to treat benign prostatic hyperplasia (Tarter and Vaughan 2006) and male baldness (Hoffmann 2003). They reduce the metabolism of testosterone to dihydrotestosterone, but affect in the same way the biotransformation of nandrolone. The drug will reduce the urinary excretion of NA to about 30%, while that of NE tends to be increased by a factor of 2–3. The presumption that the suppression of production and renal excretion of 5α-steroids such as NA may lead to false negative doping control results, because adverse findings are only reported when a threshold level of 2 ng mL^{-1} is exceeded, was therefore – disregarding the opposite impact on the 5β-steroids – more potential than real. Now, the 5α-reductase inhibitors are no longer prohibited (World Anti Doping Agency 2008b) and, as before, any shift in the steroid profile concerning the relation of corresponding 5α- and 5β-isomers of endogenous steroids will alert the analyst to have a closer look at NA and NE, if they are actually present.

7.3 Natural Nandrolone Production in Animals and Contamination in Meat

Nandrolone as a doping agent has not only been employed by people, it has also been used in connection with animal breeding and meat production (Vanoosthuyze et al. 1994). Natural nandrolone production has been proved in some species, such

as wild boar, non-castrated boars (van Ginkel et al. 1989), horses and sheep (Casson et al. 2006). The nandrolone levels are particularly high in tissues like testicles, livers and kidneys. Eating meat from animals that have been treated with nandrolone or which have endogenous nandrolone production can lead to the excretion of NA in urine (De Wasch et al. 2001; Debruyckere and Van Peteghem 1991; Debruyckere et al. 1990, 1993; Le Bizec et al. 2000). EU veterinary and food safety authorities are keeping a strict watch on this, and the likelihood of ingesting steroid-contaminated food must be considered slim. A group of experts appointed by UK Sports (Callicott and Kicman 2003) concluded that there was no significant risk involved in eating meat on ordinary sale nowadays, although it was possible to achieve NA levels that exceed the threshold value of 2 ng mL^{-1} via dishes based on offal from non-castrated boars or horses. It may therefore be wise for athletes, at any rate, to avoid such dishes.

8 Nandrolone Produced Naturally in the Body

The necessity to establish a threshold value for reporting NA as an adverse analytical finding is mainly related to a possible natural production of nandrolone followed by its metabolism in the body. The first reports of natural production of nandrolone in humans appeared more than 20 years ago (Dehennin et al. 1987, 1984). With the introduction of increasingly sensitive analysis techniques like high resolution capillary gas chromatography combined with high resolution mass spectrometry (HRGC–HRMS), in addition to more extensive sample preparation, it became possible to determine steroids like NA down to extremely low concentrations. Nowadays, the detection limits are a hundred times lower than they were 15–20 years ago. This led to the discovery that NA is naturally excreted in the urine of both women and men. There has been considerable interest in investigating the occurrence of endogenously produced NA, and by degrees many publications have appeared that describe such production, i.e. its natural origin in the body and the urinary excretion (Dehennin et al. 1999; Hemmersbach et al. 2006; Le Bizec et al. 1999, 2002a, b; Reznik et al. 2001; Robinson et al. 2001; Van Eenoo 1998, 2001). Questions arose as to whether this could result in false positive doping tests (Bricout and Wright 2004; Kohler and Lambert 2002), but analysis evaluation criteria and a reporting threshold try to resolve these problems (see Sect. 3.2).

8.1 Excretion of NA During Pregnancy

Enhanced excretion of NA has been observed during pregnancy (de Boer et al. 1993; Mareck-Engelke et al. 2000; Reznik et al. 1987). In one investigation (Mareck-Engelke et al. 2002) where 252 urine samples from pregnant females were studied, including a follow-up of five females during their whole pregnancy period, the excretion of NA rose during the pregnancy towards delivery. The first

appearance of NA above a detection limit of 0.2 ng mL^{-1} happened in week 14 of the pregnancy. NA was detected in about 60% of the samples taken. In 12% of those, the concentration exceeded 5 ng mL^{-1}, with a maximum value of 16.5 ng mL^{-1}. However, there is no risk of false positive tests, since the criteria for reporting an adverse analytical finding require the assessment of a possible pregnancy (see Sect. 3.2). This is done by determination of human chorionic gonadotropine (hCG) in the urine sample.

To find a possible mechanism for the production of nandrolone and the subsequent excretion of NA via the urine, further investigation of an NA excretion pattern in females during their menstrual cycle turned out to be of interest.

8.2 Excretion of NA During a Menstrual Cycle

Since nandrolone is produced naturally during pregnancy when great hormonal changes are observed, it was of interest to perform a more systematic investigation of the physiological excretion of NA in women. Urine samples from 12 volunteer women were collected daily throughout an entire menstrual cycle and the urinary concentration of NA in these samples was determined down to a detection limit of 0.05 ng mL^{-1}, 180 pmol L^{-1} (Hemmersbach et al. 2006). The results showed that all the women excreted NA in a characteristic pattern through their menstrual cycle.

A clear maximum in the NA concentration was observed immediately before ovulation, which is when the production of oestrogens is at its highest. The concentration in urine was significantly lower at the start of the follicle phase and at the end of the luteal phase than in mid-cycle. The timing of the maximum concentrations was clearly related to the increased mid-cycle excretion of luteinising hormone (LH), as shown in Fig. 10. However, the threshold value in a doping context, 2 ng mL^{-1}, was never reached in any of the samples. The highest concentration found was 0.8 ng mL^{-1}. A recent study with 1202 female volunteers recorded 1.7 ng mL^{-1} as the maximum urinary concentration, which needs to be compared with a corrected threshold of 2.6 ng mL^{-1} because of its high specific gravity of 1.026 (Walker et al. 2009a).

However, since the endocrine conditions in females obviously provide circumstances where natural production of nandrolone is more likely than in males, a uniform threshold value for males and females, as it was fixed in 2004, does not adequately reflect this biochemical background.

8.3 A Synthesis Path for Natural Formation of Nandrolone

Oestrogen synthesis is high during pregnancy and the mid-menstrual cycle. This led to the assumption that endogenous nandrolone is formed as a side-reaction to enzymatic aromatisation, i.e. conversion of androgens to oestrogens by the enzyme system, aromatase, as has been shown in granulose cells from pigs (Khalil et al.

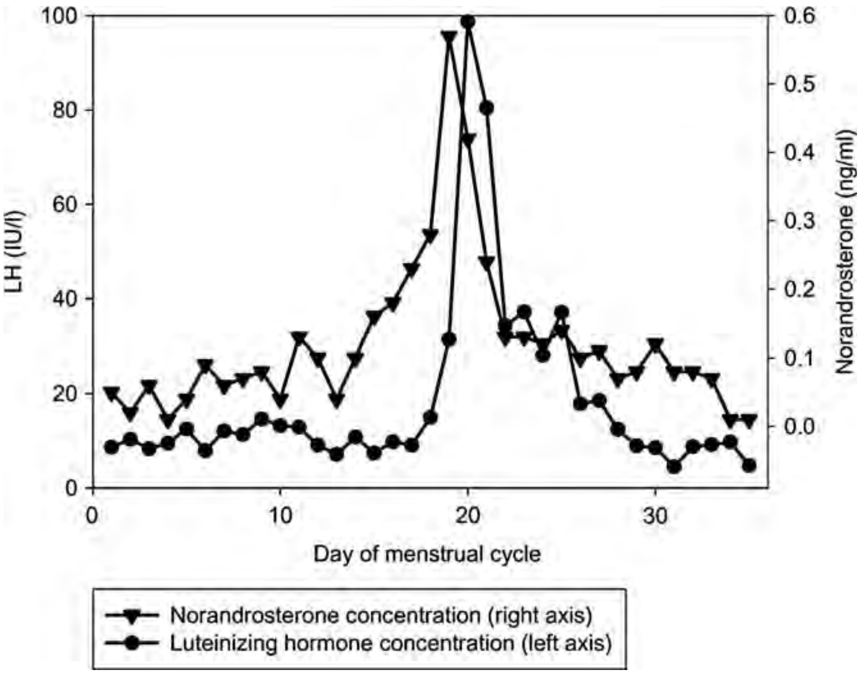

Fig. 10 Excretion of norandrosterone (*right axis*) and luteinising hormone, LH (*left axis*), through a menstrual cycle (from Hemmersbach et al. 2006)

1989), testicles from horses (Dintinger et al. 1989b) and in the uterus of horses and humans (Dintinger et al. 1989a). In order to understand the natural formation of nandrolone, the aromatisation of C-19 steroids such as testosterone and androstenedieone has to be looked into. Their aromatisation by means of the aromatase enzyme implies the loss of the angular C-19-methyl group and the formation of a phenolic A-ring (Kuuranne 2009). Several oxidation steps of the methyl group are proposed, catalysed by a cytochrome P450 enzyme complex, followed by the splitting off of formic acid. A final enolisation of the 3-keto group will result in the aromatic A-ring structure (Brueggemeier et al. 2005; Simpson 2002). The formation of nandrolone during this reaction mechanism is not fully understood, but a possible synthesis path is shown in Fig. 11. Investigations of follicular fluid have been undertaken (Lund et al. 2002) to study the aromatisation reaction. Follicular fluid contains significant quantities of aromatase and is thus a suitable medium for investigating this hypothesis, which was expressed already in early publications on the production of nandrolone in follicular fluid (Dehennin et al. 1987). The production of nandrolone and estradiol from granulosa cell cultures was studied in vitro following stimulation and inhibition of the enzyme system by, respectively, testosterone and the aromatase inhibitor, 4-hydroxyandrostenedione. The results indicate that the enzyme system, aromatase, is involved in the formation

Fig. 11 Proposed synthesis path (simplified) for the formation of nandrolone as a side reaction to the aromatisation of testosterone to estradiol

of both estradiol and nandrolone, and that nandrolone is formed as a by-product in the aromatisation reaction of androgens to oestrogens.

8.4 Physical Activity

The hypothesis of a correlation between hard training and increased excretion of NA and NE has been discussed for a long time. Many studies have been performed to cast light on whether the formation of NA can rise with physical activity (de Geus et al. 2004; Gambelunghe et al. 2002; Le Bizec et al. 2002a; Robinson et al. 2001; Schmitt et al. 2002) or metabolic stress (Reznik et al. 2001). The results are contradictory, and it is debatable whether there were enough test persons, their selection was representative, or the design of the trials was appropriate. When those studies were carried out, the possibility of in situ formation of nandrolone metabolites in urine samples (see Sect. 9) was not known and could therefore not be adequately taken into consideration. Consequently, the real potential of physical activity causing a

variation in the excretion of nandrolone metabolites remains inadequately clarified. However, current knowledge suggests that it is very unlikely that strenuous exercise alone will raise the level of NA above the threshold value of 2 ng mL^{-1}.

8.5 *Can Natural Production Be Distinguished from Doping?*

Several attempts have been made to distinguish analytically between endogenously produced NA and NA formed as a result of the administration of nandrolone or nandrolone precursors. Le Bizec et al. (2002b) proposed a hypothesis based on analysing both the glucuronide and sulphate conjugates. However, this theory could not be verified (Lund et al. 2004). The most promising attempt to distinguish between endogenous and exogenous NA was made with the help of GC-C-IRMS (gas chromatography–combustion–isotope ratio mass spectrometry). This technique has proved to be most useful for determining whether testosterone is of endogenous or exogenous origin (Shackleton et al. 1997a, b). It utilises the fact that the proportion of the ^{12}C and ^{13}C isotopes differs significantly between endogenous testosterone and testosterone of synthetic origin. Consequently, IRMS may also help to identify unambiguously the source of nandrolone metabolites in human urine samples (Ayotte et al. 2006; Hebestreit et al. 2006; Mathurin et al. 2001). This applies in the same manner to the identification of nandrolone metabolites in horse urine (Yamada et al. 2007). The challenge remains to achieve satisfactory sensitivity to be able to determine reliable carbon isotope ratio values at low concentrations of NA.

9 Changes During Storage of Urine Samples

In 2005, rare observations of NA and NE being formed from androsterone and etiocholanolone in stored urine samples leading to levels near and even above the threshold value of 2 ng mL^{-1} were reported (Grosse et al. 2005). This finding was confirmed by incubation experiments with deuterated analogues of androsterone and etiocholanolone. The subsequent detection of deuterated NA and NE clearly proved that in situ 19-demethylation had occurred (see Fig. 12). Androsterone and etiocholanolone, both terminal metabolites of testosterone, as substrates for this reaction are excreted in urine predominantly as glucuronides in the low g mL^{-1} range, i.e. roughly at a 1,000-fold excess compared to the threshold for NA. Because this conversion also takes place in the state of glucuronidation, it may exert an essential impact on nandrolone findings at a low level. The turnover was found to be significantly dependent on temperature, time and substrate concentration. As an important feature, the conversion of the 5β-isomer is favoured over the corresponding 5α-steroid by a factor of about 3, resulting in a reduced concentration ratio of NA to NE compared to that of the endogenous starting compounds,

Fig. 12 Scheme of the rare in situ formation of the nandrolone metabolites NA and NE in urine by 19-demethylation from the testosterone metabolites androsterone and etiocholanolone (Grosse et al. 2005)

androsterone and etiocholanolone. The precise nature of the driving force for this in situ process cannot be determined yet. It may be linked to an enzymatic activity presumably expressed by certain fragments of cellular or peptide origin or specific co-factors being present in those particular urine samples. This discovery meant that the potential instability of urine samples in respect of NA findings was immediately addressed and implemented into the practical work. For samples which exhibited the observed typical features of unstable urine, the criteria for reporting an adverse analytical finding were revised accordingly. It cannot be excluded that findings of NA caused by this uncommon demethylation have led to a small number of adverse reports in the years prior to this discovery. This underlines the importance of avoiding any delay in incorporating new scientific knowledge into the rules and regulations.

10 Summary

The above-mentioned factors show that a number of aspects are attached to the interpretation of a guaranteed finding of the nandrolone metabolites, norandrosterone and noretiocholanolone, in urine. Usually, only a single urine sample is available. It represents an isolated “metabolic snapshot”. Hence, the form of administration, the number of doses and the timing of intake can only be assessed to a limited extent, especially when inter-individual differences in metabolism are taken into consideration. Moreover, it is impossible to determine the precise

structure of the administered steroid. The laboratory is responsible for safeguarding the legal security of the athletes and protecting them against false positive finds. It secures proof that the doping regulations have been breached, using "state-of-the-art" analytical methods and the unambiguous and definitive identification of prohibited substances. WADA has published strict identification criteria to prove the presence of prohibited substances (World Anti Doping Agency 2003). When nandrolone metabolites are found with the help of chromatographic separation and mass spectrometric detection, further assessments are made before it can be ascertained that the athlete has taken a forbidden substance like nandrolone or its related prohormones:

– The concentration is evaluated against the threshold value of 2 ng mL^{-1}
– Possible pregnancy is excluded as regards women
– Permitted medicines that give rise to the same metabolites are excluded
– A GC-C-IRMS study may be performed
– The purity of a dietary supplement may be checked
– Possible instability during storage is excluded.

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Designer Steroids

Ray Kazlauskas

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Abstract Anabolic steroids have been studied for over 50 years and during that time numerous compounds with a variety of functional groups have been produced and many have been published. Of these only a small number have been introduced to the pharmaceutical market. WADA has continued the work begun by the IOC banning the use of these agents within sport as performance enhancing substances. Athletes, however, continue to use these anabolic steroids but tighter testing and the introduction of unannounced sample collection has made this form of cheating harder.

In order to try to evade detection, athletes who continue to dope are having to resort to the use of a far more dangerous form of drug – the designer steroid. These steroids are manufactured to closely resemble existing known compounds, but with sufficient chemical diversity to ensure that their detection by the WADA accredited laboratories is more difficult. A worrying feature of the use of these compounds is that no data is available to evaluate either the efficacy or the safety of these substances. Many such drugs are now being made in clandestine ways

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(as demonstrated by the recent BALCO case) and then passed on to athletes who become the guinea pigs determining the potential of the substances as doping agents.

Methods for the detection of these new compounds are being developed using emerging techniques such as gas chromatography or liquid chromatography attached to a variety of mass spectrometry instruments. This technology as well as vigilance by laboratories and enforcement agencies can all help in early detection of designer steroids being used for doping.

Keywords Anabolic steroids • WADA • BALCO • Supplements • Designer steroids

1 Introduction

The list of anabolic steroids banned for use by elite athletes has a long history dating back to 1974. The lists were for a long time prepared by the International Olympic Committee and then, with the formation of the World Anti-Doping Agency (WADA), the duty of deciding which substances should be banned passed on to WADA. Lists in general are finite entities requiring individual substances to be added as needed. This can be a slow process, especially when lists are reviewed once a year or less. The IOC overcame this problem by including the phrase "and related compounds" early in developing their list. This phrase later became "related pharmacologically and chemically compounds" in order to cover substances that acted in a similar manner to anabolic steroids but which would have required considerable argument about whether or were not they were related to anabolic steroids. The issue of β_2-agonists fell into such a void when it was realised that they were being abused within the European Union to increase lean muscle mass in animals in order to increase meat production and that this practise had spilled over into human sport. This prompted a change in the wording of the list from "anabolic steroid" to "anabolic agent". There occasionally occur new substances which are only covered by the term "pharmacologically related". These substances may have quite different chemical structures but are being developed for anabolic effects, e.g. SARMs (which were added to the WADA list in 2008) and so there will be need to add them to the WADA Prohibited List (WADA 2008, 2009) as individual substances or classes. Until they are added to the list, the coverage by the "related pharmacologically and chemically compounds" phrase will need to suffice. The inclusion of this phrase was an acknowledgement by the administrative bodies, even back in the very early stages in the anti-doping struggle, that science is constantly changing and new drugs are continuously being discovered and that such discoveries can be put to unintended purposes.

1.1 Exogenous Steroids

The WADA Prohibited List bans a variety of steroids but has detailed them into two separate groups – exogenous and endogenous. Both of these have important

features and quite different issues with regards to detection and so are dealt with separately. The list produced for the year 2008 has the exogenous substances listed as:

"1-androstendiol (5α-androst-1-ene-3β,17β-diol); 1-androstendione (5α-androst-1-ene-3,17-dione); bolandiol (19-norandrostenediol); bolasterone; boldenone; boldione (androsta-1,4-diene-3,17-dione); calusterone; clostebol; danazol (17α-ethynyl-17β-hydroxyandrost-4-eno[2,3-d]isoxazole); dehydrochlormethyltestosterone (4-chloro-17β-hydroxy-17α-methylandrosta-1,4-dien-3-one); desoxymethyltestosterone (17α-methyl-5α-androst-2-en-17β-ol); drostanolone; ethylestrenol (19-nor-17α-pregn-4-en-17-ol); fluoxymesterone; formebolone; furazabol (17β-hydroxy-17α-methyl-5α-androstano[2,3-c]-furazan); gestrinone; 4-hydroxytestosterone (4,17β-dihydroxyandrost-4-en-3-one); mestanolone; mesterolone; metenolone; methandienone (17β-hydroxy-17α-methylandrosta-1,4-dien-3-one); methandriol; methasterone (2α, 17α-dimethyl-5α-androstane-3-one-17β-ol); methyldienolone (17β-hydroxy-17α-methylestra-4,9-dien-3-one); methyl-1-testosterone (17β-hydroxy-17α-methyl-5α-androst-1-en-3-one); methylnortestosterone (17β-hydroxy-17α-methylestr-4-en-3-one); methyltrienolone (17β-hydroxy-17α-methylestra-4,9,11-trien-3-one); methyltestosterone; mibolerone; nandrolone; 19-norandrostenedione (estr-4-ene-3,17-dione); norboletone; norclostebol; norethandrolone; oxabolone; oxandrolone; oxymesterone; oxymetholone; prostanozol ([3,2-c]pyrazole-5α-etioallocholane-17β-tetrahydropyranol); quinbolone; stanozolol; stenbolone; 1-testosterone (17β-hydroxy-5α-androst-1-en-3-one); tetrahydrogestrinone (18a-homo-pregna-4,9,11-trien-17β-ol-3-one); trenbolone and other substances with a similar chemical structure or similar biological effect(s)."

The substances which are underlined in the above list have been added since 2004, which shows the rapid increase in clandestine use of new substances.

Previous lists often had an issue with nomenclature since the "common" name was used which was ambiguous, and so in the last few years WADA has taken the corrective action of including the chemically accepted name as well. This was particularly important when adding products recently advertised in the market place such as methyldienolone. The question quickly arose as to the positioning of the double bonds and the methyl group in this substance, as several products appeared on the market at about the same time with the same empirical formula and quite different double bond positioning.

1.2 Endogenous Steroids

The endogenous steroids were listed in the WADA List of Prohibited substances as: "androstenediol (androst-5-ene-3β,17β-diol); androstenedione (androst-4-ene-3, 17-dione); dihydrotestosterone (17β-hydroxy-5α-androstan-3-one); prasterone (dehydroepiandrosterone, DHEA); testosterone
and the following metabolites and isomers:

5α-androstane-3α,17α-diol; 5α-androstane-3α,17β-diol; 5α-androstane-3β,17α-diol; 5α-androstane-3β,17β-diol; androst-4-ene-3α,17α-diol; androst-4-ene-3α,17β-diol; androst-4-ene-3β,17α-diol; androst-5-ene-3α,17α-diol; androst-5-ene-3α,17β-diol; androst-5-ene-3β,17α-diol; 4-androstenediol (androst-4-ene-3β,17β-diol); 5-androstenedione (androst-5-ene-3,17-dione); epi-dihydrotestosterone; 3α-hydroxy-5α-androstan-17-one; 3β-hydroxy-5α-androstan-17-one; 19-norandrosterone; 19-noretiocholanolone."

The list also describes how to proceed with a finding related to an ingestion of a preparation containing a compound on the endogenous steroid list. The metabolites and isomers were also an important addition since some of these began to appear on the market as part of a series of "prohormones". Even though many of the metabolites may not have relevant activity, they are important as markers of androgen abuse and their detection indicates that a particular compound has been used.

Over the years the lists have tried to capture the many steroids which had been used medically, as well as having, over a period of time, seen the development of new compounds that have evolved either as part of the pharmaceutical industry or as new clandestine materials.

1.3 BALCO, an Insight into Conspiracy

The term "designer steroid" appears to have been coined at the time of the BALCO investigations. These occurred on 23 September 2003 when the Bay Area Laboratory Co-Operative (BALCO), owned by Victor Conte, was raided by agents from the US Internal Revenue Service Criminal Investigations Unit and the San Mateo County Narcotics Task Force. Records and materials were seized in that raid, and subsequent investigations revealed that Barry Bonds – a baseball record-breaking hitter, Kelli White – a double world sprint champion, Marion Jones – triple Olympic champion and Tim Montgomery – world 100 m record holder were listed as BALCO clients (e.g. USA Today 2007).

A prelude to this event occurred in 2002 when the UCLA WADA accredited laboratory led by Professor Don Catlin published the discovery of a new steroid, norbolethone, that appeared in the sporting arena (Catlin et al. 2002). The discovery of this compound was triggered by the appearance of a number of urine samples within that laboratory which showed extremely suppressed endogenous steroid profiles. Normal urines have a set of endogenous steroids that can be used as markers of normal steroid biosynthesis. These include testosterone (T) and epitestosterone (E) as well as their end metabolites, androsterone and etiocholanolone. The latter two normally occur at a high concentration. In the samples delivered to the laboratory these endogenous steroids were present at a low concentration without the urines being classified as dilute as determined by specific gravity measurements. Seeing unusually large numbers of these types of urine sample, the laboratory became suspicious that the athletes were taking something that suppressed steroid production. Further investigation of several urine samples

allowed the detection and confirmation that the causative compound was norbolethone. This was communicated to all laboratories and, on having a standard prepared at the National Measurement Institute (NMI) in Sydney, Australia, the detection of this substance became routine worldwide. Knowing this, athletes appear to have never used it again.

Some time before the raid on the BALCO premises the same UCLA laboratory received a syringe containing an oily material. The laboratory undertook a considerable amount of work to show that this oil contained another new steroid which they termed tetrahydrogestrinone (THG) (Catlin et al. 2004). The discovery of this substance was announced to the world through the United States Anti-Doping Agency (USADA) in September 2003 and soon afterward the reference material was prepared by NMI in Australia and distributed to all laboratories, and so, again, its detection became part of routine laboratory testing protocols. After the raid on BALCO, records showed that they were responsible for distributing the THG as a material called "Clear" which had been supplied to them through a chemist. BALCO had for some time been experimenting with designer steroids such as THG as well as mixtures of testosterone and epitestosterone which, through tests conducted at a local laboratory, were formulated to give a blend that gave normal T/E ratios after administration. The T/E ratio is a marker used within the WADA accredited laboratories to identify urines that were suspected to come from athletes taking testosterone as a doping agent. Thus it appears that BALCO was engaged in a conspiracy to undertake systematic doping through use of previously unsuspected materials (see also Fourcroy 2006).

The effects of the BALCO scandal were far-reaching within anti-doping. The directors of BALCO and the chemist (e.g. BALCO articles 2006) were prosecuted for their part in this conspiracy and were given sentences which did include a short period in detention. Information was obtained through Senatorial enquiry and through athletes informing on others involved. Some athletes were successfully sanctioned by USADA, who skilfully pieced together copious quantities of information, none of which involved an adverse finding in a urine or blood test, but which put together sufficient evidence of doping to allow a prosecution. This process for the first time led to the development of the "non-analytical positive" (USADA 2005) and has since prompted many national bodies to implement serious investigative processes within their mandate (Australian Sports Anti-Doping Authority (ASADA) Act 2006; see ASADA 2006).

The compounds uncovered in the BALCO incident, as well as other substances sold as "supplements", often appear to have been synthesised and published many years ago. As only limited data about biological activity is available, it may be of interest to see what discoveries were made in the early history of the drug industry investigating androgenic activity and the information available about their usefulness as potential designer steroids. The published data often only lists the substances as having anabolic or anti-oestrogenic activities using a particular animal or in vitro model. No information on toxicity or effects in humans is available for many published structures.

2 Anabolic Steroid Chemistry

Anabolic steroid structures are based on the male hormone testosterone, which is the main steroid responsible for the biological activity. The testosterone molecule shown in Fig. 1 has a number of positions which can be modified by addition/removal of double bonds, reduction of the keto group, and substitution using heteroatoms, halogens or addition of functional groups such as hydrocarbon chains and heterocyclic rings. Some common anabolic steroids banned for many years by the IOC and then WADA are shown in Fig. 2 to illustrate the successful changes that have been made and the drugs which have been brought to the medical field for use by androgen-deficient individuals. It is only when these drugs are abused by healthy athletes that an issue of chemical cheating occurs. The compounds linked to the BALCO scandal such as THG and norbolethone are shown in Fig. 3. These are shown together with trenbolone and gestrinone to highlight the close relationship between these molecules. This type of related compound synthesis is common in pharmaceutical chemistry where many modifications are made to an original active structure to generate new drugs with the desired or improved activity. The structure relationships for the activity are then mapped by testing the prepared compounds and finding the changes that retain the desired actions.

Both norbolethone and THG are related to the anabolic agent gestrinone which is available as a pharmaceutical product. Gestrinone has weak androgen and progestogen activity. It also possesses anti-oestrogen activity (eMIMS 2005). THG on the other hand had been reported (Death et al. 2004; Friedel et al. 2006) as having very potent anabolic activity. Since gestrinone is available as a raw material it may be a good starting material for synthesis of both norbolethone and THG. THG was prepared by hydrogenation of the acetylenic function at C17 in gestrinone and separation of the THG from dihydrogestrinone (Catlin et al. 2004; Thevis and Schänzer 2005). Norbolethone was originally synthesised as part of a total steroid synthesis (Smith 1965). It was available as a pharmaceutical in the 1960s but was discontinued and is no longer commercially available. It has been reported that norbolethone is more potent as an anabolic agent than testosterone propionate and also less androgenic (Albanese et al. 1968). These are structurally similar to other known androgens such as trenbolone (Fig. 3) and nandrolone (Fig. 2).

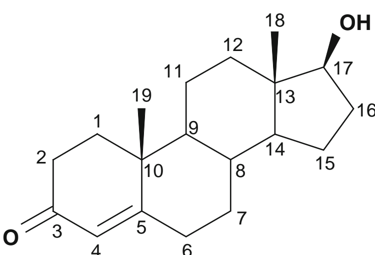


Fig. 1 Testosterone showing the numbering of the carbon atoms in the molecule

NANDROLONE

DIHYDROTESTOSTERONE

STANOZOLOL

METHANDIENONE

OXYMETHOLONE

BOLDENONE

DEHYDROCHLOROMETHYL-
TESTOSTERONE

FLUOXYMESTERONE

Fig. 2 Structures of some common steroids used available commercially

Fig. 3 Structures of designer steroids norbolethone and THG showing relationship to gestrinone and trenbolone

This relationship between gestrinone and other “designer” steroids is just one example of the types of modifications that have been based on the testosterone molecule. Early literature from the 1950s and 1960s shows numerous such examples together with data for the anabolic effects that were achieved. The literature does not however indicate why these substances were not introduced into the marketplace, especially since some had promising activities. The chemist now wishing to produce designer steroids can review these papers and find substances that will not be screened for. Of course this approach will be undertaken with almost no knowledge of the effects that the drug may have, both “beneficial” and adverse. The lesson from BALCO, where drugs were produced with very little or no appropriate human or animal studies being undertaken, is that this ethical consideration does not appear to be an issue and many steroids will be sold using athletes as the guinea pigs.

Early studies showed that certain functional groups within the testosterone molecule were essential for androgenic and anabolic activity (Klimstra 1969; Kochakian 1950, 1976). The 17β-hydroxyl group was essential but could be esterified for administration by intra-muscular injection and the biological properties were enhanced and prolonged simply due to reduction in the rate of elimination due to the slow release of the parent non-esterified steroid. Esters that are commonly used include the decanoate, undecanoate, propionate, enanthate and cypionate. Similarly the presence of the 4,5 double bond gave an increase in activity while reduction of the 3-keto group increased the androgenic activity but not the

ETHYLESTRENOL

NORETHANDROLONE

METHYLTRIENOLONE

METHYLDIENOLONE

Fig. 4 Structures of ethylestrenol, norethandrolone, methyltrienolone and methyldienolone

anabolic activity. However, removal of the 3-keto function while retaining the 4,5 double bond can retain anabolic activity, e.g. ethylestrenol (Fig. 4) marketed as Maxibolin (used in veterinary medicine). This compound can have an oxygen function reintroduced on metabolism to give 19-norandrosterone as well as metabolites of norethandrolone.

Removal of the 19-methyl group in testosterone led to the development of the 19-nor steroids such as nandrolone. It was apparently found to be as myotrophic as testosterone but with greatly reduced androgenic activity. In many cases similar functional changes to give analogues similar to those made for testosterone were studied for nandrolone leading to development of drugs such as norethandrolone (Nilevar), the 17α-ethyl analogue (Fig. 4).

The 5α stereochemistry at C5 is also important for retaining biological activity. For example, dihydrotestosterone (DHT), which is a metabolite of testosterone and is applied as a skin cream preparation such as Andractim, has the 5α stereochemistry and is considerably more potent than testosterone. However, other saturated metabolites such as androsterone and its diol analogue are not active.

Compounds which had a double bond introduced into the C1,2 position retained activity. The most orally active of these was methandienone (methandrostenolone, Dianabol, Fig. 2). It is interesting that methandienone is still one of the most popular steroids in use today, giving rise to many “positive” test results. This is

surprising since Ciba stopped manufacture of the agent in 1982; it means that it is available via an underground process, with many sources from Russia, China, Thailand and India. Addition of more double bonds into the 19-nor steroid molecule produced compounds such as methyltrienolone, trenbolone, gestrinone and recently the designer steroid THG. Methyltrienolone has not appeared as a drug for animal or human use but is often used as the reference point for in vitro biological activity. Another steroid, advertised as methyldienolone, appeared briefly on the "supplement" market but the product was suspect with no methyldienolone present in the formulation. It did contain numerous related unsaturated steroids, probably from poor manufacture.

Looking at the list of steroids that are in use today and those studied but not used commercially, it can be seen that many substituents can be added and activity retained. The compounds with addition of alkyl groups at C17 (methyl, ethyl, ethynyl) occur in preparations such as methyltestosterone, ethylestrenol and gestrinone. Addition of a methyl group at C7, C2, C1 gives compounds such as MENT (7α-methyl-19-nortestosterone), drostanolone and mesterolone (Fig. 5). MENT has very high biological activity (Dorfman 1968) and while it was studied as a possible male contraceptive (Nieschlag and Behre 2004) it has not been marketed and is very difficult to obtain. The analogous 7α-methyldihydrotestosterone was found to be more active as an androgen than testosterone (Wolf and Chang 1970). The 6α-methyltestosterone has low activity (Dorfman 1968). The analogue, 6α-methylandrostenedione, has been found in "supplements" together with androstenedione (Parr et al. 2008). Methylation in the 18 position has also produced compounds with pronounced anabolic activity. These 13-ethylgonan derivatives occur in several birth control pills having oestrogen/progestin activity, e.g. levonorgestral (Fig. 5), and have structures similar to closely related compounds THG, gestrinone and norbolethone while also have androgenic activity (McRobb et al. 2008). In fact, this tight inter-relationship between anabolic steroids, oestrogens and progestins is often seen through side effects from anabolic steroid use where some oestrogenic metabolites give rise to female characteristics. The development of these side effects in bodybuilders (and other athletes that have used steroids) have lead to the sale of compounds which have anti-oestrogen properties and which are publicised for use by steroid users to be taken at the same time to counteract unwanted effects. An example of this is the underground recommendation to take substances such as tamoxifen with steroids and this in turn led to the banning of compounds with anti-oestrogenic properties for use in sport.

The effect of addition of heteroatoms has been investigated but few compounds have reached the pharmaceutical market. The successful steroids have either chlorine or fluorine as substituents at the 4 or 9 position, such as in dehydrochloromethyltestosterone (DHCMT, oral-Turinabol), fluoxymesterone (Halotestin) (see Fig. 2), clostebol (as the acetate, Alfa-Trofodermin; Clostene; Megagrisevit; Steranabol, see Fig. 6) and 19-norclostebol (as acetate). DHCMT, which was developed for clinical use (Dörner 1965), was extensively used by the East German athletes in a state-controlled doping programme (Ungerleider 2001). Even in 2007 there is litigation against Jenapharm by some athletes from that programme for compensation

METHYLTESTOSTERONE

MENT

DROSTANOLONE

MESTEROLONE

7α-METHYLANDROSTENEDIONE

NORGESTREL

Fig. 5 Structures of some methylated steroids

for serious medical problems – infertility among women, embarrassing hair growth, breast cancer, heart problems and testicular cancer. An estimated 800 athletes developed serious ailments including a female shot-putter who took so many male hormones she decided to have a sex change (see Articles 2005). This scandal appears to be one of the first conspiracies using designer steroids, since DHCMT was developed for the East German programme and its problems are still manifest more than 50 years later. An expansive list of chlorinated and other 6-chloro substituted steroids and their activities has been published by Weichert et al. (1967), with many mainly having anti-androgenic activity.

Compounds with heterocyclic functional groups fused to the A ring are used as orally active anabolic steroids, some of which are still taken by sports persons even though they have been detectable for 20 years. The steroid stanozolol

(Winstrol, Fig. 2) was made famous by the Canadian runner Ben Johnson when he was found to have it in his system during the 1988 Seoul Olympics. This molecule contains the pyrazole ring fused to positions C2 and C3 and a 17α-methyl group and has very high anabolic activity, remaining a steroid "of choice" even today. The oral form is marketed for human use whereas an aqueous suspension for injection is used in the veterinary field. Much of the veterinary preparation is diverted for use by athletes for muscle development. A modification to the structure of stanozolol was made by introducing the 4,5 double bond to give BAS-71 (Albanese 1969; Dorfman 1968) and this compound retained activity. Recently the stanozolol analogue without the 17-methyl group has been sold on the "supplement" market as Prostanazol (see Fig. 6). Another such steroid, furazabol (Neo-ponden, Fig. 6), has an isoxazole ring instead of the pyrazole ring (Ueno and Ohta 1967), but is an uncommon steroid which was only used in Japan. The 17β-hydroxy analogue (without the 17α-methyl group) also had activity but has so far not been used in the pharmaceutical industry. The furazabol derivatives with a 16-methylene, 16β-methyl, 16α and 16β-hydroxy functional group were prepared but "did not exhibit anabolic activity comparable to that of the parent compound". Stanozolol appears to be about twice as potent as methyltestosterone but furazabol was 29 times as active orally in producing nitrogen retention in animals (Klimstra 1969).

OH
CH_3
O
Cl
CLOSTEBOL

OH
HN
N
H
PROSTANAZOL

OH
CH_3
N
O
N
H
FURAZABOL

Fig. 6 Halogenated and heterocyclic steroids

Other fused rings that have been prepared as part of steroid structure activity research include thiazoles, pyridines, pyrimidines, pteridines, oxadiazoles and pyrroles. While these may have lower activity it may still be possible that unscrupulous marketers may make and sell some of these substances.

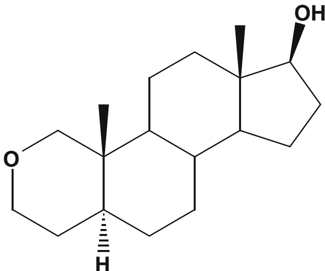
The A ring 2,3-thio-epoxide (10275-S, 2α,3α-epithio-5α-androstane-17β-ol, Fig. 7) was found to have anti-oestrogenic activity (which caused delayed implantation in the intact rat), an activity which was found to be surprisingly long-lived.

2α,3a-EPITHIO-5α-ANDROSTANE-17β-OL

2-THIA-A-NOR-5α-ANDROSTANE-17β-OL

7α-ETHYLTHIOSTANOZOLOL

SPIRONOLACTONE



2-OXA-5α-ANDROSTANE-17β-OL

Fig. 7 Heteroatom-substituted steroids studied for anabolic adrenergic activity

It also has androgenic and myogenic activity (Miyake et al. 1969; Miyake and Takeda 1967). Compounds with an A nor-heterocycle system have also been investigated (Wolf and Zanati 1969, 1970). These compounds related to 2-thia-A-nor-5α-androstane-17β-ol included heteroatoms of oxygen, selenium, tellurium disulphide, sulphone, sulphoxide. The sulphur and selenium analogues were active while the others were inactive (see Fig. 7). Further studies on A ring homosteroids containing a heteroatom showed that the sulphur derivative 3-thia-A-homo-5α-androstane-17β-ol (Fig. 7) was the most potent (Zanati and Wolf 1972), having similar activity to testosterone. The conclusion from these studies was that steric properties were dominant factors for biological activity. This was also found in the series of A ring oxa steroids in which the compound 2-oxa-5α-androstan-17β-ol (Fig. 7) was the most active (Zanati and Wolf 1971).

Substitution at C7 with alkylthio functions did not increase activity but did appear to improve myotropic activity relative to androgenic activity, especially in the 7α-thioethyl analogue of stanozolol (7α-ethylthio-17β-hydroxy-17α-methyl-5α-androstano[3,2-c]pyrazole, 7α-ethylthiostanozolol) (Kaneko et al. 1969, see Fig. 7). Other 7-thio-steroids such as spironolactone have quite different uses such as a diuretic, but also have anti-androgen activity due to their binding to the androgen receptor.

Other unusual modifications of the steroid molecule have led to a series of 5, 7-cyclosteroids such as 17β-hydroxy-17α-methyl-5,7β-cyclo-5β-androstan-3-one (Joska et al. 1968, see Fig. 8). The activity was not determined. The B-homo steroid Ba 36644 (A-nor-B-homo-7α-17α-dimethylestran-17β-ol-3,6-dione (Fig. 8) has been found to be very active (Klimstra 1969; Little 1970; Desaulles and Schärr 1967) but does not seem to have ever been used other than as an experimental drug. Recent studies into novel steroids show that interest has not waned, with production of the 13,14-seco steroids such as 17-hydroxy-13,14-seco-androst-4-en-3-one (Khripach et al. 2004) and a cyclobutane containing compound (Khripach et al. 2006) (see Fig. 8), but any activity data has not been reported yet.

This short précis of some of the anabolic steroid analogues that have been prepared clearly shows that many interesting compounds exist but with very little detailed published data on their biological activity other than some animal or in vitro testing for androgen-related mode of action. Data on activities such as toxicity and mutagenicity, which are required to be well documented in animal models before a drug can even be considered for human trials, is not available. Dealers in steroids, including those steroids present in the so-called supplements, are only interested in monetary gain and are not interested in long-term effects, or the fact that little or nothing is known about the molecule they are selling. The literature gives clues to a large variety of promising candidates for clandestine use. Most of the steroids studied in the past that have not been progressed to pharmaceutical products, have some limited information published but do not have important animal and human toxicity and side effect data published (or even had them undertaken), and so the studies are actually being conducted on the gullible/desperate community that buys products containing designer steroids. Thus, the only output of information on effects may appear in bodybuilding chat rooms and

17β-HYDROXY-17α-METHYL-5,7β-CYCLO -5β–ANDROSTANE-3-ONE

Ba 36644

17–HYDROXY–13,14–SECO–ANDROST –4–EN–3–ONE

"CYCLOBUTANE STEROID"

Fig. 8 Modified steroid ring structures

then in only barely understandable terms. Data for many compounds relating to oestrogenic, anti-oestrogenic or even anti-anabolic effects is not known, so problems such as those currently facing the older doped East German athletes will undoubtedly occur. This assumes that these modifications to the steroid molecule do not introduce an acutely toxic or life-threatening effect. in which case the data will be in the obituary columns. The athletes that take them may or may not be aware of these issues and they use them because they believe that they are not detectable.

3 Detection Methods

There are often complaints that testing lags behind the cheaters, but when the range of drugs that may be secretly used and which may provide some enhancement is taken into account, the testing laboratories have a big job to do to even try to ensure they are aware of some of the possible compounds they may need to detect. Current methods for analysis of steroids rely heavily on detection of individual drugs (Thevis et al. 2005; Thevis 2007). The detection methods were developed to give the highest sensitivity and specificity and so lack the ability to detect all of the new

drugs before their use is known. Thus, in order to cover as comprehensive a range of substances as possible. many approaches to the problem are needed.

There are two distinct ways in which drugs have been available for doping – those that are available as part of the commercial pharmaceutical industry and those that are made and sold in a clandestine manner. Clandestine usage implies a conspiracy with a number of individuals involved, which may include manufacturers, doctors, testing facilities and managers. This was seen in the East German national programme (Ungerleider 2001) and in the BALCO scandal. The conspiracy is difficult to detect as clues to usage may take some time to develop and identity of the drugs can be difficult to determined.

Commercially, steroids are produced by reputable pharmaceutical manufacturers for medical use and so will have full multiphase studies undertaken to prove efficacy and safety. However, more recently many designer steroids are sold by less reputable suppliers via internet sales. They sell designer steroids contained in "supplements" which contain compounds that have had none of the safety or efficacy studies required by pharmaceutical companies. These substances are often designed simply from similarity to published compounds and their pharmacological studies are performed by the gullible subjects who purchase them and then discuss them in chat rooms. In these cases the chemists who synthesise the compounds and the chemical companies that sell the raw materials, as well as the manufacturers, remain unknown. The purity of such substances is ill defined, often poor, which leaves considerable concern for the poor guinea pigs that are stupid enough to use them.

The contamination issues that arose late in the 1990s and well into this century are a strong warning to all about supplements. Many supplements were prepared, either by the chemical manufacturer or the supplement preparation company, with low levels of anabolic steroids present. The most common ones were 19-norandrostene-3,17-dione and 19-nor-androstene-3,17-diol, both of which were popular at the time as supplements. Both metabolise to the same products as nandrolone – 19-norandrosterone and 19-noretiocholanolone – resulting in an epidemic of nandrolone-positive results which ruined the careers of many athletes (Geyer et al. 2004; Schrader et al. 2006). The contamination may have occurred through poor practices when one batch of the steroid production was followed by preparation of materials which were appropriately sold as supplements (such as creatine). Low levels of the first batch remain in the equipment used for the next batch. Since detection of the nandrolone metabolites occurs at very low levels (less than 2 ng mL^{-1}), ingestion of low microgram levels of the analogues can give rise to an adverse finding.

Warnings against use of supplements were issued by many authorities but it took some time before manufacturers heeded these warnings and put in place appropriate strategies to combat contamination, while in the meantime the contamination issue caused considerable anxiety amongst athletes who did not know what was safe to take. Considering the general heavy use of supplements (Corrigan and Kazlauskas 2003) amongst athletes, most of which appears unwarranted, supplement use became a choice where an athlete had to decide the value of the risk, including

the possibility of returning an adverse finding in a doping control test. Often little advice was provided to them other than abstinence.

3.1 Use of Gas Chromatography–Mass Spectrometry (GC–MS)

Most anti-doping methodology up until recent times required the use of a multi-residue screening process which in turn predetermines exactly what is to be analysed. This allowed the detection of as many substances as possible with only a single extraction/sample preparation process. Thus the power of the mass spectrometer together with its software was used to allow simultaneous detection of most target steroids (and other compounds) in a single screen. The software allowed automated data analysis and a printout of the data that was relatively simple to analyse in a short time. This multi-residue detection processes required the use of a generic sample preparation protocol which covered all the range of compounds to be tested with a reasonable recovery. The method captured a wide range of compounds including neutral non-polar drugs, acidic and basic drugs. Most anabolic steroids are relatively non-polar and have similar properties which make them ideal for a multi-residue process.

The instrumentation generally used for the detection of steroids is the bench top GC–MS (often called an MSD or mass selective detector), a powerful selective detector with relatively user-friendly data reduction programmes. In order to achieve the low detection limits required for anabolic steroids (normally less than 10 ng mL^{-1}) in a fairly dirty matrix, it was necessary to use the instrument as a mass selective detector in the most sensitive mode possible, which for this instrument was the selective ion monitoring (SIM) mode. Alternatively by using a magnetic sector, high resolution mass spectrometer coupled to a GC it is possible to measure specific ions at higher resolution and so eliminate much of the background. This better selectivity coupled to the much higher sensitivity of these instruments allows the measurement of steroids and their metabolites at even lower detection limits without further cleanup of the sample (Horning and Donike 1994) and has been useful in detecting long-lived metabolites of commonly used steroids (Schanzer et al. 1996). Others have used GC–MS/MS techniques to try to increase sensitivity without further cleanup of the sample (Bowers 1997; Munoz-Guerra et al. 1977).

The bench top MSD was capable of running in two modes – full scan mode where at each time point a continuum of masses was scanned, e.g. *m*/*z* 40–650 in one mass increments, or SIM mode where a predetermined set of masses was scanned for an optimum amount of time. Due to hardware restrictions in earlier models, using SIM mode no more than 30 ions could be scanned at each time point. The data collection period (dwell time) for each ion was set such that a reasonable number of scans were obtained within any one chromatographic peak, usually no less than five scans across the peak. An example of such a set of ions is shown in Table 1 where a dwell time of 20 ms was set for all ions. If too few scans were collected then the apex of a peak would be missed and sensitivity reduced. For any substances that needed

Table 1 Typical set of groups and ions for each compound used in the GC–MS SIM analysis of steroids and other compounds

Group 1: 2–6 min *m*/*z*	Group 2: 6–10 min *m*/*z*	Group 3: 10–12 min *m*/*z*	Group 4: 12–15 min *m*/*z*	Group 5: 15–24 min *m*/*z*
86*	194	432*	446	421
356	432	435*	301	331
426	417	434	421	245
369	405	405	157	301
370	420	417	331	460
328	315	448	355	370
193	434	433	445	534
257	438	169	460	519
335	439	446	143*	389
337	429	431	449	143
82	414	430	464	495
240	236	415	466	550
361	448	206	451	254*
	358	462	468	560
	216	208	462	545
		357	447	544
		307	430	529
		412	325	157
		360	285	144
		345		435

Ions marked with an * indicate ions common to other substances within that group. The time range indicated the duration of collection of data for ions listed in that column. Usually three ions are monitored for each substance each with a dwell time of 20 ms. Group 1 has ions for terbutaline, salbutamol, probenacid, clenbuterol and benzoylecgonine; Group 2 has ions for metabolites of boldenone, methandienone and nandrolone as well as morphine, etiocholanolone, androsterone and deuterated internal standards; Group 3 has ions for testosterone, epitestosterone and deuterated standards, dihydrotestosterone, DHEA and metabolites of drostanolone, metenolone, boldenone, fluoxymesterone, trenbolone and methyltestosterone; Group 4 has ions for methyltestosterone internal standard, metabolites of norethandrolone, bolasterone, clostebol, superdrol, methyldienolone; Group 5 has ions for metabolites of norethandrolone, oxymesterone, oxymetholone, stanozolol, prostanazol and norbolethone

quantification, at least ten scans across the peak were needed. Further, in SIM mode the ions scanned could be changed a number of times within a chromatogram. A series of groups starting and ending at fixed times within the chromatogram could be established and each group would contain a set of up to three ions characteristic for each compound eluting within the time period of that group.

By cutting up a chromatographic run into short time groups of ions, many substances could be detected across a 25 minute run with data only being collected when needed for the compound of interest. The printout obtained with each analysis (Fig. 9 shows part of such a printout) using data collected for each mass measured, showed the ion intensities in one minute windows, and if the three ions expected for a compound lined up in the vertical axis at the correct retention time, then the result indicated the probable presence of that substance. The absence of three peaks that lined up was indicative of the absence of the substance within the level of detection

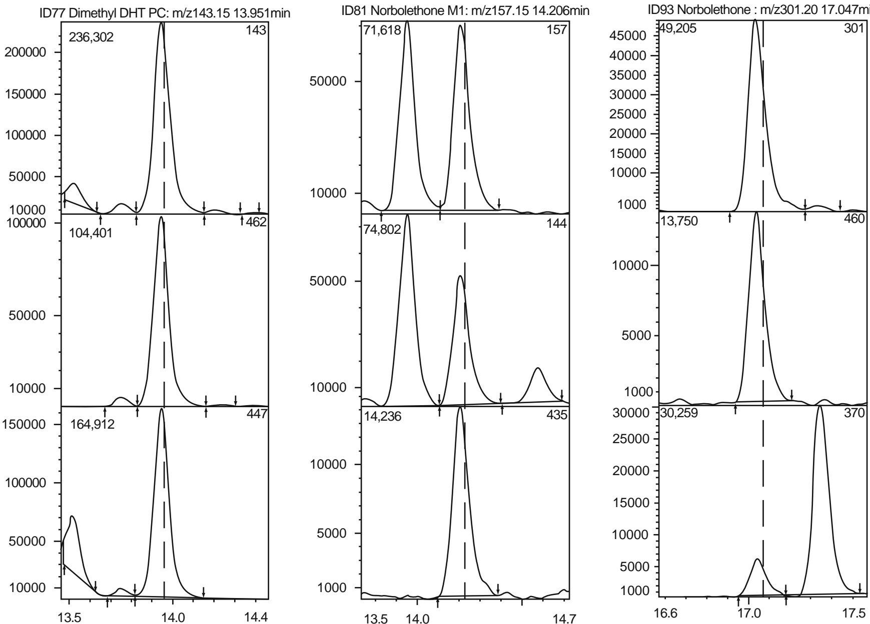


Fig. 9 Part of the printout for the GC–MS detection of steroids as TMS derivatives. Ions *m*/*z* 143, 462 and 447 were collected for Superdrol (labelled dimethyl DHT, left hand column) and ions *m*/*z* 157, 144, 435 and 301, 460, 370 were collected for norbolethone and its metabolite respectively. Thus if three ions line up at the correct retention time the sample is deemed suspicious and confirmation is required

for that substance. High sensitivity was required for anabolic steroids, including detection of any metabolites, to ensure the detection limits are in the WADA specified 1–10 ng mL^{-1} range, especially considering that the extract is a relatively crude one. The low detection level is needed to ensure that steroids which are used out-of-competition, well before the event, during the training phase, can have detection times with the longest possible retrospectivity.

The obvious drawback to this detection technique occurs when a new substance is used by athletes, as it is highly probable that the ions required for its detection will not be present in the group of ions measured in SIM mode at the retention time of the new substance or its metabolites. This is one reason why norbolethone was not detectable in the data collected during the 2000 Sydney Olympics, making it impossible to go back and re-analyse the electronic data for its absence or presence. Since samples had to be destroyed within 3 months of the Games, only the electronic data remains and this only contains the data for steroids targeted during the data collection. To subsequently include norbolethone into the steroid detection scheme it was required to obtain a standard of norbolethone, determine the recovery using the method used for sample preparation, determine the most appropriate ions for its detection and then enter the ions into the detection method at the appropriate retention time group. Only after this had been done could norbolethone use be determined.

It is important to understand the metabolism of the steroid, as well as detection of the parent drug, and to determine which is the best analyte for detection of the steroid. This entails undertaking an excretion study and collecting urine samples over a period of time. Each sample within this study is analysed for the parent drug and metabolites by comparing chromatograms for their presence after administration and absence of the compounds in samples taken before the administration. When all these studies have been completed it is possible to undertake the analysis on a routine basis, having good information about metabolism and some idea of detection limits and for how long the drug/metabolites can be detected after administration. It is then also important to obtain pure reference materials of metabolites, even though the WADA code does allow reference urine samples to be used for comparison purposes.

Norbolethone was initially detected by the UCLA Laboratory (Catlin et al. 2002) because of the observation that over a period of time a number of urine samples were being analysed which had greatly reduced levels of the endogenous steroids. This suppression of the endogenous steroid production is one of the effects that occur after anabolic steroid use, and with aroused suspicions the laboratory wisely began an investigation. They were able to obtain several urine samples which when analysed using the routine process, but using full scan mass spectrometry to obtain the data, showed several unusual unknown peaks. By analysis of the data they were able to elucidate the structure of the compound taken. The norbolethone study shows that when observations of a cluster of unusual events are made, they can lead to interesting findings even though these events are quite rare.

Recent improvements in the sensitivities of GC–MS instruments now make it possible to obtain data of sufficient clarity using full scan mode. Within NMI's WADA accredited laboratory in Sydney, using the more sensitive bench top GC–MS instruments, the steroid method is now run in full scan mode and the data obtained is similar to that previously obtained in SIM mode with similar levels of detection. This now means that for any new steroid that has a reasonable recovery using our standard sample preparation method, we can go back and interrogate the electronic stored data using retention time and ion data determined for the substance and its metabolites and ensure its detection. This retrospectivity will be very useful when information about new designer steroids is obtained. Knowledge about recovery in the method is very important, since for compounds such as THG where GC–MS gives poor data in the steroid method due to the complex interaction with the derivatisation reagent, the emerging technique of liquid chromatography – tandem mass spectrometry (LC–MS/MS) is required.

3.2 Use of LC–MS(/MS)

Over the last decade the technique of LC–MS/MS has slowly grown in importance in doping analysis (Thevis 2007). It was initially utilised for the more polar banned substances such as the diuretics and corticosteroids using the technique of

electrospray ionisation to produce charged particles. The use of electrospray ionisation (ESI) LC–MS/MS for anabolic steroid detection had been slowly introduced into laboratories as an alternative to GC–MS. In order to get good sensitivity with reduced background noise the technique uses MS/MS measurements after initial LC separation. Thus intense ions (precursor ions) formed in the ESI source are separated in MS mode, usually as the molecular ion with or without a proton ($M^{+}+1$ or $M^{+}-1$) depending on whether the instrument is working within positive or negative ion modes respectively, and then these ions are subjected to a collision-induced fragmentation with an inert gas to produce characteristic MS/MS spectra. It is then possible to measure either the full scan product ion mass spectrum or to measure only one or two specific product ions (multiple reaction monitoring (MRM) mode in a similar way to SIM in GC–MS steroid detection). The preferred technique often depends on the type of instrument used. Triple quadrupole instruments are often most sensitive in MRM mode while those having a quadrupole linked to the ion trap or quadrupole linked to a time of flight (TOF) detector will give full scan product ion spectra with good sensitivity. All these techniques are efficient in detecting a set of known steroids which are included in the screen, but suffer from the high selectivity of the technique which does not allow detection of steroids not targeted at the time. Since the precursor ion obtained for measured steroids will often be different for each steroid, it is unlikely to be the significant precursor ion in an unknown designer steroid present in the sample. Only if by coincidence the unknown steroid has the same precursor ion and product ion and also has a similar retention time as a compound measured and which is contained within the one minute time window printed, it may be detected as a spurious peak which will allow retrospective analysis of the electronic data. An example of a routine printout showing at least one MRM transition for each compound is shown in Fig. 10. This figure clearly shows the limited ability to go back and find a new substance.

Recent studies utilising TOF instruments allow the analysis of many types of compounds including a number of anabolic steroids (Georgakopoulos et al. 2007). By combining separate analyses using both GCoaTOFMS and LCoaTOFMS, most substances on the WADA banned list can be analysed in a high resolution mode. This procedure is quite useful in detection of designer steroids once it is know what steroid to investigate and readily gives good information on metabolism pathways. Since TOF instruments acquire full scan data only and can give good high resolution data, useful information relating to possible use is stored in the files. The use of the high resolution ability of these instruments reduces the background noise encountered in low resolution LC–MS scanning and so gives the ability to detect quite low levels of substances, for which the selectivity of LC–MS/MS would normally be required. The stored data can be re-interrogated at a later date, once information about new designer steroids is obtained. However the LC–MS spectrum is often very simple and mainly consists of the molecular ion. Thus interrogating the stored LCoaTOFMS data for the possible presence of a new substances may detect peaks that are different to normal “blank” urine data, but which may only stand out if present in high concentrations and then only provide a single ion

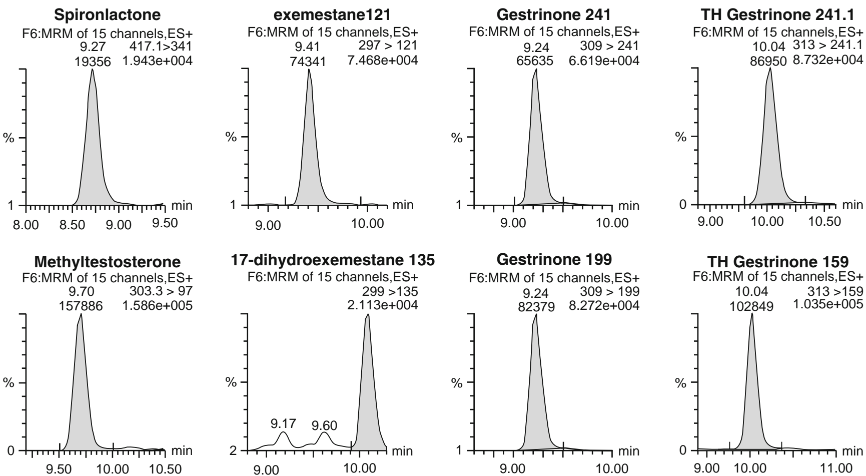


Fig. 10 Part of printout from LC–MS/MS screening for diuretics and steroids. The precursor ions measured in the region 8.5–11 min include *m/z* 417, 297, 309, 313, 303, 299 with only product ions *m/z* 341, 121, 241 and 199, 241 and 159, 97, 135 being measured in the MRM process

representing the molecular ion but giving little or no structural data. For compounds run under the GCoaTOFMS system, the full scan data will include fragmentation and so would also be informative for compound identification. The use of software that can compare profiles and detect changes would be useful for this search for designer steroids. The alternative retro-analysis at a later date for a specified substance for which the ionisation and retention time is known will provide a peak with the required molecular mass and so give an indication that it had been used. This would however be most unlikely to fit the three ion matching criterion required by WADA to prove the presence of a substance (WADA TD2003IDCR 2008).

A few methodologies that try to determine whether a steroid may be present in a urine sample, rather than first requiring knowledge about a new compound and then searching for it or its metabolites, are slowly emerging. An interesting process published recently (Thevis et al. 2005) uses the knowledge that related steroids often give MS/MS fragmentation patterns that give rise to common ion fragments. Thus for example the fragmentation of the M^{+}+H ions of testosterone (*m/z* 289), epitestosterone (*m/z* 289) and methyltestosterone (*m/z* 303) all give an ion of mass *m/z* 109. Thus by using a triple quadrupole instrument in precursor ion scan mode, chromatograms for all substances regardless of molecular weight which give an ion in the MS/MS spectrum of *m/z* 109 can be obtained and the precursor ion calculated. The base peak chromatogram in most instances will correspond to the M^{+}+1 ion of the substance giving the signal. By investigating many urine samples it is possible to obtain a library of information for endogenous steroids and other drugs using this technique. Thus if a urine sample gives a signal for the *m/z* 109 ion which does not match the retention time of any known endogenous

steroid or drug corresponding to the signal in the chromatogram, it can be investigated as a possible designer steroid, its molecular ion determined and then subsequent MS/MS data acquired and structural investigations undertaken. Thus by monitoring a relatively small number of product ions such as *m*/*z* 109, 241, 199, 227 and 187, several types of steroid structures can be followed and new designer steroids may be detected. Over time the range of ion monitored can be expanded.

Another novel approach is to use an androgen bioassay to detect the presence of a suspect substance in a human urine sample, followed by LC-QTOFMS investigations to determine its structure (Nielen et al. 2006). Reporter Gene Bioassays have been used to determine androgen, oestrogen and progestin activity in a variety of steroids (Death et al. 2004) and are logical assays for investigating designer steroids. The Nielson group developed their own system (Bovee et al. 2008) which they used for this work as well as a bioassay described by McDonnell (Gaido et al. 1997). Their process consisted of an initial sample cleanup and testing of the extracts using the bioassay. If a suspect result was obtained, the further investigation used an LC/bioassay where fractions were collected at 20-s intervals and these fractions were then subjected to their yeast-based androgen assay. This gave an androgen bioactivity chromatogram, and samples corresponding to bioactive "peaks" were analysed by LC-QTOFMS to give elemental composition of substances in the bioassay fraction. Subsequent LC-QTOFMS/MS analysis can give further information and some details of the structure elucidated. This approach to detection of designer steroids is promising but suffers from criticisms such as: background activities in urine from endogenous sources masking activities of drugs; many anabolic steroids are heavily metabolised and their metabolites (even after hydrolysis of the glucuronides) do not necessarily have androgen activity in their test system; the final difficulties in identifying the structures of bioactive substances; and, most difficult, that urine samples collected for doping control may only have very low levels of the substances left in them due to length of time after sample ingestion. This makes testing in an unexpected manner as in out-of-competition testing more likely to yield a result, as the doping would be occurring at the time of the test and concentrations of analytes could be much higher and more easily detected.

Another intriguing approach was published (Yuan and Forman 2005) where steroids extracted from serum were incubated with agarose-bound androgen receptor ligand binding domain. Compounds which will bind to the androgen receptor will remain on the beads and unbound material is washed away. Removal of bound ligands with PBS buffer which were extracted with organic solvent and analysed by GC–MS as TMS derivatives. This was successful in identifying parent drugs in serum since these often have high binding ability. The utility of this process needs to be challenged in urine since serum is not usually available in doping control, and the much higher concentrations of endogenous steroids would possibly overload the beads, which appear to have very low binding capacity more suitable for the low levels of androgens found in blood.

4 The Supplement Market

In order to try to predict which substances are likely to be found as designer steroids, several laboratories have used sources such as their Customs Officials to provide items seized at the border. These in turn often reflect the supplements sold by numerous bodybuilding supplement websites. For example Madol (Sekera et al. 2005) was received by the UCLA WADA accredited laboratory in Los Angeles and at almost the same time by the WADA accredited Montreal Laboratory which labelled it as DMT. The Canadian sample came from a Customs seizure. This compound was identified as 17α-methyl-5α-androst-2-ene-17-ol (together with the 3-ene isomer).

When one looks at websites selling supplements, especially those which appear to be operating out of the United States, it quickly becomes evident that some of the "supplements" blatantly contain steroids and are sold as having anabolic or anti-oestrogenic properties. While the United States has legislated to regulate the sale of anabolic steroids (Anabolic Steroids Control Act 2004), the vendors still persist in selling such compounds. By altering the position and types of substituents on the steroid backbone, e.g. methyl and keto groups, a wide range of designer drugs which may or may not have biological activity can be produced (as discussed earlier in this chapter). It does not appear to matter whether there is scientific evidence that these are benign, toxic or anabolic, but the vendors often provide long essays about the product as to why the use of this material is great for producing muscles or reducing side effects. Some of these "supplements" are also sold as anti-oestrogenic compounds to try to convince buyers that they will overcome the side effects of the other steroids being used. An example of such an explanation can be seen at sites such as <http://www.bodybuilding.com>, often appearing to contain "expert" chemical/pharmacological information as was provided for 1-testosterone:

> "The absence of a 4,5-double bond and its replacement with two hydrogen atoms is something that occurs naturally in the body by way of an enzyme called 5-alpha-reductase. This is the same enzyme that makes DHT (Dihydro-testosterone) from testosterone. The resulting hormone is in all cases incapable of forming oestrogen, allowing it to give a user smaller but much leaner gains, and add a look of hardness to the muscle for people with a relatively low body-fat. In most cases (nandrolone to dihydronandrolone being the exception) the new hormone is more androgenic. To sum up, it has reduced oestrogenic and increased androgenic activity. This allows for increases in strength and aggression, reduction of body-fat, and a leaner look to the physique. If we replace the dihydro structure with a 4,5-double bond, then we would see that 1-testosterone is in fact a 5-alpha-reduced version of the hormone boldenone, a testosterone analogue with an added 1,2-double bond that is characterised as being much milder than testosterone, both oestrogenically and androgenically. Which would make 1-testosterone a non-aromatizing hormone, that is androgenically milder than DHT (less aggressive on hair loss and acne), but due to its altered structure is also much more active than DHT, which is readily deactivated. So dihydro-boldenone would have been, at least from a structural viewpoint, a much better description."

And similarly for methyl-1-testosterone (presumably methylated on the 17α position) the vendor's "expert" writes:

> "Methyl-1-Tesosterone (as well as 1-Tesosterone) is a substance with no currently known pharmaceutical/medical application. Because this hormone was discovered only recently (and somewhat accidentally) it has yet to be studied by formal academia. However, substantial anecdotal evidence exists (from the bodybuilding community) that Methyl-1-Tesosterone is powerful and yields gains similar to a reasonable dosage of illegal steroids. Many past substances have come with similar claims attached, only to disappoint. However, users of methyl-1-tesosterone have noted routine gains of up to 10 lbs in lean muscle mass in a 2 week period. These gains would be considered the average (mean) amongst users, and have been seen to occur in large numbers. Methyl-1-Tesosterone, then, is most obviously a muscle building substance."

An interesting thing to note is the obvious misspelling of testosterone in the text above. This is similar to spam where misspelling circumvents search engines and thus authorities take time to find the true identity of the substance, especially when non-chemists are involved in administering the legislation. Steroids should be correctly named as derivatives of androstane. However, authorities recognise this "androstane" nomenclature quite readily and so quickly can take action to prevent sale. However, nomenclature such as alloetiocholan – or alloetiocholeve – or even as perhydrophenanthene derivatives, when used, hides the true nature of what is being sold. It is also of interest that substances sold as supplements are often listed as unavailable a short time after the first appearance on the web. This implies they have been discovered and forced to stop sale. They resurface for sale after a short while often with different trade names. Table 2 contains products sold as supplements in 2006 and which contain steroids. Many of those available in 2006 are no longer available in 2008.

The US Anabolic Steroids Control Act of 2004 should have made the sale of such compounds in the USA much more difficult. It seems however that the market in steroids continues to be a cat and mouse game. Compounds such as halodrol (Van Eenoo and Delbeke 2006) appeared in supplements such as Halodrol-50 from Gaspari Nutrition <http://www.halodrol-50.com/>, which is now advertised as being replaced by H-Drol (polydehydrogenated, polyhydroxylated halomethetioallocholane) as a substitute for the discontinued Halodrol.

The same company produced Orastane-E labelled as containing [3,2-c]pyrazole-5α-etioallocholane-17β-tetrahydropyranol, 25 mg. This material was also sold as Prostanazol by Alri, available in the USA a year ago but now appearing to be advertised out of the UK. Prostanazol is excreted as a complex mixture of hydroxylated compounds in which the tetrahydopyranol derivative is hydrolysed to the 17-hydroxyl group and then this 17-hydroxy group has been oxidised to the ketone. Monohydroxylation appears to be at the 3′-, 4- and 16α-positions (Kazlauskas 2006; Rodchenkov et al. 2006).

Superdrol (now advertised as S-drol) by Anabolic Xtreme and Methasterone by Legal Gear contain 2α,17α-dimethyl-5α-androstane-17β-ol-3-one (Fig. 11), and this compound was placed on the WADA prohibited list in 2006. Ths also appears to be discontinued but can still be part of supplements such as Super Halo. Some data on its metabolism is available and it is excreted unchanged as well as producing a metabolite, 2α,17α-dimethyl-5α-androstane-3,17β-diol (Kazlauskas 2006).

Table 2 List of supplements containing steroids bought in 2006 (many are no longer available)

Supplement 2006	Main steroid ingredient	Advertised ingredient
Hot-Rox	Androst-5-ene-3,17-diol-7-one diethylcarbonate	3,17-Dihydroxy-delta-5 etiocolane-7-one diethylcarbonate
ALRI Impact Ultra	Formestane acetate, 4-hydroxyandrost-4-ene-3,17-dione acetate	Formestane acetate
	7α-Hydroxy-DHEA acetate	7Alpha-hydroxy-dehydroepiandrosterone acetate
	7b-Hydroxy-DHEA acetate	7Beta-Hydroxy-dehydroepiandrosterone acetate
LG Methylmasterdrol	2α,17α-Dimethylandrostane-3-one-17β-ol	2a, 17a di methyl etiocholan 3-one, 17b-ol
AX Superdrol	2α,17α-Dimethylandrostan-3-one-17β-ol	2a, 17a di methyl etiocholan 3-one, 17b-ol
AX ergo max LMG	Madol (17α-Methylandrost-2-ene-17β-ol)	17-Methyl-delta-2-etioallocholane-17-ol
AX Phera-Plex	Madol (17α-Methylandrost-2-ene-17β-ol)	17a-METHYL-etioallocholan-2-ene-17b-ol
GN Novedex	Androst-4-ene-6,17-dione-3-tetrahydropyranyl ether	6, 17-Keto-etiocholeve-3-ol tetrahydropyranol
	Androst-1,4,6-triene-3,17-dione	3, 17-Keto-etiochol-triene
SAN attitude	Androst-1,4,6-triene-3,17-dione	3,17-Dioxoetioallocholan-1,4,6-triene
	Androst-4-ene-6,17-dione-3-ol	6,17-Dioxo-etiocholene-3-ol
DS ReboundXT	Androst-1,4,6-triene-3,17-dione	3,17-Dioxoetioallocholan-1,4,6-triene (diene-3)
LG Methyl 1-P	6α-Methyl-androst-4-ene-3,17-dione, including many other compounds	6-Alpha-methyl-etiocholene-3,17-dione 17-Hydroxy-6-alpha-methyl-ethyletiocholan-3,20 dione
LG Formadrol	6α-Methylandrost-4-ene-3,17-dioneAndrostenedione	6-Alpha-methyl-etiocholene-3,17-dione
AX PCT	Androstane-6-acetoxy-3-ol-17-one	6-Acetoxy-3-hydroxy-17-keto-etioallocholane
	17α-Methylandrost-1,4,6-triene-17β-ol-3-one	17a-Methyl-17b-hydroxyl-3-keto-delta 1,4,6-etioallocholtriene
AX prostanozolol	[3,2-c]Pyrazole-5α-androstan-17β-tetrahydropyranol	[3,2-c]-Pyrazole-5alpha-etioallocholane-17beta tetrahydropyranol
GN Orastan-E	[3,2-c]Pyrazole-5α-androstan-17β-tetrahydropyranol	[3,2-c]pyrazole-5alpha-etioallocholane-17beta tetrahydropyranol
Promatrix Primobolan acetate	Androst-4-ene-3,17-dione-4-ol Acetate	4-Androsten-4-ol-3beta,17beta-dione Acetate

2α,17α–DIMETHYL-5α-ANDROSTAN-17β-OL-3-ONE

ANDROST-1,4,6-TRIENE-3,17-DIONE

2α,3α-EPITHIO-17α-METHYL-5α-ANDROSTAN-17β-OL

ESTRA-4,9-DIENE-3,17-DIONE

Fig. 11 Structures of some supplement ingredients

The substance with 6α-methyl substitution (6α-methylandrostenedione, Fig. 5) was sold as Formadrol, and an ingredient in Methyl 1-P. This compound is mainly metabolised to 6α-methylandrost-4-ene-3-ol-17-one and 6α-methyl-5α-androstane-3-ol-17-one. The later metabolite has similar retention time and mass spectrum to the metabolite of drostanolone but can be easily differentiated as monoTMS derivatives (Parr et al. 2008). The supplement also contained large amounts of androstenedione.

Compounds where manipulation of the double bonds has been made to resemble drugs such as boldenone occur in products such as Novedex XI and Rebound XT which both contain androst-1,4,6-triene-3,17-dione (Fig. 11). This substance is largely excreted intact and is detectable for at least 24 h after a single dose. It has been shown to have activity as an aromatase inhibitor (acting as a suicide substrate) (Schwarzel et al. 1973; Covey and Hood 1981). The 6-phenyl analogues have also been studied (Numazawa and Yamaguchi 1999) and were potent competitive inhibitors of aromatase in human placental microsomes.

17α-methylandrost-2-ene-17β-ol (Madol) (Sekera et al. 2005) appears in a number of supplements currently available via the internet as well as some available earlier in 2006 (Table 2).

New products such as Epistane from IBE, Humaguno (Spectra Force), Epidrol (Genera Supplements) contain "2,3α-Epithio-17α-methyletioallo cholan-17β-ol" which appears to be the 17α-methylated derivative of 2α,3α-epithio-5α-androstane-17β-ol (Fig. 11) (Miyake et al. 1969). The trenbolone analogue estra-4, 9-diene-3,17-dione is also now present in many supplements.

The issue of incorrect labelling of ingredients is evident in several currently sold products. In fact, several of these appear to be reaction mixtures with poorly characterised products and it may be that the manufacturer does not know what was made! The furazabol analogue mentioned previously (Ueno and Ohta 1967) is listed as the ingredient in Furazadrol (Axis Labs) labelled as "5a-etioallocholan [2,3-c]furazan-17b-tetrahydropyranol ether" and appears to be sold as a substitute for prostanozol, which may now be more difficult to obtain and has also been placed on the WADA prohibited list. However the material in this product is not a [2,3-c] furazan and work in both the WADA accredited laboratories in Moscow and Cologne have show that the main steroid components appears to be a mixture of compounds related to danazol (unpublished data). Further, chlorinated compounds claimed to be similar to dehydrochlormethyltestosterone are listed as ingredients in several new products, but these contain a complex mixture of steroids only some of which are chlorinated. The many products that are incorrectly labelled as well as being complex mixtures of steroids show that, overall, steroids in supplement products very often contain poorly made and impure raw materials with ill defined composition, which increases the danger of using these materials.

5 Conclusion

The menu of steroids available over the internet in the guise of supplements has changed and many of the "older" steroid supplements are no longer easy to obtain or are out of stock, but still appear to cycle through website advertising for short periods of time, and are available under different names or in different countries where laws are more flexible. The supplement manufacturers will continue to evade the laws and sell steroids which are variants of the numerous compounds studied and published. Most of the web-based sales advertising is aimed at the "body-beautiful" industry to satisfy the every increasing market of those interested in bodybuilding. The advertising makes fantastic claims and tries to back it up with statements that appear to be scientifically written but often with no credible backing. By constantly monitoring the materials advertised on the web and discussed in chat rooms, it will be possible to ensure that the new products will rapidly be incorporated into any sports anti-doping testing programme. This at times is not a trivial task due to the poor production processes giving complex mixtures which may not be related to the structures listed on the label. This, coupled with emerging techniques to detect more clandestine materials in the urine collected as part of routine anti-doping programmes, will allow the WADA accredited laboratories success in the battle against the use of designer steroids in sport.

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Growth Hormone

Martin Bidlingmaier and Christian J. Strasburger

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Abstract Human growth hormone (hGH) is a proteohormone secreted by the pituitary gland. It acts through binding to the hGH receptor, inducing either direct effects or initiating the production of insulin-like growth-factor I (IGF-I), the most important mediator of hGH effects. Growth hormone is primarily known to promote longitudinal growth in children and adolescents, but has also various important metabolic functions throughout adult life. Effects of hGH on the adult organism are well established from studies with recombinant growth hormone (rhGH) therapy in growth hormone deficient subjects. In this particular group of patients, replacement of hGH leads to increased lipolysis and lean body mass, decreased fat mass, improvements in VO_{2max}, and maximal power output. Although extrapolation from these findings to the situation in well trained healthy subjects is impossible, and controlled studies in healthy subjects are scarce, abuse of hGH seems to be popular among athletes trying to enhance physical performance. Detection of the application of rhGH is difficult, especially because the amino acid sequence of

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rhGH is identical to the major 22,000 Da isoform of hGH normally secreted by the pituitary. Furthermore, some physiological properties of hGH secretion also hindered the development of a doping test: secreted in a pulsatile manner, it has a very short half-life in circulation, which leads to highly variable serum levels. Concentration alone therefore cannot prove the exogenous administration of hGH.

Two approaches have independently been developed for the detection of hGH doping: The so-called "marker approach" investigates changes in hGH-dependent parameters like IGF-I or components of bone and collagen metabolism, which are increased after hGH injection. In contrast, the so-called "isoform approach" directly analyses the spectrum of molecular isoforms in circulation: the pituitary gland secretes a spectrum of homo- and heterodimers and – multimers of a variable spectrum of hGH isoforms, whereas rhGH consists of the monomeric 22,000 Da isoform only. This isoform therefore becomes predominant after injection of rhGH. Specific immunoassays with preference for the one or the other isoform allow analysis of the relative abundance of the 22,000 Da isoform. Application of rhGH can be proven when the ratio of this isoform relative to the others is increased above a certain threshold. Because the "marker method" and the "isoform method" have a different window of opportunity for detection, complementary use of both tests could be a way to increase the likelihood of detecting cheating athletes.

Keywords Growth hormone • Recombinant • Immunoassay • Isoforms • Collagen • Bone • IGF-I • IGFBP-3 • ALS

1 Intended Pharmacological Effect

The assumption that hGH is abused in sports is mainly based on singular cases where either former athletes have confessed to abusing hGH or hGH was found in the possession of active athletes or trainers by police and customs officials. These cases, but also rumours, obscure literature (Duchaine 1983) and anonymous surveys (Rickert et al. 1992), all imply that hGH is abused because it is a performance enhancing drug and an anabolic agent. However, within the scientific literature, the effects of hGH application in normal healthy adults are far from being generally accepted. Effects of hGH on the adult organism are best studied in hGH-deficient adults, where replacement of hGH clearly shows effects on several physiological functions. However, extrapolation from these findings to the situation in healthy, well trained subjects with intact pituitary hormone secretion is difficult or impossible, and controlled studies in healthy subjects are scarce. Although robust data from studies are missing, it is obvious that several effects of hGH could potentially be useful for cheating athletes (Gibney et al. 2007), with the intended effect being dependent on the sports discipline (Wallace and Cuneo 2000):

1.1 Effects on Bone and Growth

Promotion of longitudinal growth clearly is the main effect of rhGH in children, but it also has effects on bone in later life (Ohlsson et al. 1998; Shalet et al. 1998). One might speculate that increasing height in children or adolescents could be advantageous if a career is planned in disciplines like basketball or high jumping. Although no numbers are available on the frequency of doping in children and adolescents, this issue is obviously of concern (Nilsson et al. 2005; Calfee and Fadale 2006; Buzzini 2007; Matich 2007).

1.2 Effects on Substrate Metabolism and Body Composition

Effects of hGH on substrate metabolism have been known for many years (Russell 1957). In particular, the fact that hGH is a potent lipolytic agent (Hansen et al. 2002; Schoemaker et al. 2002) makes it very interesting for the cheating athlete. Hyposomatropism reduces lipolysis in abdominal obesity (Buijs et al. 2002a). Potentially increasing muscle mass at the cost of a decrease in fat mass is definitely what many athletes want to achieve. However, from a scientific point of view it is important to keep in mind that most data were obtained from studies in hGH-deficient patients. For example, it is well documented that in hGH-deficient adults recombinant hGH replacement therapy leads to profound changes in body composition (Salomon et al. 1989; Jorgensen et al. 1994, 1996; Gibney et al. 1999), the effect being more pronounced in males than in females (Ezzat et al. 2002). In trained healthy subjects only a few controlled studies have been performed on this topic, with much less pronounced results: one study using comparatively high doses showed a decrease in body fat and an increased fat-free body weight (Crist et al. 1988). Furthermore, dose-dependent changes in body composition were observed in a double-blind placebo-controlled study in subjects without GHD with increased muscle mass (Crist et al. 1991). The small increase in fat-free mass in the GH-treated groups in another study was explained as a consequence of fluid retention or accumulation of connective tissue rather than accretion of contractile muscle protein. A placebo-controlled 6-week trial in power athletes did not demonstrate a significant effect on body composition (Deyssig et al. 1993). Recently, a 12-week double-blind placebo-controlled study in healthy women was conducted, showing a marked increase in energy expenditure and fat combustion (Lange et al. 2000). However, the participants in this study were elderly women, and it is difficult to extrapolate those finding to the situation in highly trained athletes. In conclusion, the well established lipolytic effect of recombinant hGH administration is most likely dependent on the individual body composition before treatment, and attenuated in healthy trained adults in comparison to GHD patients.

1.3 Effects on Protein Turnover, Muscle Mass and Strength

Growth hormone is known to stimulate protein turnover, and in some studies it is considered a strong anabolic agent. This might be the most important reason for its popularity in sports. Again, starting with lessons learned from treatment of hGH-deficient patients, it is obvious that hGH has an effect on muscle mass. Reduced skeletal muscle mass and muscle strength are found in GHD adult patients (Cuneo et al. 1990, 1992; Sartorio et al. 1995), and are reversible by replacement therapy with hGH (Salomon et al. 1989; Cuneo et al. 1991a; Jorgensen et al. 1994, 1996; Johannsson et al. 1997; 1999). On the other hand, a chronic excess of hGH such as in acromegaly leads to voluminous, but functionally not improved skeletal muscles (Nagulesparen et al. 1976). In an animal model of GH transgenic mice, growth hormone was less effective in increasing muscle weight than body weight, and muscle strength did not increase proportionally with muscle weight (Wolf et al. 1995). Comparing muscle contraction capacity in childhood-onset GHD patients to that of normal controls raised doubts that weakness and fatigability in GHD patients originate from the skeletal muscle itself (Bottinelli et al. 1997). In obese and normal-weight healthy women, it has been shown that hGH administration can block protein oxidation and stimulate protein turnover (Buijs et al. 2002b). However, beneficial effects of supraphysiological hGH administration on muscle strength or function have not yet been demonstrated in trained athletes (Crist et al. 1988; Yarasheski et al. 1992, 1993; Deyssig et al. 1993; Taaffe et al. 1996; Frisch 1999). In a meta-analysis of studies on hGH and muscle strength, it was concluded that overall there is no evidence of increased muscle strength with hGH in healthy trained subjects (Dean 2002), and it has been questioned if there are anabolic effects in healthy subjects at all (Rennie 2003).

1.4 Effects on Cardiac and Pulmonary Function

Many childhood-onset GHD patients have an impaired lung volume. A decreased maximal expiratory pressure was found in both childhood-onset and adult-onset GHD patients (Merola et al. 1996). Treatment with rhGH improves ventilatory function (Merola et al. 1996), oxygen uptake (VO_{2max}) and exercise capacity in GHD adults (Cuneo et al. 1991b; Nass et al. 1995). GH treatment is also used children with cystic fibrosis to improve exercise tolerance, most likely through effects on muscular, cardiovascular and pulmonary capacity (Hutler et al. 2002). However, a placebo-controlled study in intensive care unit patients requiring mechanical ventilation did not show beneficial effects of rhGH treatment on respiratory function (Pichard et al. 1996). If GHD patients are treated with GH, cardiac muscle mass and maximal cardiac output increase (Cuneo et al. 1991c; Cittadini et al. 1994), but if elevated hGH levels in acromegalic patients are reduced, cardiac function is also improved (Merola et al. 1993). The picture

remains unclear, as both GH deficiency and GH excess seem to be associated with impairments of pulmonary and cardiac function. As for many pharmacological GH effects, and also for the effects on heart or lung capacity, data are scarce in healthy trained subjects.

2 Analytical Procedures

Traditionally, peptide hormones like hGH and its peripheral mediator insulin-like growth factor I (IGF-I) are measured by immunometric methods. Those methods, introduced almost 40 years ago and routinely used in clinical diagnostics, have evolved over time, especially by the introduction of monoclonal antibodies and more sensitive, non-isotopic detection methods. Usually, peptide hormone concentrations in human circulation are in the nano- or picomolar range, making the sensitivity of the methods a crucial issue. In addition, as many proteohormones belong to larger families of hormones with structural similarity, specificity of the antibodies used in the assays is of crucial importance. Antibodies bind to a distinct, three-dimensional structure on the surface of the antigen. In case of immunoassays, this strong interaction is used to capture the analyte of interest. In all immunoassays, concentration of the analyte is determined by comparing the signal obtained in a patient's sample of unknown concentration to the signal obtained from a standard curve consisting of samples containing the analyte at known concentrations. Basically, two different types of immunoassays exist: in classical competitive immunoassays, which had been developed initially and opened the "era" of immunoassay use in routine diagnostics, the analyte in a sample "competes" with a labelled form of the same molecule ("tracer"), which is added to the sample, for binding to the antibody. The more analyte is present in a sample, the less likely the labelled tracer is to be bound by the antibody. When finally measured, the signal obtained is inversely correlated to the concentration of the analyte present in a sample. A classical representative of this type of immunoassays is the radioimmunoassay (RIA), dominating the routine diagnostics market for many years. In contrast, the more recently developed "sandwich type immunoassay" is based on two antibodies: First, the analyte in the sample is "captured" by an antibody. Thereafter, a second antibody – which has to recognise a different epitope on the surface of the analyte in spatial distance – is added to the sample. This second antibody is labelled ("detection antibody"). The more analyte is present in the sample, the more labelled detection antibody is bound. Consequently, the signal obtained by this type of assay is positively correlated to the amount of analyte present in a sample.

The technical specificities and the design of immunoassays have implications for their use in doping analytics: the current WADA International Standard for Laboratories (World Anti Doping Agency 2009) says that "Immunoassays applied for the Initial Testing Procedures and Confirmation Procedures shall use antibodies recognising different epitopes of the macromolecule analysed, unless a purification or

separation method is used prior to application of the immunoassay to eliminate the potential of cross-reactivity." Firstly, a precise description of the epitope can only be achieved when monoclonal antibodies are used, whereas polyclonal antisera consist of a mixture of antibodies and therefore involve a broader spectrum of epitopes. Clearly, the use of monoclonal antibodies must be preferred in view of the above-mentioned WADA requirement. Furthermore, as a consequence of the requirement, for each peptide hormone measured, at least two independent assays have to be developed. At least one antibody contributing to the specificity in the assays has to be replaced by another antibody, recognising the same molecule but binding to a different epitope.

Only recently, mass spectrometry has also been applied for the measurement of peptide hormones (Thevis et al. 2009). However, especially for measurement in complex biological fluids like blood, most methods published up to now still include affinity enrichment of the analyte involving immunological detection through antibodies. Those methods have also shown to be promising for analysis of GH and related peptides (Arsene et al. 2008; Bredehoft et al. 2008), and very likely will enter the field of doping detection in the near future. However, the methods currently available to detect GH doping completely rely on traditional immunoassay measurements.

3 Screening and Confirmation Strategy

There are two major problems in detecting abuse of GH (McHugh et al. 2005; Bidlingmaier and Strasburger 2007). Biochemically, it is difficult because recombinant GH is identical in amino acid sequence and hence in physicochemical properties to the major, 22,000 Da isoform of the hormone as secreted by the pituitary gland. Biologically, the large variability of circulating GH concentrations represents a major challenge. GH is secreted in a pulsatile manner, with secretory bursts being stimulated by factors like sleep and stress exercise (Healy and Russell-Jones 1997), and it has a very short half-life in circulation (15–20 min) (Holl et al. 1993).

To overcome the difficulties, two different strategies have been followed: the so-called "marker approach" investigates changes in hGH-dependent parameters like IGF-I or components of bone and collagen metabolism, which are increased after hGH injection. In contrast, the so-called "isoform approach" directly analyses the spectrum of molecular isoforms in circulation, which shows remarkable changes after injection of recombinant GH. For both methods, screening assays and confirmation assays have been developed, all based on the measurement of peptides by immunoassays. As outlined above, the assays used for "confirmation" of an adverse analytical finding have to involve antibodies recognising epitopes different from those recognised by the antibodies used in the respective "screening assays".

3.1 The Marker Approach

GH – secreted by the pituitary gland or injected in its recombinant form – leads to an increase in several hormones and other factors, which can be measured in blood samples by appropriate immunoassays. In addition to insulin-like growth-factor I (IGF-I), which is the most important mediator of GH actions in the periphery, other components like IGF binding protein 3 (IGFBP 3) and the acid labile subunit of the IGF-I/IGFBP 3 ternary complex (ALS) are also increased (Butler and Le Roith 2001; Le Roith et al. 2001). As those factors have a longer half-life than GH itself, it was investigated whether increases in these markers could be used to detect GH doping. In a large multicenter study it could be clearly demonstrated that changes in concentrations of IGF-I, IGFBP-3 and ALS seen after injection of recombinant GH clearly exceed the natural fluctuations seen in those factors after strenuous exercise (Wallace et al. 1999). In addition to the GH/IGF-I axis, factors involved in the regulation of collagen and bone turnover such as N-terminal peptide of Type III procollagens (PIIIP), collagen I carboxy-terminal telopeptide (ICTP), osteocalcin and collagen I carboxy-terminal propeptide (PICP) have also been invesitgated (Longobardi et al. 2000; Wallace et al. 2000). PIIIP and osteocalcin showed dramatic increases in circulation after recombinant GH application, with elevation above pre-treatment values persisting for up to 84 days. Endurance exercise also transiently activates bone and collagen turnover, but brief recombinant hGH administration results in a much greater increase of these markers (Wallace et al. 2000). On further investigation of the discriminatory power of the analysis of GH-dependent markers in detecting GH doping, it became obvious that measurement of more than one factor would increase the sensitivity and specificity of the test (Kniess et al. 2003). IGF-I and PIIIP in combination turned out to allow a good discrimination between GH treated and untreated subjects even several days after the injections were stopped (Powrie et al. 2007). A set of discriminant functions has been developed which-take into account that the markers vary with age and gender (Erotokritou-Mulligan et al. 2007). Various factors potentially confounding a test based on GH-dependent markers have meanwhile been investigated: to demonstrate the applicability of the marker approach in trained athletes, reference ranges for the markers have been established in this population, with slight differences from the values obtained from a normal, non-elite athlete population (Healy et al. 2005). Ethnicity was demonstrated to have only minor effects on the markers employed (Nelson et al. 2006). Finally, similar to exercise, injury also led to an increase in markers of bone and collagen turnover. However, this increase was much smaller than the increase seen after GH administration (Erotokritou-Mulligan et al. 2008). In summary, a lot of validation work was done to show that, in principle, changes in GH-dependent markers can be used to detect GH doping in sports. For all measurements used for this test method, the assays used and especially the antibodies involved are of crucial importance, as reference ranges will change with changes in the assays. Accordingly, before

widespread implementation in doping control, it has to be ensured that those assays are available in an unchanged format for a longer time, ideally produced under the control of WADA.

3.2 The Isoform Approach

Growth hormone in circulation consists of a wide spectrum of molecular isoforms: the pituitary gland secretes predominantly the 22,000 Da isoform, but also a 20,000 Da form and various smaller fragments of hGH. Furthermore, all isoforms circulate in monomeric, homodimeric and heterodimeric forms (Baumann 1999). In contrast, recombinant hGH preparations contain the 22,000 Da isoform only. Through negative feedback, pituitary secretion of all hGH isoforms is reduced when circulating hGH and IGF-I are elevated (Fig. 1) (Leung et al. 2002; Keller et al. 2007). As a consequence, after administration of rhGH, the relative abundance of the 22,000 Da isoform in circulation increases. This shift in the spectrum of molecular isoforms of hGH in circulation can be detected using specific immunoassays (Wu et al. 1999; Bidlingmaier et al. 2000). A serum sample is measured by two different sandwich immunoassays involving monoclonal antibodies with different affinities to the 22,000 Da isoform and the spectrum of other isoforms. One assay, called “recombinant assay”, preferentially translates the 22,000 Da hGH molecules into a signal, whereas the other assay, called “pituitary assay”, binds a broader spectrum of isoforms. In samples collected after administration of recombinant hGH, the result in the “recombinant assay” will be comparatively high, whereas the result for the pituitary assay is comparatively low. Consequently, the ratio calculated from the results of the “recombinant assay” over the “pituitary assay” is increased after administration of recombinant GH (Fig. 1). It is known that under physiological conditions, the isoforms are secreted in parallel

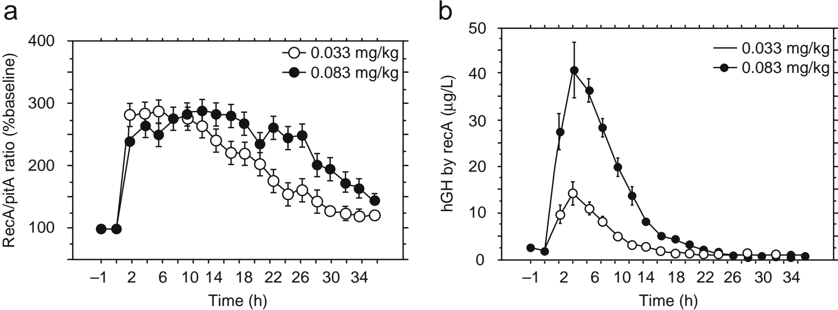

Fig. 1 Change in ratios for recombinant/pituitary (**a**) and absolute concentrations of hGH as measured by recombinant assays (**b**) before and every 2 h after injection of low- or high-dose rhGH. Ratio data (*left*) are presented as % of the mean of baseline values (–1 h, 0 h), concentrations (*right*) are given in g L^{-1}. Figure taken with permission from Bidlingmaier et al. (2009)

(Ishikawa et al. 1999). Furthermore, a potential influence of secretory peaks evoked by strenuous exercise (Jenkins 1999) was investigated in a study on the impact of exercise on the isoform composition (Wallace et al. 2001a, b). All isoforms increase during exercise, with the most pronounced initial response in the 22,000 Da GH isoform. Immediately after exercise, 22,000 Da GH decreases somewhat faster than the other isoforms. The consequence – if any – for a doping test based on investigation of isoforms would be a higher likelihood for a negative test result immediately after exercise. However, as the order of magnitude of the changes in the isoform composition was very small compared to the changes occurring after rhGH administration, it is believed that the exercise-related changes in GH secretion do not invalidate the test results after competition. It is very important to mention that – similar to the situation with the marker approach – the components of the assays used must be standardised, and continuous antibody supply is a crucial point for a more widespread use of the test. Furthermore, the principle of the isoform approach does not of course allow detection of doping with cadaveric, pituitary-derived GH, as such preparations contain a mixture of GH isoforms.

To be in accordance to the above-mentioned WADA requirements, an independent confirmatory test is necessary, and must be based on different antibodies targeting different epitopes (Fig. 2). Such assays meanwhile have been developed

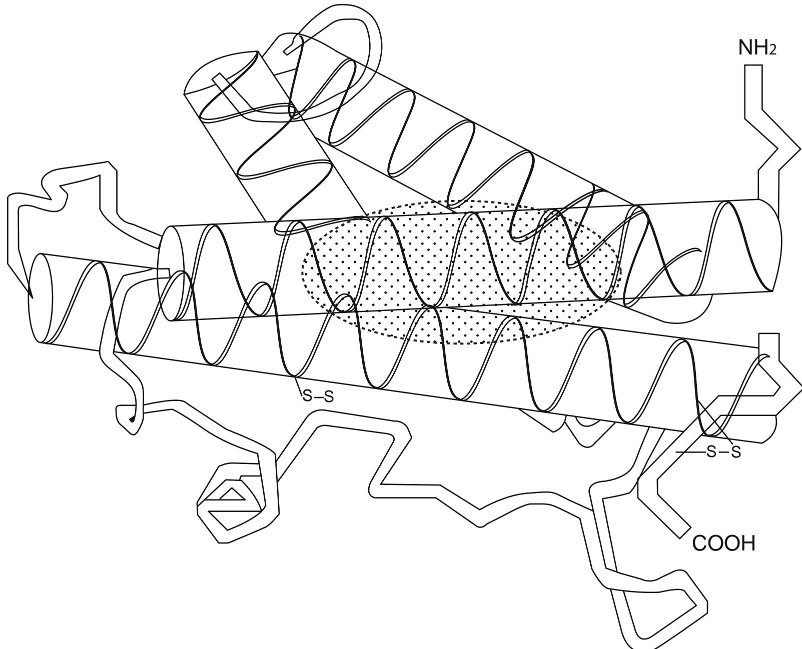

Fig. 2 The specificity of different screening and confirmatory assays is achieved by application of different antibodies (e.g. mAB AK567), binding specifically at different sites. The location of these epitopes is deduced from cross-comparison of binding affinity experiments with various hGH variants and fragments. Figure taken with permission from Bidlingmaier et al. (2009)

using new monoclonal antibodies, but the discriminatory capacity of the method could be greatly improved (Wu et al. 2002). Two independent pairs of assays, each comprising a “recombinant assay” and a “pituitary assay”, have been validated in several WADA laboratories, and were used during the Olympic Games in Athens 2004, Torino 2006 and Beijing 2008.

No adverse analytical findings have been reported so far (Saugy et al. 2006), most likely due to the short window of opportunity. It has been reported that the 20,000 Da isoform remained suppressed for up to 34 h after a single injection of 0.083 mg kg^{-1} rhGH (Keller et al. 2007), but detection is most likely during the first 24 h after the last rhGH injection. Therefore, the isoform approach will be more successful if used in unannounced out-of-competition tests.

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Mass Spectrometry-Based Analysis of IGF-1 and hGH

Mario Thevis, Michael Bredehöft, Maxie Kohler, and Wilhelm Schänzer

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Abstract Mass spectrometric approaches have been used to determine various peptide hormones in sports drug testing. While insulin-like growth factor-1 (IGF-1) and its synthetic analogs are qualitatively and/or quantitatively measured by liquid chromatography–tandem mass spectrometry after immunoaffinity purification, methods of uncovering doping rule violations with illegal applications of human growth hormone (hGH) have not yet been established using mass spectrometry-based assays. However, substantial information on the heterogeneity of hGH, splice variants and post-translational modifications with respective locations as elucidated by mass spectrometry are of utmost importance for improving currently employed immunological procedures.

Keywords 2D-Gel Electrophoresis • Doping • Sport

1 Introduction

Although mass spectrometry is one of the most frequently employed strategies for the characterization and identification of peptides and proteins in numerous fields

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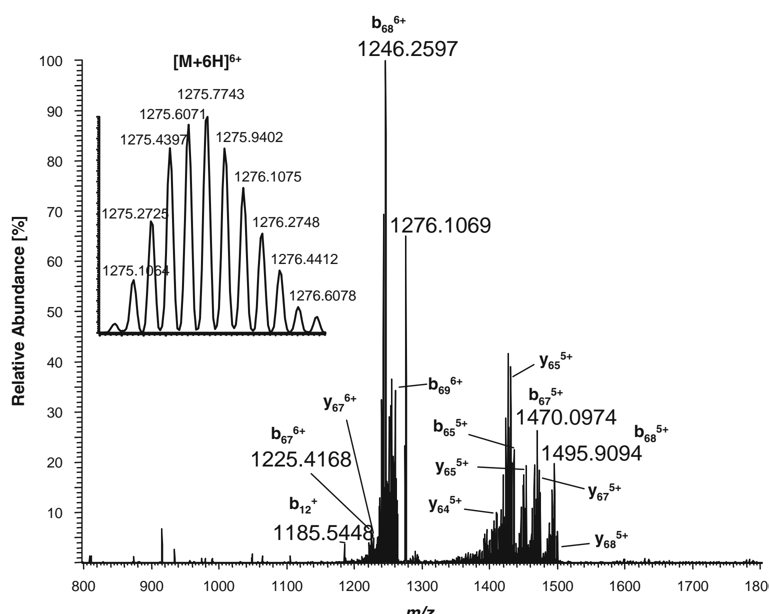
of analytical chemistry, its use for the detection of peptide hormone misuse in sports drug testing is still limited to a few examples such as cross-linked hemoglobins (Gasthuys et al. 2004, 2005; Goebel et al. 2005; Thevis et al. 2003), synthetic insulins (Thevis et al. 2005, 2006b, 2008; Thomas et al. 2007), corticotrophins (Synacthen) (Thevis et al. 2006a), human chorionic gonadotrophin (Gam et al. 2003), luteinizing hormone releasing hormone (Thomas et al. 2008) and insulin-like growth factor-1 (IGF-1) (Bredehöft et al. 2008). A major issue has been the low concentration of these and other analytes and the need to purify target compounds from blood or urine using comparatively laborious approaches. However, the enormous discriminative power of mass spectrometry has demonstrated great value and allows the unambiguous identification and quantification of substances while excluding any kind of cross-reaction due to the determination of specific molecular weights and/or diagnostic product ions derived from MS/MS experiments (Thevis and Schänzer 2007).

2 IGF-1

In contrast to many other prohibited peptide hormones, plasma concentrations of IGF-1 are comparatively high and reach levels up to 800 ng mL^{-1}. Hence, IGF-1 was one of the first candidates for detection and quantification using mass spectrometry-based assays, and several approaches were published that proved the extraordinary utility of MS for these purposes (Nelson et al. 2004; Popot et al. 2001, 2008). A first major goal was to elucidate how to disrupt the complex of IGF-1 to its binding proteins, in particular to the IGF-1 binding protein 3 (IGFBP3). The use of strong acids and ethanol as well as sodium dodecyl sulfate (SDS) were recommended to cleave the noncovalent but quite stable complexes, which result in underestimated plasma concentrations of IGF-1 if not dissociated prior to any analytical method (Nelson et al. 2004).

In addition to the natural IGF-1, synthetic analogs with supposedly higher bioavailability were studied and detection methods established, as some of them were advertised as performance-enhancing drugs. Especially the long-R^3-IGF-1 variant was reported to be misused in strength sports, and the ability of MS to discriminate between the natural IGF-1 and its extended version was demonstrated (Bredehöft et al. 2008). A comparison of product ion mass spectra of human IGF-1 and long-R^3-IGF-1 derived from multiply charged precursor ions ($[M+6]^{6+}$ and $[M+7]^{7+}$, respectively) after electrospray ionization is shown in Fig. 1. Besides the unequivocal differentiation of molecular weights by deconvolution of multiply charged intact molecules, diagnostic product ions were observed for either species with typical *b*- and *y*-ion series. The combined use of immunoaffinity purification and LC–MS/MS has enabled the qualitative and quantitative analysis of IGF-1 and its structural analogs, which represents a useful

a



b

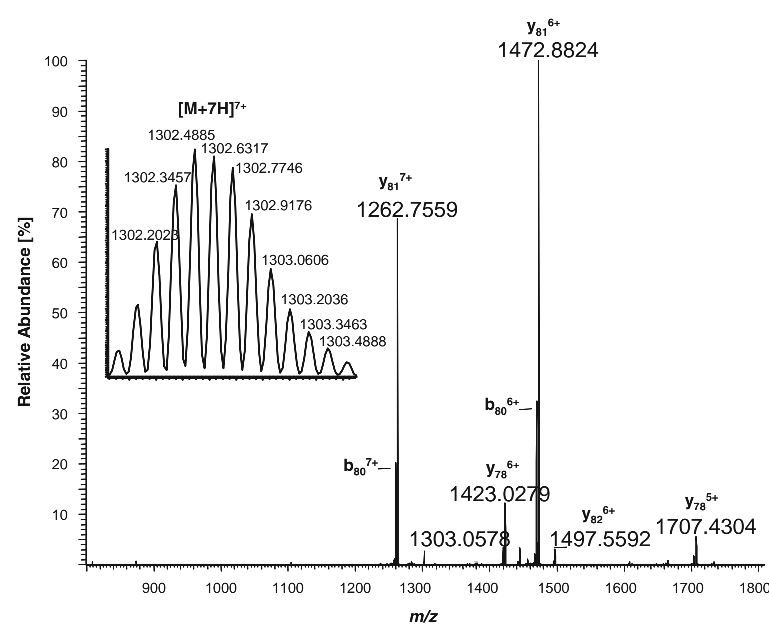


Fig. 1 ESI product ion mass spectra of multiply charged precursor ions of (**a**) $[M+6]^{6+} = 1{,}276.8$ of human IGF-1, and (**b**) $[M+7]^{7+} = 1{,}302.5$ of long-R^3-IGF-1

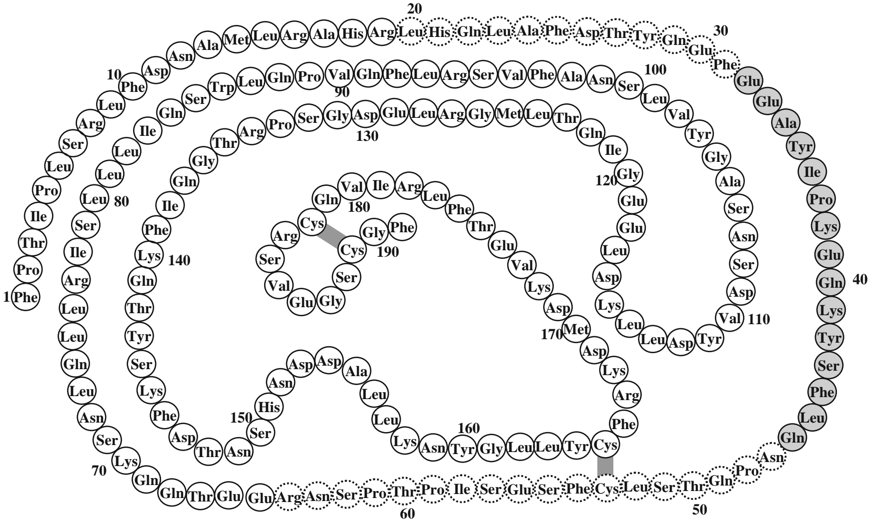


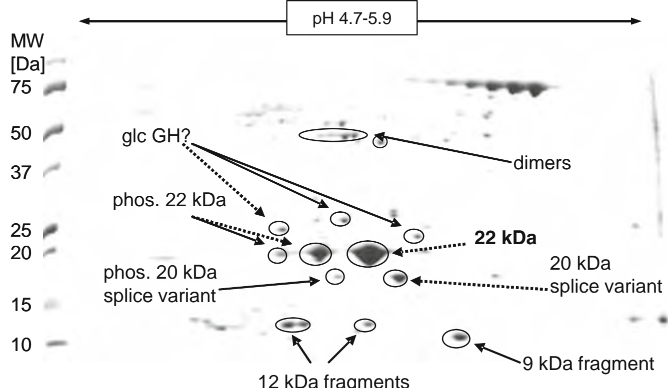
Fig. 2 The 20 kDa variant of hGH is characterized by deletion of residues 32–46 (*gray circles*) from the 22 kDa molecule. Resulting diagnostic peptides for LC–MS identification (compare Fig. 3) are signified by dotted circles

complementary procedure for measuring human IGF-1 and synthetic derivatives in doping control blood samples.

3 Human Growth Hormone

In addition to small proteins such as IGF-1, larger peptide hormones such as hGH were also studied using MS-based methods. As outlined above (Sect. 3.6), no sports drug testing assay employing mass spectrometry is currently used for hGH in terms of doping control purposes. However, full characterization of hGH and recombinantly produced GH using mass spectrometry has been reported (Giorgianni et al. 2004; Hepner et al. 2005; Kohler et al. 2007; Zhan et al. 2005). Various post-translational modifications and splice variants of hGH were determined, and recombinant versions of the main 22 kDa isoform (Fig. 2) were also reported to contain minor percentages of amino acid sequence errors such as the substitution of an arginine (R_{127}) by lysine (or glutamine) and dimethylation of lysine (K_{70}). These can possibly present a target for future doping control methods using tandem mass spectrometry-based approaches. The possibility of separating pituitary and circulating hGH isoforms by means of 2D-gel electrophoresis was recently reported (Kohler et al. 2008), and the absolutely identical migration behavior of the main and unmodified 22 kDa variant from human as well as recombinant sources was

a



b

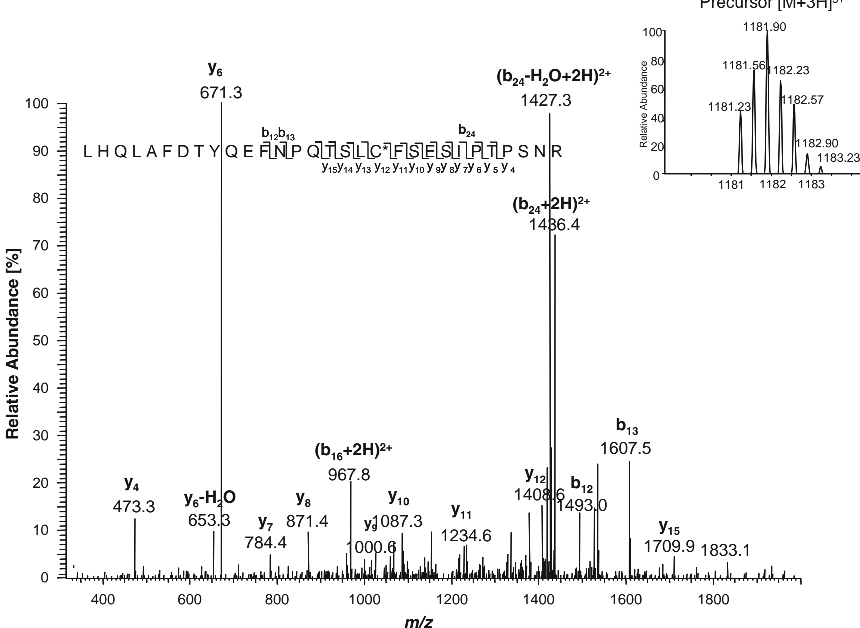


Fig. 3 (**a**) 2D-gel image of human pituitary extract, and (**b**) ESI product ion mass spectrum of a peptide uniquely characterizing the 20 kDa splice variant of hGH

demonstrated by means of 2D fluorescence Difference Gel Electrophoresis (2D DIGE), which outlined the need to search for alternative approaches to determine the presence or absence of recombinant GH. Hence, pituitary extracts were separated on 2D gels employing a pH-gradient from 4.7 to 5.9 (Fig. 3a), visualized spots were excised, enzymatically hydrolyzed and resulting peptides assigned to hGH isoforms by means of characteristic peptides (Fig. 2) and measured as well as calculated isoelectric points. Phosporylations at the amino acid residues Ser-51, Ser-106 and Ser-150, and deamidation at Asn-152, were detected and diagnostic peptides identifying the splice variant of 20 kDa were found (Fig. 3b). Moreover, the identification of a glycosylation of hGH was recently reported, which consisted of a HexHexNac*2 NeuAc modification presumably located at Thr59 (Kohler et al. 2009). Detailed knowledge of molecular differences of isoforms and synthetic analogs of target analytes is of particular importance for doping control purposes and substantiates findings obtained by methods not providing the specificity of mass spectrometry.

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Insulin

Mario Thevis, Andreas Thomas, and Wilhelm Schänzer

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Abstract Due to its versatile nature and its corresponding anabolic and anti-catabolic properties, insulin has been prohibited in sports since 1999. Numerous studies concerning its impact on glycogen formation, protein biosynthesis, and inhibition of protein breakdown have illustrated its importance for healthy humans and diabetics as well as elite athletes. Various reports described the misuse of insulin to improve performance and muscle strength, and synthetic analogs were the subject of several studies describing the beneficial effects of biotechnologically modified insulins. Rapid- or long-acting insulins were developed to enhance the injection-to-onset profile as well as the controllability of administered insulin, where the slightest alterations in primary amino acid sequences allowed the

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inhibition of noncovalent aggregation of insulin monomers (rapid-acting analogs) or promoted microprecipitation of insulin variants upon subcutaneous application (long-acting analogs). Information on the metabolic fate and renal elimination of insulins has been rather limited, and detection assays for doping control purposes were primarily established using the intact compounds as target analytes in plasma and urine specimens. However, recent studies revealed the presence of urinary metabolites that have been implemented in confirmation methods of sports drug testing procedures. So far, no screening tool is available providing fast and reliable information on possible insulin misuse; only sophisticated procedures including immunoaffinity purification followed by liquid chromatography and tandem mass spectrometry have enabled the unambiguous detection of synthetic insulins in doping control blood or urine samples.

Keywords Doping • Mass spectrometry • Sport • Peptide hormone

1 Introduction

The endocrinological importance of the pancreas for the metabolism of carbohydrates was observed as early as 1889, when von Mering and Minkowski described the symptoms of severe diabetes mellitus such as hyperglycemia and glycosuria in dogs after pancreas removal (von Mering and Minkowski 1889). A few years later, the central role of the pancreatic islets of Langerhans in producing an "autacoid" (later referred to as "hormone") affecting blood sugar concentrations was postulated by Sharpey-Schäfer in 1894. He suggested the name "insuline" in 1916, which was also proposed by de Meyer in 1909, albeit the structure, mode of action, and metabolism of the hormone were merely hypothetical at that time (Schäfer 1916). The concerted efforts of the later Nobel Laureates Frederick Grant Banting, John James Richard Macleod, Charles Herbert Best, and James Bertam Collip allowed the extraction and purification of insulin from canine and bovine pancreatic cells in 1922 and, thus, the therapy of numerous diabetics. Pure and crystalline insulin was prepared in 1926 by John Jacob Abel (Abel 1926), and Sanger and co-workers sequenced and characterized human insulin (Ryle et al. 1955; Sanger 1959, 1988), a breakthrough that enabled the biotechnological production and virtually unlimited availability of insulin for the treatment of the so-called epidemic of the twenty-first century (IDF 2006). A comprehensive review on the discovery of insulin was published in 2002 (Rosenfeld 2002).

Insulin has been recognized in sports since 1998, when the International Olympic Committee (IOC) was approached by a Russian medical officer who enquired as to whether the use of insulin was allowed only for athletes that demonstrably suffer from diabetes mellitus. Once spotlighted, insulin was added to the list of prohibited substances that became effective in 1999 (WADA 2007). Several possibly performance-enhancing properties were attributed to the misuse of insulins by healthy athletes,

and various case reports substantiated the suspicion of a widespread misuse among elite sportspersons (Dawson and Harrison 1997; Evans and Lynch 2003; Reverter et al. 1994; Rich et al. 1998; Steiner and Wagner 2002; Young and Anwar 2007).

2 Pharmacological Aspects and Mode of Action

The function of insulin in humans has many facets. The hormone has been subject of numerous studies dealing with the release, receptor binding, signaling system, and correction of deficiencies, since the storage and release of energy during feeding and fasting, which is essentially controlled by the action of insulin, is a central aspect for survival.

2.1 Endogenous Insulin Production and Storage

The biosynthesis of insulin in the β-cells of the pancreas is regulated by numerous mechanisms but primarily stimulated by the presence of glucose and augmented by cAMP. Interestingly, the secretion and utilization of insulin was not found to initiate restoration or granule formation. The biosynthetic pathway includes numerous steps and starts with the preparation of preproinsulin, which contains proinsulin and a 24 amino acid signal peptide that is liberated upon translocation into the rough endoplasmic reticulum (RER) by means of the signal peptidase (Fig. 1). The resulting proinsulin, a single chain peptide hormone with a molecular weight of 9,388 Da, establishes two intermolecular and one intramolecular disulfide bond yielding the required native structure. Insulin is derived from proinsulin by the elimination of a fragment termed C-peptide (mol wt = 3,020 Da) that connects the C-terminus of the insulin B-chain to the N-terminus of the insulin A-chain via two additional amino acid residues each (Fig. 1). Here, two pathways were demonstrated based on either the use of prohormone convertase 2 (PC2) and carboxypeptidase E (CPE) or PC1 and PC3 plus CPE to yield intermediately des-64,65 proinsulin or des-31,32 proinsulin, respectively. Subsequently, complementary cleavages give rise to insulin, a peptide hormone with a molecular weight of 5,807 Da comprising two chains with 21 (A-chain) and 30 (B-chain) amino acids that are connected via two disulfide bridges (Fig. 1). After completed biosynthesis, insulin is stored with equimolar amounts of C-peptide in mature granules of the β-cells until secretion into the blood stream. The protein possesses a strong affinity to self-association to noncovalent hexamers (Fabris and Fenselau 1999), particularly favored in the presence of Zn^{2+} ions.

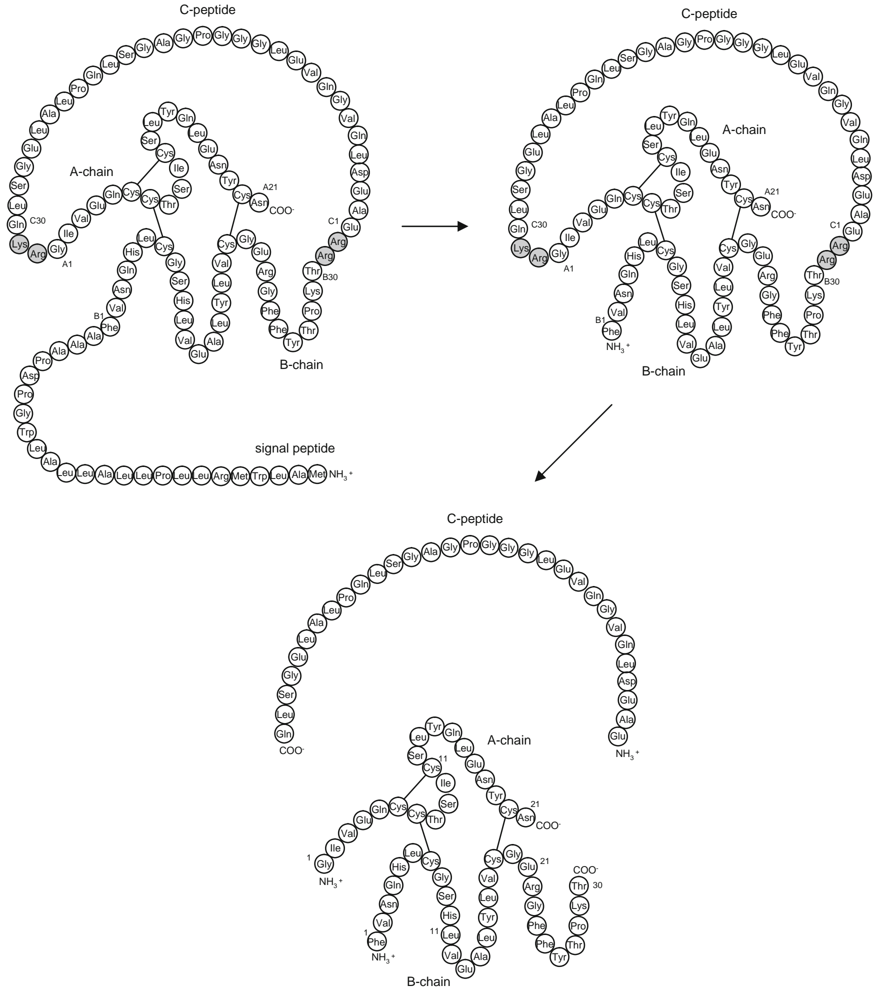


Fig. 1 Biosynthesis of human insulin

2.2 Insulin Secretion

The most important physiologic substance that regulates the secretion of insulin from pancreatic β-cells into the blood stream is glucose, and various factors are involved in a complex system necessary for controlled insulin exocytosis (Proks and Lippiat 2006). A major aspect is the link between β-cell metabolism of glucose and electrical activity (Ashcroft and Rorsman 1989). By means of the transporter GLUT-2, glucose passes through the cell membrane and undergoes metabolic processes, which cause the intracellular increase of the ATP/ADP ratio. Both substrates, ATP and ADP, affect the ATP-sensitive potassium channels (K_{ATP}),

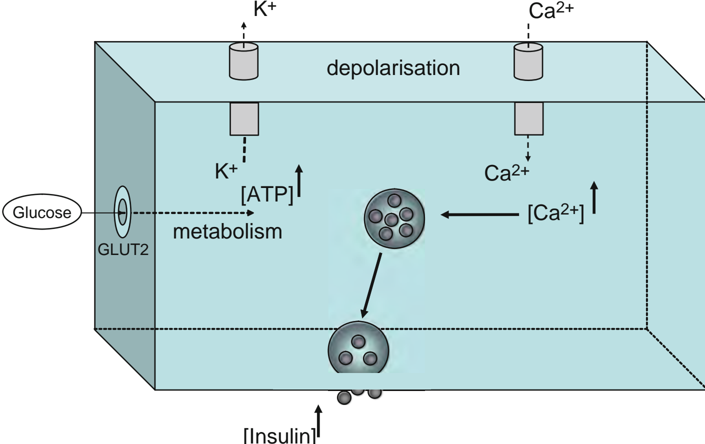


Fig. 2 Schematic overview of selected processes involved in glucose-dependent insulin secretion from pancreatic β-cells

the activity of which is inhibited or increased by elevated ATP or ADP concentrations, respectively (Ashcroft 1988). The depolarisation of the cell membrane associated with, amongst others, the inactivation of K_{ATP}, results in a calcium influx through voltage-gated calcium channels, which subsequently triggers the exocytosis of insulin granules. A schematic overview of these processes is illustrated in Fig. 2 (Mark 2002). The effect of glucose on the pancreatic β-cells is dose-related (Tillil et al. 1988), and comparison between orally and intravenously administered carbohydrates revealed a significantly greater insulin response after oral application of glucose (Shapiro et al. 1987). Major reasons for this phenomenon are summarized as the *incretin effect*, which comprises assumed glucose-sensitizing properties of peptide hormones on β-cells, e.g. the glucose-dependent insulinotropic peptide (GIP), cholecystokinin (CKK) and glucagon-like peptide-1 (GLP-1), which are released postprandially from the gastrointestinal tract (Rasmussen et al. 1990). In addition to carbohydrates, the amino acids leucine, arginine, and lysine demonstrated insulinergic activity, which is potentiated by glucose (Levin et al. 1971). This aspect had considerable impact on current recommendations for elite athletes' nutrition to optimize carbohydrate utilization (Manninen 2006).

2.3 Insulin Actions

Once secreted from the pancreatic β-cells, insulin circulates in the blood stream with an approximate half-life of approximately 12 min. Numerous tissues and organs express the insulin receptor and various actions are initiated (Sonksen 2001), some of which are of general importance as well as particular interest for elite sports.

The central effect of insulin in concert with other hormones such as glucagon or somatostatin is the control of blood glucose levels. The secretion of insulin in response to elevated glucose concentrations (e.g., postprandial) inhibits hepatic glucose production, which subsequently lowers blood glucose levels. In contrast, glucagon, which is composed of 29 amino acid residues (mol wt = 3,483 Da), is a counterregulatory hormone of insulin. It raises plasma glucose levels in response to insulin-induced hypoglycemia and plays an important role in glucose homeostasis by increasing gluconeogenesis and decreasing glycolysis. The third pancreatic hormone relevant for the control of blood glucose levels is somatostatin, a 14-residue peptide (mol wt = 1,638 Da) that exerts inhibitory effects on the secretion of insulin but not on its biosynthesis. The entire mechanism of its action has not yet been clarified but studies suggest a paracrine effect that inhibits the exocytosis of insulin from cells adjacent to somatostatin-producing D-cells (Reichlin 1983).

However, the effects of insulin on the entire organism are manifold and complex. Insulin causes for instance the translocation of GLUT-4 (the glucose transporter predominantly found in skeletal muscle and adipose tissue) from intracellular vesicles to the cell membrane and, thus, increases the rate of glucose entry for a given concentration into the target tissue. An excess of glucose transferred into cells subsequently stimulates glycogen formation (Halse et al. 2001; Yeaman et al. 2001), a fact that is of considerable interest in endurance sports where the amount of glycogen stored in muscle cells can influence athletic performance. Moreover, the (muscle) protein metabolism is significantly influenced by the *chalonic* (Rooyackers and Nair 1997; Sonksen 2001) as well as the stimulating properties of insulin (Biolo et al. 1995; Biolo and Wolfe 1993; Tipton and Wolfe 2001; Wolfe 2000, 2005). Due to the anti-catabolic effect of insulin, protein breakdown is significantly reduced and allows the preservation of contractile muscle elements. In addition, synthesis-stimulating effects were observed in numerous studies that demonstrated the anabolic properties of insulin, utilizing for instance stable-isotope labeled analogs. The anabolism, however, was strongly dependent on amino acid availability (Fujita et al. 2006; Garlick and Grant 1988; Zhang et al. 1999).

3 Clinical Applications

Insulin deficiency or the resistance of target tissues to insulin are referred to as variants of diabetes mellitus, which has become a serious contemporary health issue. According to recent estimations, approximately 380 million people will be suffering from this disease within the next two decades. Diabetes mellitus is subdivided into the categories insulin-dependent (type 1) and non-insulin-dependent (type 2), both of which can cause severe hyperglycemia. The first-mentioned version usually results from cellular-mediated autoimmune destruction of the pancreatic β-cells, which impairs or eliminates the endogenous insulin production and, thus, the availability of a key regular of blood glucose (Atkinson and Eisenbarth 2001). Type-2 diabetes is characterized by insulin resistance and, as such, a relative

insulin deficiency. The insulin produced in the β-cells of the pancreas is secreted at elevated blood glucose levels, but target tissues lack the required insulin sensitivity and the normally resulting modification of metabolism. The reasons for this particular version of glucose intolerance have not been fully clarified yet. Diabetes, mostly type-2 diabetes, now affects 5.9% of the world's adult population with almost 80% of the total in developing countries. The regions with the highest rates are the Eastern Mediterranean and Middle East, where 9.2% of the adult population are affected, and North America with approximately 8.4%. The highest numbers, however, are found in the Western Pacific, where some 67 million people have diabetes, followed by Europe with 53 million (IDF 2006).

The recombinant preparation of human insulin and synthetic analogs has been of paramount clinical relevance in order to correct hyperglycemia and counteract the severe symptoms associated with impaired blood glucose levels. One of the most critical issues of insulin substitution has been the injection-to-onset profile, which is essentially linked with insulin's tendency to self-association (vide supra). When stored in and released from β-cells, noncovalent hexamers dissociate rapidly into bioactive insulin monomers. However, subcutaneous application necessitates the consideration of so-called lag-phases, the period between injection and bioavailability, which can last as long as 45–120 min. Hence, insulin analogs were prepared that possess either rapid- or long-acting properties, both of which were accomplished by means of minor modifications to the primary structure of human insulin (Barnett and Owens 1997). During the last decade, rapid-acting insulins such as Humalog LisPro, Novolog Aspart and Apidra Glulisine were introduced that are bioavailable within 10–15 minutes after administration (Becker et al. 2005a, b; Danne et al. 2005; Lindström et al. 2002; Plum et al. 2000; Rosak 2001). The exchange of a few amino acid residues primarily at the C-terminus of the B-chain (Fig. 3) exerts repelling properties that minimize the aggregation and, thus, enable more convenient handling, improved controllability, and fast delivery of bioactive insulin. These aspects, however, are of concern for doping control authorities as these drugs might possess an even greater potential for misuse in sports than recombinant human insulin formulations. The long-acting insulins Lantus Glargine and Insulin Detemir were generated to ensure constant basal blood insulin levels. The first-mentioned bears two additional arginines at the C-terminus of the B-chain and an A-chain C-terminal glycine instead of an asparagine residue, which provokes microprecipitation after subcutaneous application and a corresponding slow and continuous release of the active ingredient into the circulation. Insulin Detemir represents the truncated des-B30 insulin, the C-terminal lysine of which is acylated with myristic acid (Fig. 3).

4 Insulin Metabolism

The metabolism of insulin has been extensively studied, and numerous articles demonstrated that the liver and kidney are the major sites of insulin degradation (Benzi et al. 1990; Duckworth 1988; Duckworth et al. 1988a, b; Duckworth and

Fig. 3 Primary structures of human insulin (**a**) and synthetic analogs: Humalog LisPro (**b**, mol wt=5,807), Novolog Aspart (**c**, mol wt = 5,826), Glulisine Apidra (**d**, mol wt = 5,823), Lantus Glargine (**e**, mol wt=6,063), and Insulin Detemir (**f**, mol wt=5,917). Modifications as compared to human insulin are highlighted in *gray*

Kitabchi 1981; Seabright and Smith 1996). Several metabolic products derived from in vitro studies were identified using ^{125}I-labeled insulin after incubation with hepatocytes or other insulin-sensitive tissues. Most metabolites originated from cleavages in the center of the A- or B-chains as well as at the C-terminus of the B-chain as obtained for instance by the insulin-degrading enzyme (IDE) (Duckworth et al. 1998). In addition, carboxypeptidases yielded several C-terminally truncated

insulin derivatives, and major urinary metabolites such as DesB30, DesB24-30 and DesB25-30 were obtained and identified (Thomas et al. 2007). As the adsorption and degradation in glomerula and postglomerula of the kidney is highly efficient, less than 1% of the intact hormone is excreted into the urine.

5 Analytical Assays in Doping Control

Mass spectrometry (MS) has been a central tool in doping control analysis due to its invaluable specificity and sensitivity (Thevis and Schänzer 2005c, 2007b, c). Compared to most other techniques, MS instruments have proved to have utmost flexibility and comprehensiveness combined with the provision of unambiguous information. Hence, most analytical assays employed in sports drug testing are based on chromatography and mass spectrometry.

5.1 Mass Spectrometry of Peptides and Proteins

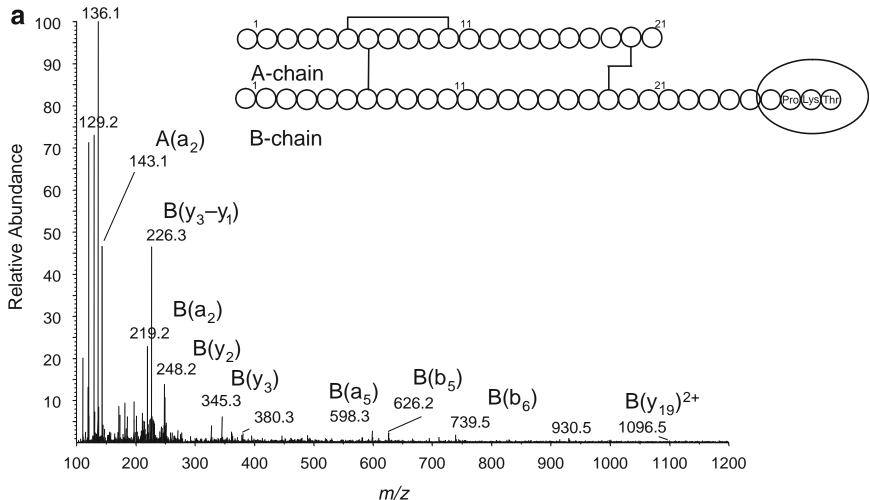
In contrast to the mass spectrometric analysis of low molecular weight compounds, peptides and proteins require soft ionization techniques such as electrospray ionization (ESI, "electrospray – wings for molecular elephants") or matrix-assisted laser desorption ionization (MALDI). The great scientific impact of these methods (Fenn et al. 1989; Tanaka et al. 1988) was honored with the Nobel Prize in 2002 for John B. Fenn and Koichi Tanaka. With ESI, the more commonly used technique in doping controls, liquids containing protonated or deprotonated molecules are sprayed by means of a capillary tip at high voltages (1 kV and higher), forming charged droplets that shrink by solvent evaporation and repeated droplet disintegration leading to very small and highly charged droplets. Finally, gas-phase ions are produced from these very small droplets as discussed in two different theories, namely *charged residue model* (Dole et al. 1968) and *ion evaporation* (Iribarne and Thomson 1976). The penetration of an imposed electric field into the liquid of the capillary leads to an enrichment of positive charges at the surface of the liquid, causing destabilization of the meniscus, formation of a cone and a so-called jet-emission of droplets bearing an excess of positive ions. The charged droplets shrink by solvent evaporation while the charge remains constant. Hence, an increase in electrostatic repulsion occurs, resulting in fission of the droplets as they reach the Rayleigh stability limit. This phenomenon continues with ongoing evaporation of solvent until very small and highly charged droplets are created. Finally, as proposed by Dole and co-workers, only one ion remains in a charged droplet, and the evaporation of solvent gives rise to a gas-phase ion (*charged residue model*). Alternatively, direct ion emission from droplets with a radius smaller than 10 nm was postulated, also generating gas-phase ions (*ion evaporation*) (Kebarle and Ho 1997). Both mechanisms lead to softly ionized molecules and, due to the

composition of peptides and proteins by numerous amino acid residues, analytes are commonly provided with more than one charge. Hence, the determination of molecular weights of protonated or deprotonated peptides and proteins requires deconvolution, and product ion mass spectra as obtained for instance by collision-induced dissociation (CID) can contain product ions at *m*/*z* ratios higher than the precursor ion due to the loss of charge(s) upon fragmentation. Efficient dissociation of peptides and proteins yields amino acid sequence information tags that allow for an identification and structural characterization of the analyte as well as various alterations, such as post-translational modifications or those originating from genetic or biotechnological mutation.

5.2 Confirmation Methods for Synthetic Insulins

Currently, several sports drug testing applications covering peptides or proteins are conducted utilizing liquid chromatography–ESI–tandem mass spectrometry (Thevis and Schänzer 2005a, b; 2007a). As insulins are considered relevant in doping controls, several assays enabling the identification of human and synthetic insulins in plasma (Thevis et al. 2005) and urine (Thevis et al. 2006) were established utilizing the identification power of immunoaffinity purification, liquid chromatography and low or high resolution tandem mass spectrometry. Insulin has been frequently mentioned as a drug of abuse in sports. The difficult aspect for doping control laboratories is the differentiation of naturally occurring insulin from that administered to artificially enhance athletic performance. Hence, the first goal was the characterization and distinction of modified rapid- and long-acting insulins from the natural human counterpart. The determination of the molecular weight as well as the unequivocal identification of the target analyte(s) is considered mandatory (Thevis et al. 2007), and product ion mass spectra of synthetic rapid- and long-acting insulins were studied to provide diagnostic and derivative-specific characteristics. The product ion mass spectra of human insulin and its synthetic analog Humalog LisPro are illustrated in Fig. 4. Despite an identical set of amino acids, diagnostic product ions derived from proline-directed dissociation (Loo et al. 1993) of the C-terminus of the B-chain allowed the differentiation of both compounds, as the ions at *m*/*z* 226 (y_3-y_1) and 217 (y_3) are indicative for human insulin and LisPro, respectively. All other insulin analogs differ by molecular weight and, thus, are readily identified as xenobiotic agents.

The isolation of insulins from biological matrices such as plasma or urine is a crucial aspect for successful detection using sensitive mass spectrometry-based assays. Hence, immunoaffinity (IA) purification was employed to selectively capture insulins with conserved B-chain regions composed of the amino acid residues B10–B15. A monoclonal antibody raised against this particular part of insulin enabled the combined extraction of all commercially available synthetic products plus animal-derived counterparts, e.g. bovine and porcine insulins. In addition, various metabolic degradation products such as the C-terminally truncated insulins



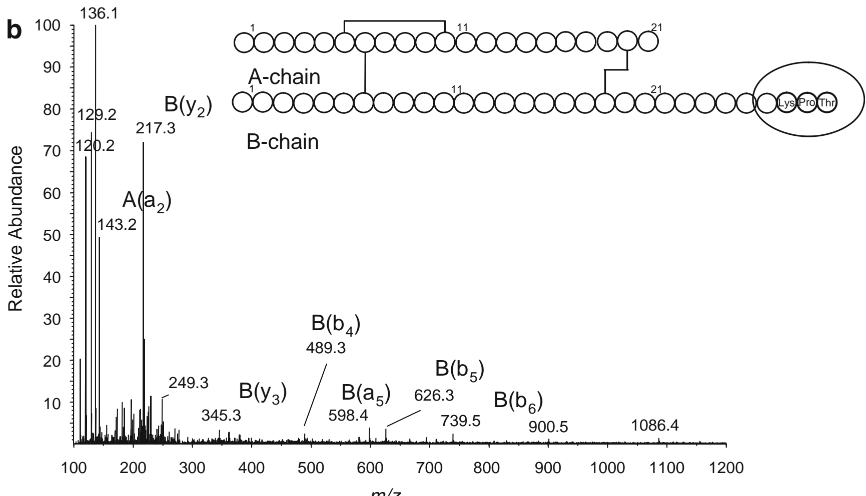


Fig. 4 Product ion mass spectra of fivefold charged molecules ($[M+5H]^{5+} = 1{,}162.5$) of (**a**) human insulin and (**b**) Humalog LisPro. Due to their composition by identical sets of amino acids, molecular weights and, thus, precursor ions are identical

were isolated using the IA approach, which can substantiate the administration of a drug in authentic urine or plasma samples. The majority of insulin modifications are located within the B-chain of synthetic analogs. Hence, the reduction of disulfide bonds of insulins was considered in order to reduce the information obtained from MS/MS spectra to the most important aspects and furthermore improve the dissociation behavior to yield more comprehensive amino acid

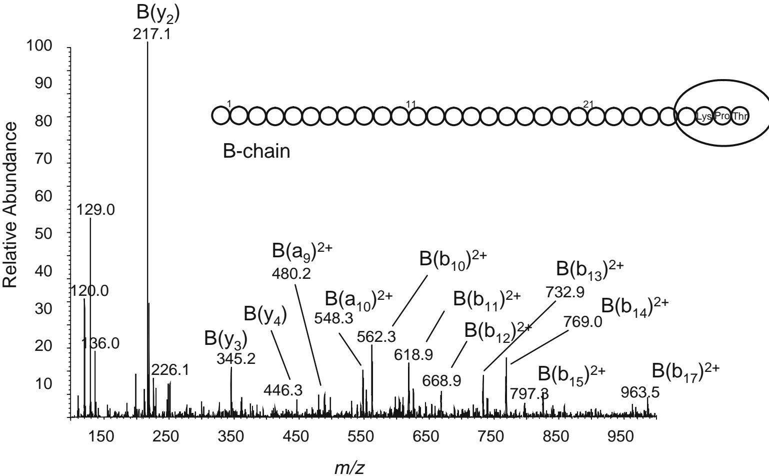


Fig. 5 Product ion mass spectrum of the fourfold charged precursor ion ($[M+4H]^{4+}$ =858.3) of the B-chain of Humalog LisPro; amino acid sequence tags encoding major parts of the analyte provide unambiguous information on the structure and identity

sequence tags. In comparison to the product ion mass spectrum obtained from intact Humalog Lispro (Fig. 4b), the corresponding spectrum obtained by CID of the B-chain only is illustrated in Fig. 5. Besides an abundant signal at *m*/*z* 217 that differentiates Humalog LisPro from human insulin, a comprehensive amino acid sequence tag from b_{10}^{2+} to b_{17}^{2+} substantiates the identity of the target analyte.

Sample preparation procedures starting from either plasma or urine are shown schematically in Fig. 6. In the case of urine specimens, preconcentration is accomplished using solid-phase extraction (SPE). Subsequently, both kinds of matrices are treated analogously using IA purification, SPE, evaporation and finally LC–MS/MS of either intact insulins or B-chains as obtained by disulfide reduction.

6 Case Reports

The misuse of insulins in strength and endurance sports has been reported several times in the past, and various cases demonstrated the preferred and combined intake with growth hormones. A daily administration of 10 IU of insulin (5 IU twice a day) over a period of 8 weeks was recently admitted by a bodybuilder, who used the rapid-acting insulin analog Humalog LisPro. Urine samples analyzed for synthetic insulins revealed the presence of the intact compound as well as its desB30 metabolite, and product ion spectra of B-chains of both analytes are shown in Fig. 7. Humalog LisPro gives rise to an abundant and characteristic y_2 product ion at *m*/*z* 217 (Fig. 7a) due to the preferred cleavage site at the N-terminus of proline located at position 29 (vide supra). The metabolite, which lacks the

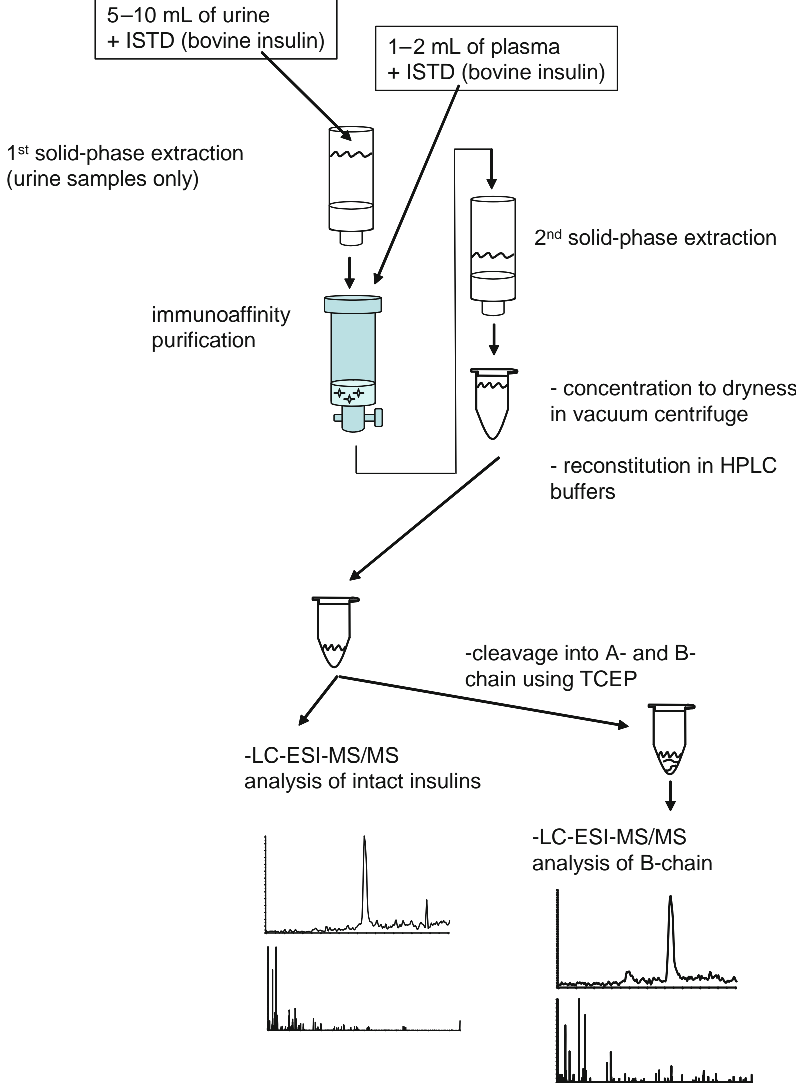
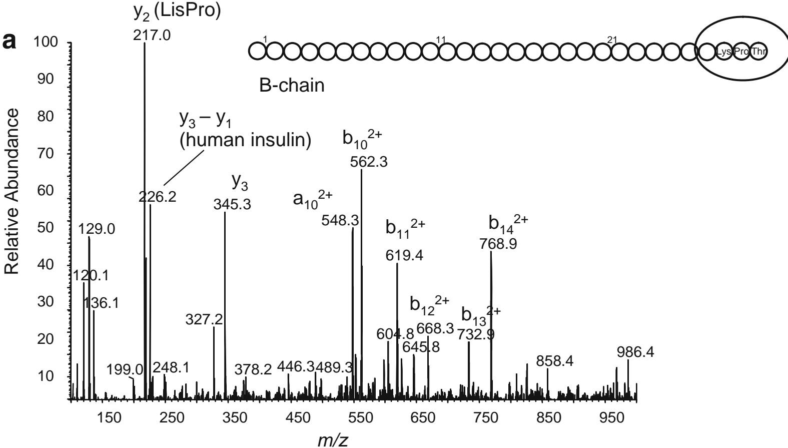


Fig. 6 Schematic overview of the sample preparation for the mass spectrometric identification of synthetic insulins in human urine or plasma

C-terminal threonine, generates an informative product ion y_1 at m/z 116 and further fragments that constitute an amino acid sequence tag (b_{10}–b_{13}) that unequivocally identifies the target compound (Fig. 7b). In addition, trace amounts of natural human insulin were detected (data not shown).

Abuse of insulin in sports is evident if xenobiotic synthetic analogs are present in urine samples of athletes who do not suffer from diabetes mellitus. Clinical tests



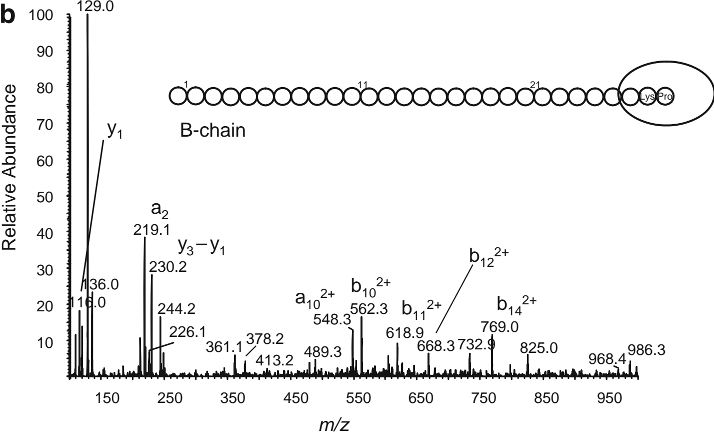


Fig. 7 Results of the analysis of an authentic urine sample provided by a bodybuilder misusing Humalog LisPro. The product ion mass spectrum of the analyte's B-chain unambiguously proves the presence of Humalog LisPro but also human insulin by product ions at *m*/*z* 217 and 226 (**a**). The product ion spectrum of $[M+4H]^{4+} = 833.2$ of the C-terminally truncated B-chain represents a urinary metabolite generated by in vivo metabolic conversion (**b**). The product ion at *m*/*z* 116 represents the y_1 ion composed by proline, which is present in the C-terminally cleaved Humalog LisPro only

that demonstrate an impaired glucose metabolism yield therapeutic use exemptions (TUEs) for diabetic athletes, and numerous cases of rapid-acting insulins in doping control samples were found between 2005 and 2006. Five specimens were found to contain Novolog Aspart, one sample Humalog LisPro and another four contained Glulisine Apidra, an insulin analog that was approved in 2006. All athletes were provided with TUEs and, thus, not sanctioned for drug abuse. However, the issue of

recombinant human insulin misuse is still present as no valid test has been introduced to unequivocally determine its application. First promising approaches were recently published based on different metabolic profiles of insulins that are subcutaneously administered and those secreted from pancreatic β-cells (Thomas et al. 2007). Forthcoming studies will have to demonstrate whether these or alternative assays will be a useful tool in the fight against insulin misuse in sport.

7 Conclusions

The peptide hormone insulin is a versatile and essential hormone for the control of blood glucose levels but is also of considerable importance to protein anabolism. Hence, it has been considered relevant in doping controls and recent studies have revealed its misuse and options for detecting intact analytes and metabolic degradation products in plasma and urine samples. Mass spectrometry plays a key role in these assays due to its sensitive and specific nature that enables discrimination between natural insulin and modified synthetic analogs even when identical molecular masses are given. However, more information, in particular on metabolic pathways, is required to cope with the problem of recombinant human insulin misuse.

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β-Adrenergic Stimulation

Peter Van Eenoo and Frans T. Delbeke

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Abstract Two groups of substances which stimulate the adrenergic system are listed as prohibited by the World Anti-Doping Agency. Stimulants are prohibited in-competition only and β_2-agonists are prohibited in- and out-of-competition. While β_2-agonists act directly on the target receptors, sympathomimetic amines can exert their action directly and indirectly. Due to differences in pharmacology but mainly due to differences in administered dose, differences in detection methods between both groups of substances exist although preparation is similar and consists of an extraction at basic pH. Gas chromatography coupled to mass spectrometry has been the detection methodology of choice for several decades. However, the importance of liquid chromatography–mass spectrometry as a

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preferred detection methodology is rapidly increasing, especially for the detection of β_2-agonists and new additions to the list of prohibited stimulants, such as modafinil. Pharmacology, metabolism and detection of both groups of prohibited substances will be discussed.

Keywords Stimulant • Beta-agonist • Chromotography • Mass spectrometry • Metabolism

1 Introduction

The prohibited list of the World Anti-Doping Agency (WADA) contains two groups of banned substances (stimulants and β-agonists) as well as one group of restricted substances (β-blockers) which exert their pharmacological action via the adrenergic system (De Boer 2000; WADA 2008a).

Both the sympathomimetic stimulants and the β-agonists act as adrenergic agonists, while the β -blockers are classified as adrenergic antagonists (Armstrong 1988; Docherty 2008; De Boer 2000; Rang et al. 2001; Van Wimersma Greidanus 2000). Hence only the actions, metabolism and detection of the first two groups will be discussed.

2 Noradrenaline and the Nervous System

The autonomic nervous system consists of the parasympathetic (controlling body functions during rest) and the sympathetic (preparing the body for the fight/flight function) branches (Van Wimersma Greidanus 2000). The nerves of the sympathetic nervous system do not link target organs directly to the spinal cord, but form synapses with a second type of neurons in the ganglia. Further differentiation can be made between preganglionic (between the spinal cord and ganglia) and post-ganglionic nerves (between ganglia and effectors). The catecholamine noradrenaline (NA), supplemented by others (Fig. 1), e.g. adrenaline (A), is the transmitter released by the terminal of postganglionic nerves, which acts on the adrenoreceptors (Armstrong 1988; Van Wimersma Greidanus 2000).

Most of the NA in the neurons is stored in vesicles, from which it can be released by substances that act on the presynaptic receptors. There is evidence that NA can

L-Tyrosine → (Tyrosyne hydroxylase) → L-Dihy droxy pheny lalanine (L-DOPA) → (DOPA decarboxylase, Aromatic L-amino acid decarboxylase) → Dopamine → (Dopamineβ-hydroxylase) → Noradrenaline → (Phenylethanolamine N-methyltransferase) → Adrenaline

Fig. 1 Biosynthesis pathway of dopamine, adrenaline and noradrenaline

regulate its own release via the auto-inhibitory feedback mechanism. After release, NA reuptake takes place or NA is slowly degraded enzymatically in the cells (Armstrong 1988; Rang et al. 2001; Van Wimersma Greidanus 2000).

There are two main mechanisms for uptake of catecholamines; uptake 1 and uptake 2. Uptake 1 is selective for NA and has a low maximum uptake rate. In contrast, uptake 2 has a high maximum uptake rate, but it can also accumulate other catecholamines, including A.

Metabolism of NA (and other catecholamines) occurs mainly via two intracellular enzymatic pathways, using monoamine oxidase (MAO) and catechol-*O*-methyl transferase (COMT), respectively. Since degradation occurs, intracellular uptake of NA precedes this degradation. MAO can oxidize monoamines to their corresponding aldehyde intracellularly; this aldehyde is then rapidly metabolized by aldehyde dehydrogenase to the carboxylic acid analog. COMT methylates one of the hydroxyl groups of the catechol from a monoamine (or its degradation products produced via the MAO-pathway) resulting in a methoxy derivative (Docherty 2008; Rang et al. 2001).

In contrast to the central nervous system, where NA is degraded to an important extent via MAO, followed by reduction of the alcohol metabolites to 3-hydroxy-4-methoxyphenylglycol, MAO and COMT are of less importance in the periphery, since NA is mostly removed by reuptake 1 (Docherty 2008; Rang et al. 2001).

3 Receptor Agonists Capacities

Catecholamines are substances containing a catechol nucleus and an amine substituent. Among the most important endogenous catecholamines are A, NA and dopamine, while isoprenaline (I) is an important synthetic catecholamine. All these catecholamines exert different effects on the body through differences in receptor agonist potencies (Armstrong 1988).

The adrenoreceptors, present in smooth muscle tissues, can be classified into α-and β-receptors based upon agonist potency, as determined by Ahlquist (Armstrong 1988; Rang et al. 2001). These main classes of adrenoreceptors can further be divided into subcategories (α_1, α_2, β_1, β_2, β_3). Although each adrenoreceptor has different functions, the main functions of α_1 are vasoconstriction and for α_2 the inhibition of NA release. The distinction of the β-adrenergic receptors is of even more significance, since the β_1-receptors are mainly found in the heart, where they increase its rate and force of contraction through positive inotropic and chronotropic effects of the catecholamines, and the β_2-receptors cause relaxation of smooth muscles in many organs. Pharmaceutical companies have tried to develop substances that selectively act on one subtype of receptor. It should be noted that such selectivity is never absolute (Armstrong 1988; Rang et al. 2001).

The potencies of the discussed catecholamines for the different adrenoreceptors are summarized in Table 1 together with possible effects of major importance to performance level.

Table 1 Target receptors, potency and effect for adrenaline, noradrenaline and isoprenaline

Receptor	Potency	Effects
α_1	A ≅ NA >>I	Vasoconstriction, hepatic glycogenesis
α_2	A ≅ NA >>I	Inhibition of NA release from ANS, contraction of vascular smooth muscle, inhibition of insulin release
β_1	I>A ≅ NA	Increased heart rate and contraction force
β_2	I>A >>NA	Bronchodilation, vasodilation, relaxation of visceral smooth muscle, hepatic glycogenolysis, muscle tremor
β_3	I ≅ NA>A	lipolysis

In sports, stimulation of the β_2-receptors offers the best possibilities for performance enhancement since they have bronchodilator and anabolic actions and enhance anti-inflammatory actions of corticosteroids (Davis et al. 2008). Those substances primarily acting directly on this receptor will be discussed mainly under "β-agonist", although it should be noted that several substances discussed under "sympathomimetic amines" could potentially also exert a direct action (Armstrong 1988; Docherty 2008; Rang et al. 2001).

Moreover, an overwhelming majority of studies on the enhancement of performance by neurotransmitters have focused on the sympathomimetic system and on the adrenergic responses. However, several prohibited substances also act on dopamine (dopaminergic system) and serotonin levels (serotonergic system) and most of the sympathomimetic amines do not act exclusively on one of these systems, but act simultaneously on several of them. Indeed, since dopamine is a precursor of NA in its synthesis pathway, exerting an effect on dopamine levels will inevitably have effects on NA (Docherty 2008; Gilber 1995; Rang et al. 2001; Van Wimersma Greidanus 2000).

4 Sympathomimetic Amines

In general these substances (Fig. 2) primarily act indirectly by influencing NA concentrations. Several modes of action (Armstrong 1988; Docherty 2008; Rang et al. 2001; Van Wimersma Greidanus 2000) can be distinguished, including:

- Release (displacement) of NA or another neurotransmitter
- Blocking the re-uptake of NA or another neurotransmitter (monoamine reuptake inhibition)
- Influencing metabolism of the catecholamines via inhibition of monoamine oxidase (MAO)
- Stimulation of post- or presynaptic receptors

Most of the sympathomimetic amines in the WADA prohibited list act mainly on the monoaminergic system. Others like strychnine, crotethamide, cropropamide, pentetrazol and nikethamide are respiratory stimulants (Hemmersbach and de la Torre 1996).

Historically, stimulants were one of the first groups of substances to be misused and they have been connected to several high-profile cases, such as the death of cyclist Tom Simpson climbing the Mont Ventoux during the Tour de France of 1967. These substances may be used to reduce tiredness and increase alertness, competitiveness and aggression. Traditionally they have been used shortly before competition to directly improve performance. Therefore, they are currently only prohibited in-competition. However, there is evidence that they are also used during training in order to increase the intensity of training.

Most substances listed as stimulants by WADA can be classified as psychomotor stimulants. This category contains amphetamine and related products, but also cocaine and the previously prohibited methylxanthines (caffeine and theophylline).

Over the period 2004–2007, more than 11% of all adverse analytical findings in doping control laboratories world-wide were still attributed to this category of substances. So, although these substances have been used and produced for several decades and might seem "old-fashioned", they are still popular and relatively widely abused (WADA 2008a).

Although some sympathomimetic amines can partly (and weakly) act directly on the nervous system, most of the stimulation is attributed to their indirect effects. These substances resemble NA to a sufficient extent to be transported into the nerve terminals via the neuronal uptake. Once inside the nerve terminals, these substances are taken into the vesicles in exchange of NA by the vesicular monoamine transporter. The released NA can then act on postsynaptic receptors.

The effects of these substances are not specific, since they partly act directly on adrenoreceptors, partly inhibit the neuronal uptake and partly inhibit MAO which degrades NA (Armstrong 1988; Docherty 2008; George 1988; Rang et al. 2001; Van Wimersma Greidanus 2000).

4.1 Amphetamines and Related Compounds

A huge number of amphetamine analogs are available on the pharmaceutical and black markets, including substances derived from phentermine, ephedrine, fenfluramine, phenmetrazine and methylenedioxymethamphetamine (MDMA, commonly referred to as ecstasy or XTC). Several of these substances have been used therapeutically as appetite suppressors, while others are particularly well known for their use in the "disco environment" (Armstrong 1988; Avois et al. 2008; George 1988).

In sports, amphetamine is still by far the most abused stimulant. Moreover, it is the main phase-1 metabolite of a high number of popular analogs (Table 2).

The dose of amphetamine plays a crucial role in the effect desired by an athlete. While aggressiveness increases with dose, elevated alertness only seems to appear at lower dosages. In general, amphetamines might have the following beneficial side-effects in sports: increased aerobic endurance capacity, acceleration, alertness,

Table 2 Overview of metabolism markers for stimulants

Metabolite	Parent drug
Amphetamine	Amphetamine, benzylamphetamine, benzphetamine, ethylmethamphetamine, ethylamphetamine, isopropylmethamphetamine, isopropylamphetamine, dimethylamphetamine, methamphetamine, furfurylmethamphetamine, selegiline, amphetaminil, clobenzorex, famprofazone, fencamine, fenethylline, fenproporex, mefenorex, mesocarb, phenatine, prenylamine, racefeine
Methamphetamine	Methamphetamine, benzphetamine, ethylmethamphetamine, isopropylmethamphetamine, dimethylamphetamine, furfurylmethamphetamine, selegiline
Phenmetrazine	Fenbutrazate, phendimetrazine, phenmetrazine, morazone
Mephentermine	Mephentermine, oxethazaine
Phentermine	Phentermine, mephentermine, oxethazaine
Norfenfluramine	Norfenfluramine, fenfluramine, benfluorex
Cathine	Cathine, cathinon, methamfepramone, pseudoephedrine, methyl-pseudoephedrine
Pseudoephedrine	Pseudoephedrine, methamfepramone, methactinon, methylpseudoephedrine
Ephedrine	Ephedrine, methylephedrine
Phenylpropanolamine	Phenylpropanolamine, amfepramone, ephedrine, etafedrine, methylephedrine
MDA	MDA, MDMA, methylendioxyethylamphetamin (MDEA)

self-confidence, endurance, muscular strength, body fat metabolism and lactic acid levels at maximal exercise (Avois et al. 2008).

4.2 Ephedrines

The presence of ephedrines in numerous over-the-counter medicines and so-called “nutritional supplements” is widespread. Therefore the urinary concentration of ephedrine, cathine and methylephedrine need to exceed a threshold level before an adverse analytical finding is issued. Until a few years ago two other ephedrines (pseudoephedrine and phenylpropanolamine, also known as norephedrine) also figured on the list of prohibited substances, but they have been removed since 2006.

In traditional medicine, the Ephedra alkaloids have been used in the treatment of allergies and asthma for over 5,000 years. The extracts of different Ephedra species contain mixtures of ephedrine, pseudoephedrine, *N*-methylephedrine, *N*-methyl-norpseudoephedrine, cathine and phenylpropanolamine, but ephedrine and pseudo-ephedrine constitute more than 80% of all alkaloids in the dried plant species (Avois et al. 2008; Soni et al. 2004; Van Eenoo et al. 2001).

Ephedrine is the most potent amongst these alkaloids and is excreted rapidly (approx. 95% within the first 24 h) and mainly unchanged after oral intake. Although currently not prohibited by WADA, high doses of pseudoephedrine

might lead to concentrations of cathine (also known as norpseudoepehedrine), a major phase-1 metabolite, exceeding the urinary threshold (Soni et al. 2004).

The widespread prevalence of these natural ephedrines in nutritional supplements, often undeclared or declared as "Ephedra extracts" or "Ma Huang extracts" on the labels, has also resulted in several cases where athletes have inadvertently tested positive (Yonamine et al. 2004).

Although many studies have indicated that the individual use of ephedrine, pseudoephedrine or phenylpropanolamine in therapeutic doses has little or no ergogenic effect (Clemons and Crosby 1993; Gillies et al. 1996; Swain et al. 1997), it should be noted that other studies, primarily where these substances are combined with other drugs (e.g. caffeine) support a potential ergogenic effect (Bell et al. 2001; Bell et al. 1998; Bell and Jacobs 1999). Not surprisingly, many athletes therefore resort to these substances to increase energy and decrease feelings of fatigue. Particularly the combination of ephedrine with caffeine (and aspirin), the so-called ECA-stack, which is largely popular in bodybuilding, might warrant further research (Avois et al. 2008).

4.3 Strychnine and Cocaine

Strychnine and cocaine are two stimulants which are not structurally related to the previously described groups.

Strychnine and cocaine are amongst the earliest substances used to enhance performance in sports. Already in the nineteenth century cyclists reportedly used cocktails of caffeine, cocaine, alcohol, ether and strychnine in endurance events and perhaps the first reported drug-related death in sports (Arthur Linton in 1896) was due to strychnine (Van Eenoo et al. 2006).

Today, strychnine and several other analeptics (e.g. nikethamide, crotethamide, cropropamide) still appear on the list of prohibited substances in sports (WADA 2008b) and strychnine is one of the few substances for which an individual minimum required performance limit (MRPL, the concentration at which a laboratory routinely should be capable of detecting a prohibited substance) of 200 ng mL^{-1} has been specified (Van Eenoo et al. 2006). These analeptics stimulate several parts of the nervous system. In sports, the increase in respiratory volume is mainly desired. Strychnine can also be present in over-the-counter natural products and excretion studies with such preparations have shown that intake of such a "supplement" can lead to detectable urinary concentrations (Van Eenoo et al. 2006).

Cocaine has been used as a medicine for many years as a local anesthetic, although it is now only seldom used as such, and known far more for its misuse outside sports. Misuse of cocaine is mainly linked with feelings of decreased fatigue and euphoria and in sports it might be used to achieve an increased alertness and elevated arousal (Avois et al. 2008). Adequate detection of cocaine use focuses on its metabolites, predominantly benzoylecgonine, which can be detected either

by gas chromatography–mass spectrometry, liquid chromatography–mass spectrometry or immunochemical methods.

4.4 Modafinil and Adrafinil

Modafinil is a relatively novel psychostimulant, claimed to be nonaddictive, used for treating excessive daytime sleepiness or narcolepsy without interfering with nocturnal sleep, attention deficit hyperactivity disorder (ADHD) and in some cases amphetamine abuse (Docherty 2008; Lopez 2006; Sweetman 2007; Tseng et al. 2005). The exact mechanism of modafinil's action in the brain is not yet fully understood. Studies have proposed that modafinil indirectly modulates the release of gamma aminobutyric acid (GABA) in areas of the brain that regulate sleep and wake cycle in both humans and animals (Tseng et al. 2005). Other studies suggest that this drug activates noradrenergic and dopaminergic systems – presumably by indirect actions at the re-uptake transporters – thereby promoting wakefulness and increasing alertness. Other studies suggest that it does not appear to have central and peripheral side-effects associated with conventional dopaminergic psychostimulants (Docherty 2008). The first doping violation involving modafinil was reported in 2003 at the Track and Field World Championships (Kaufman and Gerner 2005). Subsequently, it was prohibited in sports together with adrafinil. Modafinil was also mentioned as a drug used by the athletes in the BALCO case (known for the introduction of the designer steroid tetrahydrogestrinone) (Hatton 2007).

Adrafinil is chemically related to modafinil and acts as a central 1-adrenoreceptor stimulant and α_1-adrenoreceptor agonist.

Both modafinil and adrafinil are metabolized (phase 1) to modafinil acid. Distinction between the use of either product can be made by detecting both the metabolite and the parent drug.

4.5 Methamphetamine – Isomers: The Baxter Case

Different stereoisomers of stimulants can have very different pharmacological action. The natural and pharmacologically most potent form of ephedrine, for example, is the R-isomer, while the synthetic compound is a racemic mixture. For methamphetamine, the stereoisomer detected in urine can result in differences in sanction. Indeed, under the current WADA regulation R-methamphetamine is listed as a specified substance for which a reduced sanction can be issued if the athlete can establish that the use was inadvertent (WADA 2008b).

R-methamphetamine only has about 33% of the CNS activity of its S-enantiomer and therapeutic use would not result in significant CNS effects (Logan 2002).

In the case of methamphetamine, the possibility of inadvertently testing positive was highlighted in the Baxter case. UK Olympic Bronze Medal winner Alain Baxter was stripped of his medal at the 2002 Salt Lake City Olympic Games and suspended for 3 months after testing positive for R-methamphetamine due to the use of the American "Vicks" vapor inhaler. In contrast to the British (and other European) Vicks preparations, the US Vicks contains R-methamphetamine while the European preparation does not contain methamphetamine and hence is doping free, the use of the American analog is not (Deventer et al. 2007b). S-methamphetamine on the other hand is only available as a prescription drug or from illicit sources.

The drug S-methamphetamine is a highly addictive central nervous system stimulant that can be injected, snorted, smoked, or ingested. It is a popular drug of abuse in the United States, where it is commonly referred to as crank, crystal meth or ice and where methamphetamine laboratories are by far the most frequently encountered clandestine laboratories (Logan 2002).

While even under the current regulations Baxter would still be stripped from his medal due to the strict liability rule in WADA's World Anti-Doping Code, the period of suspension would most probably be less than the standard 2-year ban for the use of stimulants. This means of course that doping control laboratories must differentiate between both isomers (Deventer et al. 2007b).

Similar cases of inadvertent doping positive tests can frequently be encountered with other over-the counter medicines, "nutritional" supplements and homeopathic/natural preparations (although under the current strict liability rule of WADA athletes remain responsible for the presence of prohibited substances in their body fluids). Amongst the biggest risks are:

- Ephedrines, present in multiple OTC preparations for the treatment of coughs and colds as well as in nutritional supplements, often under the names Ephedra or Ma Huang (Deventer et al. 2007b; Van Thuyne et al. 2006).
- Strychnine, present in natural/herbal preparations (Van Eenoo et al. 2006)
- Cocaine, in several regions used as a preparation against altitude illness or present in traditional mate de coca teas (South America). In these cases it can be assumed that an athlete should be aware of this use (Turner et al. 2005)
- Contaminated nutritional supplements (Van Thuyne et al. 2006).

Several reports have been issued of nutritional supplements which are contaminated with prohibited substances. Over the years most of the attention has focused on the presence of anabolic androgenic steroids. However several cases have also reported the presence of undeclared stimulants as well. The reported stimulants include: phentermine, fenfluramine, ephedrines and sibutramine as well as MDMA (XTC) (Van Thuyne et al. 2006).

Phentermine, fenfluramine and sibutramine were present in supplements promoted for weight loss and were not declared on the label (Van Thuyne et al. 2006).

Before its removal from the prohibited list, caffeine was another substance often present in supplements under different names, including guarana, which has led to

inadvertent doping positives (Van Thuyne et al. 2006). Actually, caffeine has been removed from the prohibited list and has been put on the monitoring list. Indeed, besides prohibited substances, WADA also lists several substances of which the use is monitored by doping control laboratories. These results are then evaluated and will be used to either remove a substance or to put it (back) on the prohibited list (WADA 2008b; Van Thuyne and Delbeke 2006; Van Thuyne et al. 2005). The use of these substances does not constitute a doping offense. Currently, these substances are: caffeine, phenylpropanolamine and pseudoephedrine (cfr. ephedrines), bupropion (a weak blocker of monoamine re-uptake and used in treatment of tobacco dependence), pipradol (a dopamine releasing dopaminomimetic used in preparations against fatigue), phenylephrine and synephrine (WADA 2008b).

4.6 Bromantane

Bromantane is an adamantine derivate that has a bromaniline side chain. This Russian stimulant was developed as an immuno-stimulator and is reported to have serotonergic, dopaminergic and limited noradrenergic actions, similar to mesocarb (Docherty 2008; Kudrin et al. 1995; Morozov et al. 2000). Bromantane was first detected in 1996 (Ayotte and Goudreault 1999), but due to juridical problems nine doping cases at the Atlanta Olympic Games were classified without consequences for the athletes. It was included in the IOC list of prohibited substances in September 1996, shortly after the Olympic Games. Bromantane had previously been used by Russian soldiers and sportsmen, but was not a registered pharmaceutical preparation (De Rose 2008). As such, it might be regarded as (one of) the first designer doping agents, specifically synthesised/used to circumvent detection in doping control laboratories.

Initially bromantane was classified both as a stimulant as well as a masking agent, since its use can interfere with the chromatographic behavior of the GC–MS analysis of endogenous steroids. Today, bromantane is solely prohibited as a stimulant (WADA 2008b), as its masking capacities can be attributed to shortcomings in analytical capacities rather than to its inherent pharmacological action.

Bromantane is metabolized primarily through hydroxylation at C-6, although other hydroxylation sites have been reported as well (Sizoi et al. 1997; Ueki et al. 1997; van de Kerkhof et al. 1998).

4.7 Pemoline

The monoamine central stimulant pemoline (2-imino-5-phenyl-4-oxazolidinedione) is nowadays withdrawn from most markets due to its liver toxicity (Docherty 2008). Although structurally different, it has similar actions to methylphenidate and was used therapeutically for the treatment of ADHD. In the 1970s it was a widely

popular doping agent due to analytical detection problems (Ayotte 1993). In general, stimulants were/are extracted at high pH (Deventer et al. 2007b; Hemmersbach and de la Torre 1996; Van Thuyne et al. 2007; Van Thuyne et al. 2008); pemoline however is unstable under basic conditions. As a consequence the detection methods used at that time did not detect pemoline misuse, unless it was converted via an oxidation step to mandelic acid. However, mandelic acid is also formed by metabolism of styrene and other drugs and hence this technique lacked the necessary specificity. A breakthrough was achieved by Libeer and Schepens (1978), who applied an acidic hydrolysis step to form 5-phenyl-2,4-oxazolidinedione, which could then be detected via GC. This initially resulted in a high number of positive findings. In the 1990s a direct method for detecting pemoline after extraction at pH 9.0 as a TMS-derivative by GC–MS screening methods for anabolic steroids (Ayotte 1993) further raised the awareness of athletes about the detectability of this stimulant.

4.8 Methylphenidate

Methylphenidate (ritaline) is the major drug in the treatment of ADHD and is an NA and dopamine re-uptake inhibitor. It is mainly metabolized to ritalinic acid. Similar to pemoline, analytical problems associated with the detection of this substance made it a popular drug of abuse amongst athletes in the 1970s. Initially the detection of this substance was attempted with the routinely used methods, at that time TLC or GC with flame ionization detection after an alkaline extraction. However, methylphenidate is an ester which can easily be hydrolyzed at alkaline pH during extraction or on the potassium hydroxide-coated GC-column support material that was frequently used. A method for the detection of the parent drug after extraction at pH 8–9 and detection with an electron capture detector (ECD) proved to be adequately sensitive to detect misuse of methylphenidate and to catch several doping offenders in 1974 (Delbeke and Debackere 1975). Elucidation of the metabolism leading to the detection of ritalinic acid as a major metabolite further improved detection capabilities.

5 β_2-Agonists

The structure of several β_2-adrenergic agonists is shown in Fig. 2. The physiological activity of each β_2-agonist depends on its inherent activity at the receptor (Baak 2000; Rang et al. 2001; Smith 1998). All β_2-agonists including their R and S isomers are prohibited (WADA 2008b). β_2-Agonists are widely used in the treatment of asthma. For therapeutic reasons, formoterol, salbutamol, salmeterol and terbutaline can be used via inhalation by an athlete if a therapeutic use exemption (TUE) has been obtained (Kindermann and Meyer 2006). For such purposes a medical file will be needed to provide evidence of the medical condition of the

Substance	R1	R2	R3	R4	R5	R6	a-e
amphetamine	H	H	H	CH_3	H	H	
S-methamphetamine	H	H	H	CH_3	H	CH_3	
R-methamphetamine	H	H	CH_3	H	H	CH_3	
phenylpropanolamine	H	OH	H	CH_3	H	H	
cathine	OH	H	H	CH_3	H	H	
ephedrine	H	OH	H	CH_3	H	CH_3	
pseudoephedrine	OH	H	H	CH_3	H	CH_3	
methylephedrine	H	OH	H	CH_3	CH_3	CH_3	
phenmetrazine	H	OCH_2CH_2	H	CH_3		H	
mephentermine	H	H	CH_3	CH_3	H	CH_3	
phentermine	H	H	CH_3	CH_3	H	H	
norfenfluramine	H	H	H	CH_3	H	H	d: CF_3
MDA	H	H	H	CH_3	H	H	c-d: O-CH_2-O

Substance	a	b	c	d	e	R
salbutamol	H	OH	H	CH_2OH	H	$C(CH_3)_3$
clenbuterol	H	Cl	NH_2	Cl	H	$C(CH_3)_3$
terbutaline	H	OH	H	OH	H	$C(CH_3)_3$
salmeterol	H	H	OH	CH_2OH	H	$(CH_2)_6$-O-$(CH_2)_4$-C_6H_5
formoterol	H	H	OH	HCONH	H	O
fenoterol	H	OH	H	OH	H	OH
isoprenaline	H	H	OH	OH	H	$C(CH_3)_3$

Fig. 2 Examples of structures of sympathomimetic amines and β_2-agonists

athlete. Several β_2-agonists are available as different preparations. Despite a multitude of research efforts, distinction between different application routes remains a challenging task for doping control laboratories and it is customary that adverse analytical findings for this class of substances are classified without suspension, if the athlete has a TUE. So far, the only exception to this rule is salbutamol for which a threshold level has been established (WADA 2008b).

β_2-Agonists are mainly prohibited in sports because of their potential ergogenic potential in nonasthmatic athletes, although results of various studies seem to be contradictory. One of the difficulties encountered in this type of study lies in the experimental set-up and the possibility that there might be large inter-individual differences in response. It is clear from all studies that the VO_2-max will not be affected, but anaerobic performance and aerobic endurance capacity might be (Baak 2000; Kindermann and Meyer 2006).

Additionally, evidence exists that the continued use of high oral doses of clenbuterol and salbutamol can result in increased muscle strength. Therefore these substances are also classified as anabolic agents on WADA's prohibited list. On a yearly basis between 10 and 15% of all adverse analytical findings in doping control laboratories are attributed to this type of substance (WADA 2008a).

6 Detection

In doping control, samples are either collected in conjunction with a specific competition (the so-called in-competition test, IC) or at another occasion (out-of competition test, OOC). While stimulants are only prohibited in-competition, β-agonists are prohibited both during training (OOC) as well as in-competition (WADA 2008b). For both types of substances, urine is collected.

Although urine has many advantages in doping control since it can be collected repeatedly and is noninvasive, there are some disadvantages as well. One of the major disadvantages is the absence of a clear correlation between the concentration and the pharmacological activity. Urine flow and pH are only two parameters of a complete range which can distort such a correlation (Hemmersbach and de la Torre 1996).

A differentiation in detection techniques between stimulants and β_2-agonists is needed, mainly because of the differences in dose and route of administration. Indeed, in most cases the urinary concentration of stimulants will easily exceed 100 ng mL^{-1} in the period needed to monitor their misuse (period during and shortly after which they can exert stimulating effects). In contrast, several β-agonists (e.g. salmeterol, formoterol) are excreted in the low ng mL^{-1} range if administered via inhalation (Ventura et al. 2000).

For stimulants, WADA has established a MRPL of 500 ng mL^{-1}. For β_2-agonists such a limit has not yet been established, except for clenbuterol (MRPL = 2 ng mL^{-1}), but general consensus exists that such a limit needs to be far lower for β_2-agonists than for stimulants, taking into account the substantially lower concentrations at which these substances are excreted.

Most of the β_2-agonists and stimulants share several chemical characteristics (Fig. 2): they have a basic character, are relatively short-acting and although they can undergo phase 1 and 2 metabolism, they are mainly excreted as the parent drug in urine. On the other hand there are several important discrepancies as well. Indeed, the pharmacological effects of stimulants are mainly caused by relatively high doses, while small doses of β_2-agonists, administered via inhalation, can exert effects through their direct action.

For both the β_2-agonists and the stimulants, a similar sample preparation technique might show an efficient extraction recovery, but the detection technique will need to be different (Pereira et al. 2008; Spyridaki et al. 2006; Ayotte and Goudreault 1999; Trout and Kazlauskas 2004; Van Thuyne et al. 2007, 2008; Ventura et al. 2000). Several sample preparation methods have been described. In general, two basic types can be differentiated: liquid–liquid extraction (LLE) and solid-phase extraction (SPE), or a combination of them. By far the most widespread methodology is still LLE, based upon the studies by Donike and Beckett in the 1960s and 1970s. Both for stimulants and β-agonists, the sample preparation scheme consists of alkalinisation of the urine to a pH value exceeding 9.5, addition of an internal standard, followed by LLE with diethylether or *tert*-butylmethylether. This extraction can eventually be preceded by an enzymatic or chemical hydrolysis to remove glucuronic acid or sulfate groups that might have been added to the target molecule during phase 2 metabolism (Hemmersbach and de la Torre 1996; Pereira et al. 2008; Spyridaki et al. 2006; Trout and Kazlauskas 2004; Van Thuyne et al. 2007, 2008). For most stimulants and β_2-agonists, hydrolysis is however not required, and in many cases adequate detection can be performed by screening for the parent compound, although phase 1 metabolism is important for several others. After extraction the organic layer is then transferred and evaporated under a light flow of nitrogen. Because most stimulants are volatile, particular attention should be paid to this step with respect to flow and temperature (Van Thuyne et al. 2007, 2008).

Stimulants are often excreted in urine as the unconjugated parent drug. Phase 1 metabolism is limited or leads to the formation of a small number of nonspecific metabolites predominantly through *N*-dealkylation. Therefore screening methods focusing only on amphetamine, methamphetamine, phenmetrazine, mephentermine, phentermine, norfenfluramine, "the" ephedrines and 3,4-methylenedioxyamphetamine (MDA) can be quite effective (Table 1). However, the high number of parent drugs metabolized to these nonspecific substances also requires particular care during confirmation to establish the identity of the administered parent drug or a specific metabolite. Moreover, from a juridical and pharmacological point of view, the identification of a spectrum of metabolites has the advantage of demonstrating biotransformation in the body and hence avoids possible allegations of tampering (e.g. spiking a sample) (De Boer 2000; Hemmersbach and de la Torre 1996; Van Thuyne et al. 2007, 2008).

Since all sympathomimetic stimulants contain an amine function, are volatile and are expected to be excreted in relatively high urinary concentrations, the detection of these substances has focused during recent decades on capillary gas

chromatography equipped with nitrogen–phosphorous detection (GC-NPD) for screening purposes (De Boer 2000; Hemmersbach and de la Torre 1996; Van Thuyne et al. 2007, 2008).

If sample preparation and instrumental settings are adequately chosen, the (relative) retention time of a substance using GC-NPD gives a strong indication of its identity and the number of false positive initial identifications is limited. Currently this technique is still in use in most laboratories, although several stimulants are also often screened for by GC–MS in combination with other groups, e.g. narcotics (De Boer 2000; Hemmersbach and de la Torre 1996; Pereira et al. 2008; Spyridaki et al. 2006; Trout and Kazlauskas 2004; Van Thuyne et al. 2007, 2008).

For confirmatory purposes a mass spectrometric technique is required, since only this technique in combination with chromatography offers adequate certainty for unequivocal identification of a prohibited doping substance or its metabolite (Van Eenoo and Delbeke 2004). Currently GC–MS is still most often used for stimulants. A derivatisation step is required since the mass spectra of underivatised stimulants are very unspecific (Van Eenoo and Delbeke 2004). The majority of substances are derivatised using *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), *N*-methylbis(trifluoroacetamide) (MBTFA) or trifluoroacetic acid anhydride (TFAA). It should be noted that derivatisation of many stimulants with more than one functional group is often far from complete and multiple derivatives can be formed (Hemmersbach and de la Torre 1996; Van Thuyne et al. 2007, 2008). Moreover, using a stereoselective derivatisation agent, enantiomeric separation can be achieved, a prerequisite for certain stimulants (methamphetamine) to allow for a proper evaluation. In the case of methamphetamine stereoselective analysis can be achieved by pre-column chiral derivatization with *S*-(−)-*N*-(fluoroacyl)-prolyl chloride or *S*-(−)-heptafluorobutyrylprolyl chloride and GC–MS analysis (Martins et al. 2006), or by using either chiral derivatisation or chiral columns in LC–MS analysis (Kraemer and Maurer 1998).

Recently, a number of methods based upon liquid-chromatography mass spectrometry (Sect. 7) have been published for stimulants (Deventer et al. 2006, 2007b). Generally screening by LC–MS in doping control laboratories is uncommon compared to other prohibited groups, but LC–MS has shown superior detection capabilities for several more problematic stimulants, including strychnine (Van Eenoo et al. 2006), ritalinic acid (Deventer et al. 2007a, 2008), the narcoleptic agents modafinil and adrafinil (Kinney et al. 2005) or *p*-OH-mesocarb (Van Eenoo et al. 2006), the main metabolite of the Russian stimulant mesocarb.

Modafinil and adrafinil are both metabolized to modafinil acid. These substances show relatively poor chromatographic behavior by GC–MS (Pereira et al. 2008), but can easily be detected by LC–MS in the ESI mode. While modafinil and adrafinil, parent compounds, are monitored in positive ionization, modafinil acid is screened for in negative ionization mode (Fig. 3).

Adequate detection of β_2-agonists can only be achieved by gas chromatography coupled to mass spectrometry (Ventura et al. 2000) or liquid chromatography coupled to mass spectrometry (Ayotte and Goudreault 1999). These techniques

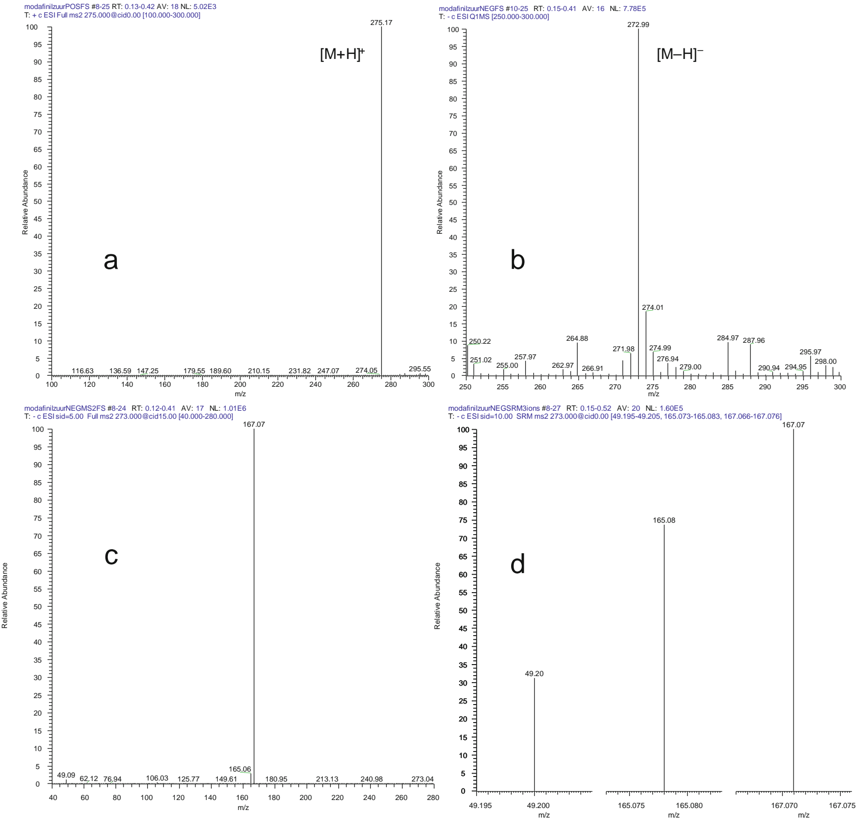

Fig. 3 LC–MS mass spectra of modafinil acid. (**a**) Full scan ESI mass spectrum in positive ionization mode; (**b**) Full scan ESI mass spectrum in negative ionization mode; (**c**) MS/MS ESI product spectrum in negative ionization mode (precursor ion *m*/*z* 273); (**d**) Selected reaction monitoring (SRM) spectrum in ESI negative ionization mode (precursor ion *m*/*z* 273)

are more sophisticated and more expensive than GC-NPD, but offer lower detection limits and improved specificity. Upon their introduction on the prohibited list, screening and confirmation of these substances has been performed by GC–MS after derivatization, often using the same method as for anabolic steroids. Several separate methods have been developed using more complex derivatisation techniques and more sophisticated instruments like high resolution mass spectrometers (HRMS) or tandem mass spectrometers (GC–MS^n) to achieve detection limits capable of confirming the minute concentrations some β_2-agonists administered via inhalation (Ventura et al. 2000). Clenbuterol has an individual MRPL of 2 ng mL^{-1} and belongs to this category of substances, similar to salmeterol, fenoterol and formoterol which have bulky side chains. Over the last decade however the number of β_2-agonists screened for by LC–MS has steadily increased and several screening methods have been developed, based on the easy protonation

of these substances. Without the time-consuming and often complex derivatisation step, the same sample pretreatment as for GC–MS methods can be applied, but the new LC–MS methodology offers improved detection limits for most β_2-agonists (Ayotte and Goudreault 1999).

For most doping agents a zero-tolerance policy is applied and hence the detection methods only need to focus on establishing the presence of a prohibited substance (qualitative analysis). For the stimulants ephedrine, cathine and methylephedrine and the β-adrenergic agonist salbutamol, however, threshold values have been set and hence a quantitative method is needed. This distinction between qualitative and quantitative analysis has several important implications with respect to method development and validation. Indeed, while for a qualitative method it is required that proof of identity is provided beyond any reasonable doubt, for a quantitative method, however, it must be shown that a threshold is exceeded, taking into account measurement uncertainty.

For identification purposes stringent criteria have been established for the current analytical techniques (Van Eenoo and Delbeke 2004). These techniques are based upon chromatography and mass spectrometry and provide sufficient selectivity to unequivocally determine the presence of a substance or its metabolites.

For a quantitative analysis, proof of correct quantitation has to be provided. This means that additional validation parameters like linearity over a measurement range of at least 0.5–2 times the threshold needs to be guaranteed, as well as accuracy, precision and reproducibility. Besides, analytical measurement, particularly for determinations at low levels in difficult matrices like urine, is susceptible to variation and inherent errors called measurement uncertainty (MU). MU is a parameter associated with the result of a measurement, that characterizes the dispersion of the values that could reasonably be attributed to the measurand. Only when a concentration of a prohibited substance exceeds the threshold, taking into account the MU, will a laboratory report an adverse analytical finding. It is obvious that MU should be kept as low as possible and several parameters can be helpful in this respect. The use of adequate (mainly deuterated) internal standards, volumetric material and high quality reference standards with traceability will keep MU under control. In general, every step in the analytical process has such an associated uncertainty. Hence, theoretically, fewer steps mean less uncertainty. In this perspective the recently developed methods for the direct quantitation of ephedrines and salbutamol (Spyridaki et al. 2006; Deventer et al. 2009) have the theoretical advantage that sample preparation is almost limited to the addition of an internal standard.

7 Liquid Chromatography Mass Spectrometry (LC–MS)

In 1906 Tswett mentioned the word "chromatography" for the first time (Tswett 1906) and ever since this technique has evolved rapidly as the most popular analytical separation technique. Within 20 years after its development, thin layer

chromatography was well established in many laboratories and in 1969, this technique was standardized by Stahl (1958). In the next decade a more modern and sophisticated version of TLC was developed: high performance thin layer chromatography (HPTLC). The real breakthrough for liquid chromatography was the development of “closed columns”, resulting in high performance/pressure liquid chromatography (HPLC). HPLC offered multiple advantages, including more precise and rapid separations.

In 1899 the principle of mass spectrometry was laid down by Thomson and in 1956 the first mass spectrometer was coupled to a gas chromatograph, resulting in the first chromatography–mass spectrometry hyphenated technique. Supported by the development of capillary columns, GC–MS rapidly developed as a popular analytical technique during the 1980s. Although HPLC offered multiple advantages to GC (e.g. no derivatisation, and suitable for thermolabile compounds), the coupling of GC to MS offered the analytical chemist unequivocal certainty of the identity of a substance, which could not be guaranteed by HPLC coupled to UV, fluorescence or other detectors.

Considerable efforts were therefore made to couple liquid chromatography systems to mass spectrometers, but this was not straightforward. Indeed, the analytes leaving the LC are dissolved in the effluent at atmospheric pressure, while the mass spectrometer operates under vacuum and detects ions in the gas phase. Therefore the interface needed to be able to remove the effluent efficiently (to reduce pressure), vaporize the solvent and ionize the analytes. In the 1980s and the beginning of the 1990s the first commercially available interfaces appeared (Deventer et al. 2008). Currently, two types are widespread for the detection of small molecules (< 1,000 Da): the electrospray ionization (ESI) and the atmospheric pressure chemical ionization (APCI) interfaces (Bruins 1998; Deventer et al. 2005, 2008; Niessen 1999; Niessen and Tinke 1995; Thevis and Schänzer 2005, 2007).

ESI requires the analytes to be ionized in the mobile phase, of which the flow enters via a hypodermic needle at atmospheric pressure, but under high voltage. At the tip of this needle the coulombic repulsion causes a liquid spray of charged droplets. This electrospray is directed to a counter electrode and a curtain of nitrogen prevents the solvent vapor from entering the sampling orifice. The solvent evaporates quickly as the droplets shrink, leading to a series of ruptures of the droplets due to the coulombic repelling forces at the droplet surface, until the electric field at their surface becomes large enough to allow the ions to desorb directly into the surrounding gas (Bruins 1998; Niessen 1999; Niessen and Tinke 1995).

In principle ESI is a relatively “soft” ionization process and therefore little or no fragmentation occurs, resulting in the detection of the pseudo-molecular ion as the sole ion. Basic low molecular weight ions (e.g. stimulants, containing an amine function) can form protonated ions which will be analyzed in positive mode giving a signal at $M+1$ $[M+H]^+$, while acidic compounds (e.g. ritalinic acid or modafinil acid) can form a deprotonated ion $[M–H]^-$ which can be detected in negative mode (Deventer et al. 2008). It should be noted that ESI is particularly susceptible to adduct formation with additives/contaminants of the mobile phase (e.g. NH_4^+, Na^+).

Moreover, ESI can be used for both low molecular weight substances and high molecular weight compounds; in the latter case multiple charged ions will be formed.

APCI has less stringent requirements for the mobile phase since the analytes do not have to be ionized prior to the infusion into the interface. Indeed, in APCI the HPLC effluent is evaporated in a heater. The vaporized analyte ions are then produced by collisions with a plasma generated by a corona discharge needle at the end of the heating coil. Since no multiple charged ions are formed, APCI can only be used for molecular weights of up to ca. 2,000 Da (Bruins 1998; Niessen 1999; Niessen and Tinke 1995).

Both ionizations types (ESI and APCI) are relatively soft, so fragmentation by the interface is very limited when these instruments are operated in full scan mode. For unequivocal identification of a prohibited substance, multiple ions are needed in a mass spectrum (Van Eenoo and Delbeke 2004) and hence these mass spectrometers are normally used as tandem mass spectrometers to allow for additional fragmentation (Fig. 3). Mainly two types of instruments can be distinguished: low mass accuracy instruments, i.e. ion trap instruments and triple quadrupole instruments, and high mass accuracy instruments, i.e. time-of-flight (TOF) mass spectrometers and the Orbitrap (Hager 2004).

Triple quadrupole instruments are tandem-in-space instruments (Hager 2004). These instruments have two independent mass spectrometers in physically different locations within one instrument. The most common MS/MS mode of operation consists of selection of a precursor ion in the first quadrupole, followed by collision induced dissociation in the second quadrupole, which acts as a collision cell where Ar as a dissociation gas collides with the precursor ion, resulting in an array of product ions. The third quadrupole (hence triple quadrupole detector) then detects these product ions. One of the most used operation modes is multiple reaction monitoring (MRM) whereby the two resolving quadrupoles monitor certain precursor-to-product ion transitions (Fig. 3). Other operation modes include: full product ion scanning, whereby the second resolving quadrupole detects all generated product ions; precursor ion scan, whereby the second resolving quadrupole is fixed at a certain product ion and the first analyzer is scanned; and constant neutral loss mode, whereby the second analyzer is set at a fixed mass difference from the first.

Ion trap instruments are tandem-in-time instruments (Hager 2004). In these instruments, the various fragmentation steps occur consecutively within the same physical trapping volume. A precursor ion is selected from the ions from the ion source by ejecting all others from the trapping volume. Next the isolated precursor ion is fragmented and a mass analysis step is performed, or additional selection and fragmentation steps are introduced. These instruments are theoretically able to perform "n" stages of mass spectrometry, hence the term MS^n (Hager 2004; Niessen 1999).

TOF instruments can generally be regarded as special types of final mass filters that are combined with triple quadrupole or ion trap technologies. In case of TOF instruments, the final mass filter is a time-of-flight analyzer which uses an electric field to accelerate the ions through the same potential, and then measures the time they need to reach the detector (Hager 2004). If the particles have the same

charge, the kinetic energies will be identical, and their velocities will depend only on their masses. Lighter ions will reach the detector first. TOF instruments show good mass assignment accuracy, high resolution and the ability to record a complete mass spectrum for each pulse of ions introduced into it (Hager 2004; Niessen 1999).

Orbitrap instruments are the latest type of mass spectrometers that have been used in doping control. The Orbitrap operates by radially trapping ions. The frequency of harmonic ion oscillations, along the axis of the electric field, undergone by the orbitally trapped ions is independent of the energy and spatial spread of the ions. These ion frequencies are measured nondestructively by acquisition of time-domain image current transients and subsequently fast Fourier transforms (FFTs) are used to obtain the mass spectra. Again, the Orbitrap mass spectrometer shows excellent resolution and mass accuracy characteristics over a good mass range (Nielen et al. 2007).

Several applications have been studied for doping analysis using each type of instruments, but for low molecular weight compounds ion trap or triple quadrupole technologies are mostly used, since for routine applications of this type of substance the resolution attained by these instruments is sufficient and their purchase cost is significantly lower.

Several screening methods have been published both for stimulants and β_2-agonists in urine and/or other biological matrices using both ion trap as well as triple quadrupole technologies using both APCI and ESI interfaces. In general, the presence of the amine function causes these substances to be easily protonated and therefore they are normally detected in the positive mode (Deventer et al. 2005, 2008; Thevis and Schänzer 2005).

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Erythropoietin and Analogs

Christian Reichel and Günter Gmeiner

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Abstract Erythropoietin (EPO), a glycoprotein hormone, stimulates the growth of red blood cells and as a consequence it increases tissue oxygenation. This performance enhancing effect is responsible for the ban of erythropioetin in sports since 1990. Especially its recombinant synthesis led to the abuse of this hormone, predominatly in endurance sports. The analytical differentiation of endogenously

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produced erythropoietin from its recombinant counterpart by using isoelectric focusing and double blotting is a milestone in the detection of doping with recombinant erythropoietin. However, various analogous of the initial recombinant products, not always easily detectable by the standard IEF-method, necessitate the development of analytical alternatives for the detection of EPO doping. The following chapter summarizes its mode of action, the various forms of recombinant erythropoietin, the main analytical procedures and strategies for the detection of EPO doping as well as a typical case report.

Keywords Doping control • Erythropoietin • rHuEPO • Isoelectric focusing • Protein mass spectrometry

1 Introduction

Approximately three decades after the initial identification, isolation, cloning and expression of the gene for erythropoietin (EPO) and the succeeding production of recombinant erythropoietin as a major achievement after almost 155 years of exploration into the nature of red blood cell production (Foote 2003), research and development of new forms and analogs of this potent protein still continues (Gaudard et al. 2003; Jelkmann 2007b). This seems to be justified by the beneficial and life saving effect of red blood cell formation for patients with severe anemia after treatment with EPO in its recombinant forms (rEPO). EPO rapidly became one of the pharmaceutical substances with the largest sales. However, according to the Donati-Report from 2005 a massive overproduction was induced, far beyond the real requirements of patients (Donati 2005; Eagleton and Littlewood 2003), estimations claim an amount of about 500,000 EPO users worldwide, based on the seized EPO preparations (Donati 2005).

Compared to the classical forms of blood doping, i.e. homologous or autologous blood transfusion, which requires substantial infrastructure and/or professional help, the increase of red blood cell mass, can be much easier achieved using recombinant human erythropoietin (rHuEPO) preparations via subcutaneous or intravenous injections. Rumors about the abuse of rHuEPO in sports arise simultaneously to the marketing of rHuEPO in 1987, even before the Olympic Winter Games in Calgary (Catlin et al. 2003). In these days, doctors and blood specialists claimed the drug may be implicated in the deaths of as many as 18 European professional cyclists in the past four years. Among the rumored sports disciplines are endurance sports like cross-country skiing, cycling, athletics or triathlon (Fisher 1991).

In 1990 EPO was added to the former IOC list of prohibited substances and is part of the WADA Prohibited List since its existence (World Anti-Doping Agency 2008; Barroso et al. 2008).

Years later, in 1998 at the Tour de France, the use of rHuEPO became obvious due to confiscations of EPO preparations as well as confessions of cyclists about

systematic rHuEPO abuse within professional cyclist teams. As a consequence a test for rHuEPO in urine was developed by Lasne and de Ceaurriz (2000), which analytically confirms the high degree of EPO doping during the 1998 Tour de France by re-analyzing samples of this competition.

The current review summarizes in brief the physiological basis of action of erythropoietins, its recombinant production, beneficial and adverse effects. The main part deals with the current status of detection of recombinant forms of erythropoietin in body fluids and ends with a typical case report.

2 Pharmacology and Mode of Action

2.1 Production of Erythrocytes

Erythrocytes as the basis of oxygen transport to target tissues arise primarily from the CD34+ pluripotent hematopoietic stem cells of the bone marrow. These progenitor cells constitute approximately 0.1% of its nucleated cells. Hematopoietic stems cells respond to a cytokine stem cell factor (SCF). The erythroid line proceeds through the shared CFU-GEMM (colony-forming unit-granulocyte, erythrocyte, macrophage, megacariocyte) precursor and undergoes a cascade of developmental stages, including erythroid burst-forming units (BFU-E) and erythroid colony-forming units (CFU-E). Stimuli for the proliferation of these cell units derive from interleukins (IL-3, IL-9) as well as from insulin-like growth factor (IGF-1). As these cells mature larger numbers of erythropoietin receptors (EPOR) are expressed, and the late BFU-E become EPO-responsive. EPO itself stimulates growth and prevents apoptosis of these cells.

The earliest microscopically identifiable erythrocyte precursor in bone marrow, the normoblast (15–20 μm) further divides itself to form basophilic and then polychromatophilic normoblasts. After extrusion of the nucleus, reticulocytes as anuclear cells are formed. Their name comes from the residual strands of RNA (reticulin) inside these cells. After a further decrease in size and loss of some reticulin reticulocytes exit the bone marrow, developing to the final stage of this process, the erythrocytes within approximately seven days starting from the early normoplasts (Israels and Israels 2003).

The life span of the red blood cells is approximately 120 days, showing a daily replacement of 0.8–1.0% (Foote 2003). The red blood cell production is in the range of 2.5×10^{11} cells per day or more than 10^{10} cells per hour (Molineux 2003).

2.2 EPO Synthesis

EPO synthesis is mediated by the transcription activator hypoxia-inducible factor-1 (HIF-1), responsible for the activation of at least 20 genes, including EPO, transferrin, the vascular endothelial growth factor (VEGF). HIF-1 binds to the

hypoxia-response element in the target hypoxia-response genes activating their transcription (Ebert and Bunn 1999). While the β-subunit of HIF-1 is expressed independently from tissue oxygenation, the oxygen-sensitive α-subunit accumulates when cells are exposed to low-oxygen stress.

The production of EPO, a ca. 30 KDa glycoprotein, is regulated by tissue oxygenation in response to hypoxia. An oxygen sensor within renal cells detects the oxygen content of the blood and the kidney regulates the amount of EPO released into the blood. In case of hypoxia the HIF-1α is translocated from the cytosol into the cell nucleus. Together with other transcription factors a complex is formed. After binding to the erythropoietin gene, transcription is forced, leading consequently to an enhanced EPO synthesis. In case of normoxic conditions, HIF-1α is deactivated by two different mechanisms, which are both oxygen dependent. These mechanisms represent the oxygen sensor. Inactivation is performed by hydroxylation of asparagin N803 in an oxygen depending reaction. As a consequence HIF-1α is no longer able to bind to the erythropoietin gene. HIF-1α is in addition a substrate of prolyl-hydroxylases, hydroxylating prolin moieties at position P402 and P564. After a cascade of further protein couplings it is inactivated by the proteasom (Aktories et al. 2005).

Approximately 90% of EPO is synthesized in renal peritubular interstitial cells (Israels and Israels 2003). In addition, the liver and the brain both synthesize some EPO, but the amount produced by these tissues alone is insufficient to maintain adequate erythropoiesis. Thus, kidney disease causes anemia due to loss of the main source of EPO production. In the fetus, the liver is the primary site of endogenous EPO production (Foote 2003).

2.3 Erythropoietin Receptor

Being a member of the cytokine-receptor superfamily the erythropoietin receptor (EPOR) was studied using a variety of methods including X-ray crystallography and nuclear magnetic resonance (NMR); it exhibits an extracellular binding region, a transmembrane region and an intracellular domain (Syed et al. 1998; Cheetham et al. 1998; Barbone et al. 1997; Dame 2003). The receptor first appears in small numbers in early BFU-E, increases in CFU-E and pronormoblasts, and declines in the late erythropoietic cells. Transmission of a signal to the intracellular domain with subsequent activation of signal transduction pathways is initiated by homodimerization of the receptor with EPO forming an EPO-EPOR complex with a 1:2 stoichiometry. When EPO binds to the extracellular portion, the receptor is activated (Philo et al. 1996).

The EPO-initiated signal transduction pathway is highly regulated, with molecules in the signaling pathway activated within minutes of EPO binding, and the signal terminated within a few hours (Israels and Israels 2003).

Figure 1 shows the rHuEPO – EPOR complex. rHuEPO appears in red color, the two receptor molecules are in blue color. Coordinates are downloaded from the

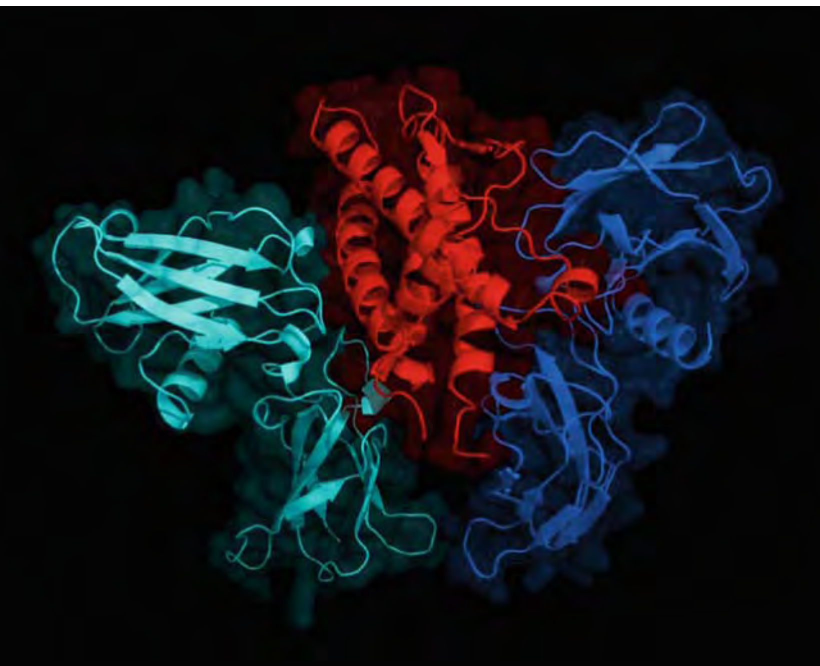

Fig. 1 Model of the rHuEPO–EPOR dimeric complex with a 1:2 stoichiometry; crystal structure data according to Syed et al. (1998)

PDB database (http://www.pdb.org; PDB Code: 1EER) and originate from the crystal structure published by Syed et al. (1998). The figure was created with PyMol 1.0 (DeLano 2007).

2.4 Isolation, Expression and Production

A significant obstacle to the development of EPO as a therapeutic agent was the difficulty in isolating and purifying adequate amounts of the hormone to allow for its characterization (Jelkmann 2007b). Miyake et al. (1977) first isolated and purified milligram amounts of EPO from 1,500 L of urine with a potency of 70,400 units/mg protein and a yield of 21% from patients with aplastic anemia, showing significant overproduction of EPO. This method allowed to partially characterizing EPO serving as basis for cloning and expression of the human gene.

Clones of the gene for EPO were inserted into CHO cells, which synthesized the 193 amino acid precursor protein, removed the signal peptide and carboxy-terminal arginine, added N- and O-linked carbohydrate to glycosylation sites, and released the mature protein into the culture medium. Immunologic, biologic, and biochemical assays showed that the recombinant hormone had the in vivo biologic activity

and was immunologically equivalent to human EPO, as revealed within the limits of the available assays (Lin et al. 1985). EPO expressed by CHO cells has a molecular mass of 30.4 kDa and contains ca. 40% carbohydrates (Davis et al. 1987, Foote 2003). Isoform-specific in vivo activity in animal studies (intraperetoneal injection into mice three times a week) was found in the early 1990, resulting in a higher response of the haematocrit for the more acidic isoforms in respect to the isoform - patterns from isoelectric focussing (Egrie et al. 1993). As a reason for this effect the differences in metabolic clearance were mentioned.

2.5 Clearance

With a half life of around 6 h EPO is cleared from the body within 1–3 days (Bressolle et al. 1997). Investigations of the pharmacokinetic properties of rHuEPO (α and β) in healthy volunteers and nephrology patients after both intravenous and subcutaneous dosing show that clearance of rHuEPO is dose dependent. At doses > 200 Ukg^{-1}, clearance of rHuEPO is approximately constant at 5 mL h^{-1}kg. At doses $<$200 Ukg^{-1}, clearance increases as dose decreases with a threefold increase in clearance observed at 10 Ukg^{-1}. This relationship appears independent of the type of rHuEPO, Epoetin alfa vs. Epoetin beta was studied. Upon multiple dosing, most of the data suggest that clearance of rHuEPO increases to approximately 10% (Heatherington 2003).

2.6 Benefits and Adverse Effects

In the mid-1980s, rHuEPO therapy was introduced as a highly efficient treatment of renal anemia being for the benefit of several millions of patients worldwide. rHuEPO headed towards the biggest market seller in pharmaceutical industry with annual sales of over 10 billion USD (La Merie 2008). The main beneficial effect is the increase in red blood cells. The erythropoietic response is dose dependent. The increase in red blood cell counts and hemoglobin is evident after approximately 2 or 3 weeks after treatment with rHuEPO. More than 90% of all patients treated with rHuEPO respond with a significant improvement of their anemia (Macdougall 2003).

Besides the treatment of renal anemia, oncology and surgery (predonated autologous blood) are among further areas of medical use (Cushner 2003).

In addition to this main focus of rHuEPO treatment, Table 1 summarizes the secondary benefits as well as the possible adverse effects.

In case of doping with rHuEPO it is assumed, that adequate medical supervision is not performed during treatment, thus leading to an increased health risk for the athletes. Regarding adverse effects of rHuEPO Lage et al. (2002) reported a case of thromboembolic complications. Depletion in iron, a risk associated with the use of rHuEPO, is faced by iron overloading leading to ferritin levels in athletes much

Table 1 Benefits and adverse effects of EPO treatment

Primary benefits	
	Increase in red blood cell counts
	Increase in tissue oxygenation
Secondary benefits	
	Increased exercise tolerance
	Normalization of increased cardiac output
	Increased peripheral vascular resistance
	Increased blood pressure (20–30% of patients)
	Decreased symptoms of angina
	Improved central nervous system function
	Reduced heart enlargement
	Reduced extreme fatigue
	Increased ability to perform daily functions of life
	Reduced risk of alloimmunization in transplant recipients
	Improved coagulation
	Reduction in myocardial ischemia
	Reduction in left ventricular hypertrophy
	Reduction in left ventricular internal dimensions
	Decreased cardiac size on chest radiograph
	Improved quality of life
	Improved brain/cognitive function
	Decreased uremic bleeding tendency
	Improved platelet function
	Improved sexual function
	Improved endocrine function
	Decreased uremic pruritus
	Enhanced immune function
Adverse effects	
	Hypertension
	Seizures/encephalopathy
	Vascular access thrombosis
	Clotting of dialysis lines
	Hyperkalemia
	Myalgia/influenza-like symptoms
	Skin irritation
	Pure red cell aplasia (anti-EPO antibodies)

higher than in the average reference population (Zotter et al. 2004). Ferritin levels even higher than 1,000 ng mL^{-1} are reported (Cazzola 2000).

3 Erythropoietin and Analogs

3.1 EPO Structure

The name “erythropoietin” was introduced by Bonsdorff and Jalavisto from Helsinki in 1948, renaming the former expression “hemopoietin” from Carnot and Deflandre of 1906 (Jelkmann 2007a).

Endogenous EPO as a typical member of the cytokine hormone family has a core structure composed of four helical bundles labeled A (residues 8–26), B (residues 56–83), C (residues 90–112) and D (residues 137–161). Those helices are connected by loops and termed the "up–up–down–down four-helical bundle topology" (Molineux 2003).

While the primary translation product of human chromosome 7q22 contains 166 amino acids, serum EPO has lost an arginine moiety at the C-terminal end leading to a 165-amino acid glycoprotein hormone with a molecular weight of 30.4 kDa that contains ca. 40% carbohydrate (Molineux 2003). Due to anomalous behavior of glycoproteins EPO migrates with an apparent size of 34–38 kDa on SDS-PAGE (Jelkmann 2003). Glycan chains are attached to 3 N-glycosylation sites (asparagines at 24, 38 and 83) as well as one O-glycosylation site (serine at 126). The carbohydrate chains of EPO are mainly responsible for its integrity and stability, being the starting point of new rHuEPO developments of hyperglycosylated analogs like darbepoetin alfa with longer serum half lives (Narhi et al. 1991).

Due to differences in the composition of the carbohydrate chains EPO exhibits microheterogeneity caused by posttranslational modifications (Choi et al. 1996). Isoelectric separation shows a variety of different isoforms (Sherwood et al. 1988; Lasne and de Ceaurriz 2000, Lasne 2001).

Sialylation of the preterminal galactose residues is required for the in vivo activity; asialo-EPO is rapidly cleared by hepatocytes; tetraantennary N-oligosaccharide chains prevent EPO from renal clearance, so the ratio of tetra- to diantennary chains is important for the in vivo activity of EPO (Jelkmann 2003).

The tertiary structure is stabilized by two disulfide bonds structurally linking together the amino terminus and the carboxy terminus at residues Cys7 to Cys161 and Cys29 to Cys33 (Osslund and Syed 2003).

3.2 EPO Analogs

According to the World Health Organization (WHO) international nonproprietary names (INN) have to be used for biological and biotechnological substances (World Health Organization 2006). The name of erythropoietin type blood factors has to be composed of a common stem (*-poetin*). For erythropoietins with the same amino acid sequence as human erythropoietin the name *epoetin* has to be used. If the amino acid sequence is different a random prefix is used (e.g. *darbepoetin*). An additional *Greek letter* indicates that there are differences in the glycosylation pattern (e.g. *darbepoetin alfa, epoetin alfa, epoetin beta, epoetin gamma, epoetin delta, epoetin epsilon, epoetin zeta, epoetin theta, epoetin iota, epoetin omega*). Due to these structural differences the serum half life of erythropoietins is different (Table 2; Deicher and Hörl 2004).

Epoetin alfa was the first recombinant erythropoietin. It has been commercially produced since 1989 and is worldwide available. It is being produced in a Chinese hamster ovary (CHO) cell line which was transfected with the human EPO cDNA/gene.

Table 2 Serum half life (h) of various erythropoietins and congeners (Deicher and Hörl 2004)

	Intravenuos	Subcutaneous
Epoetin alfa	4–11	19–25.3
Epoetin beta	8.8–10.4	24
Darbepoetin alfa	18–25.3	48.8

It is marketed under different names, e.g. *Epogen, Procrit, Eprex, Erypo, Espo* (Jelkmann 2008). Compared to Epoetin beta the degree of O-acetylated sialic acids is increased (Stübiger et al. 2005b). The amount of tetra-sialylated N-glycans is ca. 19%, about 95% of the isoforms contain O-glycans (Deicher and Hörl 2004). CHO-cell EPO also contains N-glycolylneuraminic acid (Neu5Gc) which humans cannot synthesize. According to Hokke et al. (1995) about 2% of the sialic acids are Neu5Gc. A study on the detectability of doping with Epoetin alfa was published by Breidbach et al. (2003). Significant changes in the isoform profiles were still visible after seven days in 50% of the participants.

Epoetin beta is also produced in a genetically modified CHO cell line (CHO-DN2-3α3; European Medicines Agency (EMEA) 2004). It contains a higher degree of basic isoforms than Epoetin alfa. It was first developed and produced in 1990 by Boehringer Mannheim under the name of *Recormon* and was later sold to Hoffman-La Roche. Recormon was replaced in 1997 by *NeoRecormon*. It is available only outside the USA. In Japan it is sold under the name *Epogin* (Jelkmann 2008). Since Epoetin beta contains a higher amount of basic isoforms than Epoetin alfa (Storring et al. 1998) and is thus easily distinguishable from Epoetin alfa by isoelectric focusing on slab gels (vide infra) or by capillary isoelectric focusing with whole column imaging detection (Dou et al. 2008). The amount of tetra-sialylated oligosaccharides is also higher (ca. 46%) than in Epoetin alfa (Deicher and Hörl 2004).

Epoetin omega was first marketed by Elanex Pharmaceuticals under the name of *Epomax* and was later acquired by Baxter. Epoetin omega is produced in genetically modified baby hamster kidney (BHK) cells. The amount of basic isoforms is even higher than for Epoetin beta (Pascual et al. 2004). Only 60% of the isoforms contain O-glycans. Aside from the usual complex type N-glycans a mannose-6-phosphate containing high-mannose type N-glycan was also observed (Nimtz et al. 1995).

Epoetin delta is being produced in a human fibrosarcoma cell line (HT-1080). By transfection with a virus promoter sequence the cells' own EPO gene is expressed (Deicher and Hörl 2004). Since human cells are genetically unable to synthesize N-glycolylneuraminic acid (Neu5Gc) Epoetin delta (*Dynepo*) does not contain this particular moiety (Jelkmann 2008). Compared to Epoetin alfa and beta the isoform pattern of Epoetin delta contains a slightly higher amount of the so-called alfa-isoform (vide infra). On SDS-PAGE Epoetin delta produces a very sharp band, which is unusual compared to Epoetin alfa, beta, omega, and human urinary and serum EPO (see case report below).

Darbepoetin alfa was developed by site directed mutagenesis of the original EPO gene sequence. Five amino acids were exchanged – thus leading to two additional N-glycosylation sites (Egrie and Browne 2001; Overbay and Manley 2002). Due to this hyperglycoslyation the serum half life of darbepoetin alfa could be increased about three- to fourfold compared to Epoetin alfa or beta (Macdougall 2001; Elliott et al. 2004). Darbepoetin alfa (NESP, novel erythropoiesis stimulating protein) is produced in CHO-cells and is marketed under the names of *Aranesp* and *Nespo*. Due to the altered glycosylation NESP can be easily differentiated from all other Epoetins by isoelectric focusing (IEF) and SDS-PAGE. The detection window of a single dose injection of NESP (40 μg) in urine and by using IEF was about seven days (Lamon et al. 2007a; Morkeberg et al. 2007). Ashenden et al. (2006) confirmed that microdosing can reduce the window of detection to as little as 12–18 h postinjection and recommended that authorities must adopt appropriate counter measures.

Continuos Erythropoietin Receptor Activator (CERA) is a PEGylated form of Epoetin beta (NeoRecormon), a methoxy polyethylene glycol-Epoetin beta. The glycoprotein is first expressed in CHO-cells and is then linked via an amino-group to a methoxy polyethylene glycol molecule (PEG, ca. 30 kDa). The PEGylation is either N-terminal (Ala1) or via the ε-amino group of Lys52 or Lys45 (European Medicines Agency (EMEA) 2007), leading to a molecular mass of CERA of ca. 60 kDa. Due to an altered affinity to the EPO-receptor the serum half life of CERA (sold as *MIRCERA*) is greatly enhanced (up to ca. 6 days; Jelkmann 2008). Due to the altered structure and higher molecular mass it can be easily differentiated from other Epoetins via IEF and SDS-PAGE (Fig. 2). However, only low amounts are excreted in urine.

Biosimilar Epoetins are mostly erythropoietins of the Epoetin alfa, beta or omega type, which are being produced at much lower cost due to expired patents (Schellekens 2005). Since glycosylation is not only dependent on the cell-line used for the expression of Epoetins but also on the entire biotechnological process the glycosylation patterns of biosimilars do not necessarily reflect the patterns of the originator compounds (Schellekens 2004). Today biosimilar Epoetins are manufactured and distributed worldwide and under many different names, for instance in the European Union (e.g. *Abseamed, Epoetin alfa HEXAL, Binocrit, Silapo, Retacrit*), India (e.g. *Vintor, Wepox, Eposino, Shanpoietin, Hemax*), South Africa (*Repotin,* a BHK-EPO), China (e.g. *Eposino, NingHongXin*), Korea (e.g. *Espogen*), Brazil (e.g. *Hemax, Alfaepoetina*), and Argentinia (e.g. *Hemax, Zyrop*). Differences between biosimilars and the original products can be easily revealed by IEF (Fig. 2).

While all Epoetins and their analogs (i.e. *protein based "erythropoiesis stimulating agents", ESA*) induce erythropoiesis by binding to EPO-receptors on target cells, similar effects can be also achieved by nonprotein based, i.e. *peptide based and nonpeptide based ESAs*. The amino acid sequence of EPO-mimetic peptides is not related to EPO, nevertheless they are able to bind to the EPO-receptor and activate the signal transduction cascade. *EMP-1* was the first *EPO mimetic peptide* described in 1996 and was discovered by screening a peptide phage library

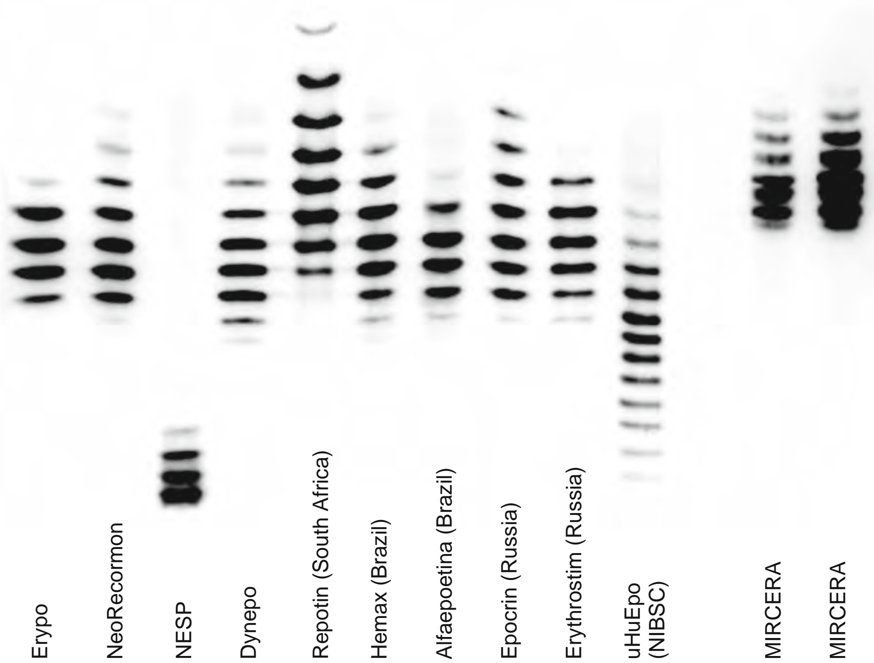

Fig. 2 IEF-profiles of various original and biosimilar Epoetins. Isoelectric focusing (IEF) was performed in the pH-range of 2–6 according to Lasne et al. (2002). The cathodic side of the gel is on the upper part of the picture
Source: Reichel et al. (2009), Wiley (permission granted)

(Bunn 2007). The peptide consists of twenty amino acids with the sequence GGTYSCHFGPLTWVCKPQGG. In order to increase the serum half life the sequence was modified, two of the peptides were dimerized via a linker and then the linker was PEGylated (e.g. *Hematide*, developed by Affymax; Stead et al. 2006). In addition, the peptide contains an internal disulfide-bridge. Currently, Hematide is in clinical phase 3. By using a similar approach for prolonging the serum half life, AplaGen Biopharmaceuticals slightly changed the amino acid sequence of EMP-1, then dimerized the peptides, and linked these dimers to hydroxyethyl starch (HES). The company named their product *HemoMer*. HemoMer is still in its developmental phase.

Other, *non-EPO-receptor based* stimulators of endogenous EPO production target the intracellular signal cascade, e.g. prolyl hydroxylase inhibitors (HIF stabilizers), GATA-2 inhibitors, or the haemopoietic cell phosphatase (HCP). The hypoxia inducible factor (HIF) is a dimeric transcription factor of the EPO gene. Under nonhypoxia conditions the cytosolic HIFα subunit is rapidly removed by hydroxylation via a HIF-specific prolyl hydroxylase (HIFα hydroxylase), ubiquitination, and subsequent degradation in proteasomes. Under hypoxia conditions the HIFα- and HIFβ-subunits dimerize and activate the EPO enhancer next to the 3′-end of the EPO gene (Jelkmann 2007c). By inhibiting the prolyl

hydroxylase, the HIF-dimer is stabilized and EPO synthesis stimulated even under nonhypoxia conditions. A stabilization of the HIFα hydroxylase can be achieved by *2-oxoglutarate analogs (HIF stabilizers)*, since 2-oxoglutarate is a required cofactor of the hydroxylase. FibroGen developed two analogs, namely FG-4383 and FG-2216 (Jelkmann 2008). However, HIF stabilizers also enhance the expression of other genes. Other, non-EPO receptor based stimulators of the endogenous EPO expression target the EPO promoter on the 5′-end of the EPO gene. The GATA-2 transcription factor inhibits EPO gene expression by binding to the EPO promoter. By inhibiting the transcription factor with so called *GATA-2 inhibitors* (e.g. K-11706, K-7174) the synthesis of EPO can be increased (Jelkmann 2008). *HCP-inhibitors* on the other hand inhibit the dephosphorylation of the JAK2 protein, which plays an important role in the EPO-receptor mediated intracellular phosphorylation cascade (Elliott 2003). However, the non-EPO-receptor based stimulators are still under development.

3.3 Production of Recombinant Human Erythropoietin

Cell line development, satisfying commercial demands and quality guidelines, and the establishment of a cell bank, providing the starting material for all lots of products manufactured, end up in a homologous population of cells. Cell culturing is done in a fermentation process, where cultured banks are grown and secrete their products into the cell culture medium under optimized nutrient and physicochemical environment. The harvesting of the bioreactor can be done continuously or in batches, and the crude reaction mixture is forwarded to further purification. Purification uses concentration, diafiltration and several types of chromatography (anion-exchange, reverse-phase, gel-filtration, immunoaffinity).

The chromatographic steps determine the isoform distribution of the final product and are essential for the analytical detection of traces of rHuEPO in urine. The last step is the manufacturing of a dosage form, usually an injectable ampoule or a syringe (Chuck et al. 2003).

3.4 Performance Enhancement

Ekblom et al. demonstrated the relationship between hemoglobin concentration [Hb], blood volume and maximal aerobic power (Ekblom et al. 1972; Kanstrup and Ekblom 1984). Ekblom and Berglund (1991) evaluated the effect of a 6 week treatment of subcutaneous injections of rHuEPO, 20–40 IU kg^{-1} body weight 3 times a week in male healthy subjects. Hemoglobin concentrations increased from an average of 152 gL^{-1} before to 169 gL^{-1} after treatment. The corresponding values for the hematocrit were 44.5% before and 49.7% after

treatment (Ekblom and Berglund 1991). Further studies confirmed the trend by showing increases in hemoglobin of up to 173 $\mathrm{g\,L^{-1}}$ and hematocrit values of up to 52%, dependent on the conditions of treatment (Audran et al. 1999; Birkeland et al. 2000; Parisotto et al. 2000a; Russel et al. 2002; Berglund 2002). Values tend to go back to baseline after approximately 4 weeks of wash-out (Parisotto et al. 2000a), but could be maintained by low dosage of rHuEPO of 20 IU $\mathrm{kg^{-1}}$ body mass three times a week (Russel et al. 2002).

During the rHuEPO treatment period VO_2max increased significantly from 4.5 to 4.9 $\mathrm{L\,min^{-1}}$. Time to exhaustion increased significantly. There was no statistically significant difference between rHuEPO treatment and blood transfusion for the change in VO_2max per gram increased [Hb].

The main conclusion is that increases in [Hb] by treatment with rHuEPO induce a similar increase in VO_2max and time to exhaustion during a standard maximal running test (Berglund 2002).

Balsom et al. (1994) administered rHuEPO in healthy male subjects. The subjects increased the [Hb] by 11% and the VO_2max by 8%. The intermittent exercise protocol (15 times 6 s exercise with 24 s rest) focused on the anaerobic performance enhancement after rHuEPO treatment. The blood lactate concentration after the eighth run and 3 min postexercise was significantly lower after the treatment period compared to before, despite the same total amount of work. These data suggest that substrate utilization during high intensity intermittent exercise is affected by enhanced oxygen availability. This is most likely due to faster phosphorylcreatine re-synthesis (PCr) during recovery periods and possibly an increased contribution from aerobic metabolism during exercise.

The benefits of increased production of erythrocytes responsible for the oxygen-carrying capacity of the blood in highly trained athletes are difficult to attain by legal means, such as continued training and altitude exposure (Parisotto et al. 2000b); thus rHuEPO is still the target doping substance to enhance performance especially in endurance sports requiring an adequate supply of oxygen to the heart and the muscles (Eichner 2007). Rumors of rHuEPO use were confirmed by seizures of rHuEPO during the Tour de France 1998, uncovering the widespread use in cycling.

During the Salt Lake City 2002 Winter Olympics three samples were reported positive for darbepoietin alfa; all three athletes were cross country skiers (Catlin et al. 2003).

Numerous other cases were reported for the misuse of rHuEPO. Table 3 summarizes the WADA annual statistics of the last 5 years for adverse analytical findings with rHuEPO.

Table 3 Adverse analytical findings for rHuEPO reported by the WADA accredited laboratories between 2003 and 2007

Year	Adverse analytical findings for rHuEPO
2003	58
2004	38
2005	16
2006	18
2007	24

4 Analytical Procedures and Instrumental Techniques

Detection strategies of doping with rHuEPO include indirect approaches using markers of enhanced or reduced erythropoiesis as well as direct detection of the recombinant isoforms (Ekblom 2000).

In the late 1990s random blood tests, able to detect abnormalities in biological parameters due to the possible use of EPO, were introduced by the International Cycling Union (UCI). These tests could not prove whether doping substances had been taken, but it was a first step in a direction which other international federations subsequently followed. Some federations like UCI or the International Skiing Federation used the data from blood tests to target athletes for a subsequent urinary test for the detection of rHuEPO. Consequently, monitoring blood parameters was the next step in order to increase the effectiveness of antidoping tests (Robinson et al. 2006).

Although this action is targeted to prevent heavy use of rHuEPO its main disadvantage is a discrimination of athletes with naturally elevated blood parameters. As a consequence individual reference ranges were introduced by the UCI in 2007 in the form of a "biological passport" including parameters like hemoglobin, reticulocytes, stimulation index (= third generation OFF-score) and haematocrit. The biological passport is based on statistical considerations and follows a Bayesian Model (Robinson et al. 2007).

At present indirect tests are discussed as basis for measures and sanctions, especially with the introduction of the biological passport by UCI.

4.1 ON/OFF Model

Starting point for the development of a blood parameter based method for the detection of doping with human recombinant erythropoietin (rHuEPO) was the preparation of the Sydney Olympic Games in 2000 (Kazlauskas et al. 2002). A rHuEPO administration trial was carried out in 1999 including 30 club level athletes. rHuEPO was administered at the rate of 50 IU kg^{-1} three times a week for 4 weeks. A wide range of blood and serum parameters were measured and statistical analysis produced two models – an "ON model" to detect those taking EPO and an "OFF" model for the washout phase (Parisotto et al. 2000a). The ON model has high sensitivity during the first part and for a few days after the end of rHuEPO administration. The OFF model becomes effective in the latter stages of the period of performance enhancement.

The ON model included reticulocyte hematocrit, serum EPO, soluble transferrin receptor, hematocrit and % macrocytes. 94–100% of r-HuEPO group members were identified during the final 2 weeks of the rHuEPO administration phase. One false positive was recorded. The OFF model incorporated reticulocyte hematocrit,

serum EPO and hematocrit and was applied during the wash-out phase. It identified 67–72% of recent users with no false positives.

As a consequence the combination of these blood test models and the direct urinary test is suggested as a powerful tool to detect rHuEPO doping.

Follow-up studies included more subjects, different ethnic groups (Caucasian and Asian origin) and lower doses of rHuEPO. More than 1,000 elite athletes of 12 countries were chosen to determine "normal" values and see the effects of ethnicity, sex, location, sport, physical effort, and injury. These studies confirmed the original findings and enabled the development of refined "second generation" ON and OFF models (Parisotto et al. 2001; Sharpe et al. 2002; Gore et al. 2003; Ashenden et al. 2003, 2004). Alternative and easy-to-determine parameters like hemoglobin or the percentage of reticulocytes were used. Additional benefits are reduced effects of storage of transportation as well as reduced dependency on cellular volume. Sets of equations were given, being nowadays incorporated into the evaluation of health tests for international federations, e.g. UCI uses the model OFF-hr ($=[\mathrm{Hb}]-60(\%\mathrm{ret})^{1/2}$) as stimulation index (Segura et al. 2007).

The ability of the second generation models to detect rHuEPO administration was generally higher than that of the previous models, particularly after injections had ceased.

The most prominent advantage of this approach remains, that the OFF model is still the only means of detecting those athletes who have stopped EPO use a week or more prior to competition. Such athletes have a significant competitive advantage and are at no risk from tests which rely on detecting rHuEPO.

More recently the third generation of models was published (Sharpe et al. 2006). Hematological values of the entire athlete's historical baseline rather than population-derived thresholds were compared as enhanced ability to detect blood doping.

This approach heightened the capacity to detect blood doping by removing within-subject variability. Longitudinal changes in either hemoglobin or the OFF-hr model score caused by recombinant human erythropoietin treatment from the natural biological fluctuations found in subjects treated with placebo were delineated.

Nowadays indirect models are included into target-testing, exclusion from competitions and concepts of the "biological passport".

4.2 Electrophoretic Methods

4.2.1 Zone Electrophoresis in Agarose Suspension

Wide and Bengtsson (1990) studied the electrophoretic mobility of human serum erythropoietin using zone electrophoresis in a 0.17% agarose suspension at pH 8.6 (0.075 M sodium veronal buffer) and compared the mobility values with the values of recombinant EPO and the second international reference preparation for human EPO (NIBSC code 67/343), which contains human urinary erythropoietin

(uHuEPO). They discovered that (1) the median charge of recombinant EPO was less negative than for the human serum EPO of healthy individuals, and that (2) uHuEPO was more negative than serum EPO. It was also observed that a subcutaneous injection of rHuEPO resulted in a shift towards less negative EPO forms in serum. They concluded that the method might be useful for the detection of a rHuEPO application in blood. In a second study Wide et al. (1995) investigated the changes in charge heterogeneity of human serum and urinary EPO after the administration of recombinant EPO in healthy males. Again, zone electrophoresis (0.1% agarose suspension) was applied. Based on the observation that rHuEPO is less negatively charged than endogenous EPO it was shown that the administration of recombinant EPO could be detected for up to 72 h in serum and for up to 48 h in urine after the last injection.

4.2.2 Isoelectric Focusing in Solution

In 1991 Tam et al. published a study investigating the differences of serum and urinary erythropoietins of three species (human, rat, mouse). Isoelectric focusing in sucrose density gradients (0–50% w/v) and carrier ampholytes in the pH-range of 2.5–5.0 were applied. A significant difference between the isoform distribution of human urinary and serum EPO was observed with a higher amount of acidic isoforms ($pI < 3.0$) in urine.

4.2.3 Isoelectric Focusing in Polyacrylamide Slab-Gels

4.2.3.1 General Description

Back in 2000 Lasne and de Ceaurriz published a method for the detection of rHuEPO-doping in human urine. The method is based on the isoelectric focusing (IEF) in a horizontal slab-gel containing carrier ampholytes in the pH-range of 2–6 (Lasne and de Ceaurriz 2000; Lasne 2001, 2003; Lasne et al. 2002). Due to the heterogeneity in the structure of the three N- and one O-glycans – resulting in mass and charge differences – IEF enables the separation of discrete isoforms. Interestingly, there is a significant difference in the distribution of the isoforms between human recombinant erythropietins and human endogenous urinary erythropoietin. The isoelectric patterns of both Epoetin alfa (*Erypo, Eprex*) and Epoetin beta (*NeoRecormon*) were shown to be quite similar and within an isoelectric point (pI) range of 4.42–5.11. Contrary to that the p*I*-values of uHuEPO were demonstrated to be more acidic (starting at p*I* 3.92) and with a more heterogeneous isoform distribution (Lasne and de Ceaurriz 2000). Usually, *Erypo* shows a maximum of ca. six, *NeoRecormon* ca. eight, and uHuEPO ca. 14 to 15 isoforms on these IEF-gels with some isoforms being more prominent that others. The application of recombinant Epoetins in a nonanemic physiological state results in the suppression of the production of endogenous erythropoietin. This consequently leads to a

continuous shift from the endogenuous to the recombinant IEF-profile depending on the serum half-life and the amount of rHuEPO applied. Based on these principles direct testing for EPO abuse in urine is possible.

The direct detection method usually starts with concentrating the urinary proteins by two ultrafiltration (UF) steps. Filters with a molecular weight cut-off of 30 kDa are used which allow an almost quantitative recovery of EPO and a much faster concentration procedure than filters with lower molecular weight cut-offs. In order to prevent UF-filters from clogging pretreatment of the urine by centrifugal sedimentation and vacuum-assisted microfiltration is mandatory. The pH-value of the urine is adjusted to 7.4 by adding 3.75 Tris–HCl buffer. A mixture of protease inhibitors is also added in order to avoid EPO-degradation by urinary proteases. About 20 mL of urine are being used and are concentrated down to ca. 20–50 μL of urinary retentate.

For the electrophoretic separation of the isoforms a 1 mm polyacrylamide slab-gel (5% T, 3% C) containing a mixture of carrier ampholytes (Servalytes 2–4 and 4–6, 1:1) at a final concentration of 2% (w/v) of each of the two ampholyte solutions and also containing 7 M urea is used. In order to stabilize the pH-gradient during focusing sucrose can be added at a concentration of 5% (w/v). The gel is cast and polymerized on a plastic backing film (ca. 260 × 125 mm) in order to make handling of the fragile polymer easier during the entire electrophoretic procedure. To establish the pH-gradient prefocusing for ca. 30 min is necessary. Phosphoric acid (0.5 M) and pH 6–8 ampholytes (2%) are used as anolytes and catholytes, respectively. However, as was shown later (Reichel et al. 2006) separation of uHuEPO, rhuEPO, and NESP can be also achieved by isoelectric focusing with carrier ampholytes in the pH-range of 3–5 instead of 2–6, preferably by using larger sized gels (ca. 260 × 200 mm). The advantage of larger sized gels is their higher loading capacity per lane together with an increased resolution. High amounts of urinary proteins in UF-retentates can result in bands with curved shape. Both, immunoaffinity purification (using a mouse monoclonal anti-EPO antibody, clone 9C21D11) or lectin-affinity purification (WGA, wheat germ agglutinin) have also been successfully applied as remedy (Lasne et al. 2002, 2007a, b).

Before applying the urinary retentates onto the gel they have to be heated at 80°C for 3 min to further inactivate proteolytic activity. For improving EPO solubility Tween-80 or Tween-20 is added at a final concentration of 1% (v/v). Standards for rHuEPO (BRP-EPO of the European Pharmacopeia Commission, NESP of Amgen) and uHuEPO (human urinary EPO of the National Institute for Biological Standards and Control, NIBSC) are used at a final amount of ca. 0.03 International Units (IU; originally, the definition of one EPO Unit (U) referred to the amount which led to the same erythopoieses stimulating effect as 5 μmol cobalt chloride in an animal experiment. Today, EPO formulations are calibrated against international reference preparations, e.g. the second international reference preparation (2nd IRP) of urinary EPO (NIBSC code 67/343) or the international standard for recombinant DNA-derived EPO (IS, NIBSC code 87/684), Jelkmann 2007b) or below on-gel, which corresponds to an absolute amount of ca. 0.20–0.25 ng or below. Estimating the EPO-concentration of the urinary retentates by ELISA is

advantageous in order to unify the applied EPO-amount on the gel and to avoid lane over- or underloading. Ready made application pieces are used for applying both standards and samples on the cathodic side of the gel. Focusing is being performed at constant wattage (1 $W cm^{-1}$ gel length) and ca. 8–10°C and for ca. 3,600–4,000 Vh. Lower temperatures have to be avoided in order to prevent urea crystallization. The interelectrode distance is between ca. 8 and 10 cm. Instead of using commercial application pieces, similar pieces can be made of blotting paper. They can be beneficial if streaking problems in the acidic region occur. The use of an applicator strip might result in lateral sample diffusion and mixing if the strip is leaking. So far, the best method for applying standards and samples onto the gel appears to be casting IEF-gels with wells for sample application (Reichel 2007, unpublished observation) as described by Westermeier (slot former technique; Westermeier 2004).

Western blotting is performed after IEF in order to detect the EPO-isoforms. The plastic backing is removed from the gel and then proteins are transferred on a PVDF-membrane by semidry blotting for 30 min using a modified Towbin buffer (25 mM Tris, 192 mM glycine). After a reduction step (5 mM DTT in PBS, 37°C) the membrane is blocked in 5% nonfat milk and subsequently incubated in a solution of the primary antibody (monoclonal mouse antihuman EPO antibody, clone AE7A5) in 1% nonfat milk (1 $\mu g\ mL^{-1}$). In order to avoid nonspecific binding of the secondary antibody a second blotting step (so-called “double-blotting” technique) is performed which aims to only transfer the bound anti-EPO antibody to a second PVDF-membrane – thus keeping the interfering urinary proteins on the first membrane (Lasne 2001, 2003). The membrane is blocked in 5% nonfat milk, incubated with the secondary antibody (a biotinylated polyclonal goat antimouse antibody) and after a washing step in a solution of streptavidin conjugated horseradish peroxidase (HRP). Enhanced chemiluminescence is used for the final detection step in combination with a CCD-camera or X-ray film for imaging. The EPO IEF-profiles are quantitatively analyzed by software assisted densitometry and interpreted according the WADA technical document (vide infra). Lasne et al. (2007b) showed that classification of IEF-profiles can be also done by linear discriminant analysis.

For the quantitative evaluation of the gel images after chemiluminescence detection special software was developed and the algorithms published (Bajla et al. 2001, 2005; Stolc and Bajla 2006).

4.2.3.2 Criteria for Positivity – WADA Technical Document TD2007EPO

EPO-IEF profiles have to be interpreted according to the criteria in WADAs technical document on the “Harmonization of the method for the identification of Epoetin alfa and beta (rEPO) and darbepoetin alfa (NESP) by IEF-double blotting and chemiluminescent detection” (TD2007EPO, World Anti-Doping Agency (WADA) 2007). Valid profiles have to fulfill a series of acceptance, identification, and stability criteria. Samples positive for recombinant EPO have to show at least

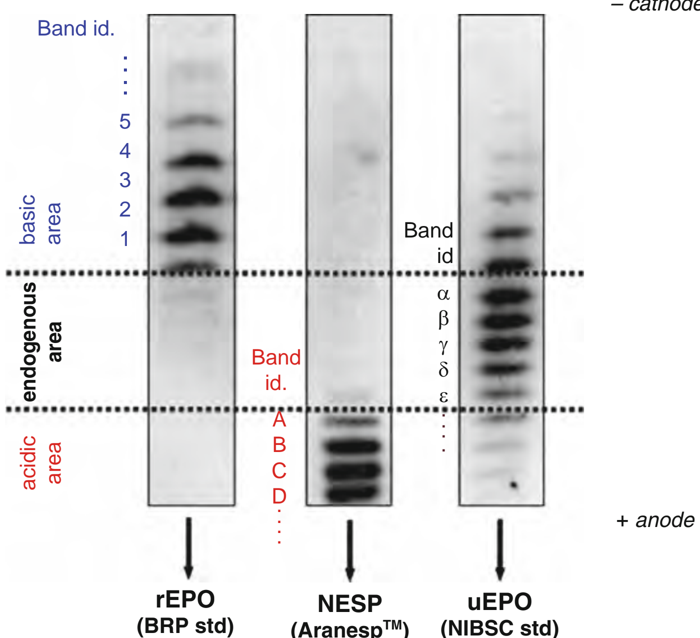

Fig. 3 Evaluation of EPO-IEF profiles according to WADA technical document TD2007EPO. The lanes are divided in a basic, endogenous, and acidic area. The band intensities are measured by densitometry. For positive samples the two most intense bands have to be in the basic or acidic area
Source: World Anti-Doping Agency (2007) (permission granted)

three acceptable, consecutive bands in the basic area which correspond to bands number 1, 2, and 3 of the reference standard. In addition, the two most intense bands have to be consecutive and bands number 1, 2 or 3. Their desitometric intensity must be higher than the intensity of any band in the endogenous area (approximately twice or more). Similar criteria were developed for the bands in the acidic area with the most intense bands being bands C and D (Fig. 3).

Samples which show a suspicious profile during the screening procedure have to be confirmed by independently repeating the entire procedure together with a *stability test*. Aim of the stability test is to prove that there is no enzymatic activity in the suspicious urine sample which might cause a shift in the IEF-profile (Imai et al. 1990; Belalcazar et al. 2006). This is being tested by adding the standards for recombinant EPO and NESP to a small fraction of the urine. After overnight incubation at 37°C no changes in the isoform distribution of these standards have to be detectable.

The following figure (Fig. 4) shows a comparison between the IEF-profiles of a BRP/NESP standard mix and the stability test of two urine samples spikes with the same BRP/NESP mix. The isoform profile of the urine sample given in lane (B) shows no shift in the positions of the bands compared to the lane of the BRP/NESP

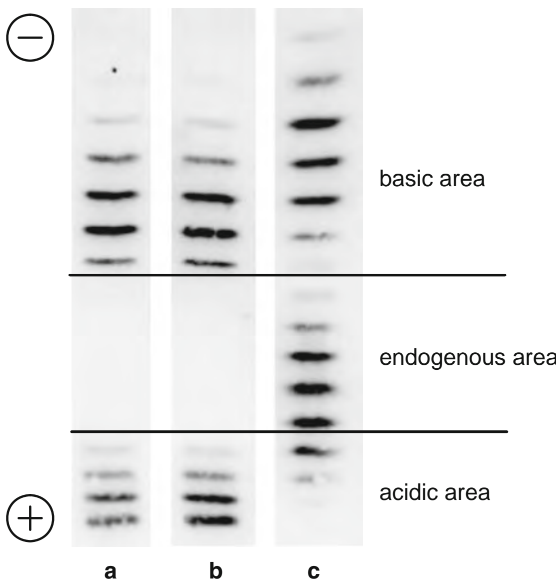

Fig. 4 Urine stability test: Lane (**a**) shows a BRP/NESP standard mix, lanes (**b**) and (**c**) show urine samples spiked with BRP/NESP and incubated overnight at 37°C

standard mix lane (A). The isoform profile of lane (C) clearly shows a shift of the bands and band intensities, especially in the acidic area of the lane. It thus confirms the presence of enzymatic activity, which is able to alter the band positions (Belalcazar et al. 2006).

Only samples fulfilling the criteria of the technical document for both the screening and confirmation procedures are reported as adverse analytical findings. After strenuous physical exercise some urine samples show an endogenous IEF-profile which is shifted towards the basic area. However, these atypical profiles do not fulfill the criteria of positivity (Abellan et al. 2007; Berglund et al. 2003).

Lasne et al. (2005) demonstrated that this method can also be used for detecting rHuEPO and Darbepoietin alfa administration in race horses. Bartlett et al. (2006) showed the applicability of the method for the detection of human EPO in canine samples. The analysis of approximately 6,000 grayhound sport samples resulted in the detection of one positive sample for rHuEPO.

4.2.3.3 Specificity of the Antibody

Since 2005 the specificity of the primary antibody used for the detection of EPO (clone AE7A5) has been quite controversially discussed in public and in various peer-reviewed publications. The monoclonal antibody is directed against the first 26 amino acids at the N-terminus of the processed amino acid sequence (Sytkowski 2004; Sytkowski and Fisher 1985). While some authors showed nonspecific binding on SDS-PAGE (Beullens et al. 2006; Franke and Heid 2006; Khan and Baker 2007;

Delanghe et al. 2008) and 2D-gels (Khan et al. 2005) no interferences could be observed for the IEF-method as used in doping control. Only one protein which (partly) focuses in the basic region of the pH 2–6 (or pH 3–5 large sized) gels was observed. Its existence was well known under experienced performers of the EPO-IEF method and was also documented in published form (Rabin et al. 2006, Lasne 2006, Lasne et al. 2007a). However, its isoforms focus outside the p*I*-range used in the evaluation procedure for EPO-doping control. The protein (zinc alfa-2-glycoprotein) was later identified by mass spectrometry and immunological methods (Reichel 2008). Binding only occurs if high amounts of this protein are present in urine. An immunoaffinity purification step of the urinary retentate can be performed before isoelectric focusing, thus providing additional specificity. Care must be taken that the antibody selected for the purification step does not alter the distribution of the EPO-isoforms in the sample. Lasne et al. (2007a) issued a protocol using a mouse monoclonal anti-EPO antibody (clone 9C21D11) and demonstrated no changes in the EPO IEF-profiles due to the purification step.

4.2.4 SDS-PAGE

In contrast to isoelectric focusing SDS-PAGE separates proteins according to their apparent molecular mass. Sodium dodecyl sulfate (SDS) imposes a negative net-charge on proteins resulting in a more or less uniform charge density of the protein molecules. Consequently, their migration in an electrophoretic gel with defined pore size is only dependent on the molecular mass. It was demonstrated by SDS-PAGE that uHuEPO and rHuEPO (Epoetin alfa and beta) differ in their apparent molecular masses on SDS-PAGE. Under reducing conditions the average molecular mass of uHuEPO is ca. 34 kDa, the one of Epoetin alfa and beta ca. 36–38 kDa (e.g. Kung and Goldwasser 1997; Desharnais and Ayotte 2007; Reichel 2007). Since EPO is a highly glycosylated protein with a glycoform mass distribution (Neusüss et al. 2005) bands on SDS-PAGE are broader than for nonglycosylated proteins (Stübiger et al. 2005b). Consequently the bands of uHuEPO and rHuEPO (Epoetin alfa and beta) are slightly overlapping, but with a clear difference in the average molecular mass. Due to their higher mass, NESP (ca. 44–45 kDa) and MIRCERA (ca. 69–78 kDa) are perfectly separated from each other and from uHuEPO and rHuEPO. Dynepo (ca. 36 kDa) has a similar mass as Epoetin alfa and beta but produces a very sharp and hence very characteristic band on SDS-PAGE (see case report, Fig. 10).

By combining SDS-PAGE with an upstream immunoaffinity purification step the abuse of various forms of recombinant EPO can be detected in urine (Desharnais and Ayotte 2007; Reichel 2007, 2008; Kohler et al. 2008). Due to the high protein content of the urinary ultrafiltration retentates (as prepared for the IEF-method) they cannot be directly used for SDS-PAGE. The method works especially well for Dynepo and NESP. Usually, total acrylamide concentrations in the range of ca. 8–12% T are used. However, while the majority of CHO-cell produced EPOs shows a higher molecular mass on SDS-PAGE than uHuEPO, the mass of

BHK-EPO (Epoetin omega) is comparable to uHuEPO. Interestingly, the molecular mass of human serum EPO on SDS-PAGE is comparable to the mass of uHuEPO (Masuda et al. 1994; Skibeli et al. 2001) while the IEF-profiles are different (Lasne et al. 2007a).

4.2.5 2D-Electrophoresis

2D-electrophoresis (2D-PAGE) combines the techniques of IEF and SDS-PAGE. Proteins are first separated according to their charge by IEF and are then transferred to an SDS-PAGE gel and separated according to their molecular mass. The advantage of the method is an increased resolution due to the two-dimensional separation (Schlags et al. 2002). A comparative study of the performance characteristics of recombinant erythropoietins (Epoetin alfa, beta, and NESP) on 2D-PAGE was performed by Caldini et al. (2001). For focusing IPG-strips (immobilized pH-gradient gels) in the pH-range of 3–6 and 3–10 were used. It was shown that for Epoetin alfa and beta the molecular mass of the isoforms was increasing with decreasing p*I*. A drawback of the IPG-based methodology is that proteins with very acidic isoforms (p*I* below 3) cannot be properly resolved. Hence, only two isoforms of NESP could be detected by Caldini et al. On carrier ampholyte gels at least six isoforms are visible for NESP (e.g. on pH 2–6 gels or pH 3–5 large sized gels). A remedy would be the use of IEF in carrier ampholyte tube gels for 2D-PAGE. This technique was used in the early days of 2D-electrophoresis but is more complicated and less reproducible than the IPG-technology.

Based on the 2D-PAGE technique Khan et al. (2005) developed a method for the detection of EPO-doping. Instead of concentrating the urines by ultrafiltration an acetonitrile based protein precipitation step was used. After reduction and alkylation the samples were desalted by diafiltration and then focused on pH 3–5 IPG-strips. 6–5% T SDS-PAGE gradient gels were used for the second dimension. However, the entire procedure led to some nonspecific binding of the anti-EPO antibody (as used for IEF-method, clone AE7A5) to isoforms of four proteins (alfa-1-antichymotrypsin, alfa-2-thiol protease inhibitor, alfa-2-HS-glycoprotein, and Tamm Horsfall glycoprotein). However, it was not possible to show any interference of these proteins in the IEF-method as used in doping control (Rabin et al. 2006).

4.3 Chromatographic Methods

Differences in the glycan structure of recombinant and endogenous erythropoietins are largely responsible for the different electrophoretic behavior of these proteins (Imai et al. 1990; Watson and Yao 1993; Belalcazar et al. 2006). In order to differentiate between rHuEPO and uHuEPO chromatographic methods for oligosaccharide-profiling with fluorescent detection of the labeled oligosaccharides or

with pulsed amperometric detection were developed (e.g. Tsuda et al. 1988; Rice et al. 1992).

Skibeli et al. (2001, 2003) used some of these chromatographic methods for investigating the glycan structures of three different recombinant erythropoietins (Epoetin alfa, beta, and omega) and compared them with human serum EPO. The oligosaccharides were released by PNGase F, which selectively cleaves N-linked glycans. Subsequently, the glycans were purified by solid-phase extraction and fluorescence-labeled with 2-aminobenzamide (2-AB). For the separation both normal-phase HPLC as well as anion exchange chromatography were used. Due to its polar functional groups normal phase chromatography separates neutral and charged glycans largely according to their hydrophilic properties. By using this technique Skibeli et al. were able to demonstrate characteristic elution profiles for each of the investigated erythropoietins. Performing weak anion exchange chromatography allowed charge state analysis with assignment of the 2-AB labeled oligosaccharides to five categories (neutral, mono-, di-, tri-, and tetra-acidic glycans). All four EPOs showed characteristic differences, with the most prominent being the complete lack of tetra-sialylated glycans in human serum erythropoietin (Table 4). Treatment of the 2-AB-labeled glycans with neuraminidase from *Arthrobacter ureafaciens* revealed that these charge differences were attributable to sialic acids.

Llop et al. (2007) used this approach in combination with several other methods for the characterization of the *N*-glycans of electrophoretically separated isoforms of BRP-rHuEPO (a standard of the European Pharmacopea Commission containing a mixture of Epoetin alfa and beta) and NESP. 2D-PAGE was used for the separation of the isoforms. The released N-glycans (PNGase F digest) were purified by graphitized carbon column (GCC) chromatography, labeled with 2-AB and then again separated with weak anion exchange and normal-phase HPLC. The 2-AB labeled glycans were also analyzed by MALDI-TOF mass spectrometry. Interestingly, the individual isoforms showed a considerable heterogeneity with the more acidic isoforms (i.e. migrating to the anode) containing the highest amount of tetraantennary tetra-sialylated glycans. On the other hand, isoforms, which focused at a higher pH, comprised the majority of the under-sialylated glycan structures.

So far, these methods led to no test for the detection of doping with recombinant erythropoietins.

Table 4 Charge state analysis of N-glycans of various erythropoietins (Skibeli et al. 2001)

Glycoprotein	Neutral glycans (%)	Mono-acidic glycans (%)	Di-acidic glycans (%)	Tri-acidic glycans (%)	Tetra-acidic glycans (%)
Human serum EPO	20	9	48	23	0
Epoetin alfa	4	5	41	31	19
Epoetin beta	3	2	17	32	46
Epoetin omega	3	10	34	32	21

4.4 Mass Spectrometric Methods

In order to ionize and transfer biological macromolecules such as glycoproteins into the gas-phase soft ionization techniques have to be applied. *Fast atom bombardment (FAB)* combined with various HPLC-methods was the earliest mass spectrometric method used for the characterization of urinary and recombinant human erythropoietins (Sasaki et al. 1987, 1988; Recny et al. 1987; Nimtz et al. 1993). Sasaki et al. (1987, 1988) investigated the carbohydrate structures of CHO-cell expressed rHuEPO and compared them with uHuEPO by FAB-MS. Intact proteins were first digested by endoproteinase Lys-C and then separated by reverse phase HPLC. N-linked glycans were released by treatment with PNGase F. They found that the oligosaccharides on Asn83 of their CHO-cell expressed rHuEPO mainly consisted of tetraantennary structures without N-acetyllactosamine repeats, while the saccharides on, e.g. Asn24 contained a mixture of bi-, tri-, and tetraantennary structures with or without these repeating units. The major component of the O-glycan on Ser126 was identical for uHuEPO and rHuEPO and was described as Neu5Acα2→3Galβ1→3(Neu5Acα2→6)GalNAc. Nimtz et al. (1993) performed similar experiments on EPO expressed in recombinant BHK-21 cells and observed that tetraantennary chains with or without N-acetyllactosamine repeats could be detected only on Asn38 and Asn83 with almost all of the di- and triantennary oligosaccharides being located on Asn24. Only 60% of the molecules were glycosylated on Ser126. Recny et al. (1987) used FAB-MS to demonstrate that both the CHO-cell rHuEPO and uHuEPO no longer contained the arginine residue on position 166 as originally deducted from the DNA-sequence of the human EPO-gene.

Due to the low sensitivity FAB-MS was replaced in the early 1990s by more efficient soft ionization techniques for the analysis of biological macromolecules like EPO. Since then, matrix-assisted laser desorption/ionistation mass spectrometry (MALDI-MS) and electrospray ionization mass spectrometry (ESI-MS) have been extensively used for the characterization of erythropoietins. Both mass spectrometric methods have been mostly used in combination with separation techniques like HPLC or capillary electrophoresis (CE). Stuctural studies were performed on the level of the intact protein as well as on its peptides/glycopteptides and purified glycans.

Measurement of the molecular mass of *intact glycoproteins* by MALDI-TOF mass spectrometry is usually performed in the linear mode of the mass spectrometer. The main reasons for using this mode instead of the reflector mode are its higher sensitivity for high mass compounds like proteins and a decreased susceptibility of labile compounds to an energy-induced decay (e.g. loss of sialic acids of glycoproteins). Due to the way the MALDI-experiment is optimized (e.g. matrix selection, laser energy, etc.) and the instrument is calibrated (internally, externally) different molecular masses are obtained. Usually, *sinapinic acid* is the preferred matrix for mass spectrometry of intact proteins, but not necessarily glycoproteins. Zhou et al. (1998) determined a molecular mass of *m*/*z* 28,707 for the singly charged molecular

ion (positive ion mode) for rHuEPO expressed in CHO-cells. According to the amino acid sequence the calculated mass should be only 18,396 Da. Hence it was concluded that the carbohydrate content is approximately 35.9% (erroneously the cDNA-derived amino acid sequence containing the C-terminal Arg166 was used). The applicability of measuring intact masses of CHO expressed erythropoietins (Exprex, NESP) for doping control purposes was investigated by Stanley and Poljak. About 1 pmol of each gylcoprotein was used. Measurements were performed in positive ion mode and with *caffeic acid* as matrix. Both proteins showed a broad mass distribution, which is typical for glycoprotein spectra. The peak maximum observed in the spectrum of Eprex was at *m*/*z* 29620 for the singly charged molecular ion (positive ion mode) and with a peak width ranging from *m*/*z* 26,000 to 33,500 (which corresponds to a carbohydrate content of 29.9 and 45.5%). The peak maximum for NESP was at *m*/*z* 36954. It was concluded that for doping control purposes the sensitivity should be at least in the low femtomol but preferably attomol range. Hence, direct measurement of the intact glycoprotein masses – even if the proteins were deglycosylated – would not be sensitive enough for fulfilling this requirement.

Studies on the influence of matrix, pH and composition of the matrix solution, and laser power were studied by Sottani et al. (1997). A solution of *ferulic acid* in a mixture of acetonitrile/water/formic acid ($pH \leq 1.8$) performed better than a solution of sinapinic acid in a mixture of acetonitrile/water/trifluoroacetic acid ($pH \leq 2.3$). By keeping the laser power as low as possible (ca. 30% above the threshold of ionization) eight subpeaks became visible on the broad molecular ion peak. These subpeaks appeared to differ by the mass of one sialic acid each (*m*/*z* 291). The *m*/*z* of the most intense peak was $28{,}673 \pm 0.1\%$, the highest observed mass was ca. 30 kDa. Stübiger et al. (2005b) compared the sensitivity of different MALDI-matrices, MALDI target surfaces, and sample preparation techniques for measuring the masses of three intact recombinant erythropoietins (Epoetin alfa, Epoetin beta, and NESP). By using a very hydrophobic surface instead of the usual stainless steel MALDI target surface an increased sensitivity was obtained, roughly in the range of 50–100 fmol. Ferulic acid and sinapinic acid performed best. Another useful matrix was *super-DHB* (a mixture of DHB and 2-hydroxy-5-methoxybenzoic acid). The average molecular mass was 29.8 ± 0.3 kDa for Epoetin alfa and beta and 36.8 ± 0.4 kDa for NESP with sinapinic acid as matrix. Discrete glycoforms could be detected in the spectra after enzymatic removal of the sialic acids (Fig. 5). In yet another approach, Giménez et al. (2007) evaluated different matrices (sinapinic acid, 2,5-dihydroxybenoic acid (DHB), super-DHB, 6-aza-2-thiothymine (AAT), 2′,4′,6′-Trihydroxyacetophenone (THAP), and *nor*-harmane), sample preparation techniques, and laser energies for rHuEPO and NESP. The best performance was achieved with a combination of *DHB* and vacuum drying. Peak maxima for rHuEPO and NESP were detected at, e.g. *m*/*z* 30,208 and 37,716, respectively.

However, so far no MALDI-TOF mass spectra of highly purified intact human urinary erythropoietin in direct comparison with the mass spectra of intact recombinant erythropoietins have been published. Only spectra of rather impure uHuEPO preparations were obtained (Yu et al. 2005).

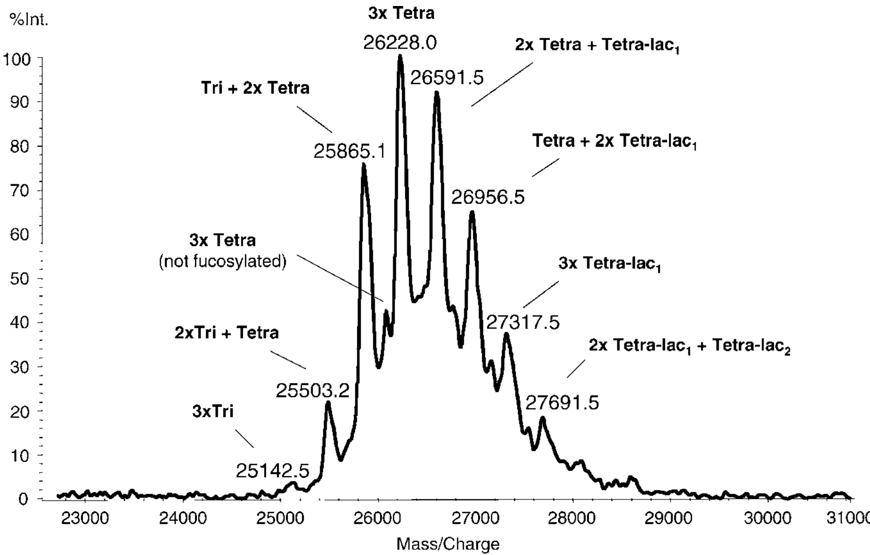

Fig. 5 MALDI-TOF mass spectrum of Epoetin alfa after enzymatic de-sialylation. Sinapinic acid was used as matrix, about 1 pmol of protein was applied on target. The relative abundance of the major tri- and tetraantennary N-glycoforms with or without N-acetylactosamine repeats (lac) is detectable
Source: Stübiger et al. (2005b), Wiley (permission granted)

A more detailed structural characterization of erythropoietin could be achieved by performing MALDI-TOF mass spectrometry on the level of *peptides/glycopeptides* or glycans. This strategy also resulted in an increase in sensitivity if compared to mass spectrometry of intact proteins. Enzymes like trypsin, Lys-C, or Glu-C were typically used for cleaving the intact protein. The resulting peptide/glycopetide mixture was either spotted directly on MALDI target plates (Zhou et al. 1998) or separated and fraction-collected by various techniques before the MALDI-experiment (e.g. off-line LC-MALDI, CE-MALDI). Rahbek-Nielsen et al. (1997) used this approach for profiling the glycopeptides of human urinary erythropoietin. After reduction and alkylation uHuEPO was digested by endoproteinase Lys-C. Reverse phase HPLC on a C8 column was used as separation method. After dyring the collected peptides/glycopeptides in a Speedvac they were dissolved in 50% methanol containing 1% acetic acid. Either *α-cyano-4-hydroxycinnanmic acid* (4HCCA) or *2,5-dihydroxybenzoic acid* (DHB) was used as matrix. All N-glycosylation sites (Asn24, Asn38, Asn83) showed fucosylated complex-type glycans. Tetraantennary glycans were mostly found at Asn38 and Asn83 while Asn24 contained a mixture of bi-, tri- and tetraantennary glycans. Three types of O-glycans were found at Ser126 composed of hexose (Hex), N-acetylhexosamine (HexNAc), and sialic acids (SA) – namely HexNAc-Hex, HexNAc-Hex-SA, and HexNAc-Hex-2SA (i.e. HexNAc-Hex with one or two sialic acids).

A reverse phase microscale sample purification technique combined with MALDI-TOF mass spectrometry was used by Stübiger et al. (2005a) for characterizing the N- and O-glycopeptides of recombinant erythropoietins (Epoetin alfa, Epoetin beta, NESP). After enzymatic digestion glycopeptides were separated by combining a C18 ZipTip peptide/glycopeptide trapping strategy with a stepwise elution procedure. O-glycopeptides were less hydrophobic than N-glycopeptides, hence eluted mainly in the 3% acetonitrile fraction. The eluent was a solution of 1 mM ammonium acetate (pH 7.0) containing 3, 15, and 50% acetonitrile. Subsequently, the isolated glycopetides were analyzed in positive linear mode using DHB as matrix. A different degree of sialylation and O-acetylation of the sialic acids was observed for the various recombinant erythropoietins. These characteristics already allowed a differentiation between the three products. Unfortunately, a direct comparison with the N- and O-glycopeptides of uHuEPO was not possible due to the lack of a highly purified uHuEPO standard. However, the outlined strategy would be interesting for doping control purposes.

MALDI-TOF mass spectrometry can be also used for characterizing only the oligosaccharide part of glycoproteins. After enzymatic or chemical cleavage the released *glycans* are separated from the proteins, derivatized, and further separated by chromatographic methods. Mo et al. (1998) used PNGase F for releasing the glycans of rHuEPO and 4-aminobenzoic acid 2-(diethylamino) ethyl ester (ABDEAE) for derivatization. RP-HPLC on a C18 column was used for separating and purifying the derivatized glycans. *DHB* was used as matrix for high resolution MALDI-TOF experiments. Typically, 50–200 fmol of glycans were necessary for assigning structural details to the obtained mass spectra. A similar approach was applied by Kanazawa et al. (1999). After PNGase F digestion oligosaccharides were separated from the remaining protein by C18 RP-HPLC and subsequently labeled with 2-aminobenzamide (2-AB). Separation of the 2-AB labeled glycans was achieved by ion exchange chromatography on DEAE column chromatography. *2′, 4′, 6′-trihydroxyacetophenone monohydrate* (THAP) and DHB were used as matrices for sialylated and neutral oligosaccharides, respectively. Llop et al. (2007) used this method for characterizing the glycan structures of erythropoietin isoforms. After separating rHuEPO and NESP by 2D-PAGE and subsequent PNGase F digestion the released N-glycans were purified by miniaturized graphitized carbon column (GCC) chromatography using packed GELoader tips. After 2-AB labeling oligosaccharides were analyzed by MALDI-TOF using negative ion mode and DHB as matrix. A considerable heterogeneity within each isoform was observed with the more acidic isoforms containing the highest amount of tetraantennary tetrasialylated glycans (vide supra). A slightly modified approach was introduced by Yuen et al. (2003): the PNGase F released N-glycans were fluorescent labeled with 4-aminobenzoic acid (4-ABA) and then separated by anion-exchange (diethylaminoethyl, DEAE) chromatography followed by normal phase (Amide-80) HPLC. The fractions were mixed with DHB-matrix and analyzed on a MALDI-TOF mass spectrometer. Changes in the N-glycan structures of CHO-EPO produced under different culture conditions were observed. Besides the well known and well characterized complex-type N-glycans sulfated

or phosphorylated high-mannose type N-glycans were also detected. Nimtz et al. (1995) identified a mannose-6-phosphate containing oligomannosidic N-glycan on BHK-21 cell expressed erythropoietin. The structural characterization was done by NMR.

While MALDI-TOF mass spectrometry remains an off-line method – even if operated, e.g. in on-line LC-spotting mode (LC-MALDI-TOF-MS) – *electrospray ionization mass spectrometry* (ESI-MS) can be operated in either on-line (e.g. coupled to HPLC or CE) or off-line (e.g. classic static (infusion) nanospray) mode. Similar to MALDI-MS glycoproteins can be analyzed on the level of the intact protein, the glycopeptides/peptides, and the oligosaccharides. The majority of the investigations on *intact erythropoietin* using electrospray ionization were done in on-line mode. Rush et al. (1995) used a triple stage quadrupole mass spectrometer which was on-line coupled to a reverse phase HPLC (LC-ESI-MS). A linear gradient of acetonitrile (2–90%, 15 min) was used for eluting both the intact and neuraminidase treated rHuEPO off the column. The obtained electrospray mass spectrum (*m*/*z* 1,600–2,400) of the intact molecule was quite complex and without useful information. However, by cleaving off the sialic acids the spectrum showed distinct peaks and became less complex. It was concluded that the micoheterogenity of rHuEPO was partly due to the sialic acids and that more detailed information could only be gained on the level of the glycopeptides.

In a series of experiments Neusüss et al. (2005), Balaguer et al. (2006), Balaguer and Neusüss (2006a, b) used capillary electrophoreses coupled to an electrospray-time-of-flight mass spectrometer (CE-ESI-TOF-MS) for characterizing the glycoforms of intact recombinant erythropoietins (Epoetin alfa and beta). A total of 44 glycoforms and ca. 135 isoforms could be distinguished for the rHuEPO reference preparation of the European Pharmacopeia (BRP-EPO). This heterogeneity was also due to O-acetylation of sialic acids. A different degree of acetylation was observed for recombinant erythropoietins of different suppliers. The lowest mass measured for a BRP-EPO glycoform was 27,261.7 and the highest 32448.0. Figure 6 shows the mass distribution of Epoetin alfa and beta glycoforms analyzed by CE-ESI-TOF-MS (Balaguer and Neusüss 2006b).

However, about 50 ng of rHuEPO were necessary for the characterization. The authors concluded that the sensitivity of the method is still too low for testing the low amount of EPO contained in biological fluids (urine, serum), which is typically in the range of ng L^{-1}. Additionally, the injection volume due to the CE-method had to be rather low (50 nL), thus requiring EPO concentrations in the $\mu g\,\mu L^{-1}$ range. Yu et al. (2005) used CE coupled on-line to an ESI-ion trap mass spectrometer for comparing intact rHuEPO and uHuEPO. Unfortunately, the purity of the uHuEPO standard was very low. An ESI-single quadrupole instrument was employed by Sanz-Nebot et al. (2003) in combination with CE for characterizing intact rHuEPO. Again, the purity of the EPO formulation was not high enough. The presented ESI-spectrum showed high amounts of a polymer (probably residuals of the detergent present in the BRP-EPO standard used). De Frutos et al. (2003) compared the profiles of CHO-EPO (BRP-EPO standard) and purified uHuEPO after capillary zone electrophoresis. Eight isoforms were detected for the

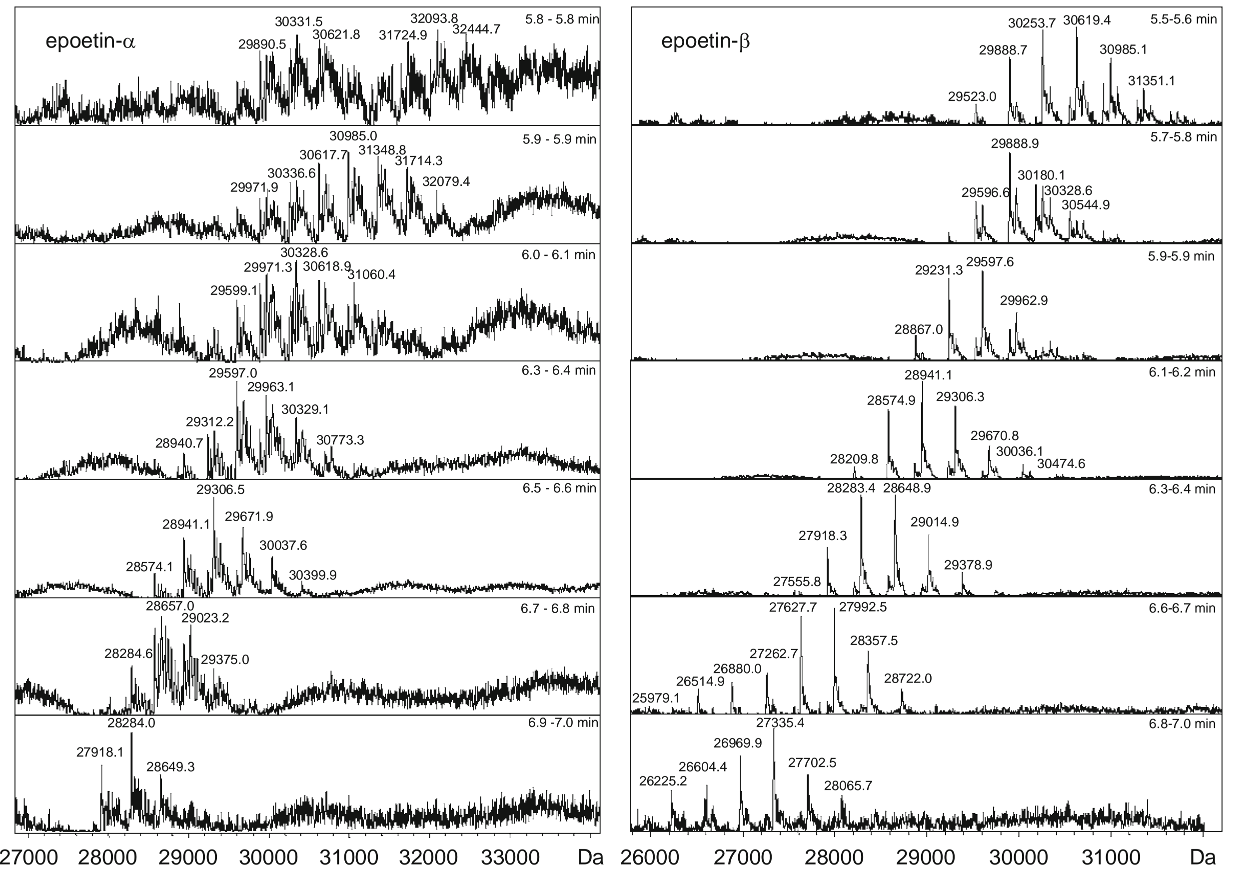

Fig. 6 Glycoform analysis of Epoetin alfa (Eprex) and beta (NeoRecormon) by CE-ESI-TOF-MS. Time windows mainly represent different sialic acid isoforms

Source: Balaguer and Neusüss (2006b), Vieweg (permission granted)

recombinant EPO but only six isoforms for the urinary protein. Unfortunately, the detection was not done by mass spectrometry but only by measurement of the UV absorption at 214 nm.

Due to the microheterogenity and the tendency of ESI to produce a series of different charge states for each molecular ion in the gas phase ESI-spectra of intact glycoproteins – like erythropoietin – become highly complex. For detailed structural studies by ESI-MS investigations on the glycopetide or glycan level are the method of choice. Linsley et al. (1994) digested rHuEPO enzymatically (trypsin and Lys-C, respectively) and characterized the four *glycopeptides* after RP-HPLC separation on C18 or C4 columns. Due to its small size the carbohydrate structure of the O-glycopeptide could be easily deducted without having to first isolate the glycan. Interpretation of the mass spectrum was accomplished by following the sequential losses of N-acetyl neuraminic acid, hexose, and N-acetyl hexosamine. ESI-MS/MS spectra obtained by collision-induced dissociation (CID) of selected ions were also used for deducting the glycan structure. However, direct ESI-MS or ESI-MS/MS of the three N-glycopeptides was less informative than for the O-glycopeptide. Hence, degylcosylation and analysis of the released glycans was performed. Rush et al. (1995) used a similar approach as Linsley et al. (Glu-C and trypsic digest, RP-HPLC separation) but also collected the fractionated glycopeptides for off-line infusion experiments. They observed that O-acetylation of N-acetylneuraminic acids (Neu5Ac) was a common feature of all four glycosylation sites, and that both mono- and di-O-acetylated N-acetylneuraminic acids were present. In total, 52 different N-linked oligosaccharide structures were identified. N-glycolylneuraminic acid (Neu5Gc) was present in small amounts on all N-glycosylation sites. Both authors used triple stage quadrupole mass spectrometers for their studies. In yet another approach Zhou et al. (1998) compared on-line LC-ESI-MS with on-line CE-ESI-MS for characterizing rHuEPO glycopeptides. They concluded that due to the limited sample volume analysable on CE-columns (typically in the nanoliter-range) the sensitivity of the system was lower than for the LC-system. Typical eluents used by these authors for separating the peptide/glycopeptide mixtures on RP-HPLC were composed of acetonitrile, water, and TFA (trifluoroacetic acid), and were run in gradient mode.

Ohta et al. (2001) achieved selective mapping of the rHuEPO glycopeptides by changing from the usual acetonitrile/water/TFA gradient to an acetonitrile/water/ammonium acetate gradient. A C18 RP-column was used in combination with a triple stage quadrupole mass spectrometer. Solvent A was 1 mM ammonium acetate in water, pH 6.8, and solvent B was 80% acetonitrile containing 1 mM ammonium acetate. For elution a multi-step gradient from 1 to 36% solvent B was run. Positive and negative ionization modes were compared (Fig. 7). While for the 0.05% TFA containing eluents both peptides and glycopeptides were eluted the switch to ammonium acetate selectively eluted the glycopeptides. The separation was better at pH 6.8 than at pH 4.0. Also, due to the negative ion mode an increase in the sensitivity for the N-glycopeptides was achieved. By using this approach it was found that Asn38 and Asn83 contained fucosylated tetrasialylated tetraantennary oligosaccharides with 0–2 N-acetyllactosamine repeating units. Asn24 contained

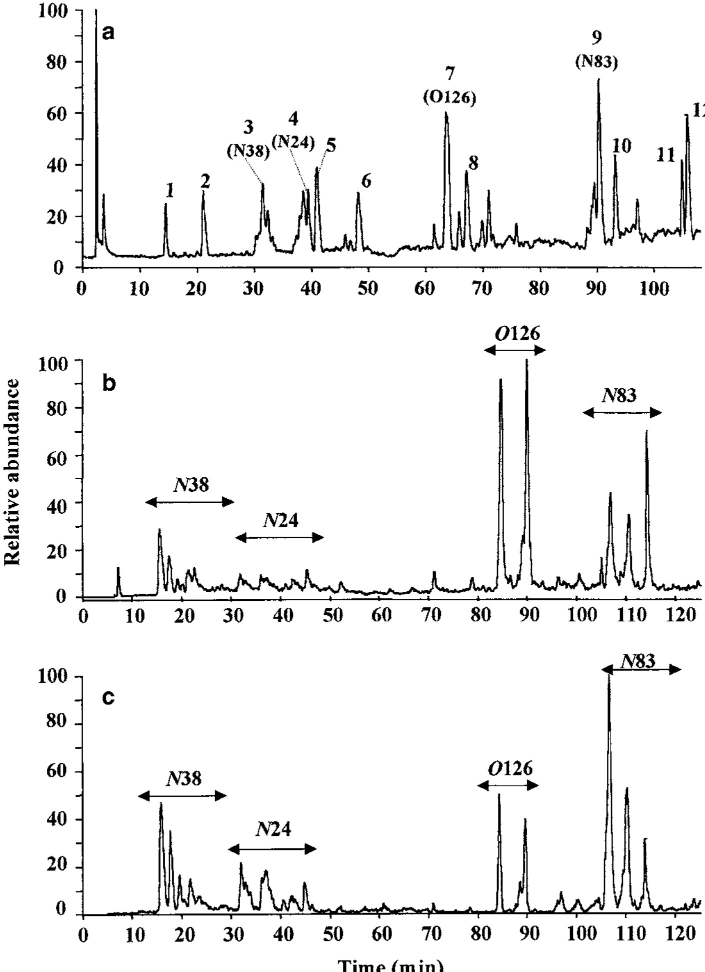

Fig. 7 Selective mapping of rHuEPO glycopeptides. By using 1 mM ammonium acetate (chromatograms B and C) instead of 0.05% TFA (chromatogram A) in a gradient consisting of acetonitrile and water glycopetides could be selectively eluted from a C18 RP-column. An increase in sensitivity was observed for N-glycopetides when operating the mass spectrometer in negative ion mode
Source: Ohta et al. (2001), Elsevier (permission granted)

a mixture of bi-, tri-, and tetraantennary glycans with 2–4 sialic acids and 0–2 N-acetyllactosamines. These results perfectly matched the results of earlier studies (e. g. Sasaki et al. 1988; Linsley et al. 1994; Rush et al. 1995). Small amounts of Neu5Gc were also detected. Subsequently, Ohta et al. (2002) used this method for differentiating between three rHuEPO preparations expressed in CHO- and BHK-cells. Site-specific differences in acetylation, sialylation, and sulfatation were detected. Mono- and disulfated tetraantennary oligosaccharides were found on

N24, N38, and N83 of CHO- and BHK-EPO. However, BHK-EPO had a higher amount of sulfated glycans.

Capillary zwitterionic-type hydrophilic interaction chromatography (ZIC-HILIC) coupled to an ESI linear ion-trap TOF (ESI-LIT-TOF-MS) was used by Takegawa et al. (2008) for profiling the N- and O-glycopeptides of CHO-cell expressed and Glu-C digested rHuEPO. The eluent contained acetonitrile, water, and 10 mM ammonium acetate. The mass spectrometer was operated in negative ion mode. 105 N- and eight O-glycopeptides could be detected in a single run. In an attempt to decrease the amount of rHuEPO necessary for glycopeptide analysis Groleau et al. (2008) applied nanoflow HPLC-chip ESI-MS/MS. However, amounts of digested EPO in the pmol range were still required for their approach.

Due to differences in the amino acid sequence of human and equine erythropoietins the abuse of recombinant human erythropoietins for horse doping can be detected (Singh and Gupta 2007). ELISA-methods originally used for measuring human EPO in human serum or plasma have been used for detecting doping of horses with rHuEPO (Kearns et al. 2000). Stanley and Poljak (2003) analyzed Epoetin alfa and NESP by MALDI-TOF-MS, ESI-MS, and ESI-MS/MS, and concluded that for equine and canine doping control the proteins should be first deglycosylated and enzymatically digested (Glu-C) in order to detect characteristic human sequences. Guan et al. (2007) published an LC-ESI-MS/MS method for detecting rHuEPO and NESP in equine plasma. It is based on immunoaffinity extraction of both proteins from horse plasma and subsequent tryptic digestion of the eluted fraction. Two short peptides which were characteristic for rHuEPO and NESP – and hence allowed a differentiation between human and equine EPO – were selected for designing a sensitive mass spectrometric method. The limit of detection for both substances was 0.1 ng mL^{-1} of equine plasma. However, since the selected peptides could not differentiate between rHuEPO and NESP a second method was developed (Guan et al. 2008). This time two longer tryptic glycopeptides were selected which contained the five altered amino acids of NESP. After immunoaffinity purification the extracted rHuEPO and NESP were digested by trypsin and PNGase F in order to remove the MS-interfering glycans. The achieved sensitivity of the new method was comparable to the first method but also allowed a differentiation between the two pharmaceuticals.

On-line structural analyzes on the level of erythropoietin *glycans* by ESI-MS or ESI-MS/MS have been mostly performed in combination with graphitized carbon column liquid chromatography (GCC–LC). RP-HPLC-MS of oligosaccharides requires the derivatization of the carbohydrates with hydrophobic compounds (e.g. 1-phenyl-3-methyl-5-pyrazolone, 2-aminopyridine) for increasing the interaction with the RP-phase. Subsequent purification of the derivatives is time consuming and not quantitative (Kawasaki et al. 1999). Contrary to that, graphitized carbon columns are able to also retain and separate less hydrophobic compounds like oligosaccharides and oligosaccharide alditoles without the need of derivatization. Kawasaki et al. (2000) used GCC-LC/MS and GCC-LC/MS/MS for structural studies on CHO-EPO and its N-glycans. The N-linked oligosaccharides were either globally analyzed or on the level of the glycopeptides. For global

analysis glycans were released by PNGase F, the protein was precipitated with ethanol, and then the supernatant was dried and reduced with $NaBH_4$. The oligosaccharide aldioles were separated using a gradient of acetonitrile containing 5 mM ammonium acetate. Mass spectrometry was performed on a triple stage quadrupole mass spectrometer, which was operated in negative ion mode. For site specific oligosaccharide analysis rHuEPO was enzymatically digested with Glu-C and then the glycopetide containing fractions were collected after C18 RP-HPLC separation. Oligosaccharides were liberated from the peptides by treatment with glycopetidase A and then reduced with $NaBH_4$. It was found that Asn24, 38, and 83 contained di- and trisialylated tetraantennary glycans and that only Asn24 contained di- and trisialylated tetraantennary glycans with additional N-acetyllactosamines. Subsequently, the method was used for analyzing the *N*-linked oligosaccharides of BHK-EPO (Kawasaki et al. 2001). It was shown that some of the N-glycans were partly mono- or disulfated – a feature which the authors also detected in some EPOs expressed in CHO-cells. ^{1}H-NMR analyzes of the sulfated tetraantennary oligosaccharide indicated that the sulfate group was located at the C-6 position of the GlcNAc in the GlcNAcβ1-4Manα1-3 branch. In 2002 Kawasaki et al. used this technique of sugar mapping by LC–MS for comparing three different rHuEPOs with each other (BHK-EPO, two CHO-EPOs). Significant differences in the degree of sialylation, acetylation, and sulphatation were detected and could be used to distinguish between these three rHuEPO products. Also in 2002, Itoh et al. transferred the method to microbore GCC-LC-MS in order to gain sensitivity. A variation of the technique was introduced by Yuan et al. (2005) and allowed quantitative analysis of mono- and oligosaccharides. Carbohydrates were isotope-labeled by reaction with hexadeuterium-2-aminopyridine (d_6-AP) and used as internal standards. By labeling the oligosaccharides of the analyte with d_0-AP and the standard with d_6-AP relative quantification was obtained in a single run.

4.5 *Proteolytic Digestion of EPO*

Confessions of endurance athletes have recently revealed the use of proteolytic agents like proteases for the manipulation of their urine samples (Siegmund-Schultze 2007). These substances are easily available and in widespread use as e.g. washing powders. Once dropped into the urine sample as powder or grain, endogenous as well as exogenous proteins are digested and thus detracted from further detection. Lamon et al. (2007b) demonstrated this effect by adding increasing amounts of trypsin to a urine sample containing endogenous EPO, and also studied the efficiency of protease inhibitors. Thevis et al. (2007) published an analytical strategy for the detection of the manipulation with several proteases using the determination of elevated protease activities in urine samples, followed by the visualization of protein degradation and identification of proteases using bottom-up sequencing approaches based on in-gel digestion of separated enzymes followed by capillary liquid chromatography–Orbitrap tandem mass spectrometry.

4.6 *Miscellanous*

4.6.1 Epo Detection Using MAIIA Technology

The membrane-assisted isoform immunoassay (MAIIA) is a combination of two techniques, namely a membrane-based chromatographic technique for the separation of isoforms of a protein together with an immunoassay technique for the detection of the separated isoforms (Lönnberg and Carlsson 2000, 2001a,b, 2006). Both steps are performed on one single usage device. For the chromatographic separation either an ion-exchange membrane or a membrane with a lectine zone are used. The liquid flow across the separation zone is entirely driven by capillary forces supported by a buffering pad and an adsorbent sink.

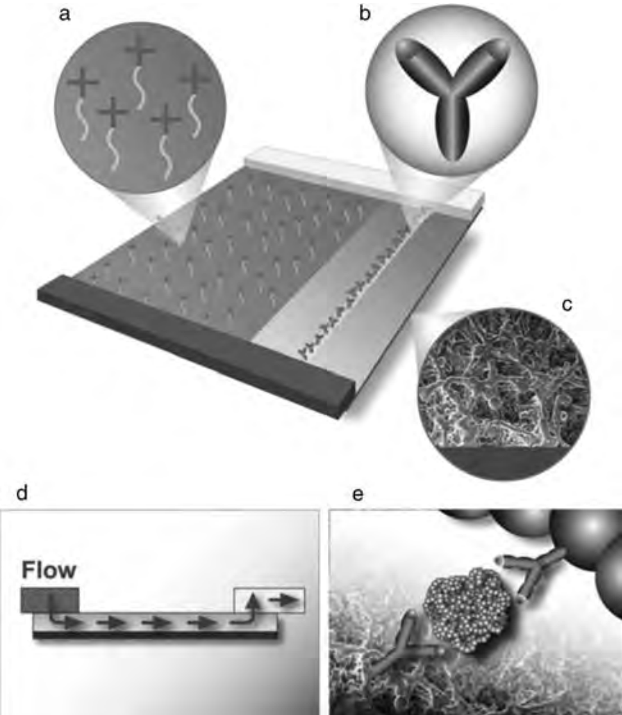

Fig. 8 The MAIIA-device is composed of a chromatographic zone (membrane-based anion-exchanger or lectin zone) (**a**) and a detection zone (**b**) on a porous membrane (**c**). A constant liquid flow across the membrane is achieved by applying a buffer pad (*gray*) and adsorbent sink (*white*) at the start and end of the separating zone (**d**). EPO-isoforms are separated by their differing interaction with the separating material. The immunoassay-like detection zone combines an immobilized anti-EPO antibody for capturing the separated EPO-isoforms with a carbon black labeled detection antibody (**e**). The flow towards the detection zone is achieved by applying a new set of pads orthogonally to the direction of the separation
Source: Lönnberg and Carlsson (2006), Elsevier (permission granted)

For detecting the separated isoforms the membrane is rotated 90 degrees and then the isoforms are driven again by capillary forces across a detection zone. It contains an immobilized anti-EPO antibody (Fig. 8). For visualization, a carbon black labeled detection antibody is added. The resulting isoform profile is scanned and then evaluated by densitometry (Lönnberg and Carlsson 2006). For the detection of EPO in urine the sample has to be first immunoaffinity-purified on an anti-EPO immunoaffinity column (e.g. monolith) and then applied on the chromatographic zone. Careful selection of the antibody is necessary in order to enrich the entire set of EPO-isoforms present in the sample.

5 Case Report: Detection of Dynepo Administration

Although prohibited both in- and out-of competition, the main focus for rEPO-detection was initially suggested to find its principle application in out-of-competition testing, especially in endurance sports (Lasne and de Ceaurriz 2000). Several examples during, e.g. the Olympic Games and the Tour de France proved its applicability in in-competition testing, too.

The following figures provide an example reported as adverse analytical finding for the presence of recombinant erythropoietin. Analytical data as obtained by isoelectric focusing (Fig. 9) and SDS-PAGE (Fig. 10) are shown. The gel image after isoelectric focusing, double blotting, and chemiluminescence detection

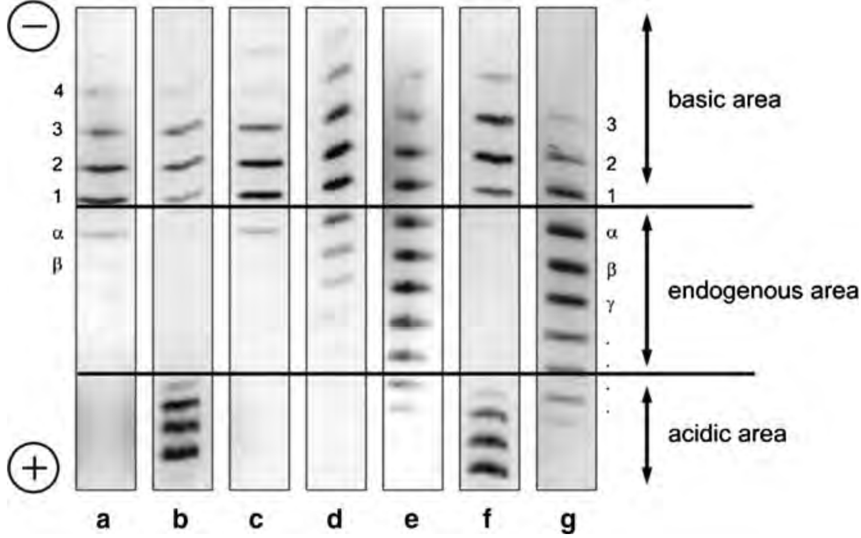

Fig. 9 Gel image after isoelectric focusing, double blotting, and chemiluminescence detection: athlete's sample (**a**), stability test (**b**), Epoetin delta (**c**; Dynepo), positive control sample (**d**), negative control sample (**e**), BRP/NESP standard mix (**f**), NIBSC uHuEPO standard (**g**)

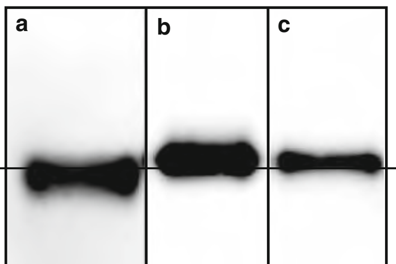

Fig. 10 SDS-PAGE image of (**a**) a negative control (NIBSC uHuEPO), (**b**) a positive control (Epoetin delta, Dynepo), and (**c**) the athlete's sample (same as shown in Fig. 9)

(vide supra) fulfilled the criteria of positivity according to the WADA technical document TD2007EPO. It contained the athlete's sample (A), the stability test (B), a standard of Epoetin delta (C; Dynepo), which corresponds in the band profile to the athlete's sample, a positive control sample (D), a negative control sample (E), a BRP/NESP standard mix (F), and the NIBSC uHuEPO standard (G). Compared to the IEF-pattern of the BRP standard (bands in the basic region of lane F) the pattern of Dynepo (C) showed a more intense band alfa (Segura et al. 2007).

The image obtained after SDS-PAGE, double blotting, and chemiluminescence detection (Fig. 10) included amongst others a negative control (A; NIBSC uHuEPO), the Epoetin delta standard (B; Dynepo), and the athlete's sample (C). The band of the athlete clearly corresponded to the band of the Epoetin delta standard but not to the negative control.

In conclusion these data clearly confirm the presence of a recombinant form of erythropoietin in the urine of the athlete. SDS-PAGE analysis in particular supported the evidence that the adverse analytical finding was caused by the application of Dynepo or a Dynepo-like preparation.

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Blood Transfusion in Sports

Sylvain Giraud, Pierre-Edouard Sottas, Neil Robinson, and Martial Saugy

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Abstract Blood transfusion is an effective and unmediated means of increasing the number of red blood cells in the circulation in order to enhance athletic performance. Blood transfusion became popular in the 1970s among elite endurance athletes and declined at the end of the 1980s with the introduction of recombinant erythropoietin. The successive implementation in 2001 of a direct test to detect exogenous erythropoietin and in 2004 of a test to detect allogeneic blood transfusion forced cheating athletes to reinfuse fully immunologically compatible blood. The implementation of indirect markers of blood doping stored in an Athlete's Biological Passport provides a powerful means to deter any form of blood transfusion.

Keywords Blood transfusion • Doping • Antigenic pattern • Athlete's Biological Passport

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Abbreviations

ABPS	Abnormal blood profile score
BT	Blood transfusion
HGB	Hemoglobin
RBC	Red blood cell
RET%	Percentage of reticulocytes
rhEPO	Recombinant human erythropoietin

1 Introduction

Blood transfusion (BT) is the process of transferring blood or a blood-based product into the circulatory system of a person. Allogeneic or homologous BT refers to the transfusion of blood of another person compatible for ABO and Rhesus D blood groups, whereas autologous BT or auto-transfusion is the reinfusion of the individual's own stored blood.

In the 1970s, it was demonstrated that BT significantly improves physical performance (Ekblom et al. 1972), due to increased red blood cell (RBC) mass and therefore augmented oxygen transport capacity. Several studies in the 1980s confirmed the gain observed from auto-transfusion (Eichner 2007). Following a long list of recognized BTs in elite endurance sports in the same period – particularly during the 1980 and 1984 Olympics – BT methods were banned by the International Olympic Committee for the 1988 Olympics. This period coincides with the appearance of recombinant erythropoietin (rhEPO) from molecular biotechnology. Since rhEPO is easier to administer than BT – no logistically challenging blood storage and reinfusion – rhEPO became the doping method of choice in the 1990s for athletes who wished to artificially increase their RBC mass. It was only in 2001 with the introduction of a method for direct detection of rhEPO in urine (Lasne and de Ceaurriz 2000) that BT doping practices regained interest among cheating athletes. In the 2000s, there are consistent records substantiating the strong resurgence of BT in endurance elite sports (Lippi and Banfi 2006). Some international federations recording medical data on a regular basis observed notably among some athletes very unusual ferritin increases in combination with elevated OFF scores (Gore et al. 2003)[1]. This strongly suggests BT practices rather than EPO abuse. Since 2004, this has been confirmed by numerous athletes convicted of allogeneic BT abuse (Arndt and Kumpel 2008).

[1] The OFF score is a multiparametric marker of altered erythropoiesis that was developed to detect the wash-out phase of rhEPO doping.

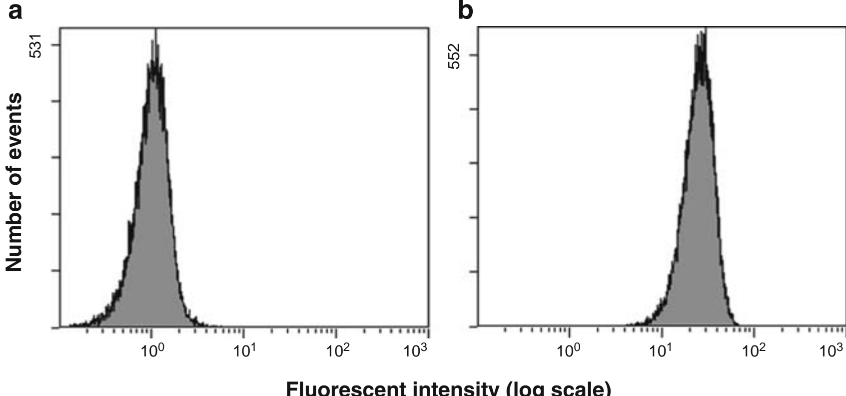

Fig. 1 RBCs furnished by the serology industry were stained using anti-Jk[a] antibody. The histograms represent the fluorescence intensity for nonexpressing (**a**) and expressing (**b**) RBC population. The presence of a single peak in both histograms indicates a single RBC population, hence no allogeneic BT

2 Detection of Homologous Blood Transfusion

Today, there are almost 300 RBC surface antigenic determinants or blood group specificities recognized by the International Society of Blood Transfusion (Daniels 2006). RBC surface antigens are macromolecules anchored in the lipid bilayer of the RBC envelope; most of them are glycoproteins. In a serological test, RBC surface antigens are identified for the determination of a specific blood group. The presence or absence of a blood group is traditionally evidenced by a visual agglutination of RBCs.

In the case of homologous BT, blood compatible for ABO and Rhesus D groups is transfused, but in most cases differences remain for minor RBC antigens. Thus, the presence of a mixed blood group expression pattern is characteristic of a mixed RBC population[2]. Traditional hemoagglutination assays are inefficient for the detection of homologous BT, since the distinction between a minor population and the main population is unmanageable visually in clumped RBCs. Hopefully, fluorescence-based flow cytometry labeling of antibodies directed against blood groups is able to give a well-defined image of the RBC antigenic expression pattern (Garratty 1990, Nelson et al. 2003). Figure 1 presents the histogram returned by the fluorescence intensity channel after the analysis of a negative control by flow cytometry.

[2]Two different RBC populations are either the result of an allogeneic blood transfusion or chimerism. A human chimera is a person composed of two genetically distinct types of cells. Chimerism can be detected in DNA testing. The frequency of RBC chimerism is estimated to be lower than 1:1,000,000 (Giraud et al. 2008). A longitudinal follow-up also allows discrimination between the transient effect of homologous BT and the steady state distinctive of chimerism.

Short after the introduction of the rhEPO test in urine, the principles for the detection of homologous BT by fluorescence-based flow cytometry were established (Nelson et al. 2002). The validation of the test, however, took two additional years to reach the quality standards – forensic analysis and objective interpretation – mandated by the World Anti-Doping code. The validation included:

– The substantiation of the response of the antibodies that were primarily designed for hemoagglutination tests and not for flow cytometry;
– The definition of a minor population without using any quantification (Sottas et al. 2006), because a blood mixed with a known amount of minor population could lead to different percentages of the minor population depending on which antibodies were used (Voss et al. 2007);
– The verification that the flow cytometry signal was the result of a unique RBC and not from a small aggregate of RBCs;
– The determination of the sensitivity and specificity of the method via a single-site blinded study with 140 samples (Giraud et al. 2008).

The test uses up to 12 antibodies: C, c, E, e, K, P_1, Fy^a, Fy^b, Jk^a, Jk^b, S and s. Internal and external controls are added to the analysis of all blood samples. External controls are used to check the specificity and identification capabilities of the antibodies. The process of setting up external controls includes staining and flow cytometry analysis of RBCs furnished by the serological industry. Each RBC population phenotype is thus fully characterized a priori. Negative controls with expressing or nonexpressing RBCs as well as positive controls with expressing and nonexpressing RBCs are then prepared and analyzed. On the other hand, the aim of internal blank controls is to show that the immunofluorescent signal of the tested sample is exclusively the result of the expression of a specific blood group.

Figure 2 shows a typical distribution of the fluorescence intensity channel obtained from two external, positive controls. The presence of two peaks in the

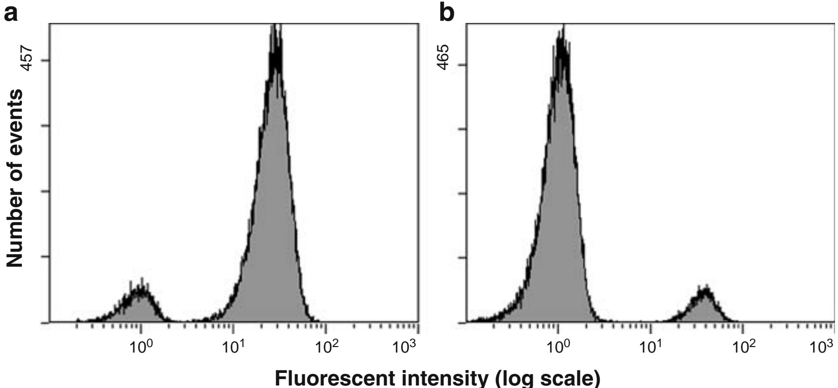

Fig. 2 Mixes of RBCs furnished by the serology industry were stained using the anti-Jk^a antibody. Histograms represent the fluorescence intensity for (**a**) a mix of RBC with main population expressing Jk^a, and (**b**) a mix of RBC with the main population nonexpressing Jk^a. The presence of two peaks in the histograms indicates a mixed RBC population

histograms is characteristic of a bimodal antigenic expression, hence a mixed RBC population. The presence of two peaks in only one of the histograms – of potentially 12 histograms – is a sufficient condition to show the presence of a mixed RBC population. However, following the recommendations of the World Anti-Doping Agency, more than one antibody must give a signal showing a mixed RBC population to declare the sample positive. The chance of detecting two samples with identical antigenic expression pattern by this screening strategy is reportedly in the range of 1:200. The described procedure has a sensitivity to less than 1% of the RBC minor population, so that the time detection window can reach several months after the transfusion of one unit of blood (Giraud et al. 2008). The first positive cases of allogeneic BT were reported in 2004.

3 Indirect Testing of Blood Transfusion

Analysis of RBC antigenic expression patterns by flow cytometry is able to reveal the presence of mixed RBCs populations in transfused recipients. To date, there is however no recognized test if the donor and the recipient have an identical expression pattern of minor RBC antigens, such as in the case of autologous BT. Current research on direct testing of fully immunologically compatible BT is based on the detection in the athlete's blood or urine samples of exogenous substances spread during the withdrawal, storage and reinfusion of the blood. Metabolites of plasticizers leaked from blood bags and residues of solvents used for cryogenic storage of the blood are some examples. It is however believed by most that a direct testing approach is inappropriate, because it is based solely on the detection of features dependent on the process, and not on the substance itself[3]. For this reason, research has mainly focused in recent years on the development and validation of indirect markers of autologous BT. Indirect markers of auto-transfusion include:

- Traditional markers of altered erythropoiesis (Parisotto et al. 2001, Sottas et al. 2006);
- The total mass of hemoglobin (Schmidt et al. 2005; Pottgiesser et al. 2007, Prommer et al. 2008);
- Markers of degradation during storage, such as 2,3-bisphosphoglycerate levels;
- Markers of alteration of the red cell membrane structure;
- Several gene-expression levels and transcriptional markers;
- Various proteomic and metabolomic approaches;
- Markers of neocytolosis.

The development and validation of all these indirect markers are at various stages of advancement. In the long run, validated markers may be included in the so-called Athlete's Biological Passport (Sottas et al. 2008a, b). The passport is an individual

[3] For example, unscrupulous athletes will find a way to store blood in a bag that does not release additives and plasticizers.

electronic document that contains the biomarker test history in order to use the athlete as his/her own reference, as well as additional information to increase the sensitivity and specificity of the marker (confounding effects, heterogeneous factors ...[4]). In the Athlete's Biological Passport, substantial deviations from the athlete's baseline of a biomarker may highlight either a pathological condition or doping, providing in both cases a good reason to withdraw the athlete from competing.

A promising marker of any form of transfusion is the total mass of hemoglobin (Prommer et al. 2008). In contrary to traditional markers of altered erythropoiesis, the total mass of hemoglobin is independent of changes in plasma volume shifts. This has advantages both in testing logistics – the athlete can be tested independently of his/her state of hydration – and in sensitivity to blood doping – the variance arising from plasma volume shifts is removed. An adult male endurance athlete has a hemoglobin mass in the range of 1 kg, with high between-subject variations (Schmidt and Prommer 2005). Since one blood unit of 450 ml (or ~ 200 ml of packed RBCs after removal of the plasma) contains about 60 g of

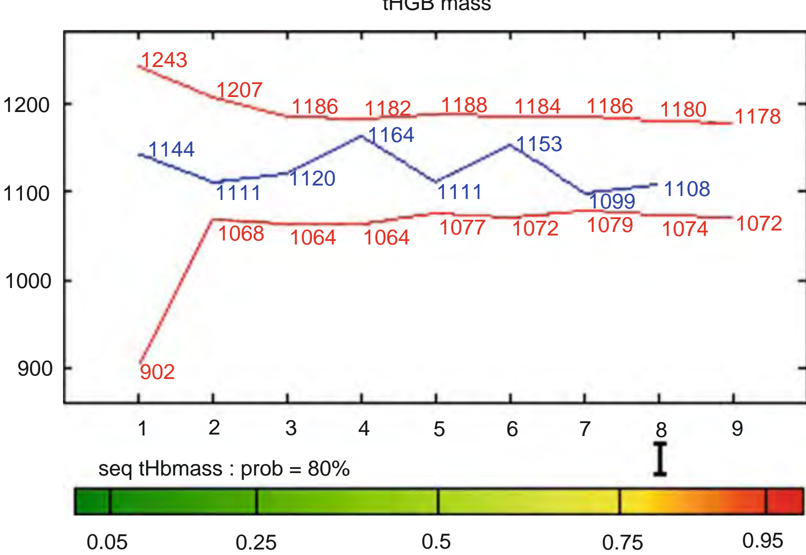

Fig. 3 Longitudinal follow-up of the total mass of hemoglobin for an elite male endurance athlete. *Center line*: results of 8 in- and out-of-competition tests. *Upper and lower lines*: individual limits for a specificity of 99%, for an analytical error of 1.6% and a within-subject variance of biological origin of 56 g^2. The athlete has a mean mass of hemoglobin of 1,126 g. The deviations from the mean have not exceeded 40 g. The sensitivity to the transfusion of one blood bag is higher than 50% for this athlete with this model

[4] An example of confounding effect for indirect markers of altered erythropoiesis is exposure to altitude, therefore the altitude time-profile of the athlete is stored in the passport. Heterogeneous factors may include physiological characteristics such as gender, age, ethnic origin and some genotyping information. Additional markers specific to pathological conditions can also be used, for example markers of injury or inflammation.

hemoglobin, a BT increases the total mass of hemoglobin by the same amount[5]. The total mass of hemoglobin is known to be a very stable marker in a one-year period for adult, healthy athletes who stay at low altitude (Prommer et al. 2008). Within-subject variations were found to be significantly lower than the change induced by the BT of one blood bag. A longitudinal follow-up of the total mass of hemoglobin of an athlete tested during one year, several times in competition, is shown in Fig. 3.

4 An Example of a Convicted Athlete

Figure 4 shows the histograms obtained by flow cytometry for a professional athlete tested four times in 20 weeks to homologous BT. At week 0, the presence of two peaks indicate a mixed RBC population, hence a possible transfusion of allogeneic

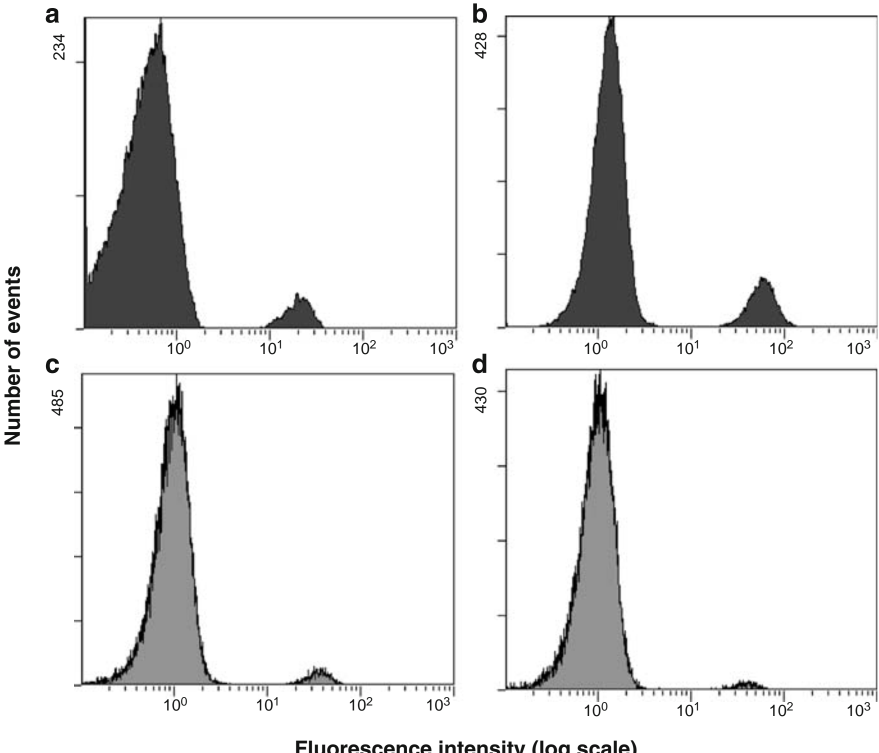

Fig. 4 Follow-up of a convicted athlete with time. RBC were stained for the complete antibody panel, but only the results obtained with the Jk^a antibody are presented here. The histogram show the fluorescence intensity at week 0 (**a**), 6 (**b**), 10 (**c**) and 20 (**d**)

[5] On condition that the blood was properly stored, with still efficient RBCs.

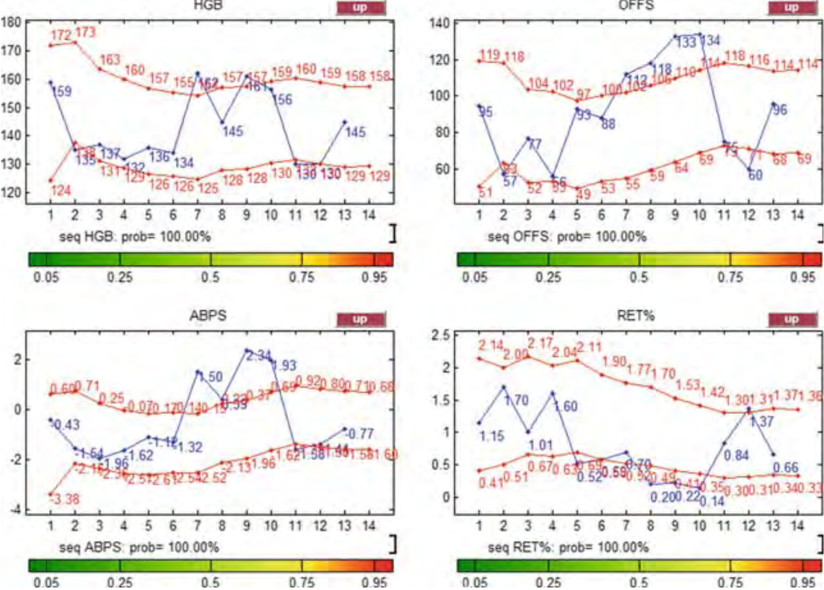

Fig. 5 Longitudinal follow-up of a male endurance athlete who tested positive to homologous BT. Three indirect markers of blood doping: hemoglobin (HGB), OFF-score (OFFS) (Gore et al. 2003), Abnormal Blood Profile Score ABPS (Sottas et al. 2006) and percentage of reticulocytes (RET%). *Center line*: 13 tests carried out during four years. *Upper and lower lines*: individual limits in function of athlete's test history and other information in his passport (Sottas et al. 2008b). All tests pre-competition at the exception of tests #5, #8 and #13 that were performed in-competition

blood. The slight increase of the minor population from week 0 to week 6 suggests that an additional allogeneic BT has been performed between week 0 and 6, maybe from the same donor. The additional results obtained at weeks 10 and 20 show a decrease of the minor population: this is typical of a natural RBC wash-out. The nonsteady behavior of the minor population in this time-profile indicates that the athlete is not a chimera. This behavior supports the evidence of homologous BT.

Figure 5 presents the longitudinal follow-up of the markers hemoglobin (HGB), the OFF score, the Abnormal Blood Profile Score (ABPS)[6] and the percentage of immature red blood cells (RET%), for the same athlete. The athlete was tested 13 times in four years. The histograms A, B, C, D presented in Fig. 4 were obtained from the blood samples number 9, 10, 11 and 13, respectively. The homologous BT test was not carried out on the other blood samples. Extreme variability in the passport confirms the act of doping. The large variability seen pre-competition in

[6]ABPS is a multiparametric marker of altered erythropoiesis using up to 12 blood parameters. ABPS has been developed from pattern classification techniques enabling the detection of EPO and blood transfusion doping (Sottas et al. 2006).

the production of new RBC suggests that this athlete also abused rhEPO during the two first years of tests (tests #1 to test #8). The highly abnormal increase of the parameters from tests #12 and #13 – tests are 11 days apart – may be the result of an autotransfusion, since this increase is not corroborated by an increase of the minor population in the graph D of Fig. 4.

5 Discussion

In order to avoid being tested positive in urine following an rhEPO cure, some top level athletes adapted their doping practices. They returned to “old” doping practices such as autologous blood transfusion. This was a fairly simple way to increase the total red blood cell mass without being worried about the actual urinary rhEPO test. Biological indirect blood makers enabled scientists to foresee this strategy (Robinson et al. 2007). The publication and validation of the homologous BT test confirmed the scientists doubts. Rapidly, to avoid both the urinary EPO test and the blood homologous BT test, some athletes combined rhEPO injections and autologous BT. The possibility of taking rhEPO during the dead season (winter for cycling, summer for skiing), to withdraw blood, store it for a long time and reinfuse it during the competition period made it clear that autologous BT would be the doping practice of choice. Unfortunately, up to now, there is no direct method to detect autologous blood transfusion doping. On the other hand, the Athlete’s Biological Passport based on a individual profiling of indirect markers of altered erythropoiesis combined with intelligent out-of-competition urine tests will make lives very difficult for athletes willing to transfuse blood.

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The Athlete's Biological Passport and Indirect Markers of Blood Doping

Pierre-Edouard Sottas, Neil Robinson, and Martial Saugy

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Abstract In the fight against doping, disciplinary sanctions have up to now been primarily based on the discovery of an exogenous substance in a biological fluid of the athlete. However, indirect markers of altered erythropoiesis can provide enough evidence to differentiate between natural variations and blood doping. Forensic techniques for the evaluation of the evidence, and more particularly Bayesian networks, allow antidoping authorities to take into account firstly the natural variations of indirect markers – through a mathematical formalism based on probabilities – and secondly the complexity due to the multiplicity of causes and confounding effects – through a distributed and flexible graphical representation. The information stored in an athlete's biological passport may be then sufficient to launch a disciplinary procedure against the athlete. The strength of the passport is that it relies on a statistical approach based on sound empirical testing on large populations and justifiable protocols. Interestingly, its introduction coincides with

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the paradigm shift that is materializing today in forensic identification science, from archaic assumptions of absolute certainty and perfection to a more defensible empirical and probabilistic foundation.

Keywords Blood doping • Indirect markers • Athlete's biological passport • Evaluation of scientific evidence • Bayesian inference

Abbreviations

3G	Third generation
ABPS	Abnormal blood profile score
BN	Bayesian network
CDF	Cumulative distribution function
CSCQ	Swiss Quality Control Center
CV	Coefficient of variation
EPO	Erythropoietin
Hct	Hematocrit
Hgb	Hemoglobin
IGF-1	Insulin growth factor-1
OOC	Out-of-competition
PIO_2	Partial pressure of inspired oxygen
PREC	Pre-competition
RET#	Reticulocyte count
RET%	Reticulocyte percentage
rHuEPO	Recombinant human erythropoietin
WHO	World Health Organization

1 Introduction

Thanks to technological progress, new and increasingly effective medical drugs are developed at an unprecedented pace to preserve human health. Sometimes, the same therapeutic substances are diverted to be used by athletes as performance enhancers. Technological advances also result in the production of substances with molecular structures that are similar if not identical to substances produced naturally by the human organism. In the fight against doping, the distinction between endogenous and exogenous substances becomes difficult and in certain cases impossible (Ashenden 2002).

Indirect doping markers provide an interesting alternative. Instead of focusing on the detection of exogenous substances in biological matrices, indirect markers

reveal key modifications in biological parameters induced by the doping product used by the athlete. Hematocrit (Hct) and hemoglobin (Hgb) are classical indirect markers of blood doping. They can reveal abnormal modifications in erythropoiesis even though the actual cause remains unknown: pathological origin, blood transfusion, recombinant erythropoietin (rHuEPO), growth factors, etc. Since the advent of automated blood analyzers, all the necessary blood parameters can be measured quantitatively, yielding a complete hemogram at the location of the competition in less than a minute after blood collection (Robinson et al. 2005).

In recent years, there has been some ambiguity in the justifications for measuring blood parameters in sports. For instance, the official objective of the introduction of the hematocrit rule in 1997[1] was to protect the health of athletes. In practice, however, it is evident that this rule was a deterrent to the abuse of rHuEPO which was undetectable by direct means at the time. Any infringement of the hematocrit rule, and that of any other rule relying on indirect biological parameters, has not been considered as a violation of the world antidoping code for many years.

The goal of this work is to demonstrate that indirect markers may nevertheless constitute sufficient evidence to prove doping. Indeed, we believe that in certain cases of suspected doping, an athlete may be subjected to disciplinary action based solely on indirect blood marker measurements. This is possible because sufficient sensitivity and specificity can be reached by combining various information stored in the so-called *athlete's biological passport*. A number of developments have contributed to such a methodological leap: the introduction of multiparametric markers specific to blood doping (Parisotto et al. 2001; Gore et al. 2003; Sottas et al. 2006), the inclusion of heterogeneous factors (Gore et al. 2003; Robinson et al. 2007) such as gender, age, ethnic origin (Sharpe et al. 2002) as well as exposure to altitude (WHO 2001), the use of athlete's own previous measurements as basal levels (Sharpe et al. 2006; Sottas et al. 2007), thus using the athlete as his or her own reference (Malcovati et al. 2003), adoption of standardized protocols for blood sample collection and extensive use of external quality control procedures that reduce pre-analytical and analytical variability (Robinson 2007), probabilistic inference techniques to evaluate scientific evidence (Taroni et al. 2006; Sottas et al. 2007, 2008a), and the inclusion of information about the prevalence of doping in the specific population under consideration (Sottas et al. 2008b).

2 The Multiparametric Approach

Some blood markers such as Hgb and Hct respond to virtually all forms of blood doping almost by definition: blood doping aims at increasing oxygen transport to the muscles. However, as soon as these classical markers were introduced in 1997, they showed a major limitation: a high sensitivity to changes in the plasma volume. For

[1] Any racing cyclist whose result is above 50% is declared ineligible and is excluded from the race.

example, an unscrupulous athlete can mask abnormally high Hgb levels by simple hemodilution.

In the 1990s, the use of rHuEPO was shown to affect other markers such as the immature red blood cells known as reticulocytes (RET), serum erythropoietin (EPO) concentrations, and soluble transferrin receptors (Audran et al. 1999). However, the discriminating power of these markers remains insufficient to detect rHuEPO with certainty. A large-scale study, conducted in anticipation of the Sydney Olympic Games, focused on indirect markers of rHuEPO doping (Parisotto et al. 2001). Some of these markers that respond differently to rHuEPO administration were combined to yield multiparametric markers with specificities and sensitivities optimized for rHuEPO detection. For example, the OFF score, a marker that combines Hgb and RET%, is specifically used to detect the interruption of rHuEPO administration.[2]

A second generation of multiparametric markers were developed in 2003 (Gore et al. 2003). In 2006, all the data contained in a single blood profile of an athlete were actually combined (Sottas et al. 2006) using specific pattern classification techniques (Duda et al. 2001). The *Abnormal Blood Profile Score* (ABPS) is a universal multiparametric marker that responds to modifications in 12 different indirect markers that may result either from rHuEPO administration or from blood transfusion. ABPS is sensitive to rHuEPO independently of the actual timing of administration (Sottas et al. 2006). However, in practice, only seven markers are usually used to determine ABPS and the results can still be largely affected by hemodilution. It is unfortunate that valuable markers such as EPO and the absolute number of reticulocytes (RET#)[3] are not taken into account in multiparametric markers.

3 Heterogeneous and Confounding Factors

Every indirect marker has an associated variability which has a biological origin – heterogeneous factors – or which results from the athlete's activities – confounding factors. This variability may be significantly reduced if one understands the nature of the factors that affect the marker under study. For example, the World Health Organization (WHO) has proposed different Hgb and Hct thresholds in the diagnosis of anemia depending on age, gender, altitude and specific physiological states such as pregnancy (WHO 2001). In a recent development (Robinson et al. 2007), the roles played by these factors as well as those of other variables more specific to

[2] As of 2008, the OFF score remains among the best indirect markers of blood doping. Even though it was originally developed to detect solely the interruption of rHuEPO doping, it is also sensitive to slow, long-acting erythropoietic agents such as insulin-growth factor 1 (IGF-1), human growth hormone (hGH) and microdosing with rHuEPO.

[3] The RET# marker offers an enormous advantage: it responds both to rHuEPO microdoses and to hemodilution, the latter actually reinforcing the RET# drop.

the antidoping (e.g. type of sport) have been formalized to improve detection of blood doping. By taking into account the athlete's individual characteristics, the assessment becomes highly individualized and all the variability stemming from heterogeneous and confounding factors can be removed.

Factors that have been studied in detail include gender, ethnic origin, type of sport and age (Sharpe et al. 2002; Gore et al. 2003), altitude and smoking (WHO 2001), and inter-instrument bias (Ashenden et al. 2004; Robinson et al. 2005). Two types of factors can be distinguished: those that are time-dependent and those that are time-independent. The first type, such as altitude and type of instrument used, includes variables that change over time and from one blood test to another. These time-varying factors can affect longitudinal follow-up. The second type, such as gender and ethnic origin, is specific for a given athlete. For example, female blood contains on average 16 gL^{-1} less hemoglobin than male blood. In both cases, the causal relationship between a factor and a marker requires a validated model. Such models are typically built by specifically analyzing the effects of a given factor (WHO 2001; Sharpe et al. 2002; Ashenden et al. 2004; Robinson et al. 2005).

The hypotheses underlying a model can – and should – always be questioned. However, because current regulations do not take into account the effects of exposure to altitude, athletes that live or train regularly at high altitudes may be unjustly penalized. It may well be impossible to develop a model that takes into account all types of variations stemming from exposure to altitude,[4] but a reductionist model is always better than none. An ideal model would lead to the removal of all the variability that originates from altitude changes, an imperfect – but realistic – model the removal of a part of that variability, no model no removal at all. Since a high specificity is fundamental in antidoping, using no model can be highly detrimental to the sensitivity. A model that is based on the distribution of altitudes during the two weeks preceding the test may offer a reasonable alternative.

4 Longitudinal Follow-Up

The development and the validation of a biological marker traditionally relies either on a statistical description of an endogenous substance measured in a population or on a longitudinal evaluation of a series of tests repeated on the same individual. A longitudinal study is of interest when the intra-individual variability of the biological marker under consideration is lower than the corresponding inter-individual variability. The relevance of a longitudinal follow-up can thus be

[4]For example, an athlete may spend 8 days training at sea level during the day and sleeping at an altitude higher than 2,000 m and then 6 days at an altitude of 800 m. Such a pattern is extremely complex and simplification is unavoidable.

assessed quantitatively by measuring the ratio of intra-individual to inter-individual variabilities (Harris 1974).[5] All of the known blood doping markers display a ratio that confirms the usefulness, if not the necessity, of a longitudinal follow-up (Sharpe et al. 2006; Sottas et al. 2008b). This fact supports the claim to define ranges of reference values that are specific for a given individual and not necessarily specific for a given population (Malcovati et al. 2003). From a statistical point of view, this amounts to the elimination of the inter-individual variability of the biological marker under study, the athlete becoming his or her own reference.

While methods of analysis of time series have been applied to different domains such as econometry or signal processing for some time (Hamilton 1994), methods aimed at analyzing individual values of blood doping markers have been developed only recently (Sharpe et al. 2006; Robinson et al. 2007; Sottas et al. 2007, 2008a, b). The structure of these methods may show slight differences, but all require a prior assessment of intra-individual variability of the marker under consideration. For example, the *third generation* (3G) model relies on the use of a unique value of non-inter-individual variations, presupposing that this value is the same for every athlete (Sharpe et al. 2006). In order to be able to use this model, one or more preliminary basal measurements are needed to characterize a given athlete's mean value. The 3G model can be used as an internal model in a Bayesian network (Sottas et al. 2008b).

The evaluation of the sensitivities of different markers becomes problematic in a longitudinal follow-up. Indeed, the sensitivity depends on the types of states represented in the sequence (which is indeed what we would like to know). If a sequence is composed of profiles corresponding to nondoped states and of other profiles corresponding to a unique doped state, such as in the case of rHuEPO microdoses, ABPS is generally superior (Robinson et al. 2007; Sottas et al. 2008b). In contrast, nonuniversal markers such as reticulocytes or the OFF score may be more sensitive if several different doped states exist in the same sequence. This may be the case when a blood pouch is removed and the blood is then reinfused, as typically carried out in clinical studies. In this latter example, depending on the time of sample collection, ABPS may be quite high, but remain constant in both cases, since the two states are abnormal. In contrast, the OFF score would switch from a low to a high value, with an improved sensitivity. Since the doping status of an athlete is not known a priori, the situation is clearly different from that in a clinical study and several different markers must be measured simultaneously. The analysis of a global sequence (see the second paradigm below) leads however to a better concordance between the different markers.

[5] This terminology may be misleading: intra-individual variations are not restricted to biological variations occurring in an individual. They include all variations with the exception of inter-individual variations, whether their origin is biological or analytical. Non-inter-individual variations may be a better term in the current context.

5 Standardized Protocols for Blood Sample Collection and Analysis

A biological passport is valid only if the conditions under which samples are collected, transported and analyzed obey strict rules (Robinson et al. 2004, 2005). Such compliance is necessary to reduce pre-analytical and analytical uncertainties. Much work has been accomplished to date to evaluate and quantify all the possible analytical errors that may occur in a laboratory. At the same time, a rigorous evaluation of the different types of analyzes of blood parameters was conducted when accreditations were developed for laboratories devoted to the fight against doping (World Anti-Doping Agency accreditation and/or ISO 17025). Modern hematological devices are robust, precise and offer a high performance with relatively small uncertainty. More importantly, the errors that may still occur can be characterized and quantified (Ashenden et al. 2004). Indeed, thanks to years of experience, the sources of potential errors in hematological data collection have been traced to poor management and lack of rigor in the pre-analytical conditions. Our recommendations, listed below, reflect this knowledge and are particularly helpful in reducing possible variations in plasma volumes due to posture (Leppänen and Gräsbeck 1998; Robinson 2007). The collection of a blood sample is optimal under the following conditions:[6]

1. The phlebotomist must be qualified, rigorous and possess good stress management skills.
2. Prior to blood collection, the athlete must answer the following questions:
   (a) Has he or she had a blood transfusion in the last 3 months? If yes, when and what volume was transfused?
   (b) Has he or she lost blood (hemorrhage) or given blood (blood donation) in the last 3 months? If yes, when and what volume was lost or given?
   (c) Has he or she spent any time at high altitudes (>1,000 m) during the last 2 weeks? If yes, when and at what altitude?
   (d) Has he or she used a hypoxia tent (>1,000 m) during the last 2 weeks? If yes, when and what PIO_2 was used?
3. The phlebotomist must choose a single type of equipment: one single supplier of materials, identical volume for all tubes, identical type of needle (gauge, model, etc.).
4. The athlete must not have been engaged in a strong physical activity in the last 2 h preceding the blood collection.

[6] Not all conditions should necessarily be met. The higher the number of conditions fulfilled, the smaller the variability of the marker. If one condition is not met, the component of variance should be adapted accordingly.

5. Eating and drinking excessively must be avoided during the last hour preceding blood drawing.
6. A resting period (sitting position) of at least 10 min must precede blood drawing.
7. The athlete must remain seated during blood drawing.
8. Venous blood must be collected exclusively from the antecubital fossa.
9. Blood drawing time (when the tourniquet is applied) must not exceed 45 s.[7]
10. The tube or tubes containing the blood samples must be homogenized and labeled appropriately.
11. The athlete and the phlebotomist must accept and sign the transcript describing the blood drawing.
12. The phlebotomist must check the site of blood collection (absence of hemorrhage) and must ascertain that the athlete is in good condition and may resume his or her activities.

When blood is collected with the objective of conducting both an antidoping test (detection of blood transfusion, human growth hormone, synthetic hemoglobin, etc.) and to establish a blood profile, two tubes (tube A and tube B) must be collected.[8] It is imperative that the two tubes contain blood from the same blood drawing, and therefore the same vein. If it is impossible to fill the second tube (tube B), the entire blood drawing procedure must be repeated. This precaution is necessary because the blood parameters of the second sample (tube B) may be slightly altered if the time spent applying the tourniquet or drawing blood is too long. If the first blood drawing fails (i.e. the needle misses the vein), two other drawings may be attempted.

The procedure described above yields blood samples of optimal quality, suitable for biological passport analyses. The experience gained by the Swiss Antidoping Laboratory during the last 12 years shows that less than 1% of the athletes must be subjected to a second blood drawing. As soon as the blood drawing procedure is completed, the samples must be sent to the accredited laboratories without delay and under the following optimal conditions (Robinson et al. 2004, Robinson 2007):

1. Rapid shipping (analytical turnaround time < 36 h).
2. The blood samples must be refrigerated ($2°C < T < 12°C$). It is important to avoid high variations in temperature.
3. A temperature recorder is recommended to indicate whether the samples were subjected to a thermal shock.
4. A secure box must be used for shipment.

[7] This is particularly important for quantitative tests, such as Hgb and Hct, used to establish the athlete's biological passport.

[8] Only one tube is necessary if blood is drawn only to establish a blood profile.

6 Internal and External Quality Control Systems

The principal recommendation that can be made to the various sports federations wishing to conduct blood analyses as a means of biological follow-up is to choose laboratories that are accredited by the World Anti-Doping Agency and/or that have ISO 17,025 accreditation. Ideally, these laboratories should use the same type of hematological analyzer. This is important to prevent data dispersion due to lack of standardization among the different technologies used to carry out the different analyzes, in particular reticulocyte analysis (Ashenden et al. 2004). The laboratories must analyze their internal quality controls and their samples in the same manner.

Three-level internal quality controls should be analyzed twice successively before any sample analysis. The only controls that are acceptable are those provided by the manufacturer of the hematological analyzer. One of the three-level controls is then systematically analyzed throughout the sequence (once every 30–50 blood samples). At the end of the analysis of the entire sequence of blood samples, the three-level controls are once again run to ensure that the device is stable and precise.[9] Fresh blood (seven consecutive analyses) should be analyzed regularly, whenever the instrument is transported. If the CV of Hct or Hgb is above 1.5% or if the CV of the percentage of reticulocytes is above 15%, it is not recommended to conduct any blood analysis.

The blood samples should be homogenized (15 min on a roller mixer) and then analyzed twice using the same analyzer. Data from the first analysis are retained only if the following conditions are met:

1. The absolute difference between the two Hgb values $\leq 0.1\ \text{g}\,\text{dl}^{-1}$
2. The absolute difference between the two Hct values $\leq 0.6\%$
3. The absolute difference between the two RET% $\leq 0.15\%$ if the first value $\leq 1.0\%$ and $\leq 0.25\%$ if the first value $> 1.0\%$.

If one ore more conditions is not met, the two analyses must be repeated.

Identical external quality controls must be provided to all the laboratories. For example, the Swiss Quality Control Center (CSCQ), acting on behalf of the International Cycling Union, provides control samples 12 times per year. Each control is analyzed seven consecutive times in order to determine the precision of measurements and the mean value (which is communicated to CSCQ to determine its exactitude). If one or more of the communicated measurements falls outside the range defined by the CSCQ, the laboratory where the data have originated is immediately notified and must take corrective measures (such as revision of equipment, calibration, etc.). External quality controls are analyzed upon reception, with the analyses taking place in the laboratory or in the field (when the analyzer has been transported to the site of competition). By doing so, it is possible to demonstrate that the analyses conducted inside and outside the laboratory are identical.

[9]This procedure makes it possible to calculate the mean and the coefficient of variation (CV) and to compare the data to the target values provided by the manufacturer of the controls.

7 A Bayesian Approach for the Evaluation of Evidence

The causal relationship between a doping activity (the cause) and the induced modification in the blood parameters (the effect) can be formalized and graphically represented by a network (Fig. 1a). The goal is to establish whether an athlete is doped by examining his or her indirect parameters. This type of problem goes against the causal direction and the only logical reasoning that may apply here is Bayesian reasoning (Gelman et al. 2004). If an athlete receives a blood transfusion (the cause), the value of Hgb increases (the effect). If a model that links cause and effect is available (for example the transfusion of one blood pouch results in a 10 g L^{-1} Hgb increase), Bayes' theorem can be used to follow the direction that is

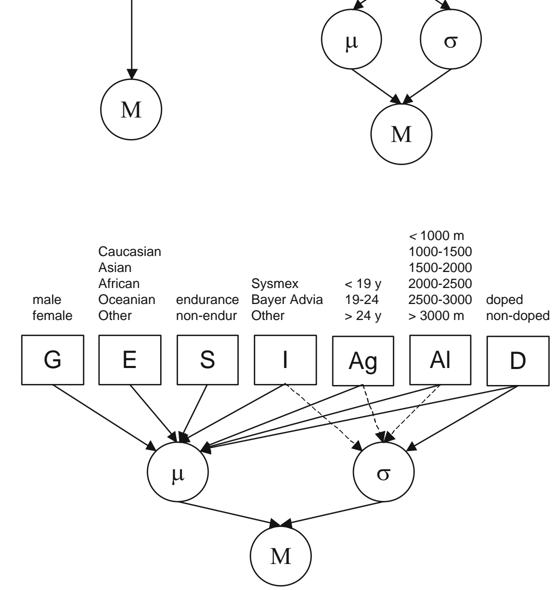


Fig. 1 Bayesian networks for the evaluation of the evidence with the indirect marker of blood doping *M*. Each rectangle presents a discrete variable, each circle a continuous variable, each arrow a causal relation. (**a**) *D* represents the doping status of the athlete. Doping (the cause) has an effect on the marker (the effect) and the goal is to know if the athlete is in category *doped* or *nondoped* in light of the result of the marker *M*. This problematic goes against the causal direction and only Bayes' theorem handles this point. (**b**) A longitudinal approach may be modeled by making explicit two variables: the expected mean and standard deviation of the sequence of *M* values. (**c**) Heterogeneous factors known to influence the result of the marker (G = gender, E = ethnic origin, S = sport, I = instrument, Ag = age, Al = altitude), their respective classes and causal relations. Gender, ethnic origin and sport are fixed factors, instrument, age and altitude are time-varying factors

opposite to that of causality and to determine whether an increase in Hgb may be the result of a transfusion or is caused by natural variations. In such a Bayesian network (Fig. 1a), D is a dichotomic variable with two states (doped; nondoped), and M a continuous variable representing the result of a measurement of some blood marker. According to Bayes' theorem, the causal relationship between M and D may be formulated as follows:

$$P(D|M) = \frac{P(M|D) \cdot P(D)}{P(M)} \tag{1}$$

where the formulations are given as probabilities. $P(D|M)$ represents the probability of being in state D as a function of the value of marker M. $P(\cdot|\cdot)$ denotes a conditional probability density function and $P(\cdot)$, a marginal distribution,[10] the same notation being used for continuous density functions and discrete probability mass functions. $P(M|D)$ represents the probability of measuring the result M knowing that the athlete is in state D. For example, if M is Hgb and $D = 0$ a nondoped state, $P(Hgb|D = 0)$ is well represented by a normal distribution with a mean of 149 $\mathrm{g L^{-1}}$ and a standard deviation of 9 $\mathrm{g L^{-1}}$ for a population of Caucasian male athletes aged 19–24 and residing at low altitudes measured with an Advia instrument (Sharpe et al. 2002).

The advantage of a Bayesian approach resides in the possibility of using the conditional probability function $P(M|D)$ (a cause-to-effect relationship is much easier to establish than the reverse effect–cause relation) to determine $P(D|M)$, the function that is actually of interest in the fight against doping. Because the marginal $P(M)$ only plays a normalizing role, the knowledge of $P(M|D)$ and $P(D)$ is sufficient to characterize the entire system (Gelman et al. 2004). Expression (1) lies at the heart of any Bayesian inference method: the task always consists in defining the $P(M|D) \cdot P(D)$ model and in carrying out the necessary calculations to determine $P(D|M)$. A decision rule can then be applied based on the posterior distribution $P(D|M)$. The prior distribution $P(D)$ represents the probability for the athlete to be in the state D before any testing. Today, all decision rules are based solely on the specificity[11] of a marker, with the underlying assumption that the athlete is nondoped (in accordance with the principle of presumed innocence). In a Bayesian approach, this presumption amounts to defining $\Pr(D = 0) = 1$; $\Pr(D = 1) = 0$.[12] The validity of this hypothesis is discussed below in terms of forensics, taking into account the notion of prevalence of doping.

[10] The terms *distribution* and *density* are often used interchangeably in mathematics.

[11] As a reminder, specificity refers to the capacity for a marker to correctly identify negative cases, i.e. nondoped athletes. Specificity equals one minus the rate of false positives.

[12] To avoid confusion, the notation $\Pr(\cdot)$ is used to represent the probability of a state or of an event, whereas $P(\cdot)$ represents the density of probability.

8 Bayesian Networks

A Bayesian network (BN) is a graphical probabilistic model (Taroni et al. 2006), in which the causal relationships between the variables of interest are represented as probabilities. In other words, the fact that one or more causes are observed does not necessarily mean that the corresponding result or results are necessarily obtained, but that only the probability of realization is affected. BNs are particularly interesting because they make it possible to formalize knowledge in a distributed and flexible fashion and can thus help model the complexity arising from the diversity of indirect causes. Every causal relationship in a BN is itself a model represented by a conditional probability density function. The complete BN can be thus viewed as a model of models.

The BN of Fig. 1a and the corresponding equation (1) may be extended to analyze all the data gathered during a longitudinal follow-up (Sottas et al. 2007, 2008a, b; Robinson et al. 2007). Indeed, by implementing a two-level hierarchical Bayesian network, it is possible to define variables that express inter-individual and non-inter-individual variations (Fig. 1b). If one assumes that a sequence of individual values of marker M follows a Gaussian distribution,[13] the variables μ and σ representing the mean and the standard deviation are sufficient to characterize the sequence. In this case, the effect of doping is not seen directly as a modification of the value of a marker, but rather indirectly as a modification of the mean and of the standard deviation of this marker. The formulation is as follows:

$$P(M, D, \mu, \sigma) = P(M|\mu, \sigma) \cdot P(\mu|D) \cdot P(\sigma|D) \cdot P(D) \tag{2}$$

where the distribution of the joint probability $P(M, D, \mu, \sigma)$ is made explicit. $P(M|\mu, \sigma)$ is given by the normal distribution $N(\mu, \sigma)$. Similarly, $P(\mu|D)$ follows a normal distribution with a variance equal to the inter-individual variance of the marker. Different distributions have been proposed for $P(\sigma|D)$. For example, the hypothesis of a universal non-inter-individual variance (as is the case in 3G) implies a degenerate distribution with a single value for the variance. In contrast, if one believes that the intra-individual variation is not necessarily the same in all athletes, a log-normal distribution may be preferred (Sottas et al. 2007). In both cases, however, the variance components must be evaluated using control reference groups of athletes. Traditional Bayesian inference techniques can then be used to estimate the marginal distributions of interest as a function of the available information (Gelman et al. 2004). Typically, a sequence $\{M_1, M_2, ..., M_n\}$ may be analyzed in a reiterative procedure with the successive addition of an M_i measurement as new evidence. By doing so, the posterior distribution $P(\mu, \sigma|\{M_1, ..., M_i\})$

[13] No evidence against normality has been found for Hgb, OFF score and ABPS if time-dependent factors are taken into account and if samples are taken at least 5 days apart (Sharpe et al. 2006, Sottas et al. 2008b). The non-linear response of ABPS to changes in hematopoeisis may however lead to a departure from normality if the athlete changes altitude.

is updated and made to include all the information present in the sequence $\{M_1, ..., M_i\}$ necessary to determine the mean and standard deviation for the athlete. When the number of measurements n is large, the $P(\mu, \sigma|\{M_1, ..., M_n\})$ distribution ends up representing a single state: the true mean and standard deviation of the individual under consideration.

The structure of a BN is sufficiently flexible to take into account the effects of heterogeneous factors. Let H represent a set of heterogeneous factors. For example, the fact that Hgb is 16 $\mathrm{g L^{-1}}$ lower in female blood (Sharpe et al. 2006) can be easily formalized by a dichotomic variable G with states [male; female] and a model $P(\mu|G)$. $P(\mu|G = 1)$ would follow a normal distribution with a mean of 149 $\mathrm{g L^{-1}}$ and $P(\mu|G = 2)$ would follow a normal distribution with a mean of 133 $\mathrm{g L^{-1}}$ (the standard deviation being 9 $\mathrm{g L^{-1}}$ in both cases). The BN represented by the graph in Fig. 1c includes this factor and its causal relationship, as well as ethnic origin E, type of sport S, age Ag, altitude Al and type of analyzer I. In this representation, the heterogeneous factors are assumed to be independent (the graph contains no arrows between any two factors) with an additive effect on the variable μ. The athlete's biological passport typically includes these heterogeneous factors in addition to the measurements obtained for the athlete in the past. The system is then fully characterized by the joint probability distribution $P(M, \mu, \sigma, D, H)$ and the causal relationships given by the network of Fig. 1c (gender $G \in$ [male; female], ethnic origin $E \in$ [Caucasian; Asian; African; Oceanian, other], $S \in$ [nonendurance; endurance], $Ag \in$ [<19; 19–24;>24] years, $Al \in$ [<1,000; 1,000–1,500; 1,500–2,000; 2,000–2,500; 2,500–3,000; >3,000] m, $I \in$ [Sysmex; Advia; other]). If the states of the heterogeneous factors are known (for example we know that the athlete has a Caucasian origin, i.e. $E = 1$), the BN is said to contain evidence nodes. If for any factor information is incomplete or nonexistent, it is possible to choose a prior distribution of the variable that represents this factor. For example, if data about the altitude are incomplete or if the athlete has changed altitudes often, such as in the case of a *live high–train low* training, a prior distribution may be set, taking into account a distribution of altitudes based on data that are actually known. Consider the following situation: a training camp in the Alps with 12 h training sessions in a valley at an altitude of 500 m but lodging in a high altitude station at night (12 h at 2,200 m). In such a case, the prior distribution will be [50%; 0%; 0%; 50%; 0%; 0%] for discrete states defined as [<1,000; 1,000–1,500; 1,500–2,000; 2,000–2,500; 2,500–3,000; >3,000] m. In comparison to a single altitude state, a wider distribution is obtained for the possible values of the marker. The BN thus helps the decision maker to make the best possible use of the information that is available in his or her decision-making process.

9 Prevalence of Doping

If one does not restrict the definition of prevalence to its medical connotation, prevalence of doping may be defined as the proportion of doped athletes in a given population of athletes at a particular moment in time. Surveys based on

questionnaires completed by athletes (Scarpino et al. 1990) and the frequency of sensationalist articles in lay sports literature may give some sense of the extent of doping, but this information cannot be used to seriously estimate its prevalence. In contrast, the BNs presented here (Fig. 1) may yield an estimation of prevalence of blood doping in a target population (Sottas et al. 2008b). Two conditions must be met, however:

– The number of tested athletes in the population must be large; ideally a blood sample should be collected and analyzed for each athlete.
– The effect of blood doping (rHuEPO, blood transfusions, hormones, etc.) on the blood markers must be known and quantifiable specifically for the population that is being studied. In other words, it should be possible to define a model defined by $P(M|D = 1)$.

With respect to the first condition, the prevalence of doping may be estimated when an important fraction of the athletes taking part in a given competition are tested before it begins. The precision of the estimation depends on the discriminating power of the marker that is being studied: for example, about a hundred measurements are needed to obtain a good estimation of the prevalence of rHuEPO doping using ABPS (Sottas et al. 2008b). The second condition can be met if one relies on (1) data obtained in clinical studies where volunteers were doped, and (2) data obtained from samples collected from athletes whose doped status is absolutely certain.[14]

The cumulative distribution function (CDF) obtained with the help of the BN for the ABPS marker is shown in Fig. 2. Both the nondoped state $D = 0$ (green) and the doped state $D = 1$ (red) are shown, using data from athletes doped with low doses of rHuEPO or transfusions (Sottas et al. 2008b). Any population of athletes with the same characteristics should display an empirical CDF positioned between the two CDFs as a function of the prevalence of blood doping. In this particular case, the characteristics are as follows: male athletes aged between 20 and 24 years (30%) or above 24 years (70%), exposed to an altitude $<$1,000 m with blood samples analyzed with a Sysmex instrument. Assuming that the target population is distributed uniformly between the population $D = 0$ and the population $D = 1$,[15] the prevalence may be estimated by Bayesian inference after introducing as evidence the set of ABPS measurements obtained from the target population. In mathematical terms, this amounts to a determination of the marginal $P(D)$ by integrating over all the M values measured in the target population.[16] Other methods can be used to evaluate the prevalence using the CDFs and the

[14]Such a situation may occur if the athlete is tested positive for rHuEPO in the urine or for homologous blood transfusion and if an analysis of indirect blood markers has been carried out at the same time.

[15]This hypothesis is wrong if for instance the prevalence of doping is higher in athletes with ABPS that is naturally lower compared to athletes with high ABPS.

[16]A mathematical formulation may be found in the book by Gelman et al. (2004) ((1.3), page 8)

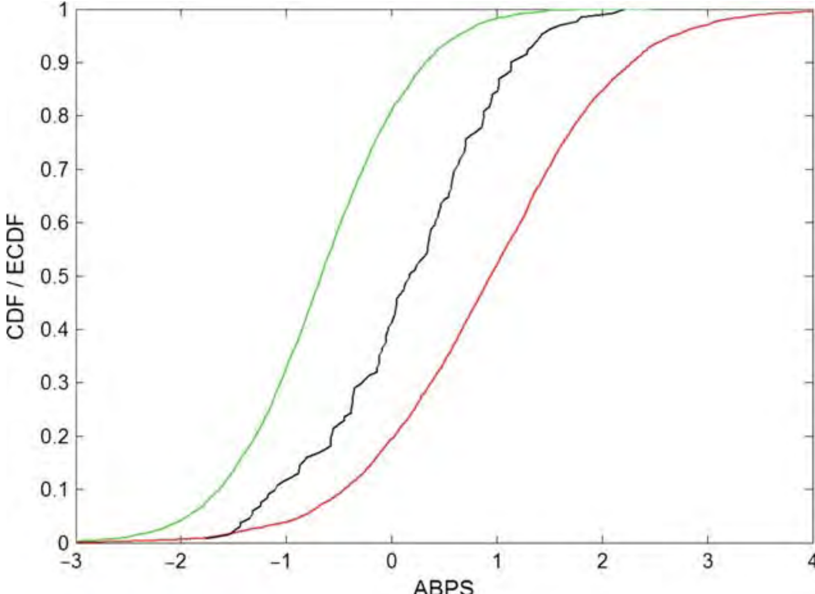


Fig. 2 Cumulative distribution function of ABPS for (1) a reference group of undoped athletes (*left*), (2) 131 athletes tested before an international competition in 2008 (*centre*), (3) athletes who doped with low doses of rHuEPO or blood transfusion (*right*). The significant departure of the center curve from the left curve suggests that a high number of athletes (prevalence of 52 [43–60]%) were doped in that population

ECDF.[17] One such method has the advantage of being visual: it merely requires one to calculate the ratio of two surface areas, the area between CDF $D = 0$ and the ECDF and the area between CDF $D = 0$ and CDF $D = 1$. Similarly, if one excludes sampling variations, the maximum difference between the ECDF and CDF $D = 0$ represents a minimal bound of the true prevalence. This latter approach is particularly interesting because knowledge about the type of doping, as described above, is no longer required. This method leads to minimal prevalence of 44% for the competition shown in Fig. 2 (75% of the athletes have a ABPS value superior to –0.1 whereas only 31% of nondoped Caucasian athletes should present such high values). Because all of these methods do not take into account the possibility of hemodilution, the corresponding estimates of prevalence are minimal estimates of the true prevalence. Fortunately, an underestimate can only lead to a decrease in sensitivity, unlike an overestimate which may cause a loss of specificity (see below).

In today's antidoping field, all rules pertaining to indirect markers are based on the notion of a threshold of specificity. From the point of view of forensic sciences,

[17] These methods are usually based on maximum likelihood techniques applied to a distance measure between two distributions, such as the Kolmogorov–Smirnov distance.

this constitutes a *false-positive fallacy* (Aitken and Taroni 2004). This sophism is in fact a particular case of the more general *prosecutor's fallacy* that results from a misunderstanding of the notion of multiplicity of tests: an increase in the number of tests on a population with zero prevalence causes an increase in the probability of obtaining a false positive. Currently, we cannot exclude the possibility that all the positive cases relative to a given rule that is based on some threshold of specificity are not in fact merely false positives. This logical fallacy is particularly troublesome in the light of the significant increase in the number of antidoping tests.

In an antidoping testing situation, the decision maker will display a different judgment if he or she knows that there are no doped athletes in a population or that a large fraction of the population is actually doped. By introducing the prevalence of doping, one does not need to consider false positives but the true probability that the athlete is actually doped.[18] Thus, knowing that there is a threshold of specificity of 99.9% does not tell the decision maker anything about the proportion of true positives among all the measured positives (every case could be a false positive). In contrast, a threshold of 99.9% that pertains to a posterior probability that an athlete is doped (the positive predictive value) signifies that, on average, 999 cases out of 1,000 are genuinely doped. Two qualitative scales linking this probability to the likelihood of doping have been proposed (Sottas et al. 2008b), based on the Jeffreys–Evett scale for the value of the evidence and on Hummel's scale as used in paternity testing (Aitken and Taroni 2004).

Regardless of the actual level of evidence required for taking disciplinary action, the results obtained from a BN should be considered together with all the other elements pertaining to the physiological and/or pathological conditions of the athlete. In forensic sciences, a suspect may well be identified thanks to a DNA test, but any judge would agree that in spite of an extremely high value of the DNA evidence, a conviction cannot rest on this type of data alone if other elements are diverging. Similarly, the BN should not be the only element that prompts a decision: an abnormal profile (punctual or longitudinal) as found by the BN gives a sound basis to initiate a thorough assessment of the case carried out by experts in the field (mainly hematologists in the case of blood doping). Only this expertise will provide a qualitative interpretation of the quantitative value of the evidence returned by the BN.

10 Sequence Analysis: Two Paradigms

In the antidoping field, two type of methods of sequence analysis can be distinguished:

- Analysis of a specific measurement as a function of previous results obtained for the athlete;
- Analysis of a complete sequence as a function of other control sequences.

[18] In statistics, the positive predictive value is among the most important parameters in decision making. It is the only statistical entity that measures the probability that a positive test actually proves the tested hypothesis, such as the fact, in our case, that an athlete is truly doped.

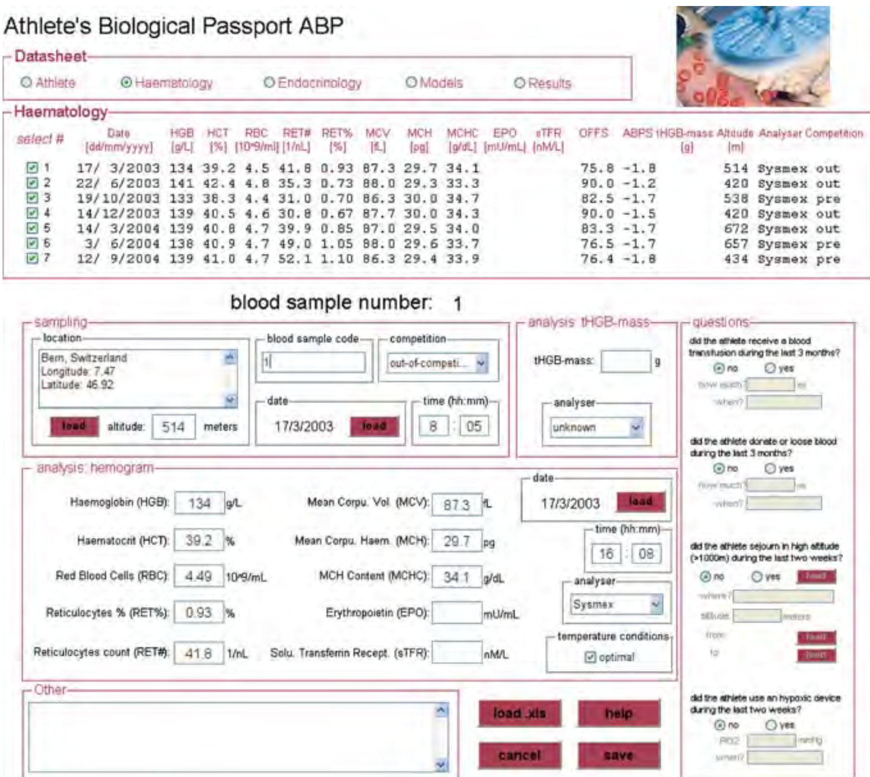
The first method merely allows the detection of an abnormal value in a sequence using one or more basal values from the athlete as reference. A typical application is a test that immediately precedes a competition to prevent the positively tested athlete from participating (a no-start rule). In the second scenario, the entire sequence is compared to sequences obtained from athletes with an ascertained doped or nondoped status, such as athletes that voluntarily participate in a clinical study. A positive result in this case may lead to temporary exclusion (2–3 months) if the objective is to redefine the biological passport by conducting new tests. The sanction may be heavier depending on the likelihood of doping.[19] The second approach should be preferred because in addition to revealing one or more abnormal values, it can also uncover abnormal variance or combination of results. This second method is thus far superior to the first one (Sottas et al. 2008b).

In practice, the sensitivity of the first method is optimal when the values that are passed refer only to the state $D = 0$. Thus, if it can be assumed that blood doping is more probable before a competition (PREC) than out-of-competition (OOC), the only results that should be used as reference values are those obtained during OOC tests, whether announced or unannounced. The sensitivity of the second method is optimal when the values are distributed among the two states $D = 0$ and $D = 1$. The best strategy is then to aim for 50% PREC tests and 50% OOC tests, 50% announced tests and 50% unannounced tests. The second method should be favored for optimal sensitivity.

11 Two Examples

A software application called *Athlete's Biological Passport* is available from the authors upon request. In the first example, the athlete is a male Caucasian endurance athlete tested seven times in 18 months (Figs. 3 and 4). Figure 3 shows a screen shot of the hematological data sheet of the software, together with a detailed view of the first blood profile. The results of the analysis of sequences of Hgb, OFF-score, ABPS and Ret% are shown for a specificity of 99%. For example, for the first test for Hgb with default parameters (between-subject variance of 57.15, within-subject variance of 28.22, a population mean of 146 gL^{-1} for the modal group, an increase of $+2$ gL^{-1} for endurance athletes), the distribution of expected values is normal with a mean equal to $146+2=148$ gL^{-1} and a variance equal to $57.15+28.22=85.37$. This can be written $P(M_1|D = 0, H = [1, 1, 2, 1, 1, 1, 1]) = \mathrm{N}(148, \sqrt{85.37})$. For a specificity of 99% and two-tailed limits (this corresponds to percentiles 0.5% and 99.5%, or to ± 2.58 the standard deviation), the limits for a first test are $148 + -2.58 * \mathrm{sqrt}(85.37) = [124; 172]$ g L^{-1}. Adding then M_1=134 gL^{-1} as new evidence, Bayesian inference leads to the posterior distribution $P(\mu|D = 0, H = [1, 1, 2, 1, 1, 1, 1], M_1 = 134) = \mathrm{N}(138.4, \sqrt{18.89})$ for the mean.

[19] A low level of evidence, such as 50%, may be chosen for the purpose of targeting the athletes who may then be tested for the presence of rHuEPO in the urine, homologous blood transfusion and/or human growth hormone. This is true in both paradigms.



select #	Date [dd/mm/yyyy]	HGB [g/L]	HCT [%]	RBC [10^9/ml]	RET# [1/nL]	RET% [%]	MCV [fL]	MCH [pg]	MCHC [g/dL]	EPO [mU/mL]	sTFR [nM/L]	OFFS	ABPS	tHGB-mass [g]	Altitude [m]	Analyser	Competition
1	17/ 3/2003	134	39.2	4.5	41.8	0.93	87.3	29.7	34.1			75.8	-1.8		514	Sysmex	out
2	22/ 6/2003	141	42.4	4.8	35.3	0.73	88.0	29.3	33.3			90.0	-1.2		420	Sysmex	out
3	19/10/2003	133	38.3	4.4	31.0	0.70	86.3	30.0	34.7			82.5	-1.7		538	Sysmex	pre
4	14/12/2003	139	40.5	4.6	30.8	0.67	87.7	30.0	34.3			90.0	-1.5		420	Sysmex	out
5	14/ 3/2004	139	40.8	4.7	39.9	0.85	87.0	29.5	34.0			83.3	-1.7		672	Sysmex	out
6	3/ 6/2004	138	40.9	4.7	49.0	1.05	88.0	29.6	33.7			76.5	-1.7		657	Sysmex	pre
7	12/ 9/2004	139	41.0	4.7	52.1	1.10	86.3	29.4	33.9			76.4	-1.8		434	Sysmex	pre

Fig. 3 Screen shot of the application *Athlete's Biological Passport* for a Caucasian male endurance athlete tested seven times pre- and out-of-competition. *Top*: the data sheet specific to hematology shows a summary of the test results. *Bottom*: detailed view of the information stored for the first blood sample

We see that a single baseline value has lead to the removal of about two-thirds of the between-subject variance (from 57.15 to 18.89). With a universal within-subject variance, the distribution of expected values for the second value M_2 is then $P(M_2) = \mathrm{N}(138.4, \sqrt{18.89 + 28.22})$, with a 99% two-tailed interval of $138.4 + -2.58 * \mathrm{sqrt}(18.89 + 28.22) = [121; 156]$ g L^{-1}. This athlete does not show any (single) suspect result for Hgb, OFF score and ABPS. The last value corresponds to the limit that should be applied to a subsequent test if the athlete is tested with a Sysmex analyzer at a low altitude. Here, the upper and lower boundaries were obtained from previous values of the sequence without taking into account the fact that the measurement may have been taken OOC or PREC. For a no-start rule, we advise to use only the four profiles measured OOC to define a basal level. In addition to the results of the analysis of one value in function of previous individual values, Fig. 4 also shows the result of the analysis of the full sequences. A value of 21% (as found for HGB) represents the 21st percentile of the distribution $P(\{M_1, ..., M_7\}|D = 0, H = [1, 1, 2, 1, 1, 1, 1])$.[20] A high value, e.g.

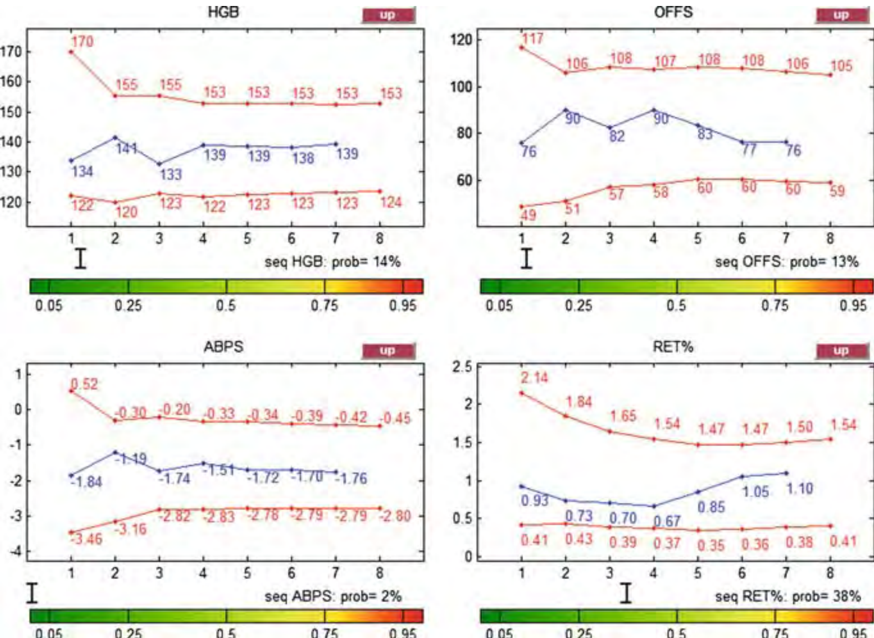


Fig. 4 *Center lines*: sequences of Hgb, OFF-score, ABPS and Ret% for the biological passport of Fig. 3. *Upper and lower lines*: two-tailed threshold limit values as found by the BN of Fig. 1c for a specificity of 99%. The last value, here 153 $\mathrm{g L^{-1}}$, corresponds to the limit that applies for a subsequent test (if the athlete sojourned at low altitude and was measured with a Sysmex). A OFF score higher than 106 (the limit for this athlete) can be reached with a value as low as 149 $\mathrm{g L^{-1}}$ for Hgb and as high as 0.5% for reticulocytes. There is much less opportunity for the athlete to dope with these values as compared to the population-based limits of 170 $\mathrm{g L^{-1}}$ and 0.2% that were applied for many years by the Union Cycliste Internationale. The lower bar shows the result of the analysis of the full sequence in order to detect not only one single abnormal value but also a succession of abnormal values or an abnormally high variance

99%, suggests a departure from what is expected for nondoped ($D = 0$) athletes with characteristics $H = [1, 1, 2, 1, 1, 1, 1]$.

The second passport represents a Caucasian endurance male athlete who trains regularly at high altitudes (Fig. 5). The analysis of the full sequences (85th and 75th percentile for Hgb and OFF score respectively, Fig. 5a) suggests that no doping product has been administered. However, if the altitude variations were ignored (Fig. 5b), the values would have increased to 99.43 and 89%, respectively. The first high value indicates that the variations in the Hgb sequence have an origin that

[20]For the ease of interpretation, the likelihood function as expressed in the formula (3) of a previous article (Sottas et al. 2008a,b) has been converted in a probability density function that is independent of the length n of the sequence. The likelihood follows a gamma distribution function with scale $1/n$ and shape $n/2$.

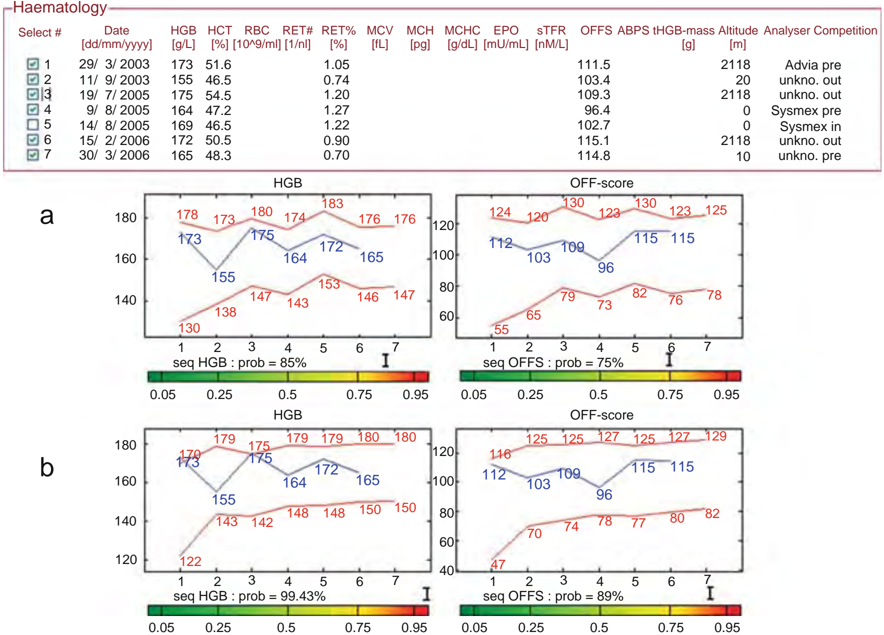


Fig. 5 Hematological data for a Caucasian male athlete who trains regularly at high altitudes, as well as (**a**) the results of the BN when the altitude has been taken into account, (**b**) the results when the altitude has not been taken into account

cannot reasonably be explained by the BN, and that an external factor may be the cause (here, the altitude).

12 Discussion

The level of evidence provided by indirect blood doping markers can be sufficiently high to launch a disciplinary procedure against an athlete. The departure from the principle of detecting an exogenous substance towards the principle of detection based on indirect markers is similar to the paradigm shift taking place in forensic sciences (Saks and Koehler 2005). The notions of uniqueness and perfection are being replaced by empirical and probabilistic arguments (Aitken and Taroni 2004). Today's forensic and legal experts have dismissed the ideas of *absolute certitude* and *exact science* and prefer to focus on the evaluation of scientific findings (Berger and Berry 1988; Evett 1996; Aitken and Taroni 2004; Saks and Koehler 2005; Taroni et al. 2006). Forensic scientists accompany their findings with appropriate explanations and thus contribute to avoiding an unjustified impression, in particular among nonexperts, of precision and conclusive force. Every element constituting scientific evidence must be incorporated into others and/or corroborated by additional evidence. A probabilistic formalism makes it possible to evaluate the evidence

associated with every scientific finding or combination of findings. DNA-based identification is an interesting example: in spite of the fact that it fails to provide absolute certainty, it is a very powerful method of identification because it relies on a statistical approach based on population genetics and a plethora of empirical tests. The methodology described in this work follows the same probabilistic framework using hematological data of populations and a large number of empirical tests.

In order to use indirect blood doping markers optimally, some aspects must still be clarified. Certain blood parameters, such as Hgb, may decrease as a result of an expansion of the plasma volume caused by a sustained effort over several days. A model describing the causal relationship between *effort* and *marker* remains yet to be validated. Consequently, it is currently impossible to use indirect blood markers as evidence of doping after several days of competition (for example during the second week of a major cycling tour). Similarly, no model of serial correlation exists today for measurements separated by less than five days: particular care is required for any additional measurement obtained within such a time interval.

The approach presented in this work is applicable to all biological markers, including markers of disease and of exposure, and markers associated with a response to the administration of a particular substance. In the antidoping field, the total hemoglobin mass (tHgb-mass) (Schmidt and Prommer 2005), insulin growth factor-1 (IGF-1) and genetic markers of altered erythropoiesis (Varlet-Marie et al. 2004) offer great promise as indirect markers of the abuse of erythropoiesis stimulating agents. In particular, tHgb-mass has the huge advantage of being independent of plasma volume variations, so that it can be applied at any time independently of the hydration status of the athlete, such as just after a competition to detect autologous transfusion. Even though these markers are obtained from different analytical protocols, there is no technical and/or scientific restriction to combining them in a more sensitive marker: a multiparametric marker combining tHgb-mass, reticulocytes, IGF-1 and EPO (all are known to be very stable for an individual person) is conceivable to detect most (if not all) forms of blood doping.

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Masking and Manipulation

Rosa Ventura and Jordi Segura

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Abstract The list of prohibited substances in sports includes a group of masking agents that are forbidden in both in- and out-of-competition doping tests. This group consists of a series of compounds that are misused in sports to

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mask the administration of other doping agents, and includes: diuretics, used to reduce the concentration in urine of other doping agents either by increasing the urine volume or by reducing the excretion of basic doping agents by increasing the urinary pH; probenecid, used to reduce the concentration in urine of acidic compounds, such as glucuronoconjugates of some doping agents; 5α-reductase inhibitors, used to reduce the formation of 5α-reduced metabolites of anabolic androgenic steroids; plasma expanders, used to maintain the plasma volume after misuse of erythropoietin or red blood cells concentrates; and epitestosterone, used to mask the detection of the administration of testosterone. Diuretics may be also misused to achieve acute weight loss before competition in sports with weight categories. In this chapter, pharmacological modes of action, intended pharmacological effects for doping purposes, main routes of biotransformation and analytical procedures used for anti-doping controls to screen and confirm these substances will be reviewed and discussed.

Keywords Doping control • Masking agents • Diuretics • Probenecid • Epitestosterone • Plasma expanders • 5α-Reductase inhibitors

1 Introduction

The prohibited list of substances in sports includes a group of masking agents that are forbidden in both in and out of competition doping tests (World Anti-Doping Agency, WADA 2007). This group consists of a series of compounds with wide differences in chemical structures and pharmacological activities. They are not considered performance-enhancing drugs; however they are misused in sports with the objective of masking the administration of other doping agents. This group includes diuretics, epitestosterone, probenecid, 5α-reductase inhibitors and plasma expanders.

The mechanisms of action responsible for the masking effects are completely different. In most of the cases, the masking action is based on reducing the concentration of the doping agents and/or metabolites in urine, either by urine dilution or by a reduction in the excretion of the drugs in urine by different mechanisms. In other occasions, the masking effect is based on interferences in the parameters used by anti-doping control laboratories to detect the administration of a doping agent.

In this chapter, pharmacological modes of action, intended pharmacological effects for doping purposes, main routes of biotransformation and analytical procedures used for anti-doping controls to screen and confirm these substances will be reviewed and discussed.

2 Diuretics

2.1 *Pharmacological Mode of Action of Representative Compounds*

Diuretics are drugs widely used in clinical practise to adjust the volume and/or composition of body fluids in a variety of clinical situations, including hypertension, heart failure, renal failure and nephrotic syndrome (Jackson 2005). Diuretics increase the renal excretion of water and electrolytes, as a consequence of their disturbing action on the ionic transport in the nephron. They act by interfering with the tubular reabsorption of sodium and may modify renal handling of other cations, anions and uric acid, leading to an increase in their renal excretion which is accompanied by water elimination.

Diuretics may be classified according to their chemical structure, their mechanism and primary site of action in the nephron, or their diuretic efficacy (Table 1, Fig. 1) (Jackson 2005; Lant 1985a, b).

Regarding the kidney area where the diuretic activity takes place, those diuretics with primary action in the proximal tubule include the carbonic anhydrase inhibitors, such as acetazolamide and diclofenamide, which are sulfonamide derivatives. Their diuretic efficacy is low, causing the excretion of less than 5% of filtered sodium, and for this reason they have a limited role as diuretics in sport.

Maximum efficacy (excretion of more than 15% of filtered sodium) is reached with those drugs which have their major activity in the ascending limb of the loop of Henle by inhibition of the sodium/potassium/chloride electroneutral system; this group includes sulfonamide derivatives such as furosemide, bumetanide and piretanide, and phenoxyacetic acids, such as etacrynic acid.

In the early portion of the distal tubule, sodium chloride reabsorption is impaired by benzothiadiazine diuretics and related compounds (chlorthalidone, indapamide) as their primary site of action; they are considered medium efficacy diuretics (excretion of 5–10% of the filtered sodium).

The major site of action of the named potassium-sparing diuretics (spironolactone, amiloride and triamterene) is the late distal tubule and the collecting duct, where they inhibit the exchange of sodium for potassium and hydrogen; these drugs have low diuretic efficacy and differ chemically and in their mechanism of action.

In addition, there is also a group of low efficacy diuretics whose mechanism of action is based on the osmotic effect (osmotic diuretics).

2.2 *Intended Pharmacological Effects*

Diuretics have been included in the prohibited list of substances in sports since 1988 (WADA 2007). It was demonstrated that they may be misused in sports for

Table 1 Pharmacological classification of diuretics, main metabolic pathways and percentage of the dose excreted unchanged in urine

Compound	Main metabolic pathways	References	Unchanged (%)	References
1. Low efficacy diuretics:				
1.1. Potassium-sparing diuretics:				
Spironolactone	– Loss of side chain in position 7 – Oxidation of the side chain – Hydroxylation – Conjugation	Abshagen et al. (1976); Karim et al. (1976a)	0	Abshagen et al. (1976); Karim et al. (1976a, b)
Amiloride	–		33–43	Williams et al. (1987)
			36	Sabanathan et al. (1987)
Triamterene	– Hydroxylation of the benzene ring – Conjugation with sulfate	Hasegawa et al. (1982) Gilfrich et al. (1983); Sorgel et al. (1985); Gundert-Remy et al. (1979)	4–12	Williams et al. (1987)
			7	Hasegawa et al. (1982)
			5	Gilfrich et al. (1983); Sorgel et al. (1985)
			4–5	(Gundert-Remy et al. 1979)
1.2. Carbonic anhydrase inhibitors				
Acetazolamide	–		98	Chapron et al. (1985)
2. Medium efficacy diuretics: Benzothiadiazides and related compounds				
Bendroflumethiazide	Unknown		30	Beermann et al. (1977)
Benzthiazide	–		10	Welling (1986)
Chlorthalidone	Unknown		34–53	Riess et al. (1977)
			25–70	Fleuren et al. (1979a)
Hydrochlorothiazide	–		70–95	Beermann et al. (1976)
			65–72	Beermann and Groschinsky-Grind (1977)
			50–60	Patel et al. (1984)
			30–64	Williams et al. (1987)
			46	Sabanathan et al. (1987)
Indapamide	– Breakdown of the amide bond	Chaffman et al. (1984)	7	Chaffman et al. (1984)

	– Conjugation with glucuronic acid and sulfate			
3. High efficacy diuretics: loop diuretics				
Bumetanide	– Hydroxylation in butyl side chain	Halladay et al. (1977)	35–44	Feit et al. (1973)
			55	Halladay et al. (1977)
	– *N*-debutylation		27–37	Brater et al. (1983b)
	– Conjugation with glucuronic acid		58–69	Holazo et al. (1984)
Etacrynic acid	– Glutathione conjugation	Dirks and Sutton (1986)	ND	
Furosemide	– Loss of the side chain linked to the amino group (artefact ?)	Andreasen et al. (1981); Boles Ponto and Schoenwald (1990); Cutler and Blair (1979); Smith et al. (1980)	48	Andreasen et al. (1981)
	– Conjugation with glucuronic acid		20–88	Boles Ponto and Schoenwald (1990)
Piretanide	– Hydroxylation of the pyrrolidine ring	Clissold and Brogden (1985); Hepner et al. (1984)	45	Brater et al. (1983a)
	– Unknown pathways		60–74	Clissold and Brogden (1985)

ND: no quantitative data

Fig. 1 Structures of some diuretics

two main reasons. First, in sports with weight categories, to achieve fast acute weight losses before competition in order to allow competition in categories of lower weight, and second, to mask the ingestion of other doping agents by reducing their concentration in urine. This effect may be accomplished either by increasing the urine volume, or by increasing the urinary pH (carbonic anhydrase inhibitors) and, thus, reducing the excretion in urine of basic doping agents. Diuretics may be also used to relieve the water retention induced by long treatments with anabolic androgenic steroids (e.g. in bodybuilders).

Weight loss produced by diuretics has been described in different studies evaluating the effect of hypohydration and electrolytic alteration during exercise (Caldwell 1987; Caldwell et al. 1984a, b; Armstrong et al. 1985). Caldwell et al. (1984a, b) described a 4.1% reduction in bodyweight after a furosemide dose of 1.5 mg kg^{-1} in weightlifters, wrestlers, judokas and boxers. Armstrong et al. (1985) induced a 2% weight loss with furosemide in long-distance runners. The increase in urine volume after diuretic intake can be an indirect marker of weight loss: increases of urine volume between 800 and 1,600 ml in 3–4 h can be achieved after therapeutic doses of furosemide, bumetanide or piretanide (Clissold and Brogden 1985; Delbeke and Debackere 1986a).

The effects of diuretics on the excretion of other drugs have been extensively studied by Delbeke and co-workers (Delbeke and Debackere 1985, 1986a, b, 1988, 1991a, b). Acetazolamide administration reduces the excretion of basic doping agents, such as the stimulants mephentermine, phentermine, ethylamphetamine and amphetamine, due to an increase in urinary pH (Delbeke and Debackere

1985, 1986a, b). Urine concentrations of these compounds can be reduced below the detection limits of the routine anti-doping tests. Moreover, longer half-lives in the body can produce an increase in the metabolism of some of the compounds (Delbeke and Debackere 1985). High-efficacy diuretics, such as furosemide and bumetanide, only act by diluting the urine without effects on drug disposition (Delbeke and Debackere 1985, 1986a, b, 1991a, b).

2.3 Biotransformation

Metabolism and urinary excretion of diuretics must be considered for doping control purposes. The main metabolic pathways and the percentages of the dose excreted unchanged in urine described for some diuretics after pharmacokinetic studies in humans using different routes of administration and different doses are listed in Table 1. Most diuretics are excreted unchanged in urine to a variable extent: from 99% of the dose for acetazolamide to 4–12% for triamterene. Nevertheless, some compounds such as spironolactone are nearly completely metabolised. Spironolactone is excreted in urine as canrenone, which has also diuretic activity, and other sulfur-containing metabolites.

2.4 Analytical Procedures, Screening and Confirmation Strategy

The family of diuretics includes compounds with wide differences in molecular structure (Fig. 1) and, in consequence, in physico-chemical properties. Partition coefficients octanol/water and p*K*a values for some diuretics are presented in Table 2 to illustrate this variability. Taking into account the chemical nature of their functional groups, diuretics may be classified into four sub-groups as indicated in Table 2.

As indicated in the previous section, most diuretics are excreted unchanged in urine to a variable extent (Table 1) and, therefore, procedures to screen for diuretics can be addressed to the detection of the parent drug for most of the compounds. Only for some compounds (e.g. spironolactone), the methods should be addressed to the detection of the metabolites.

Due to their use in therapeutics, extensive methodology has been described for the detection and quantitation of single diuretics; however the number of screening methods for the whole group is more limited. Liquid–liquid extraction procedures have been the most widely described to isolate diuretics and metabolites from the urine matrix. Two different strategies have been followed to extract all the compounds (acidic, basic and neutral compounds) from urine. One of them is the application of two separate liquid–liquid extractions at acidic and basic pH. In some studies, the extracts are mixed afterwards, but poor detection limits have been reported for some of the compounds (Cooper et al. 1989; Deventer et al.

Table 2 Classification of diuretics according to their acid-basic behaviour. Values of log *P* (Hansch and Leo 2008) and p*K*a of some diuretics

Acid/basic behaviour	Compound	Log *P*	p*K*a	References
1. Basic diuretics	Amiloride	ND	8.7	Lant (1985b)
	Triamterene	0.98	6.2	Lant (1985b)
2. Neutral diuretics	Canrenone	2.68	–	
	Spironolactone	2.78	–	
3. Weakly acidic diuretics	Acetazolamide	−0.26	7.4, 9.1	Maren (1956)
			7.2, 9.0	Moffat et al. (2004)
	Diclofenamide	1.03	7.4, 8.6	Moffat et al. (2004)
	Bendroflumethiazide	1.19	9.0	Henning et al. (1981a, b)
	Benzthiazide	1.46	6	Henning et al. (1981b)
	Chlorthalidone	ND	9.35	Fleuren et al. (1979b)
	Hydrochlorothiazide	−0.07	9.5, 11.3	Henning et al. (1981a, b)
4. Strongly acidic diuretics	Bumetanide	0.12	3.6, 7.7	Orita et al. (1976)
	Etacrynic acid	−0.81	3.5	Lant (1985a)
	Furosemide	−0.83	3.8, 7.5	Orita et al. (1976)
	Piretanide	ND	4.1	Lant (1985a)

Log *P* is defined as the logarithm of the partition coefficient octanol/water, except for: spironolactone and canrenone (octanol/phosphate buffer pH 7.4); diclofenamide, bumetanide and etacrynic acid (partition coefficient diethyl ether/phosphate buffer pH 7.4, transformed to octanol/aqueous phase according to the equation proposed by Hansch and Leo (2008)); and bendroflumethiazide and benzthiazide (octanol/phosphate–citrate buffer pH 6.5).
ND: no data

2002; Tsai et al. 1991). In others, the extracts were analysed separately (Morra et al. 2006). The second strategy is the use of a single extraction at neutral or alkaline pH with the salting-out effect. Extraction at pH 7 with diethyl ether and anhydrous sodium sulfate to promote the salting-out effect allowed the recovery of most of the compounds, although amiloride was not extracted (Park et al. 1990). However, the use of a more polar solvent (ethyl acetate) with sodium chloride for the salting-out effect and alkaline pH has proven to be useful for the detection of all the compounds (Ventura et al. 1993).

Solid-phase extraction has also been evaluated. Sep-Pak C_{18} columns with a mixture of diethyl ether and methanol as elution solvent have been employed to screen for diuretics (Amendola et al. 2003; Park et al. 1990). However, it is difficult to achieve single solid-phase extraction conditions to recover all the compounds of the group due to the differences in polarity, as was shown using different Bond-Elut columns (octadecyl, octyl, ethyl, cyclohexyl, phenyl, cyanopropyl) (Campíns et al. 1991). A column switching procedure using octadecyl cartridges directly coupled to LC detection has also been reported with the same limitations due to the differences in polarity (Campins and Falcó 1994). However, these solid-phase extraction procedures can be successfully used to detect single compounds or a group of them with similar characteristics (Barroso et al. 1997; Campins and Falcó 1993; Salado and Vera-Avila 1997).

Isolation of diuretics from urine by adsorption on a polystyrene resin (XAD-2) and elution with methanol has been used before derivatization and GC–MS analysis (Carreras et al. 1994) or before LC–MS analysis (Thieme et al. 2001). In this case,

the lack of specificity of the extraction procedure is compensated by the use of selective techniques for separation and detection such as GC/MS or LC–MS/MS. Goebel and co-workers (Goebel et al. 2004) described the use of solid phase extraction using Nexus columns with extraction recoveries greater than 80% for the diuretics under study.

Direct injection of the urine sample has also been described using LC–MS/MS analysis (Politi et al. 2007) or capillary electrophoresis coupled to MS analysis (Lu et al. 2007).

Both LC with DAD (Ventura et al. 1993) and GC–MS after methylation (Carreras et al. 1994; Hagedorn and Schulz 1992; Lisi et al. 1991, 1992; Ventura 1994) were used to screen for diuretics in urine in the past. The use of LC avoids derivatization, which is for diuretics a time-consuming step, and offers a more comprehensive screening than GC–MS due to the inability to form suitable derivatives for GC analysis of some of the compounds. In recent years, the availability of robust and reliable mass spectrometric detectors for LC has promoted the use of LC–MS systems for screening and confirmation of these compounds (Deventer et al. 2002, 2005; Garbis et al. 1998; Goebel et al. 2004; Kolmonen et al. 2007; Politi et al. 2007; Sanz-Nebot et al. 2001; Thieme et al. 2001; Thörngren et al. 2007; Ventura et al. 2008).

Capillary GC–MS was the method of choice to screen for diuretics before the introduction of LC–MS systems. The need for derivatization was the main limitation factor. Due to the inability of silylating reagents to form stable derivatives with sulfonamide groups, methylation was used to derivatize most of the diuretic compounds (Ventura and Segura 1996). Trimethylsilylation with MSTFA has been described for specific compounds such as triamterene or amiloride (Ventura 1994). Three main methylation procedures have been described for screening purposes: extractive methylation, pyrolytic methylation, and methylation with methyl iodide in acetone. Comparison of the three methylation procedures for analysing diuretics revealed that derivatization with methyl iodide in acetone is the most comprehensive methylation method and the best choice for screening purposes (Carreras et al. 1994). Extractive and pyrolytic methylation were found to be faster and more effective procedures for some compounds and can be of interest for confirmation purposes.

Methylation with methyl iodide in acetone and dry potassium carbonate allows the derivatization of amine functions, such as those of triamterene, in addition to carboxylic acids, sulfonamide groups, and alcohols. The main drawback is the long incubation time needed to derivatize diuretics with sulfonamide or amino functions (2–3 h), in contrast to the derivatization of compounds with only carboxylic acid functions, which can occur without incubation (Ventura 1994). Fast methylation reactions and improvement in detection limits were achieved using microwave-assisted derivatization in comparison with thermal incubation (Amendola et al. 2003).

Extractive methylation consists of the extraction of the organic acid as an ion pair with a quaternary ammonium salt from the alkaline aqueous phase into an aprotic organic solvent (containing the methylation reagent, methyl iodide) where

the methylation reaction occurs. Extractive methylation was directly applied to the urine sample (Beyer et al. 2005; Lisi et al. 1991, 1992). The quaternary ammonium salt was removed to avoid interferences during GC analysis and premature loss in column efficacy. An efficient clean-up procedure based on solid-phase extraction with a macroreticular acrylic copolymer of the organic extract obtained after extractive methylation was used by some authors (Lisi et al. 1992).

In pyrolytic methylation, the residue obtained after the extraction procedure is dissolved in the methylation reagent, normally a quaternary ammonium hydroxide solution, such as trimethylaniline hydroxide, tetramethylammonium hydroxide, or a mixture of both. The reaction occurs in the injector of the gas chromatograph, which is kept at high temperature. For some diuretics a high degree of methylation has been described (Hagedorn and Schulz 1992).

Regarding GC separation, in recent years the use of fast GC with short columns and high carrier gas velocities resulted in a drastic reduction of the analysis times (5 min), while resolution was maintained (Brunelli et al. 2006; Morra et al. 2006).

Electron impact (EI) ionisation is the preferred ionisation technique when using GC–MS. EI mass spectra with high diagnostic value are obtained, and three ions are monitored for each compound for screening and confirmation purposes (Ventura and Segura 1996). GC–MS with negative chemical ionisation was also used in some cases to improve sensitivity, although mass spectra with less fragmentation were obtained (Ehrhardt 1992). Using fast GC–MS, electron capture negative ionisation has recently been demonstrated to be more sensitive for most of the compounds except for bendroflumethiazide (Morra et al. 2006).

LC separation of diuretics has been usually performed with octadecylsilane columns. A substantial reduction of the analysis time was achieved using columns with particles of small size (3 or 1.7 μm particle size) (Ventura et al. 1993, 2008) compared to columns of 5 μm particles. The reduction of the particle size allows the reduction of the column length to obtain the same chromatographic efficacy and, thus, a reduction in the analysis time. Analysis of diuretics and other acidic compounds can be accomplished with a total run time of 5 min, using columns of 1.7 μm particle size (Ventura et al. 2008). Fig. 2 presents results obtained after analysis by LC–MS/MS, using a 1.7 μm column, of a positive control urine containing different diuretics and other acidic compounds.

Mobile phases containing an acidic aqueous buffer and acetonitrile as organic modifier have been commonly reported (Ventura and Segura 1996). Propylamine (De Croo et al. 1985) or an ammonium salt (Ventura et al. 1993) have been added to the acidic aqueous phase to improve the chromatographic behaviour of diuretics with amino groups (amiloride, triamterene). Gradient elution is needed to obtain adequate run times, due to the differences in polarity of the different diuretics.

LC–MS analysis of diuretics was studied early on using thermospray and particle beam interfaces (Ventura et al. 1991; Ventura 1994). Procedures with suitable sensitivity were developed, however application to routine analysis was impeded by the technical limitations of these interfaces. In recent years,

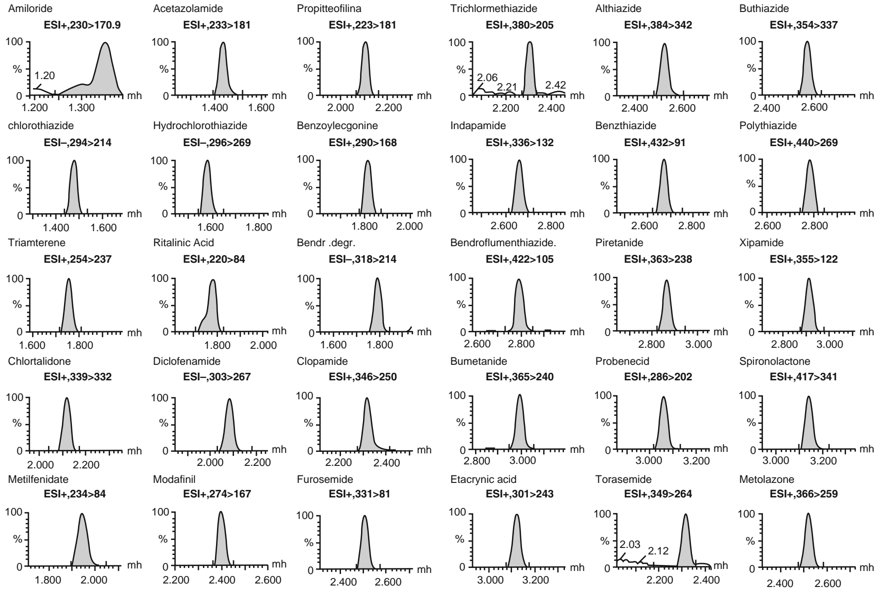

Fig. 2 Results obtained after analysis by LC–MS/MS of a urine with different diuretics, probenecid and stimulants spiked at a concentration of 200 ng ml^{-1}. 7-Propyltheophylline (1 μg ml^{-1}), was used as internal standard (Ventura et al. 2008)

comprehensive screening methods based on LC–MS/MS using an electrospray interface have been developed (Garbis et al. 1998; Sanz-Nebot et al. 2001; Thieme et al. 2001); (Deventer et al. 2002, 2005; Goebel et al. 2004; Politi et al. 2007; Thörngren et al. 2007; Ventura et al. 2008). The screening of diuretics is performed using Multiple Reaction Monitoring (MRM) and monitoring at least one transition for each compound.

In electrospray ionisation, the ionisation mode (positive or negative) is an important step in the analysis. Depending of the composition of the mobile phase, the formation of positive or negative ions is favoured. Using mobile phases containing acetonitrile and water with 1% of acetic acid or ammonium acetate (Deventer et al. 2002, 2005; Thevis et al. 2002; Thieme et al. 2001), the positive ion mode was favoured for basic and neutral compounds, however negative charged ions are mainly formed for acidic diuretics. However, using mobile phases at more acidic pH (water with formic acid), the positive ion mode was preferred for most of the compounds, including most of the acidic diuretics (Goebel et al. 2004; Ventura et al. 2008). For this reason, for any comprehensive screening both modes of ionisation must be used, and either the mass spectrometer is capable of switching between positive and negative ionisation modes in a single analysis or two analyses per sample have to be performed. Mass spectrometric behaviour of thiazide-based diuretics after electrospray ionisation and collision-induced dissociation has been extensively studied (Thevis et al. 2002).

Another strategy that has recently been demonstrated to be useful for screening of diuretics is the use of LC with an electrospray interface coupled to a time-of-flight (TOF) MS (Kolmonen et al. 2007). The identification was based on retention time, accurate mass and isotopic pattern.

An important point to take into consideration in the analysis of diuretics is the stability of some of the compounds in solution and in urine matrix (Thieme et al. 2001, Goebel et al. 2004). Hydrolysis of benzothiadiazine diuretics was observed and the rate of the reaction is mainly dependent on storage conditions and the individual compounds. The products are dependent on the structure of the benzothiadiazine and some compounds share the same hydrolysis product. For this reason, it is also recommended to monitor the hydrolysis products during the screening step in doping control.

Another instability process observed is the oxidation of the sulfide side-chain substituents (althiazide, benzthiazide, polythiazide) to sulfoxides and sulfones, caused by impurities in the organic solvent used for extraction (ethyl acetate). This effect can be diminished by elimination of the impurities by distillation of the organic solvent (Thieme et al. 2001).

3 Epitestosterone

3.1 Potential as Masking Agent

The abuse of testosterone in sport has long been screened by measuring the ratio between testosterone (T) and epitestosterone (E) in the urine (the so-called T/E ratio) (Donike et al. 1984). The approach is based on the relatively unaffected excretion of epitestosterone even in the presence of the administration of testosterone itself. In the 1980s a threshold ratio of 6 for T/E was established as the decision point to suspect testosterone abuse. The threshold was subsequently increased to 10, or lowered to 4 as it is in the 2008 WADA list of prohibited substances. From the above, it is evident that any procedure that is able to increase the amount of epitestosterone in the urine will be able to reduce the T/E ratio, thus making the result less detectable for a suspected testosterone administration. Accordingly, the sport authorities early on included the administration of epitestosterone among the prohibited practises in sport under the heading of potential manipulation. A concentration of epitestosterone higher than 200 ng ml^{-1} in urine is considered an adverse doping finding. However, the observation that some individuals may have either a higher or a lower natural T/E ratio is shifting the pressure toward individual longitudinal follow-up of natural values for a given subject instead of population-based fixed thresholds (Sottas et al. 2008, 2009). It is known that some of these differences in natural values of T/E ratio are mainly due to polymorphism for testosterone glucuronidation (*UGT2B17* gene) (Schulze et al. 2008) and that this behaviour is more prevalent is some populations (e.g. Orientals) than others (e.g. Caucasians), as shown in Fig. 3 (de la Torre et al. 1997).

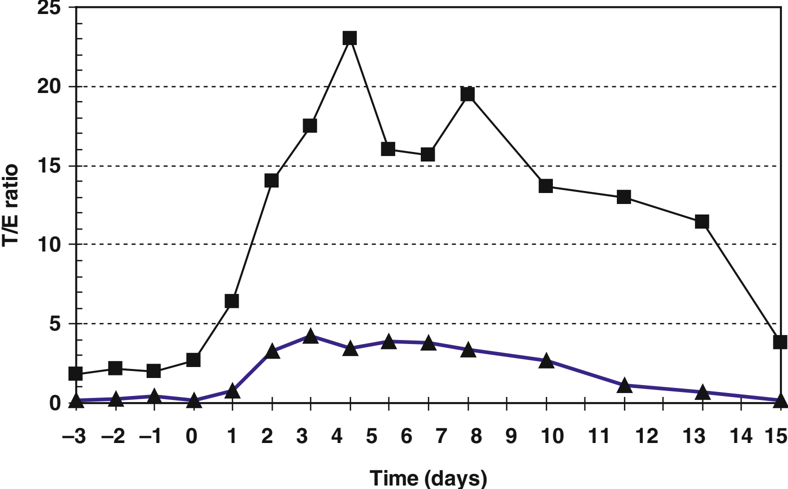

Fig. 3 Evolution of testosterone to epitestosterone (*T*/*E*) ratio after administration (day 0) of 250 mg testosterone enanthate to Oriental ($n = 6$; closed triangles) and Caucasian ($n = 6$; closed squares) subjects. Adapted from de la Torre et al. (1997)

3.2 Biological Disposition

Epitestosterone (17α-hydroxy-4-androsten-3-one) has the same structure as testosterone except for the different position (α instead of β) of the hydroxyl group in position 17. In humans, the interconversion of testosterone and epitestosterone is negligible, as shown by studies involving isotopically labelled testosterone (Wilson and Lipsett 1966). The exact biochemical pathway responsible for the biosynthesis of epitestosterone is not known, although at least half of its production is carried out in the testes (Dehennin 1993), probably from a precursor 5-androsten-3β,17α-diol. The contribution of the adrenal glands appears to be relatively modest (Kicman et al. 1999; chapter 3, this volume). The fact that androstenedione has been postulated as an epitestorone precursor (Brooks and Giuliani 1964; Catlin et al. 2002) may be relevant in cases of androstenedione administration. The enzyme responsible is probably 17α-hydroxysteroid dehydrogenase (Bellemare et al. 2005). Further data on the metabolism and biological activity of epitestosterone may be found in the review by Starka (2003).

As for testosterone, epitestosterone is excreted in urine mainly as a 17-hydroxy-glucuronide. Both the production rate and the excretion rate are lower for epitestosterone than for testosterone (Starka 2003). The effects of several factors on the excretion of *T* and *E* glucuronide in urine may be of concern in the framework of sports drug testing. Pubertal stage seems to influence rate of excretion but not the *T*/*E* ratio (Raynaud et al. 1993). Some components of nutritional supplements claimed to have some effects on disposition of testosterone, such as *Tribulus terrestris* (Saudan et al. 2008), or others such as β-hydroxy β-methyl butyrate

(Slater et al. 2000) have not shown a significant effect on *T*/*E* ratio. The main concern over nutritional supplements remains their potential contamination with different androgenic compounds (Geyer et al. 2004). Ethanol, but at very high doses, seems to have some effect on increasing *T*/*E* ratio, especially in females (Falk et al. 1988; Karila et al. 1996).

3.3 Testing for Epitestosterone Abuse in Sport

The method routinely used to analyse for the epitestosterone content of a urine is the same used for the determination of testosterone and other endogenous steroids in routine doping analysis. Briefly (Jimenez et al. 2006), samples are submitted to solid–liquid clean-up followed by extraction of unconjugated *T* and unconjugated *E* with *tert*-butyl methyl ether (thus obtaining the free fraction). The remaining aqueous phase is hydrolized with β-glucuronidase and extracted at alkaline pH with *n*-pentane or other appropriate solvent (thus obtaining the originally conjugated fraction). *T* and *E* are separated by GC/MS after derivatization (enol-trimethylsylyl (TMS) derivatives). Some modifications suggested for the GC/MS approach involve different extraction procedures or derivatizations (He et al. 2005; Stopforth et al. 2007).

Studies have shown the stability of *T* and *E* glucuronides and the *T*/*E* ratio when stored in sterile (Jimenez et al. 2006) or freeze-dried (Jimenez et al. 2004) urine at 4°C or −20°C for up to 22 months. Other studies suggest adding sodium azide for storage at higher temperatures (Saudan et al. 2006). Experiments carried out generating deliberate microbial contamination in urines showed that *E* glucuronide may be partially hydrolized in parallel to *T* (but with no change in the *T*/*E* ratio) and that *E* itself is not metabolically generated by the microorganisms.

In order to avoid potential losses due to the hydrolysis and derivatization steps for the analysis of *T* and *E* glucuronides, the use of LC is being increasingly developed. Initial attempts of limited sensitivity based on UV detection (Gonzalo-Lumbreras et al. 2003) have been superseded by LC/MS methods of higher sensitivity and specificity. In one of the approaches (Pozo et al. 2008), two different strategies were tested for sample preparation: direct injection after filtration and acidic liquid–liquid extraction. Full concordance with the GC/MS reference method result was observed. Some strategies to reduce the formation of adducts when using ion trap MS/MS instrumentation have been proposed (Cowan et al. 2008).

Isotope ratio between ^{13}C and ^{12}C may be used, as routinely done for *T*, as a way to demonstrate an exogenous origin of the drug found in the urine. In contrast to the methods proposed for *T*, which can be addressed to either *T* itself but usually to the more abundant metabolites androsterone, etiocholanolone and the androstanediols, the method for *E* is addressed to *E* itself. The goal is to observe if there is a difference between the delta value (related to ^{13}C and ^{12}C content) for *E* and the delta value of an endogenous marker (Cawley et al. 2004). This fact implies usually

an additional step of HPLC fractionation to purify and concentrate *E*. In a study involving 456 healthy males (Aguilera et al. 2002), it was verified that the approach is useful because the mean delta values observed were significantly different from those of synthetic epitestosterone. In the case of simultaneous administration of *T* and *E* (a potential situation in actual doping cases), isotope ratio measurements for *T* metabolites would confirm the administered cocktail approach.

Apart from the analysis of *E* to detect its potential abuse as masking agent, the determination is useful as *E* is one of the important analytes defining the natural steroid profile of an individual. The biological passport for athletes, recently being introduced, and the possibility of detecting other manipulations such as urine sample substitutions (Thevis et al. 2007b), reinforces the values of its determination.

4 Probenecid

Probenecid (4-(dipropylsulfamoyl)benzoic acid) is widely used as a uricosuric agent in the treatment of chromic gout and as an adjunct to therapy with penicillins and other antibiotics to enhance plasma antibacterial concentrations (Cunningham et al. 1981; Burke et al. 2005).

Probenecid is a competitive inhibitor of the active transport of organic acids in the renal tubule and other organs. Its action produces a decrease in the concentration in urine of some acidic compounds and, in consequence, an increase in their plasma concentration. This pharmacological effect is used in the treatment with penicillins and other antibiotics to increase their permanence in human body. Other drugs that have been shown to be affected by the inhibitory action in the tubular transport of probenecid are non-esteroidal anti-inflammatory drugs and diuretics (Cunningham et al. 1981; Burke et al. 2005).

Probenecid also produces an increase in the excretion of uric acid as a consequence of the inhibition of its tubular reabsorption. In humans, uric acid is widely reabsorbed from the tubule to the tubular cells by the active transport. Probenecid is a competitor with uric acid for this transport system, and inhibits the reabsorption of uric acid.

The misuse of probenecid in sports was detected in 1987 in routine anti-doping controls. It was detected in urine samples with abnormally low concentrations of endogenous steroids. In controlled studies, Geyer and co-workers (Geyer et al. 1993) demonstrated that the administration of probenecid produced a substantial reduction in the excretion of both endogenous (testosterone, epitestosterone, androsterone, etiocholanolone and others) and synthetic (norandrosterone, noretiocholanolone) androgenic steroids. As those compounds are mainly excreted in urine as glucuronic acid conjugates, the action produced by probenecid is probably due to its inhibitory effect on the active transport of organic acids. The excretion of other metabolites such as 6β-hydroxymetandionone, present mainly in free form in urine, was not affected by the concomitant administration of probenecid. A small diuretic effect of probenecid was also observed.

In man, the biotransformation of probenecid involves oxidation of alkyl chains (*N*-dealkylation, formation of primary and secondary alcohols, oxidation of the primary alcohol group to carboxylic acid function) and conjugation with glucuronic acid. From 76 to 88% of the dose is recovered in urine in 4 days after oral administration. The main metabolite is the conjugate with glucuronic acid, accounting for 38 to 55% of the dose (Melethil and Conway 1976). The metabolites resulting from oxidation of the side chains represent between 10 and 15% of the dose each. About 5–10% of the dose is excreted in urine as unchanged drug.

In anti-doping control, the detection of probenecid misuse is performed together with the screening of other acidic drugs, such as diuretics (Fig. 2). Although it is mainly excreted as a conjugate, the high doses administered allow the detection of the unchanged drug in routine screening procedures without additional enzymatic or chemical hydrolysis (Ventura et al. 1993, 2008).

5 Inhibitors of 5α-Reductase

5.1 Pharmacological Aspects and Intended Use for Doping Purposes

The study of the androgen-related endogenous steroids excreted in urine provides important information for the longitudinal follow-up of athletes, allowing the identification of subjects suspected of having received anabolic steroid doping agents. In general the follow-up is based in the calculation of ratios between different metabolites appearing in urine. Some of the more important ratios imply the quotient between the concentrations of 5α and 5β androgen-related compounds. It is obvious that a pharmacological manipulation in the conversion of any parent compound into its 5α metabolite(s) will affect the interpretation of results and is considered as a masking practise in doping control. Thus, 5α-reductase inhibitors, used in males to treat benign prostate hyperplasia, prostate cancer or alopecia, clearly belong to this group of compounds (Geyer et al. 1999; Marques et al. 1999).

Benign prostatic hyperplasia is a leading disorder of the ageing male population (Tiwari 2007), obstructing the proximal urethra and disturbing the normal urinary flow and further quality of life of the patients. Prostate disease development is associated with increased expression of isoenzymes (type-1 and type-2) of 5α-reductase and a logical treatment is to use inhibitors of this enzymatic activity. The recognition of the central role of the 5α-reduced metabolite of testosterone, dihydrotestosterone (DHT), in prostatic disease has lead to the introduction of 5α-reductase inhibitors by suppressing DHT synthesis (Andriole et al. 2004). Sometimes the 5α-reductase inhibitors are therapeutically associated with α_1-selective blockers (Bhardwa et al. 2007). The two presently leading compounds of the 5α-reductase inhibitors family are finasteride, a type-2, and dutasteride, a dual type-1 and type-2 inhibitor.

The satisfactory effect of 5α-reductase inhibitors on benign prostatic disease has increased interest in a role for the prevention and treatment of prostate cancer as well. A prostate cancer prevention trial showed that finasteride significantly decreased the 7-year risk of prostate cancer in man with prostate specific antigen (PSA) of 3 ng ml^{-1} or less, while a similar study with dutatseride is on-going (Tindall and Rittmaster 2008). 5α-Reductase inhibitors are now components of multimodal therapy for all stages of prostate cancer (Hudak et al. 2006). Some of the adverse effects described for this family of compounds are reproductive events such as decreased libido, impotence and ejaculatory dysfunction (Andriole et al. 2004) although some conflicting opinions exist (Canguven and Burnett 2008).

A major concern for sport is the use of 5α-reductase inhibitors for a clinically minor issue but relevant for the social life of some subjects due to their applicability to treat alopecia, and specifically male baldness. The pharmacological basis is also the inhibition of the 5α-reductase enzyme. Finasteride is especially popular for this application and for some years some athletes were receiving the compound without realising its potential as a masking agent in sports drug testing. However, since 2005 the WADA included 5α-reductase inhibitors (exemplified with finasteride, 17β-*N*(tert-butyl)carbamoyl)-4-aza-5-androst-1-en-3-one, and dutasteride, 17β-*N* (2,5-bis(trifluoromethyl)phenylcarbamoyl)-4-aza-5-androst-1-en-3-one) in the list of masking agents and the prohibition of their use has been made widely known.

Under the administration of finasteride, the excretion of 5α-steroids (androsterone, 5α-androstane-3α,17β-diol, allotetrahydrocortisol, 11β-hydroxyandrosterone and DHT) decreased, while the excretion of 5β-steroids increased or did not change (Geyer et al. 1999; Thevis et al. 2007a). The outcome was obvious decreases in the ratios between epimeric 5α- and 5β-steroids which lasted for more than 8 days for both finasteride (Geyer et al. 1999) and dutasteride (Simoes et al. 2005). Interestingly, no effect on the testosterone/epitestosterone (T/E) ratio, routinely used to detect testosterone abuse, has been observed (Geyer et al. 1999; Simoes et al. 2005). After discontinuation of a (chronic) use of finasteride, the recovery of the 5α/5β ratios was back to normal (Simoes et al. 2005).

Two other classical determinations in sports drug testing may be affected by 5α-reductase inhibitors reinforcing their potential use as masking agents. One of them is the detectability of the administration of DHT itself. Although the concentration of the administered drug will not change, other parameters used to suspect its administration will be altered (Donike et al. 1995; Kicman et al. 1995; Southan et al. 1992) thus complicating the interpretation of results. The second relevant determination affected by 5α-reductase inhibitors is the detection of 19-norandosterone, marker of the administration of nandrolone, norandrostenedione or norandrostenediol, all of them prohibited substances in sport. In fact, the excretion of the marker 19-norandrosterone metabolite is extensively suppressed (Marques et al. 1999; Thevis et al. 2007a). This fact may lead to false negative doping controls because urine specimens are to be declared positive only if a concentration of 2 ng ml^{-1} is exceeded, according to WADA. In addition, the suppression in the production of 5α-metabolites of other synthetic steroids may lead to additional false negative results.

5.2 Analytical Determinations

Indirect evidences of the administration of 5α-reductase inhibitors are easily achieved through the determination of the ratios between epimeric 5α- and 5β-steroids (e.g. androsterone/etiocholanolone or 5α-androstane-3α,17β-diol/5β-androstane-3α,17β-diol ratios). However, unquestionable proof of its administration is the detection of the compounds or their metabolites.

ω-Carboxy-finasteride is the main urinary metabolite of finasteride (Carlin et al. 1992) and is the primary target for the detection of finasteride administration. It is excreted unchanged in urine and its detection is normally performed by LC–MS/MS using electrospray ionisation, with a previous liquid–liquid or solid-phase extraction without additional hydrolysis steps (Simoes et al. 2005; Thevis et al. 2007a; Ventura et al. 2008).

On the other hand, due to the pharmacokinetic properties of dutasteride, neither dutasteride nor its metabolites have been detected in urine (Simoes et al. 2005). In order to find direct proofs, other biological samples are needed (faeces or plasma) (Ramakrishna et al. 2004). Consequently its detection in urine is only possible at present by evaluating the steadiness of the 5α/5β ratios in longitudinal studies.

6 Plasma Expanders

6.1 Pharmacological Action

Plasma expanders are clinically used to expand the volume of blood plasma, which is important in some emergency clinical situations such as acute hypovolaemia or shock because of blood or fluid loss (e.g. in surgery). In sport, given the fact that some endurance sport federations (cycling, cross country skiing, etc.) do not allow athletes to compete with high haematocrit and haemoglobin levels, some athletes use plasma expanders to try to mask the benefits obtained from erythropoietin administration or illegal transfusions of red cell concentrates. The goal is to temporary reduce their haematocrit or haemoglobin values, which are tested by those federations to give an athlete permission to compete. Plasma expanders may be used also to enhance performance in a situation of dehydration. They were included in the IOC List of Prohibited Substances in the year 2000 by the International Olympic Committee (IOC) and are maintained there now by the WADA. First reports involving the detection of the use of plasma expanders in sport came from Skiing World Championships in Lahti (Finland) in 2001.

The colloid osmotic pressure of plasma proteins is the main factor for the retention of intravascular volume. Colloid solutions have been developed and used over the past 80 years as expanders of the intravascular space (Mehvar 2000; Roberts and Bratton 1998). In clinical settings, large blood volume deficits are replaced by colloid solutions, which may contain albumin, gelatine, dextran or

hydroxyethyl starch (HES). They increase the intravascular volume by resorption of interstitial fluid (Adams et al. 1998). Clinically, as important as the selection of the product is the choice of the infused volume (Vercueil et al. 2005). The main risk with plasma expanders is associated with acute allergic reactions (anaphylaxis). Sometimes also minor effects such as nausea, fever or chills appear.

6.2 Molecular Characteristics

Chemically, the term dextran applies to polysaccharides produced by bacteria growing on a sucrose substrate, containing a backbone of D-glucose units linked predominantly $\alpha1\rightarrow6$. Clinically used dextrans usually have lower molecular weight than native dextrans. They are obtained by depolymerization of native dextrans or by synthesis. The main formulations are dextran 40 and dextran 70 with average molecular weight around 40 and 70 kDa, respectively. Hydroxyethyl starch refers to a starch derivative of undetermined exact composition. It is comprised of more than 90% amylopectin (non-linear $\alpha1\rightarrow4/\alpha1\rightarrow6$ polymer of D-glucose) with *O*-ether linkages to the extent that a substantial percentage of the –OH groups present in the polymer have been converted to $-OCH_2CH_2OH$ groups.

Important characteristics of synthetic colloids are their concentrations, their molecular weight and the degree of substitution. In case of HES this results in different combinations depending on the mean molecular weight of the product (130, 200, 350, 670, . . . kDa) and on the mean number of hydroxyethyl residues per glucose unit (0.4, 0.5, 0.6, 0.75, . . .). The pharmacokinetics of HES also depends on those characteristics (usually indicated as mean molecular weight/mean residues per glucose unit). Interesting comparisons of several formulations (130/0.4 vs. 200/0.5 vs. 350/0.6 vs. 670/0.75) may be found elsewhere (Jungheinrich et al. 2002; Jungheinrich and Neff 2005; Mishler et al. 1981; Sander et al. 2003; Waitzinger et al. 2003). Accumulation in the body is minimal when using lower molecular weight formulations. Thus, for doping purposes, those compositions would probably be preferred.

6.3 Simple Screening Approaches

In order to precociously detect the presence of urines potentially positive for glucose-based plasma volume expanders, several simple colorimetric methods have been proposed. A direct reaction of urine with I_2/I^- (Avois et al. 2004) with measurement of red colour at 490 nm in a microplate was useful for HES if the reading is made immediately after iodine addition. Other proposed colorimetric methodologies involve the acidic hydrolysis of HES or dextran to generate free glucose-derived units. A further simple addition of Benedict's reagent allows the

oxidation of glucose and the detection of reducing sugars by formation of coloured precipitates of Cu(I) oxide (Simoni et al. 2008; Sook et al. 2006). Oxidation of glucose may be also linked to a secondary reaction with a chromogenic agent (*o*-toluidine) with the consequent formation of a blue to violet coloration that can be visually evaluated (Mazzarino and Botre 2008). Alternatively, glucose derivatives obtained after hydrolysis may be dehydrated to [5-(hydroxymethyl)]-2-furaldehyde for dextran and its hydroxyethyl derivatives for HES, which in a subsequent reaction with anthrone (9,10-dihydro-9-oxo-anthracene) give rise to a green colour measurable at 620 nm (Gutierrez Gallego et al. 2005). All those procedures may result in a substantial number of false positives by the presence of other saccharides (e.g. saccharose) in athlete's urines.

6.4 Chromatographic and Mass Spectrometric Analysis

Chromatographic analyses of plasma volume expanders have been proposed either as screening or as confirmation procedures. Thin-layer chromatography of hydrolyzed HES or dextran gives a profile of spots characteristically different from negative urines (Guddat et al. 2004; Simoni et al. 2008). Other approaches have

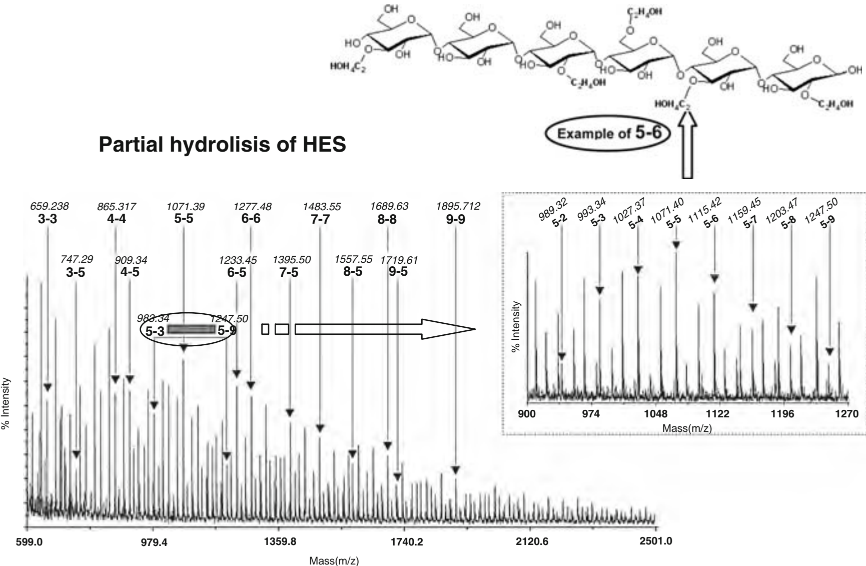

Fig. 4 Mass spectrum (matrix-assisted laser desorption ionisation (MALDI) – time-of-flight (TOF) of a partially hydrolized urine containing HES (450/0.7). Ions corresponding to oligosaccharides of 1–15 linked glucose units and containing from 0 to 13 total hydroxy ethyl residues were identified. As an example of assignment, all the ions (sodiated, positive) of those oligosaccharides bearing five hydroxyethyl residues are indicated in the insert corresponding to the limited mass range (900–1300*m*/*z*) (e.g. 5–6 corresponds to a hexasaccharide bearing five residues). Adapted from Gutiérrez Gallego and Segura (2004)

been described involving totally, partially or non-hydrolyzed (Guddat et al. 2004) urines and using different combinations of gas chromatography or liquid chromatography with mass spectrometric techniques. For total hydrolysis acidic treatment is usually performed, sometimes with partial clean-up (Deventer et al. 2006), while for partial hydrolysis, either short-time acidic, enzymatic (dextranase to generate isomaltose from dextran) (Guddat et al. 2005) or both (Son et al. 2006) types of hydrolyses are carried out. The chromatographic detection of the total or partially hydrolyzed saccharides may be carried out by derivatization to form monosaccharides-trimethylsilyl (Thevis et al. 2000a), disaccharides-acetyl (Guddat et al. 2005), monosaccharides partially methylated alditols-acetyl (Thevis et al. 2000b) derivatives; or without derivatization (Deventer et al. 2006; Guddat et al. 2004). Gas chromatography with mass spectrometric detection or liquid chromatography with tandem mass spectrometry are the most usual methods for final identification.

An approach not involving chromatography is the direct analysis of partially hydrolyzed HES or dextran by means of matrix-assisted laser desorption ionisation (MALDI) – time-of-flight (TOF) mass spectrometry (Gutiérrez Gallego and Segura 2004). A sample as small as 20 μl of urine may be hydrolyzed with trifluroacetic acid and analysed directly by MALDI-TOF. The multiple ion mass spectrum allows the identification of each one of the multiple oligosaccharides formed during the partial hydrolysis and also the number of hydroxyethylderivative substitutions for each one of those small oligosaccharides (see Fig. 4). Thus, even the degree of hydroxyethyl substitution of the HES abused may be approximately known.

7 Conclusion

There exist multiple possibilities for masking the evidences of doping in sport. In this chapter those more relevant from the pharmacological point of view have been described. They are based on different principles and include substances with wide differences in physico-chemical properties. Accordingly, different analytical strategies have been developed for their detection.

The expansion of the list of prohibited substances and methods to other challenging areas such as recombinant peptides, hormone antagonists and modulators, or gene doping, to name but a few, will increase also the possibility for masking their administration based on totally different pharmacological grounds from those described so far. Permanent surveillance of the prevalence of abused products and deep knowledge of potential pharmacological manipulation rationales will be necessary to cope with this evolving problem.

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Hormonal Growth Promoting Agents in Food Producing Animals

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Abstract In contrast to the use of hormonal doping agents in sports to enhance the performance of athletes, in the livestock industry hormonal growth promoters ("anabolics") are used to increase the production of muscle meat. This leads to international disputes about the safety of meat originating from animals treated with such anabolics.

As a consequence of the total ban in the EU of all hormonal active growth promoters ("hormones") in livestock production, in contrast to their legal use [e.g. of five such hormones (17β-estradiol, testosterone, progesterone, trenbolone and zeranol) as small solid ear implants and two hormones as feed additives for feedlot heifers (melengestrol acetate) and for swine (ractopamine) in the USA], the regulatory controls also differ sharply between the EU and the USA.

In the EU the treatment of slaughter animals is the regulatory offence that has to be controlled in inspection programs. In the USA testing for compliance of a regulatory maximum residue level in the edible product (muscle, fat, liver or kidney) is the purpose of the inspection program (if any).

The EU inspection programs focus on sample materials that are more suitable for testing for banned substances, especially if the animals are still on the farm, such as urine and feces or hair. In the case of slaughtered animals, the more favored sample

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materials are bile, blood, eyes and sometimes liver. Only in rare occasions is muscle meat sampled. This happens only in the case of import controls or in monitoring programs of meat sampled in butcher shops or supermarkets.

As a result, data on hormone concentrations in muscle meat samples from the EU market are very rare and are obtained in most cases from small programs on an ad hoc basis. EU data for natural hormones in meat are even rarer because of the absence of "legal natural levels" for these hormones in compliance testing. With the exception of samples from the application sites – in the EU the site of injection of liquid hormone preparations or the site of application of "pour on" preparations – the hormone concentrations observed in meat samples of illegally treated animals are typically in the range of a few micrograms per kilogram (ppb) down to a few tenths of a microgram per kilogram. In the EU dozens of illegal hormones are used and the number of active compounds is still expanding. Besides estrogenic, androgenic and progestagenic compounds also thyreostatic, corticosteroidal and β-adrenergic compounds are used alone or in "smart" combinations.

An overview is given of the compounds identified on the EU black market. An estimate is also given of the probability of consumption in the EU of "highly" contaminated meat from the application sites in cattle. Finally some data are presented on the concentration of estradiol in bovine meat from animals treated and not treated with hormone implants. These data are compared with the recent findings for estradiol concentrations in hen's eggs. From this comparison, the preliminary conclusion is that hen's eggs are the major source of 17α- and 17β-estradiol in the consumer's daily "normal" diet.

Keywords Beef • Pigs • Poultry • Hormones • Anabolics • Food • Public Health • Consumer • Economics • Trade dispute • Law • Hazard • Outrage

1 Introduction

Hormonal active growth promoters can be used very effectively for promotion of muscle growth in farm animals, especially bovine ruminants, veal calves and swine (Berende and Ruitenberg 1983; Lu et al. 1976; Meissonnier and Mitchell-Vigneron 1983; Organization 1982).

For the protection of consumers and for the benefit of international trade, a total ban on anabolic agents for growth promoting purposes in slaughter animals has been effective in The Netherlands since 1961, in Belgium since 1962–1969, in all Benelux Countries since 1973 and in the European Union since 1988.

A restricted controlled use of some specified anabolics is legalized, e.g., in the USA, Canada, Australia, New Zealand and in some countries in South America, Asia and Africa. Up to the present, β-agonists (Hanrahan 1987; Stephany and Ginkel 1996) are all banned for growth promoting purposes except ractopamine, e.g. in the USA and zilpaterol, e.g. in South Africa.

As a consequence of the 1988 total ban in the EU of all hormonal active growth promoters ("hormones") in livestock production, in contrast to the legal use of some hormones in the USA, the regulatory controls also differ sharply between the EU and the USA (Stephany and Ginkel 1996).

The differences in approach and attitude towards the "hormone problem" in the different parts of the world in the last decade resulted in many trade conflicts between the EU and the USA, amongst others. Most recently the World Trade Organization (WTO) gave its final verdict in a long-lasting "hormone arbitration case" between the EU and USA and Canada in favor of the two North American States.

In the EU the treatment of slaughter animals is the regulatory offence that has to be controlled in inspection programs. In the USA testing for compliance of a regulatory maximum residue level in the edible product (muscle, fat, liver or kidney) is the purpose of the inspection program (if any).

The different opposing aspects of the "hormones in meat problem" are characterized by legal issues (e.g. illegal use of undefined drugs from black markets) administration routes (solid implants versus liquid cocktails), acceptance levels (residue tolerance vs. zero tolerance), specimens applied for testing (residues in edible tissues or in excreta), location of controls (slaughterhouse or farm), legal responsibility (Ministries of Agriculture or of Public Health), conflicts of interests (producers vs. consumers lobby) and different technical (harmonized methods vs. harmonized performance criteria) or logistic (laboratory hierarchy, quality assessment) organization of residue analysis.

2 Hormones in Use as Growth Promoters

In the USA (Community Reference Laboratory (CRL) 2009a, b, US Environmental Protection Agency 2009) five hormones are authorized as the active component of solid ear implants (17β-estradiol – as such or as benzoate, testosterone – as such or as propionate, progesterone, trenbolone acetate and zeranol) (see Table 1) and two hormones as feed additives: melengestrol acetate (MGA) for feedlot heifers (Berende and Ruitenberg 1983; Lu et al. 1976; Meissonnier and Mitchell-Vigneron 1983; World Health Organization 1982) and ractopamine for swine (Marchant Forde et al. 2003).

In the EU in the past three decades, dozens of illegal hormones have been used (see Table 2) (Koole 2009). This has been demonstrated amongst others in a continuing series of European International Symposia and Conferences, such as the six EuroResidue Conferences on Residues of Veterinary Drugs in Food organized under the auspices of the Federation of European Chemical Societies (FECS) Division of Food Chemistry and the six so-called "Ghent Symposia" on Hormone and Veterinary Drug Residue Analysis.

The number of active compounds is continuously changing, as observed by the investigating EU National Reference Laboratories (NRLs). Besides estrogenic, androgenic and progestagenic compounds, thyreostatic, corticosteroidal and β-adrenergic compounds are also used alone or in "smart" combinations.

Table 1 Examples of anabolic steroid and Zeranol implants commonly authorized outside the European Union

Registered trade name	Active ingredient					To be used for
	E2	**T**	**P**	**TB**	**Z**	
Synovex S	+		+			Steer, bull
Synovex H	+	+				Heifer
Synovex Plus	+			+		Calf, bull, steer
Implix BM	+		+			Bull
Implix BF	+	+				Heifer
Implus S	+		+			Steer
Implus H	+	+				Heifer
Compudose[a]	+					Calf, steer
Revalor	+			+		Calf, bull, steer
Torrevex S	+		+			Bull, steer
Torrelor	+			+		Bull, steer
Finaplix H				+		Heifer, cow
Ralgro					+	Steer, bull, calf, sheep, lamb
Magnum					+	Cattle
Steer-oid	+		+			Steer
Heifer-oid	+	+				Heifer
Calf-oid	+		+			Calf
Component E-C	+		+			Bull
Forplix				+	+	Calf
Proferm					+	Cattle, sheep

Bold printed are single "hormone" implants. E2: 17β-estradiol or its benzoate; T: testosterone or its propionate; P: progesterone; TB: Trenbolone acetate; Z: Zeranol

[a]The carrier for this implant is silicone rubber. This means that the implant can be fully recovered at any time.

It has to be stressed that from this qualitative picture nothing can be concluded about the actual use of these compounds. Even recently a new β-agonist has been detected in a feed sample (Nielen et al. 2003), or the anti-diabetic drug glyburide combined with nortestosterone in an illegal cocktail (Blokland et al. 2004). One of the reasons for this is often the targeted sampling of the animals. The percentage of misuse ranges dramatically from (apparently) zero use in some Member States to an extensive use in others. This picture also changes from time to time. A realistic overall estimate for the use of these compounds in the European Union based on results from annual regulatory residue testing programs could be in the range of 5–15%.

In these annual regulatory residue testing programs, protein hormones like the somatropins bST, pST and oST are not included. Also no control occurs on the use of hormonal gene doping although some experiments are ongoing (Haisma et al. 2004; Reiter et al. 2007).

3 Pharmacology and Withdrawal Times

Only for the two xenobiotic implant compounds trenbolone acetate and zeranol allowed, among other, in the USA and for the three feed additives (MGA,

Table 2 Examples of hormone active components of illegal anabolic preparations as ascertained in the last three decades in the European Union and as found in application sites

Biological effect	Natural steroids	Xenobiotic steroids	Other xenobiotic anabolics
Substances with estrogenic effect	Estradiol[a]	Ethynylestradiol mestranol	Diethylstilboestrol (DES)[a] dienoestrol[a] (DE) hexoestrol[a] (HEX) zeranol
Substances with androgenic effect	Testosterone[a] nortestosterone[a] (= nandrolone) boldenone[a]	Nortestosterone[a] (= nandrolone) methyltestosterone trenbolone[a] boldenone[a] chlortestosterone[a] vinyltestosterone methylboldenone stanozolol methandienone quinbolone norethandrolone ethylestrenol fluoxymesterone norethisterone 1-testosterone[a]	
Substances with progestagenic effect	Progesterone hydroxyprogesterone	Medroxyprogesterone (MPA)[b] chlormadinone (CMA)[b] melengestrol (MGA)[b] megestrol[b] pregnenedione fluorogestone acetoxyprogesterone algestone[a]	
Substances with glucocorticoid effect	Cortisone cortisol	Dexamethasone[a] betamethasone[a] clobetasol	

(*continued*)

Table 2 (continued)

Biological effect	Natural steroids	Xenobiotic steroids	Other xenobiotic anabolics
		triamcinolone[a]	
		beclomethasone[a]	
		methylprednisolone[a]	
Substances with thyreostatic effect			Thiouracil (TU)
			methylthiouracil (MTU)
			propylthiouracil (PTU)
			tapazol (TAP)
Substances with β-adrenergic effect			Clenbuterol
			clenproperol
			clenpenterol
			clencyclohexerol
			carbuterol
			brombuterol
			salbutamol
			salmeterol
			mabuterol
			mapenterol
			terbutaline
			fenoterol
			cimaterol®
			cimbuterol
			ractopamine®
			zilpaterol®

[a]These substances are often administered as esters (propionates, benzoates, laurates, cypionates, etc.) which breakdown after administration into "free" anabolics and their corresponding carboxylic acids
[b]Always administered as acetate ester

ractopamine and zilpaterol) allowed in some parts of the world as growth promoters are some pharmacological data available. Here, however, the situation is much more complex than in human sports doping (World Anti-Doping Agency 2009) or horse (Wagner et al. 2008) and greyhound racing because one not only has to deal with adult male and female subjects but also with young (e.g. veal calves) and with a variety of different species (e.g. ruminants such as bovines, swine, poultry and fish).

The main pharmacological effect of a compound as growth promoter is its purpose. This purpose is legally or illegally to obtain more edible muscle meat for less money. Edible here means more kilograms of meat per kilogram feed, preferably more tender (e.g. beef) and/or leaner (e.g. pork) than without the use of growth promoter(s). So here economics plays a leading role!

The illegal use of thyreostatics as growth promoters in bovines is even more profitable because here the producer is selling additional water as meat to the consumer. For the legal growth promoters no fixed withdrawal times are laid down. For the implant preparations a withdrawal time is judged to be unnecessary because the implantation sites (the ears) are cut off during slaughter. For the feed additives withdrawal times of a few weeks are sometimes recommended to reach the Maximum Residue Level.

For the EU illegal growth promoters, nothing is known regarding withdrawal times. However, because it all is economics no legal or illegal user will use unnecessarily long withdrawal times.

4 Control Strategies

It all began within the European Union in 1980–1981 by the finding of residual diethylstilbestrol (DES) in baby food in various Member States. The finding of this human carcinogen caused a lot of political, trade and consumer problems. Triggered by a continuous series of residue scandals with illegal "anabolic hormones" in cattle and swine, the European Commission (EC) decided to adopt, instead of methods, analytical criteria for screening and confirmation and/or reference purposes (EC Scientific Veterinary Committee 1984). The Commission developed integral analytical strategies for residue analysis of veterinary drugs and contaminants in food of animal origin (Heitzman 1992, 1994; Stephany and Ginkel 1996). In 1986 Council Directive 86/469/EEC concerning the examination of animals and fresh meat for the presence of residues was implemented to enforce uniform application throughout the EU of measures to ensure that meat was free from undesirable residues (Council of the European Communities 1986). For that purpose within the past 15 years a system was developed based on four fundamental corner stones and controlled by a series of hierarchically linked European Union and National Reference Laboratories. Routine or field laboratories involved in the annual residue monitoring programs are coordinated and controlled per EU member state (at present 27) by at least one National Reference Laboratory (NRL) designated by the National Government. The NRLs are supported, advised and

controlled by four Community Reference Laboratories (CRLs) designated in 1991 (Stephany et al. 1996). The overall objective of the CRLs (at present much more than the original four) (European Commission 2009a–c) and NRLs (Community Reference Laboratory (CRL) 2009) is to improve the quality, accuracy and comparability of the results at official control laboratories.

Most recently Council Directive 2008/97/EC has been published amending Council Directive 96/22/EC concerning the prohibition on the use in stock farming of certain substances having a hormonal or thyreostatic action and of β-agonists, to exclude companion animals from the prohibition (Council of the European Communities 2008).

In the USA at present there are no approved laboratories operating for the testing of residues of hormonal growth promoters (AOAC International 2009). Worldwide there are only a very few laboratories acting as NRLs and being accredited by WADA at the same time.

The EU inspection programs focus on sample materials that are more suitable to test for banned substances, especially if the animals are still on the farm, such as urine and feces or on hair. In the case of slaughtered animals are the more favored sample materials are bile, blood, eyes and sometimes liver or kidney. Only in rare occasions is muscle meat sampled. This happens only in the case of import controls or in monitoring programs of meat sampled in butcher shops or supermarkets (see Table 3). As a result data on hormone concentrations in muscle meat samples from the EU market are very rare and are obtained in most cases from small programs on an ad hoc basis. Some representative results from recent decades in the European Union are summarized in Table 4. EU and USA data for natural

Table 3 Sample material to be examined for "hormones" or their metabolites to monitor the illegal EU use of hormonal anabolic agents

Sample material to be analyzed	At the farm	At slaughter	In the shop or at import
Urine	+	+/–	–
Feces	+	+	–
Hair	+	+	–
Bile	–	+	–
Thyroid[a]	–	+	–
Eyes (retina)[b]	–	+	–
Application site	–	+/–	0/–
Under skin	–	+/–	–
Muscle	–	+/0	+/0
Liver	–	+	+
Kidney	–	+	+
Fat	–	+/0	+/0
Feed	+	–	–
Stomach content	–	+/–	–
"Cocktails" & implants	+	–	–
Sludge & manure	+/0	–	–

+ Suitable; **0** less suitable or low availability; – not available. In countries where the use of hormones is legalized, only muscle, liver, kidney and/or fat is sampled at the slaughterhouse
[a]Only suitable for anti-thyroids
[b]Only suitable for β-agonists

Table 4 Results of residue analysis for illegal "hormones" in beef, liver and edible fat sampled at random from butcher and retail shops and supermarkets in the European Union by Inspection Services or Consumers' Organizations

Year of sampling	Country	Sample	Number of samples	Violating findings %	"Hormones" found
1989–1993	NL	Minced beef	249	3.2	MP & NT
1991–1992	BE	Minced beef	51	9.8	CT & NT
1993–1999	NL	Fat	430	0.5	CT & MP
1994	EU	Entrecote	1183	1.6	17 Androgens & two gestagenes
1994	EU	Liver	936	10	Clenbuterol

For all steroids found in these programs the limit of detection was 1–2 ppb. Concentrations of detected "hormones" were in general well below 5 ppb. MP = medroxyprogesterone, NT = nortestosterone (= nandrolone), CT = chlortestosterone. For the 1994 EU program see Remy and Debeuckelaere (1994). No analysis for estradiol was included, that was performed in the USA study (Stephany and André 2000)

hormones in meat are even rarer because of the absence of "legal natural levels" for these hormones preventing regulatory testing for compliance.

With the exception of meat from the application sites – in the EU the site of intra-muscular injection of liquid hormone preparations *or* the site of application of trans-dermal "pour on" preparations – the hormone content observed in 250 g of meat of illegally treated animals is typically less than 250 ng per steak. At the application site the content per steak can easily rise into the range of milligrams of the applied preparation with injectable "hormones", but will be less than 3,000 ng with pour-on preparations.

Around 1990 the probability of consumption of bovine meat that is "highly" contaminated by injectable "hormones" was estimated for EU Member States using such illegal "hormones" as less than 1 in 70,000 in the case of a 250 g steak. As an example, it has been estimated that only 5% of consumers in the Netherlands have a probability of eating an injection site once in their life. The other 95% will not encounter an injection site at all in their lifetime.

The only more extensive trans-EU consumers' at random study in 1994 with 1,183 samples of bovine steak ("entrecote") revealed the presence of trace levels of 17 androgenic and two gestagenic "hormones" in 1.6% of the samples (Remy and Debeuckelaere 1994). A trans-USA consumers' at random study in 1999 with 103 samples of bovine meat revealed the presence of trace levels of MGA in about 75% of the samples. About 20% of the samples also showed traces of trenbolone. However, no zeranol or other "hormones" were found (Stephany and André 1999, 2000).

As EU-EFSA (European Commission 2009) still considers 17β-estradiol as the most risky growth-promoting residue in meat production, because according to their opinion it is a complete carcinogen, we have compared the 17β-estradiol content of beef and hen's egg. From at random studies in 1998 and 1999 with meat

Table 5 17β-Estradiol intake via EU hen's eggs and US cattle meat

	17β-Estradiol in Dutch hen's eggs microgram *per kg (ppb)*	17β-Estradiol in High Quality "clean" Harmone Free Cattle US beef microgram per kg *(ppb)*	17β-Estradiol in Medium/Low Quality domestic US beef *microgram per kg (ppb)*
0% = minimum	0.06	<0.01	<0.01
50% = median	0.13	<0.01	0.02
90%	0.22	0.02	0.08
95%	0.25	0.02	0.10
99%	0.35	0.03	0.25
100% = maximum	0.38	0.04	0.27
Average	0.14	0.004	0.030
Standard deviation	0.07	0.009	0.046
Number of samples	25	97	102[a]
Amount (ng)	Per 50 g egg	Per 250 g steak	Per 250 g beef
Average	6.8	1	7.5
Median	6.5	<2.5	5
90%	11	5	20
95%	13	5	25

[a]103 samples minus 1 outlier of a few ppb
EU CRL data: Stephany et al. (2004)

imported from the USA to the EU or obtained from the US domestic market (Stephany and André 1999, 2000), it is estimated that the median dietary intake of 17β-estradiol via a 250 g steak of "Hormone-Free Cattle" is less than 2.5 ng and via 250 g "beef" of "Hormone-Treated Cattle" is 5 ng. This has to be compared with the recently found median dietary intake of 17β-estradiol of 6.5 ng via a 50 g hen's egg (Table 5) (Rossum et al. 2000; Stephany et al. 2004). From this comparison the preliminary conclusion is that hen's eggs are a major source of 17β- (and 17α-) estradiol in the daily "normal" western diet.

Often the academic question has been asked whether athletes consuming edible tissue from hormone-treated meat can be found positive in sports doping controls (Debruyckere et al. 1992, 1993a, b). This question was discussed by scientists in 1993 during the second International Symposium on Drugs in Sports at Lillehammer (Debruyckere et al. 1993a, b). During an IAAF arbitration doping case in 1997 about the "Ben Johnson" steroid, an athlete claimed that he was positive because he had eaten meat in the European Union. As an expert witness, the author demonstrated that the consumption probability of an effective doping dose by eating an injection site under a regular sports doping control scheme was less than 1 in 6 million. That was judged as negligible by the IAAF arbitration court. So the answer is no, with the exception of clenbuterol where poisoning of consumers has been reported in, e.g., Spain (beef) and China (pork). It is more likely that athletes eating boar tissue (non-castrated male swine) can text positive for nortestosterone (see chapter 6, this volume) naturally present in boars.

This leads to another forensic interfering interpretation factor in the misuse of anabolic steroids in the meat industry. Some species (ruminants) contain naturally small amounts of α- and β-nortestosterone and/or boldenone. Others like swine

contain naturally only the β-isomer, extra to the natural occurrence of both isomers of testosterone and estradiol in all species (Le Bizec et al. 2008).

The European Commission insists on special "hormone-free" farming programs in any country exporting food of animal origin to the European Union (Serratosa et al. 2006). However, public media are cooking up "hormone findings in food" and so a series of scandals arise. Here not the regular scientific definition of a risk applies but the alternative definition:

$$\text{Risk} = \text{Hazard} + \text{Outrage}.$$

This will continue as modern analytical chemical residue techniques become more and more sophisticated down to the nanogram per kilogram (ppt) level and by various kinds of "omics" technologies (Kootstra et al. 2007; Stolker and Brinkman 2005; Stolker et al. 2007; Le Bizec et al. 2008).

An overview on the social impact of hormones in food is given by Blokland et al. (2004) and one on the legal aspects of hormones in food by Sabbe and Beken (2002) and Sabbe and Cruysberghs (2004). Here the crucial question about a numerical value for the error probability that represents the legal phrasing "beyond any reasonable doubt" still has to be answered. For the validation statistics of confirmatory analytical chemical methods the answer to that question is crucial (Stoev and Michailova 2004).

5 Conclusions

It is concluded that

- The "hormones in meat problem" should be evaluated in relation to all facts about the actual total dietary intake of "hormones", e.g. from meat (products), poultry, milk, dairy products, eggs and fish (products) taking into account also the effects of various methods of food production and/or of "household" cooking.
- The EU has a large and *in general* adequate network of residue laboratories for regulatory inspection for illegal "xenobiotic growth-promoting hormones". However, such residue analyses is performed with non-edible sample material, often at the farm level.
- At present in the EU there is only a very restricted number of adequate laboratories operational for regulatory residue analyses of "hormones" in edible products, especially muscle meat. In the USA, e.g., at present there is *no* adequate laboratory operational for residue analyses of "hormones" in food animals or any of their products.
- In the EU (or anywhere else) there exists *no* adequate regulatory database with relevant and updated reliable "state-of-the-art" information about the levels of natural and xenobiotic "hormones" in common food commodities of animal origin.

Note: Any opinions, conclusions and recommendations expressed in this paper are those of the author and do not necessarily reflect the official views of the International Bodies referred to.

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Some Aspects of Doping and Medication Control in Equine Sports

Ed Houghton and Steve Maynard

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Abstract This chapter reviews drug and medication control in equestrian sports and addresses the rules of racing, the technological advances that have been made in drug detection and the importance of metabolism studies in the development of effective drug surveillance programmes. Typical approaches to screening and confirmatory analysis are discussed, as are the quality processes that underpin these procedures. The chapter also addresses four specific topics relevant to equestrian sports: substances controlled by threshold values, the approach adopted recently by European racing authorities to control some therapeutic substances, anabolic steroids in the horse and LC–MS analysis in drug testing in animal sports and metabolism studies. The purpose of discussing these specific topics is to emphasise the importance of research and development and collaboration to further global harmonisation and the development and support of international rules.

Keywords Horseracing • Drug metabolism • Doping analysis

1 Introduction

This chapter focuses on doping control in horseracing, but the principles apply to many animal sports, particularly greyhound racing. Many people will be familiar with the idea that some human athletes may seek performance improvements through the misuse of drugs. Horseracing is also concerned with drug induced performance enhancement but there are other drug related threats to the integrity of the sport. Performance impairment through the use of sedating drugs is a major concern as it is more predictable to slow a horse through the use of "stoppers" than it is to make it reliably gallop faster. Inside knowledge of animals that have been sedated in a race confers advantage when betting on the outcome of that race. This form of doping can therefore be perpetrated by individuals who are not directly connected with the horse (or greyhound) affected.

The welfare of competing horses is also more strictly regulated in many countries throughout the horseracing world than their human athlete equivalents. Horseracing jurisdictions that are signatories to the International Agreement on Breeding and Racing, published by the International Federation of Horseracing Authorities (IFHA), insist that horses must compete on their natural abilities, with no influence at all from drugs. This extends to medication that might allow an animal to compete on race day. For example, the use of painkillers to mask pain is not permitted. In many countries animal welfare is protected by legislation, so animal sports must comply with the local law. Absolute protection of animal welfare is critical to continuing social acceptance of animal sports in many countries in the twenty-first century.

The range of prohibited substances in horseracing and greyhound racing is therefore even wider than the equivalent list of prohibited substances in human sports. The list of prohibited substances in horseracing is defined differently from

Table 1 Prohibited substances according to Article 6 of the International Agreement on Breeding, Racing and Wagering published by the International Federation of Horseracing Authorities (http://www.horseracingintfed.com)

- Substances capable at any time of acting on one or more of the following mammalian body systems:
  The nervous system
  The cardiovascular system
  The respiratory system
  The digestive system
  The urinary system
  The reproductive system
  The musculoskeletal system
  The blood system
  The immune system except for licenced vaccines
  The endocrine system
- Endocrine secretions and their synthetic counterparts
- Masking agents

the list for human sports published by the World Anti-Doping Agency (WADA). In horseracing, prohibited substances are defined on the basis of their physiological action (see Table 1).

The finding of a prohibited substance means a finding of the substance itself or a metabolite of the substance or an isomer of the substance or an isomer of a metabolite. The finding of any scientific indicator of administration or exposure to a prohibited substance is also equivalent to the finding of the substance.

The history of doping control in horseracing can be traced back several centuries and includes a case in England in the nineteenth century where an individual was actually executed for doping a racehorse with arsenic. The twentieth century saw the widespread application of analytical chemistry to detect and therefore to control doping in horseracing. These developments were designed to counteract manipulation of gambling and to protect the integrity of racing and therefore the confidence of the "honest" punter.

Horseracing is generally understood to be a competition where the horses are controlled by a jockey while racing along a course with or without some form of obstacle that the horses are required to jump. Variations on "flat" (without hurdles) and "jump" (with hurdles) racing can be found around the world. Horseracing in Great Britain is regulated by the British Horseracing Authority (http://www.britishhorseracing.com). Similar regulatory organisations exist in most countries where horseracing is a popular sport.

However, equine sport is not confined to horseracing.

Harness racing is popular in many parts of the world. In this form of equine competition the horses race with a particular gait (stride pattern and frequency) while pulling a two-wheeled carriage, controlled by a driver rather than a jockey.

Equestrian sports such as show jumping, dressage and eventing are popular around the world with many tiers of competitors from local to national and international levels. Indeed, some of these sports are part of the Olympic Games

and come under the international jurisdiction of the Federation Equestre Internationale (http://www.fei.org).

The principles of doping control in all of these horse sports are broadly similar, although there may be greater focus on particular areas of welfare and/or integrity that arise from the specific form of competition. For example, Article 142 of the General Regulations of the FEI deals specifically with "Abuse of Horses" and includes, among other things, prohibition of abnormally sensitising or desensitising any part of a horse. The finding of capsaicin in samples taken from several horses during the 2008 Olympic Games may be considered under this category, as the substance is recognised as having hypersensitising properties as well as being used for pain relief.

2 Technological Advances in Drug Detection

The pace of change in both the nature of the drug threat and the technology developed to detect it has accelerated dramatically in the last 30 years. The pharmaceutical industry has been developing a greater range of drugs with increasing potency. The technological challenge facing laboratories involved in doping control in horseracing has therefore been to develop screening tests that can detect increasing numbers of substances (and their metabolites) as well as deliver much lower limits of detection.

It is interesting to note how the technologies that have been available have been applied to deliver these twin aims.

In the 1980s high performance liquid chromatography (HPLC) with ultraviolet (UV) spectrometric detection typically gave microgram per millilitre sensitivity and allowed the development of multi-analyte screening tests for tens of compounds in a single chromatographic analysis.

Bench-top instruments to deliver gas chromatographic separation linked to single quadrupole electron impact mass spectrometry (EI GC–MS) became widely available in the 1990s. This technology was rapidly adopted and methods were widely developed because of the technique's ability to deliver nanogram per millilitre sensitivity, coupled with large databases of mass spectral libraries. Careful selection of appropriate chemistry during sample preparation could give a single GC–MS analysis that covered hundreds of different substances.

The development and adoption of robust LC–MS systems since 2000 currently means that laboratories can routinely detect concentrations in the picogram per millilitre range if required.

This means that in just over 20 years a typical horseracing doping control laboratory has been able to increase testing sensitivity a million-fold! Mixing and matching these automated instrument techniques with careful selection of sample preparation chemistries can give a screening protocol capable of indicating the presence of thousands of different substances. Typically this means preparing several different extracts from the urine or blood sample and then subjecting these extracts to different instrumental analyses.

Previous limitations on sensitivity often meant that relatively large volumes of sample (typically tens of millilitres) were required to carry out a particular screening test. The sample itself is generally consumed by the testing process so, in turn, this limited the number of tests that could be applied to a single urine or blood sample. Interestingly, the technical capability to identify substances at much lower sensitivities has also been helpful in reducing the volume of sample required. This allows more separate aliquots to be extracted from a single urine or blood sample. This in turn leads to broader coverage by applying more tests.

These gains in sensitivity are critical to control the misuse of potent substances and the modern drug delivery systems that are intended to deliver lower doses of drug to a targeted area of the body. However this increased sensitivity is not intended to detect decreasing amounts of the more old-fashioned drugs that are still given in relatively large doses. In recent years the regulatory authorities have had to develop new policies to address the control of therapeutic substances that can legitimately be used for welfare during training of horses but are prohibited during competition.

3 Knowledge of Drug Metabolism and its Significance to Drug Surveillance Programmes

An effective doping control programme needs a considerable amount of knowledge about the metabolism of prohibited substances. In particular, what goes into the body isn't necessarily what comes out. Urine was the sample matrix most commonly used for doping control in the latter part of the twentieth century. There are a number of reasons for this including:

- Relative ease of collection (non-invasive)
- Metabolites excreted in urine provide additional evidence of administration
- Relatively higher concentrations of parent drug and/or metabolite than in blood
- Distribution and binding to specific sites while active in the body meant that the elimination phase was the most realistic target for doping control

It is therefore important to know exactly which substances are likely to be excreted in urine following a drug administration so that:

(a) The screening test is designed to detect the appropriate molecule(s) – not necessarily the parent substance
(b) Knowledge of the elimination time course will inform the time "window" when detection is required

Without this information it becomes much more difficult to design an effective and efficient screening protocol.

Drugs developed for legitimate human medicine undergo lengthy studies to determine their metabolic profile. Many of these studies are published or at least can be made available for legitimate scientific application, such as the internationally regulated doping control in sport. There is simply not as much published

literature on drug metabolism in the horse as there is for human athletes. In addition, many substances that might be misused in horseracing are not even licenced for legitimate veterinary therapeutic use. So, although the generic metabolic mechanisms for Phase I and Phase II metabolism are well understood, there is a constant need for administration studies. Horseracing in the UK has traditionally invested significant sums annually on research into drug metabolism, the effect of novel delivery systems and new analytical technology and methods. The results of this research are shared with the international doping control community through publication and meetings, such as those organised by the Association of Official Racing Chemists (AORC), e.g. the International Conference of Racing Analysts and Veterinarians.

Administration studies also provide samples for comparison with regulatory test samples in those instances where metabolites are not commercially available for use as reference standards.

4 Quality Systems and Drug Control in Sport

Doping control in horseracing must conform to similar standards to those applied in human sports. First and foremost the testing process must be trusted by the stakeholders and viewed as fair. Although there are elements that must be regarded as sensitive in order to maintain a credible deterrent for any participant that is tempted to cheat, the system must be as transparent as possible to the majority of participants and stakeholders who compete fairly as a result of their dedication and determination to get the best from the natural talent and ability of the thoroughbred racehorse and the jockey.

A robust quality system should be applied to all aspects of the testing process from sample collection through to reporting results. Consideration must always be given to the fact that a regulatory process needs to withstand close scrutiny in disciplinary hearings or legal challenges.

A clear sample record should be maintained from the point of collection through to disposal of the sample once the process is complete. The collection procedure is generally outside the laboratory's control but the relevant regulatory authority should have clear procedures to record and document sample collection. In common with most regulatory testing in sport, horseracing samples are anonymised for the laboratory process by using coded identification. It is clearly important that the regulatory authority procedure links the original identity of the horse with its code.

The testing laboratory can assist this part of the process by providing sample collection kits for individual horses that contain uniquely identified bar codes and tamper evident bags to ensure the collection process and subsequent transport of the sample to the laboratory is carried out in a manner that clearly records and identifies the sample and protects it from a breach of integrity.

Once the sample arrives at the laboratory it is important to create individual sample records that track progress through the testing protocol. Use of electronic records and a Laboratory Information Management System (LIMS) can simplify this part of the process and make it easier to store and retrieve individual records.

Accreditation and audit by an independent organisation provides assurance to both the testing laboratory and to the authorities that use its services. In horseracing the most widely accepted standards are the International Standard ISO 17025, General Requirements for the Competence of Testing and Calibration Laboratories, and the Accreditation Requirements and Operating Criteria for Horseracing Laboratories issued by the International Laboratory Accreditation Cooperation (ILAC-G7) in 1996 (recently revised and reissued in June 2009). Compliance with these standards is monitored by a regular internal audit programme and by an annual inspection by a national accreditation service, such as the United Kingdom Accreditation Service (UKAS).

The laboratory Quality System will ensure that all elements of delivering the testing service are controlled and monitored appropriately. This will include:

- Use of documented, controlled testing methods
- Regular use of Quality Control samples to ensure the methods are working to the relevant specification
- Properly trained staff
- Maintenance of equipment to ensure good working order
- Clear records for all aspects of the testing service
- Appropriate selection of reagents and reference standards

A modern laboratory Quality System can do much more than simply examine the procedures in current use. Doping control laboratories are always under pressure to develop new solutions to changing circumstances. The Quality System should encourage the people in the laboratory to look for more effective and efficient ways to improve the testing service. This will require even closer working relationships with the regulatory authorities. Lessons can be learnt from other sectors that have faced similar issues. For example, the automobile manufacturing industry radically changed processes in the latter part of the twentieth century, developing a number of tools to help instil a culture of continuous improvement. The challenge for laboratories in the twenty-first century is to maintain the scientific expertise required to meet the challenges of doping control while creating a culture of change across all people involved.

5 Typical Laboratory Testing Process

Historically urine has been considered the most appropriate sample matrix for doping control. Many prohibited substances are eliminated via the urine, often with higher concentrations than would be found in blood. Also, the presence of metabolites in urine may provide additional information and evidence to support the conclusion that a horse has been administered a prohibited substance. Technological advances mean that some of the historical disadvantages of blood as a testing matrix are now being addressed and the reliance on urine is likely to become balanced with other sample matrices in the future.

Most doping control laboratories operate a two stage testing process.

5.1 Screening

The first stage (screening) is designed to indicate suspicious samples and to eliminate negative samples as quickly as possible. Typically, less than 5% of samples tested will be suspicious so it is important to concentrate on these as quickly as possible.

The screening process must be capable of detecting the very wide range of substances considered to be prohibited in horseracing. If a prohibited substance is present in urine it is likely to be at a concentration much lower than many normal urinary components. It is therefore important to isolate the prohibited substances, usually by adopting several different sample preparation steps. Solid phase extraction (SPE) is a versatile technique that offers many different chemical binding properties according to the functional groups attached to the silica support. The laboratory can design a multi-stage SPE process that produces several different isolates for each sample. It is relatively simple to design SPE processes to use polar/non-polar or acid/base interactions to separate groups of substances. More complex, multiple interactions can be used to refine the extraction process to improve efficiency of recovery of individual substances or groups of substances and to obtain "cleaner" background signals by removing more natural components. SPE can be automated to confer organisational efficiencies in terms of staff and time.

Once the chosen number of extractions has been carried out and a number of isolates obtained, the next stage in the screening process is to carry out a number of instrumental analyses designed to indicate the presence of prohibited substances. Again, a number of different analyses will be performed to detect the broadest possible range of substances. Currently the most common screening instrumental techniques employed will utilise a combination of GC–MS, LC–MS and immunoassay. The exact deployment of these techniques will depend on the degree of capital investment by the laboratory, the local scientific skills and national approaches to particular groups of substances.

However, each of these techniques may be deployed in a variety of ways to detect the maximum range of prohibited substances. For example, GC–MS screening can be used in full scan mode with all the integrated peaks subjected to a library search using large databases of known prohibited substances. The technique can also be used in a targeted mode with ion chromatogram "windows" designed to indicate the presence of individual substances. A combination of these two approaches using multiple analytical test runs can detect a wide variety of substances, provided they are amenable to GC analysis and the relevant ionisation technique(s) employed. This often involves a chemical derivatisation step to make the drug molecule more amenable to GC separation.

LC–MS technology is becoming more widely adopted as a screening technique, typically offering improved sensitivity, faster run times per sample than GC–MS analysis and eliminating the requirement for derivatisation. A current disadvantage of LC–MS screening is a relative lack of database libraries that can be used for

automated comparison with a test sample result. It is likely that these will be developed in future making library searching more efficient.

Enzyme linked immunosorbant assay (ELISA) has been widely used for many drug screening applications, including doping control in horseracing. It is easily automated and can provide high throughput batch analysis. However, the technique lacks the specificity provided by mass spectrometry. This and other immunological techniques have been particularly developed recently to address the larger molecules that can be used as doping agents, such as erythropoietin (EPO) and growth hormone (GH).

In any case, the deployment of a multi-test screening process utilising a combination of these (or other) techniques will generate a large amount of data that requires interpretation by expert scientists to decide whether the screening results from a sample are all negative or whether there is something suspicious that requires further investigation.

If all the screening test results are negative then the sample will be reported to the relevant regulatory authority.

However, if any of the screening tests indicate a suspicious finding then the sample is subjected to the second stage of the testing process. This is referred to as the confirmatory analysis.

5.2 Confirmatory Analysis

A new portion of the original sample is prepared to look specifically for the suspicious substance. Typically the confirmatory test will involve a specific extraction stage designed to isolate the substance from the sample matrix. The resulting isolate is often analysed using chromatographic separation linked to mass spectrometric detection. The results are compared to the concurrent analysis of a reference standard of the suspected substance. The combination of chromatography and mass spectrometry confers a high degree of specificity, providing identification based on the molecular structure of the substance.

If the data from the test substance is comparable to that obtained from the reference standard then the presence of a prohibited substance is reported to the relevant regulatory authority.

6 The Approaches Adopted by the Racing Industry to Address Endogenous Substances and Substances of Dietary Origin

In the early 1980s there was considerable pressure upon racing authorities to introduce methods to distinguish "normal" levels of certain endogenous substances and substances of dietary origin from the levels arising through their administration. This pressure was primarily due to three factors:

- Certain laboratories demonstrated their ability to detect salicylic acid in all post-race urine samples;
- The detection of nandrolone and its metabolites in extracts of normal colt urine; and
- The significant number of theobromine positives arising from contaminated manufactured feed. In 1980 Ireland had 29 positives for theobromine and in 1981 in the UK there were 32 positives. There was a further spate of positives within the UK in 1985.

Salicylic acid is a natural ingredient in feed, nandrolone, at the time, was considered an endogenous steroid and theobromine is a contaminant in manufactured feed. One question facing the authorities was whether these three problems could be addressed by a single approach. With an endogenous substance there will always be a natural level in the body and the only possible approach to this issue is a threshold value. Regarding contaminated feed it has been stated that this problem should be addressed by feed manufacturers rather than racing authorities or laboratories. The salicylic acid problem could have been addressed by restricting feed containing salicylic acid but this was not viable for the racing industry and, again, the only possible solution to salicylic acid was a threshold.

These issues had received considerable attention by European racing analysts, administrators and veterinarians over a 3-year period leading up to the sixth International Conference of Racing Analysts and Veterinarians in Hong Kong in 1985. At a meeting in September 1985, the European analysts representing France, Ireland, Spain, United Kingdom and West Germany made certain recommendations to address the problem of endogenous substances and substances of dietary origin. The meeting concluded that the authorities should adopt specified thresholds for prohibited substances of dietary and endogenous origin, and values were agreed by the five participating countries for salicyclic acid, nandrolone and arsenic. These recommendations were summarised in the proceedings of the sixth International Conference of Racing Analysts and Veterinarians, Hong Kong (Smith 1987).

The threshold concept was also discussed in depth at the sixth International Conference of Racing Analysts and Veterinarians in Hong Kong in 1985 where Moss et al. (1987) presented a paper on “normal” concentrations of salicyclic acid in post-race horse urine samples from the UK. By this time, precedent had already been established for the threshold value concept. Agriculture Canada had written regulations for thoroughbred racing for salicylic acid such that a sample was suspicious at 500 $\mu g\ mL^{-1}$ and that 750 $\mu g\ mL^{-1}$ was the official level for a positive. Also the IOC Medical Commission had set a threshold for caffeine at 15 $\mu g\ mL^{-1}$. There was also a proposed IOC threshold for testosterone (testosterone/epitestosterone ratio = 6) in urine. Thus, the precedent had been set to address these types of problem by a single approach, that of thresholds, and this was in accordance with the conclusions of the European analysts. In discussion at the sixth International Conference of Racing Analysts and Veterinarians, Hong Kong, Manfred Donike also stated “I can only stress the idea of accepting thresholds for more and more compounds”. This was also an opinion expressed in the report of

standardising policies in Europe presented at the conference business session (Smith 1987).

The concept of thresholds eventually received international approval and two approaches were adopted by the industry to determine these values depending upon the type of problem:

- The determination of the concentration of the analyte of interest in urine and/or plasma in a statistically significant population of post-race samples (Method 1); or
- Performing feeding experiments and selecting concentrations from the top end of the permissible range (Method 2).

6.1 Salicylic Acid Threshold

Salicylic acid was the first substance to be addressed by the threshold approach and thresholds were determined in both Canada and the UK. The approach adopted in Canada (Beaumier et al. 1987; Beaumier 1995) was to perform feeding experiments to determine a threshold (Method 2). The Canadians also determined the levels of salicylic acid in a number of post-race urine samples.

In the UK Method 1 was used, i.e. the determination of the concentration of salicylic acid in post-race urine samples (Moss et al. 1987). The distribution of salicylic acid in post-race urine samples in the UK at that time is shown in Fig. 1.

Performing a log transformation on the values provided a Gaussian distribution. From the mean of this Gaussian distribution and the standard deviation, it is possible to determine the probability of a normal value exceeding a given value using the equation as shown in Fig. 2.

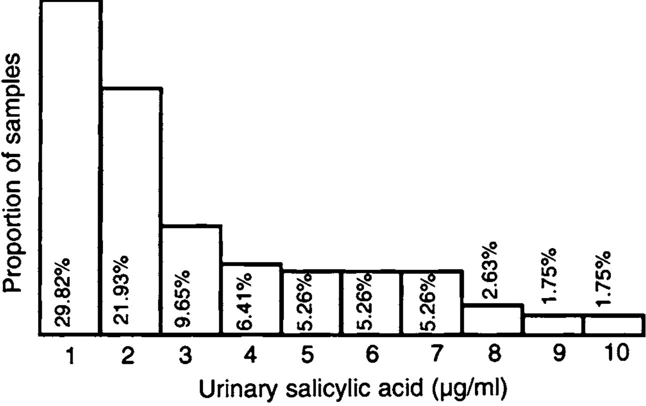

Fig. 1 Distribution of salicylic acid levels in post-race urine samples from thoroughbred racing in Great Britain in early 1980s

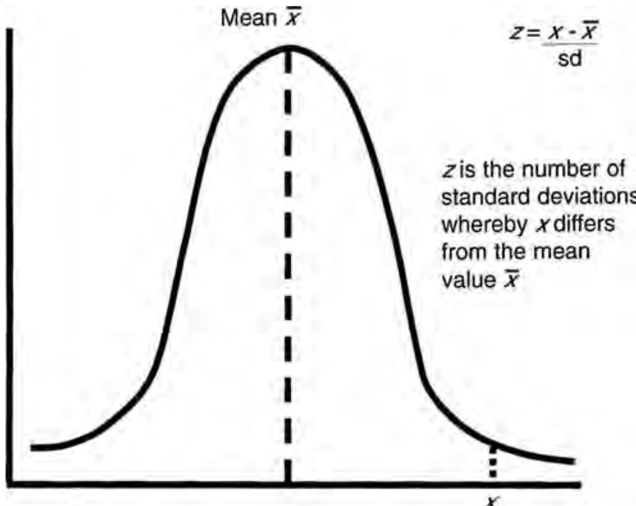

Fig. 2 A Gaussian distribution and the equation to determine the probability of a normal value exceeding a given value

Table 2 Probability of a normal value exceeding a determined threshold

Threshold	Probability of exceeding threshold
Mean + 3 sd	1 in 741
Mean + 3.09 sd	1 in 1,000
Mean + 3.29 sd	1 in 2,000
Mean + 3.54 sd	1 in 5,000
Mean + 3.72 sd	1 in 10,000
Mean + 3.89 sd	1 in 20,000
Mean + 4 sd	1 in 31,574

The probabilities of a normal value exceeding a determined threshold at various standard deviations from the mean value are shown in Table 2.

From the feeding experiments, the Canadians defined a threshold for salicylic acid at 750 μg mL^{-1}. Using the UK approach the threshold at 3.89 sd was 650 μg mL^{-1}. This compared favourably with the value obtained by the Canadians using a different approach and the threshold of 750 μg mL^{-1} was eventually adopted internationally. The fact that very similar values were obtained using two distinct approaches probably established the precedent of setting thresholds whereby there was a 1 in 10,000 (mean + 3.72 sd) to 1 in 32,000 (mean + 4 sd) probability, or higher, of a normal value exceeding the threshold.

6.2 Arsenic Threshold

Crone (1995) has discussed the arsenic threshold in detail. Arsenic, as a doping agent, received considerable attention in the early nineteenth century. During the period 1807–1812 a certain Daniel Dawson attempted to dope horses at Newmarket

and Doncaster with arsenic. Sadly his attempts resulted in the death of a number of horses and he was hanged for his crimes in 1812. Arsenic continued to be used as a "stopper" but more widely as a skin tonic for horses. As it is ubiquitous in nature, a threshold was needed to distinguish between normal levels and levels that had arisen abnormally. The Hong Kong laboratory determined arsenic levels in about 4,000 presumptively negative post-race samples (Crone 1995). The cube root transformation of the data gave a Gaussian distribution and the threshold determined at 4 sd from the mean was calculated to be 0.234 $\mu g\ mL^{-1}$; this was rounded up to 0.3 $\mu g\ mL^{-1}$.

6.3 Nandrolone Threshold

Following the detection of nandrolone and its major metabolites in extracts of normal colt urine samples in 1982 (Houghton et al. 1984), there was an urgency within the industry to develop a method to detect the administration of proprietary preparations of this anabolic agent to entire male horses. Clearly, this was an issue to be addressed by a threshold and two approaches were possible:

- To determine the concentration of nandrolone or a metabolite in the biological fluid of interest (the absolute threshold approach); or
- To determine the ratio of the endogenous substance or a metabolite in the biological fluid of interest to a structurally related substance which is not a metabolite (the ratio approach).

A precedent had already been established in human athletics for the use of the ratio approach (testosterone/epitestosterone ratio) and this was adopted to address nandrolone. Nandrolone and its major metabolite, 5α-estrane-3β,17α-diol, had been detected in extracts of normal colt urine (Houghton et al. 1984). Another steroid, 5(10)-estrene-3,17-diol, was also detected as a constituent of the extracts of normal urine from entire male horses. This is not a metabolite of nandrolone and the possibility of monitoring the ratio of estrane-3,17-diol to 5(10)-estrene-3,17-diol was investigated. If nandrolone is administered to entire male horses, the urinary concentration of estrane-3,17-diol will increase; whereas the concentration of 5(10)estrene-3,17-diol will remain constant, or possibly decrease, if the administration of nandrolone induces a negative feedback on steroidogenesis in the horse.

This theory was tested by administration of nandrolone to entire male horses (Houghton et al. 1986a) and shown to be effective in monitoring the administration. Both estrane-3,17-diol and estrene-3,17-diol are present as glucuronic acid conjugates and a very simple approach was adopted to monitor the ratio. Urine was enzyme hydrolysed with *Helix pomatia* and extracted with ether. The ether extract was dried, the ether removed and the residue derivatised to form the *t*-butyltrimethylsilyl derivative. The peak area ratio of the major ions (M^{+}–57) of the two steroid diols, *m*/*z* 449 and *m*/*z* 447, was then monitored. This approach was used to determine the ratio in a statistically significant population of post-race urine

samples from entire male horses. Again, a log transformation of the data produced a Gaussian distribution and a threshold was established at a ratio of 1 whereby there was a 1 in over 10,000 (mean + 3.72 sd) probability of a normal value exceeding this ratio. Thus, for a number of years this was a method dependent threshold. 5α-Estrane-3β,17α-diol was available as a reference standard but the stereochemistry of the 5(10)-estrene-3,17-diol was, at the time, unknown. This was eventually determined and the appropriate reference compound, 5(10)-estrene-3β,17α-diol, synthesised. Thus the method dependency was removed; the availability of the two steroids as reference standards allowed laboratories to develop quantitative methods or determine the relative response factors using alternative methods.

More recently as a result of studies in France (Dehennin et al. 2007) and international collaboration between a number of laboratories throughout the world, an alternative to this threshold value has been proposed, an absolute concentration threshold for 5α-estrane-3β,17α-diol isolated from the glucuronic acid fraction in urine at a concentration of 45 ng mL^{-1}.

6.4 *Theobromine Threshold*

In addressing the problem of feed contaminated with theobromine, the Horseracing Forensic Laboratory, UK (now HFL Sport Science) worked with Dalgety Agriculture. Dalgety investigated quality control with regard to producing compressed feed and concluded that theobromine can be excluded at concentrations above 0.8 mg kg^{-1}. Experiments were then conducted on feed contaminated with theobromine at 11.5, 6.6 and 1.2 mg kg^{-1}. The feed regimen was 7 kg/day for four days of contaminated compounded cubes. A quantitative method for measuring theobromine in urine was developed and the maximum urinary concentrations of theobromine determined after feeding. The maximum concentrations after feeding with 1.2 mg kg^{-1} of contaminated feed to three horses were 0.56, 0.55 and 0.54 μg mL^{-1}. The total dose of theobromine over the four days to the three horses was 32.9, 31.7 and 33.6 mg. These studies are described by Haywood (Haywood et al. 1990) and summarised by Houghton (Houghton 1995). On the basis of these results a pragmatic approach was taken and a threshold established at 2 μg mL^{-1}.

Theobromine and salicylic acid are not the only prohibited substances that occur naturally as chemical constituents of plant material. The pharmaceutical industry has a long history of developing potent medications from plant extracts, e.g. morphine and related opiates from poppy seeds for use as narcotic analgesics. The feeding of thoroughbred racehorses has developed from simply feeding natural materials such as oats and grass into an increasingly complex international business to prepare compound feed and nutritional supplements. Ingredients for compound feed or supplements may be sourced from all around the world and the risk of inadvertent contamination with plant material that contains a prohibited substance

has increased. Many racing countries have experienced "outbreaks" of positive findings of morphine in racehorse urine samples that have been traced to feedstuff that was contaminated with poppy seeds (Ginn et al. 2001; Kollias-Baker et al. 2002; Scott et al. 2005; Wynne 2005). To date an international threshold for morphine has not been agreed, due in part to the potent nature of this substance. It is also a substance that can be kept out of compound horse feed if good quality control procedures are adopted by feed manufacturers.

Other pharmacologically active substances that occur naturally in plant material include (but are not limited to) the following:

- Reserpine
- Scopolamine
- Atropine
- Valerenic acid.

The consideration of such substances as prohibited or not can vary in different regulatory jurisdictions throughout the world. Participants should always be fully aware of the regulatory rules of the sport in which they compete and should also ensure they know of any risks associated with their feeding regimen.

6.5 The Carbon Dioxide Threshold

The threshold for carbon dioxide in plasma was driven by concern about the misuse of bicarbonate solutions in an attempt to buffer the effects of exercise induced metabolic acidosis arising during a race and thus to delay fatigue in the competition horse. New Zealand was the first country to adopt such a threshold in 1991 (Irvine 2000). Subsequently the Australian racing laboratories generated significant data to support the adoption of an international threshold of 37 mmol L^{-1}, reduced to 36 mmol L^{-1} following the 12th International Conference of Racing Analysts and Veterinarians, held in Vancouver in 1998 where a whole session was dedicated to Alkalinising Agents. The control of bicarbonate (and other alkalinising agents) continues to be a challenge to equine sports across the world into the twenty-first century, as the continuing scientific investigations into this substance demonstrate (Auer et al. 1993, 2001; Kallings and Persson 1995; Tang and Crone 1995; Reilly et al. 1996; Irvine et al. 2000; Schuback et al. 2000; Vine 2000; Scarth et al. 2001; Brooks et al. 2001; Collins et al. 2005; Stenhouse et al. 2007; Arthur et al. 2007).

6.6 Threshold Values and Article 6 of the International Agreement on Breeding, Racing and Wagering

The thresholds for salicylic acid, nandrolone, theobromine and arsenic were introduced internationally into the Rules of Racing in the late 1980s. Article 6 of the

International Agreement on Breeding, Racing and Wagering states that threshold values can only be adopted for:

- Substances endogenous to the horse;
- Substances arising from plants traditionally grazed or harvested as equine feed; and
- Substances in equine feed arising from contamination during cultivation, processing or treatment, storage or transportation.

Subsequent to the establishment of the four threshold values in the late 1980s, as predicted, threshold values were established for other substances and, currently, Article 6 lists ten substances (Table 3).

Of particular interest is the need for the introduction of threshold values for the anabolic steroids testosterone and boldenone. When the initial studies on the metabolism of anabolic steroids in the horse commenced in 1974, four proprietary preparations were available for veterinary use: esters of testosterone, nandrolone, boldenone (1-dehydrotestosterone) and trenbolone. Of the four parent steroids at the time only testosterone was regarded to be of an endogenous nature. The radioimmunoassay screening procedure developed for testosterone in the 1970s was not sensitive enough to detect endogenous levels in the urine of the castrated male horse (gelding) and the female horse (filly), thus a threshold value was considered not necessary. Abuse of testosterone was detected by a positive response to the radioimmunoassay screening procedure and confirmation of the presence of testosterone and its metabolites in urine by GC–MS. The introduction of GC–MS screening methods to replace radioimmunoassay in the 1980s and the increase in the sensitivity of mass spectrometric techniques resulted in the detection of low nanogram levels of testosterone in all gelding and filly urine samples and the

Table 3 Internationally agreed threshold values as published in Article 6 of the international Agreement on Breeding and Racing (2008)

Threshold name	Threshold
Arsenic	0.3 μg total arsenic per mL in urine
Boldenone	0.015 μg free and conjugated boldenone per mL in urine from male horses (other than geldings)
Carbon dioxide	36 millimoles available carbon dioxide per litre in plasma
Dimethyl sulphoxide	15 μg dimethyl sulpoxide per mL in urine, or 1 μg dimethyl sulphoxide per mL in plasma
Estranediol in male horses (other than geldings)	The mass of free and conjugated 5α-estrane-3β,17α-diol to the mass of free and conjugated 5(10)-estrene-3β,17α-diol in urine from male horses (other than geldings) at a ratio of 1
Hydrocortisone	1 μg hydrocortisone per mL in urine
Methoxytyramine	4 μg free and conjugated 3-methoxytyramine per mL in urine
Salicyclic acid	750 μg salicyclic acid per mL in urine, or 6.5 μg salicyclic acid per mL in plasma
Testosterone	0.02 μg free and conjugated testosterone per mL in urine from geldings, or 0.055 μg free and conjugated testosterone per mL in urine from fillies and mares (unless foal)
Theobromine	2 μg theobromine per mL in urine

requirement of the threshold value. Subsequently, nandrolone and its major metabolite were shown to be present in extracts of urine from the male horse (Houghton et al. 1984). Further increases in the sensitivity of mass spectrometric techniques early this century have allowed for the routine detection of low picogram levels of urinary analytes and boldenone has been detected as a "normal" constituent in extracts of urine from some male horses, again resulting in the need to establish a threshold value (Dehennin et al. 2003; Ho et al. 2004).

Dehennin et al. (2003) developed a sensitive, quantitative GC–MS method for the analysis of extensively purified urinary extracts to determine concentrations of testosterone and boldenone in normal urine samples from the male horse. The mean concentration of boldenone in male horses ($n = 156$) was 0.336 ng mL^{-1} (minimum 0.02, maximum 1.51 ng mL^{-1}). Ho et al. (2004) analysed urinary extracts by LC–MS/MS and reported the presence of 17β-boldenone sulphate in extracts of normal urine from the male horse; boldenone was not detected in urine from untreated castrated male horses or female horses. An absolute threshold concentration of 0.015 μg mL^{-1} has been established to control misuse of boldenone and its esters in male horses

In addition, a threshold for 3-methoxytyramine to control administration of levodopa, dopamine and possibly *l*-tyrosine has been accepted and adopted within Article 6 (Wynne et al. 2001a, b).

6.7 Managing Threshold Values

The substances in Table 3 fall into four categories:

- Endogenous substances: nandrolone, hydrocortisone, testosterone, boldenone, carbon dioxide
- Natural products of feed: dimethyl sulphoxide, salicyclic acid
- Environmental contaminants in feed: arsenic
- Contaminants in manufactured feed: theobromoine

The natural levels of endogenous substances tend to show a skewed distribution. Log, square root and cube root transformations have been used to obtain Gaussian distributions. For certain substances, e.g. hydrocortisone and testosterone in the filly, it was possible to obtain a Gaussian distribution of the data using more than one transformation (e.g. for hydrocortisone the log and cube root transformations gave Gaussian distributions). The industry is then faced with the dilemma of which of these transformations is most appropriate in establishing a threshold. Popot et al. (1997) have compared the calculated hydrocortisone levels at various probabilities for both the log and cube root transformations (Table 4).

The values obtained at the same probability level are significantly lower for the cube root transformation. The choice for the threshold in this case was made on the basis of providing the least risk to the horseman, i.e. the log transformed data.

Table 4 The hydrocortisone threshold: Comparison of $\log x$ and $x^{0.33}$ thresholds

Probability of exceeding the concentration	Urine concentration (ng mL^{-1})	
	$\log x$	$x^{0.33}$
10^{-2}	326	222
10^{-3}	611	315
10^{-4}	1025	410
10^{-5}	1606	506

This leads to the question of how the industry should assess an appropriate degree of risk for thresholds. Clearly, thresholds must not be low enough to be breachable when no offence has been committed. Also, the effectiveness of a threshold may be assessed from detection periods following administration of proprietary preparations of the threshold substance. For example if, following administration of testosterone ester preparations, the threshold only allowed detection of the administration for 2–3 days this would be totally inappropriate. If the threshold allowed detection for 15–20 days this would be acceptable.

The industry has established additional measures to support findings of threshold substances. Article 6 of the International Agreement on Breeding, Racing and Wagering states "For any finding of a prohibited substance of endogenous nature, the Horseracing Authority may decide either itself or at the owner's or trainer's request to examine the horse further".

6.7.1 Isotope Ratio Mass Spectrometry

Within human sports, supportive evidence can be obtained from the use of additional analytical techniques. For endogenous substances, a number of laboratories working in this area now use the measurement of stable isotope ratios ($^{13}C/^{12}C$) to provide evidence for administration of endogenous hormones. The method is based on the premise that the relative abundance of stable carbon isotopes ($^{13}C/^{12}C$) for steroids in animals is closely related to the carbon source in their diet. Also a synthetically produced compound will be isotopically similar to the carbon source from which it was made. These two carbon sources may be isotopically distinct. This offers the possibility, therefore, that carbon isotope ratio analysis can be used to identify administered hormones against a background of endogenous molecules.

Over the last 50 years or so, isotope chemists have perfected methods to measure stable isotopic compositions to a very high precision. Thus small variations in measurements can be resolved and these are quoted differentially (compared with an internationally agreed standard) where:

$\delta^{13}C = [^{13}C(\text{analyte})/^{13}C(\text{standard}) - 1] \times 1{,}000$ in per mil (parts per thousand) units (symbol ‰).

The accuracy of the technique is constrained by the ability of the analysts to isolate and pre-treat samples chemically and convert them (by combustion) to CO_2 for study in dedicated mass spectrometers.

The modern variant of the carbon isotope ratio (IR) technique combines gas chromatography (GC) with mass spectrometry (MS) via a serial combustion interface, (GC–IR–MS). The integrated methodology can provide high precision (±0.3%) on small samples (nanogram quantities) of individual specific compounds separated rapidly from mixtures.

GC–IR–MS has become the industry standard method for compound specific isotope analysis (CSIA) with applications proliferating by the day. The method is now well established as a suitable means of addressing the challenge of detecting and confirming endogenous steroid abuse in human sports and in livestock (Horning et al. 1998; Aguilera et al. 1999; Ferchaud et al. 1998; Ferchaud et al. 2000; Mason et al. 1998). However, to date there are only a limited number of applications in thoroughbred racing. The technique has been applied to studies on hydrocortisone in the horse (Aguilera et al. 1997; Kinoshita et al. 2007), the administration of human chorionic gonadotrophin to the stallion (Houghton et al. 2000) and the detection of abuse of nandrolone (Yamada et al. 2007).

The concept of thresholds is now well established for endogenous substances and substances of dietary origin in both equine and human sports. However, within the thoroughbred racing industry, is a requirement counter-analysis in a second, nominated laboratory may be used as part of the confirmatory process. Thus the industry realised that with the threshold value substance, it faced the challenge of managing the agreement between laboratories for quantitative measurement. This issue was addressed by a Forum Discussion Group at the 11th International Conference of Racing Analysts and Veterinarians held in Brisbane in 1996. The delegates agreed it was necessary to adopt an international collaborative programme designed to improve the consistency of performance between laboratories. Elements of this programme to be addressed were:

- The initiation of comparative studies between laboratories for threshold substances analysis.
- Regular implementation of quality assurance exercises;
- Undertaking positive steps towards achieving accreditation;
- Applying the use of standardised reference materials and quality control samples;
- Organised research into sample stability and shipment studies;
- Agreeing on a the application of a standardised approach for the determination of the uncertainty of measurement;
- Adoption of a simple and pragmatic approach by analysts in reporting their results and by regulators in accepting, for example, that a counter laboratory need do no more that report its actual findings; and
- Regulators must accept that significant variations of reported results are a fact of analytical life and are likely to be reduced over a relatively long period only with considerable commitment of resources.

Over the intervening period the majority of these issues have been addressed. The majority of laboratories outside the USA are now accredited to ISO 17025 and new threshold values are determined through international collaboration with well

organised ring tests. The results of the studies are reported to the Advisory Council on Prohibited Substances for the IFHA for circulation to all interested stakeholders in the racing industry for comment prior to their submission to the Executive Council for the IFHA and the IFHA General Assembly for approval and inclusion in Article 6.

7 An Approach for the Control of Therapeutic Substances in Thoroughbred Horseracing

In thoroughbred racing in Europe, over the past decade, there has been growing concern that the way post-race drug control procedures are conducted may lead to inappropriate control of legitimate veterinary medication. A central reason for concern is the realisation that many drug infringements may not be due to intentional malpractice, but are more likely to be caused by residues of normal therapeutic medication or dietary or environmental contamination. Furthermore, these concerns are not restricted to European racing; other racing authorities around the world have initiated debate on these issues.

With few exceptions, the current policy for post-race testing is for high throughput screening analysis. This means that the same powerful analytical processes are used to look for all substances, regardless of their potencies or their abilities to affect race results. Under the policy of "zero tolerance", once a substance has been detected, no matter how insignificant the quantity, it must always be reported to the racing authorities. This may leads to the horse being automatically disqualified and the trainer having to face a disciplinary enquiry.

In the absence of official solutions, it was considered likely that unofficial solutions would creep in which could make the racing authorities vulnerable to criticism. Thus in 1998 the European Horserace Scientific Liaison Committee (EHSLC) began to examine these and related problem. At this time, the EHSLC comprised analysts, veterinarians, veterinary pharmacologists and administrators representing racing authorities within the five member nations; France, Germany, Great Britain, Ireland and Italy. Currently steps are underway to expand the membership of EHSLC.

The aim in 1998 was to develop a fair and effective approach for controlling substances commonly used for treating racehorses and harmonisation of this control across the five member nations of the EHSLC through agreed limits of the sensitivity of screening procedures. The determination and publication of detection times corresponding to these agreed screening limits would then allow practising veterinarians to apply good veterinary practice in the use of the therapeutic substances to treat thoroughbred horses.

A review of "positive findings" within Europe over the period 1993–1997 showed that for the 97,451 samples analysed, 431 (0.44%) were reported to contain prohibited substances. Of these, 332 contained drugs with market authorisation for

use in the horse. As a result, representatives from the five EHSLC countries confirmed the need to establish sensitivity limitations for certain substances during post-race analysis, whilst maintaining the current stringent policies for others. In other words, it is desirable to control pharmacological effects for some (mainly therapeutic) substances on race days, whilst continuing to look for evidence of exposure to drugs whose use is unacceptable in horseracing.

7.1 Pharmacokinetic/Pharmacodynamic Assessment of Irrelevant Drug Concentrations in Equine Plasma and Urine

Where sensitivity limitations were appropriate, it was agreed that they should be established according to appropriate scientific principles, by reference to a full pharmacokinetic/pharmacodynamic (PK/PD) evaluation. To provide a full pharmacokinetic/pharmacodynamic evaluation, a system of drug modelling was recommended (Toutain and Lassourd 2002) which relies on the calculation of the effective plasma concentration (EPC) of a drug based upon detailed pharmacokinetic studies. From this concentration it is possible to calculate the plasma concentration which would be ineffective and hence irrelevant. The irrelevant urine concentration (IUC) can be calculated from the irrelevant plasma concentration (IPC). The application of the model to literature-based data for pharmacokinetic studies of various drugs in horses has been published by Toutain and Lassourd (2003).

In order to be applicable to the model, the drug must act systemically, i.e. the pharmacological effect should be directly related to the plasma concentration. Thus, local anaesthetics and substances administered intra-articular or by inhalation are not considered suitable candidates. Nevertheless, in some instances, a PK/PD approach for non-systemically acting substances can be applied to indicate minimum analytical performance requirements. In addition it was considered that:

- Substances which exert their delayed effect by a cascade of indirect mechanisms are generally not considered suitable candidates unless the effect of interest can be clearly related to plasma or urine concentrations;
- The presence of two or more pharmacologically related substances (cocktails) invalidated the model; and
- The presence of drug plus a diuretic invalidates the model.

A collaborative research programme was then undertaken by the racing laboratories of the five nations of the EHSLC to generate reliable pharmacokinetic data for a number of therapeutic substances. Using the above criteria, drugs were chosen for study on the basis of their regular use in the treatment of racehorses. The laboratories of the Hong Kong Jockey Club and the Jockey Club of Southern Africa also participated in this experimental programme.

7.2 Administration and Analytical Assay Validation Studies

There was unanimous agreement within the EHSLC that the pharmacokinetic data must be established according to strict scientific principles, and guidelines for the animal administration and analytical studies were agreed and followed by all participating laboratories in the collaborative research programme.

These guidelines apply to:

- Test substance
- Animal administrations
- Sample collection
- Assay validation
- Sample storage
- Sample analysis
- Data analysis.

Regarding animal administration studies, the guidelines recommend:

- Careful control of test substance
- A single pilot administration study
- Main administration study – minimum of six animals
- Administration studies completed in a short time-frame
- Regular sample collection (plasma)
- Appropriate urinary collection period.

The PK/PD model recommended by Toutain and Lassourd (2002) requires quantitative analysis of both plasma and urine samples. Development and validation of appropriate quantitative methods for the drugs of choice prior to performing the animal administration studies is thus essential and the recommended assay validation procedures cover:

- Details for preparation of calibration curve
- Testing for linearity
- Assay validation
- Intra- and inter-assay precision
- Accuracy
- Specificity
- Sensitivity
- Selection of an appropriate limit of quantification (LOQ).

The guidelines also provide recommendations for sample analysis following the administration studies:

- Samples to be analysed as rapidly as possible after collection
- Appropriate sample storage conditions to be applied
- Analyte stability to be monitored
- Appropriate quality control samples to be included in each batch analysis.

Following these guidelines, pharmacokinetic data was generated for a number of drugs including phenylbutazone, flunixin, carprofen, ketoprofen, meloxicam, eltenac, dipyrone, vedaprofen, furosemide, meclofenamic acid, dembrexine, detomidine and naproxen.

7.3 The Irrelevant Urinary Concentration and Risk Management

The Irrelevant Urinary Concentration (IUC) of the drug is then determined by applying the PK data to the Pierre-Louis Toutain model. In order to establish a provisional screening limit based on the IUC, the five member nations of the EHSLC agreed an ordinal scale. Risk management is then applied, with the discussions involving representatives of all the five member nations of the EHSLC, to apply the IUC to a point on the ordinal scale. Risk management is the process of weighting policy alternatives to accept, minimise or reduce the assessed risk and, on the basis of this process, to select and implement the appropriate option regarding prevention, control or regulatory measurers. Factors considered by the risk management group included, but were not limited to, the extent of use of the drug in equine veterinary practice and its potential to affect the welfare of the horse or to improve its performance.

7.4 The Determination of Detection Times

The final step in this overall process is the determination and publication of detection times corresponding to the final agreed screening limits in order to allow practising veterinarians to apply good veterinary practice in the use of the therapeutic substances to treat thoroughbred horses and to harmonise the control of the drug across the member nations of the EHSLC. The provisional agreed screening limit, determined by the risk management exercise to apply the IUC to a point on the ordinal scale, provides an indication of the method sensitivity required for the detection time studies. As with the pharmacokinetic data, the detection times are determined on a sound scientific basis according to EHSLC agreed guidelines.

A minimum of six horses is preferred for each detection time study. The administered drug must be a proprietary preparation available for equine veterinary use or a preparation of a drug commonly used in the treatment of competition horses. The drug/preparation must be administered at a recommended dosing regimen used in equine veterinary practice. If several dosing regimens exist, the highest dose/longest duration of treatment should be selected.

Following the administration, the collection period for biological fluids of interest (urine and/or blood) should be over a time period that ensures samples containing the drug, at concentrations below the provisionally determined screening sensitivity limit, are available from all horses in the study. For the analysis of the biological fluids, ideally the analytical method used should have the sensitivity to effectively detect the drug at a concentration corresponding to the next point

lower on the ordinal scale than the provisionally agreed screening limit for the drug. If this is unattainable, the analytical method should effectively detect the drug at a concentration corresponding to half the provisionally agreed screening limit in the biological fluid under study. The analytical methods developed must be validated according to accepted practices. Analysis of all post-administration samples by this approach provides a full excretion profile for the drug in the biological fluid under study and allows detection times to be determined for a range of proposed screening limits.

The detection time is defined as the first observed time point at which urine samples collected from all horses in the study conducted according to the recommendation of the EHLSC (a given medicinal product, manufacturer's recommended dosage regimen, etc.) are negative, i.e. the concentration in the biological fluid under study is below the proposed screening sensitivity limit. Taking into consideration the detection times over a range of sensitivity limits for the drug under study, risk management is again applied to agree a final sensitivity limit for the control of the drug, with the sensitivity limit being applied at the screening level and published by European regulatory authorities for a number of therapeutic substances.

In the framework of risk analysis/risk communication, the detection time is a piece of information released by racing authorities to stakeholders to assist professionals (veterinarians) to recommend a withdrawal time for a given horse. This allows the veterinarians to apply good veterinary practice in the use of the therapeutic substances to treat thoroughbred horses. The overall process provides a fair and effective approach for controlling substances commonly used for treating racehorses and allows the five member nations of the EHSLC to harmonise on the control of these substances through agreed limits of the sensitivity of screening procedures.

Discussions are underway to extend this approach across a wider international front. The International Federation of Horseracing Authorities (IFHA) has given its full support for the international harmonisation of the control of substances commonly used to treat racehorses through the use of internationally agreed screening limits and published detection times. At the time of publication, the Advisory Council on Prohibited Substances to the IFHA is driving this initiative. The Advisory Council is seeking to adopt the screening limits and detection times determined in the EHSLC studies for international acceptance and then to extend the EHSLC list to cover other commonly used substances.

8 Anabolic Steroids in the Horse

This section and the LC-MS section have been reproduced in part from, "Anabolic steroids : Metabolism, doping and detection in human and equestrian sport, Kicman AT, Houghton E and Gower DB", In Steroid Analysis, Eds HJ Makin and DB Gower, 2009, Springer by kind permission of the authors, editors and publishers. In the 1970s, proprietary veterinary preparations of anabolic steroids were available

for testosterone (T), 19-nortestosterone (19-NT), boldenone and trenbolone. The metabolism of these steroids was studied in horses (Houghton 1977; Houghton and Dumasia 1979, 1980; Dumasia and Houghton 1981, 1984, 1988; Dumasia et al. 1983) to identify key analytes for the development of gas chromatographic–mass spectrometric (GC–MS) confirmatory analysis procedures; initial efforts were concentrated upon the study of the metabolism of 19-NT (nandrolone).

Following administration of the parent steroid (19-NT) to horses along with its radiolabelled analogue, determination of urinary radioactivity showed 80% of the administered material was excreted in 24 h (Houghton 1977). Aliquots of the post-administration urine samples were hydrolysed with *H. pomatia* enzymes (mixed glucuronidase and sulphatase) and the metabolites extracted with ether. The residues from the ether extracts were derivatised (methoxylamine/trimethylsilyl, MO-TMS) and analysed by gas chromatography–mass spectrometry; two metabolites were identified as isomers of estrane-3,17-diol and estran-3-ol-17-one.

These initial metabolism studies were performed in castrated males horses (geldings) and screening and confirmatory analysis methods to provide effective control of the abuse of nandrolone-based anabolic steroids were developed as a result of these studies for geldings and female horses. Radioimmunoassay was used for screening (Jondorf 1977; Jondorf and MacDougall 1977; Jondorf and Moss 1978) and a GC–MS confirmatory method was developed based upon the identification in urine of the major metabolite, the isomer of estrane-3,17-diol (Houghton et al. 1978). Due to the significant concentrations of testosterone in urine from male horses, the radioimmunoassay screening could not be applied and the GC–MS confirmatory analysis procedure was adapted and applied on a limited basis to control anabolic steroids in male horses.

However, detailed studies of urinary steroid profiles for entire male horses (Houghton et al. 1984a) using GC–MS demonstrated the presence of traces of 19-nortestosterone, isomers of estrane-3,17-diol and isomers of 5(10)-estrene-3, 17-diol in extracts of urine from untreated animals; these steroid had not previously been detected in urinary extracts from untreated castrated animals. The detection of the parent steroid, 19-nortestosterone and its major metabolite, estrane-3,17-diol, in extracts of normal urine of the male horse precluded their use in drug control in sport and an alternative approach had to be adopted: a threshold value, the estrane-3,17-diol to 5(10)-estrene-3,17-diol ratio.

As a result of these findings in male horses, more detailed studies for nandrolone, testosterone, boldenone and trenbolone were performed. These studies allowed for the identification of metabolites in the separated glucuronic acid and sulphate conjugate group fractions. Following the administration of radiolabelled nandrolone, testosterone, boldenone, and trenbolone (parent drugs, not esters) to the horse, the rates of urinary excretion of radioactivity varied markedly in the 0–24 h period, as did the distribution of radioactivity between the glucuronic acid and sulphate conjugate group fractions (Table 5).

For nandrolone, boldenone and trenbolone the major portion of the urinary excretory products were conjugated with glucuronic acid, whilst for testosterone, the major portion of the radioactivity was in the sulphate fraction.

Table 5 Distribution of radioactivity between the glucuronic acid and sulphate conjugate group fractions following administration of anabolic steroids to horses

Steroid	% Radioactivity excreted in 0–24 h urine		
	% in urine	% in Glucuronic acid fraction	% in sulphate fraction
Nandrolone	60–65	50–60	23–29
Testosterone	23–26	18–20	62–65
Boldenone	32–36	60–62	25–30
Trenbolone	48–50	60–65	22–25

For nandrolone, isomers of estrane-3,17-diol and 16-hydroxylated metabolites were detected in the glucuronic acid and sulphate conjugate group fractions with the 17α-hydroxy metabolites present mainly in the glucuronic acid fraction. The major metabolite was an isomer of estranediol and its stereochemistry was determined as 5α-estrane-3β,17α-diol (Houghton et al. 1989). 19-NT-17β-sulphate was a major metabolite in the sulphate conjugate group fraction (Dumasia and Houghton 1984).

A similar metabolic profile was obtained for testosterone with isomers of androstane-3,17-diol and 16-hydroxylated steroids distributed between the glucuronic and sulphate conjugate group fractions and the parent steroid, testosterone, present only as a sulphate conjugate (Dumasia and Houghton 1981).

The metabolism of 1-dehydrotestosterone (Boldenone) proved to be more complex. The 1,4-diene-3-one structure of the A-ring appears to stabilise the steroid to reductive metabolism and the major Phase I metabolic pathway results in formation of the 17α-epimer. However, partial and complete reduction of the 1,4-diene-3-one functionality coupled with metabolism at C-17 produces a complex series of minor metabolites (Dumasia et al. 1983, Dumasia and Houghton 1988). These metabolites were primarily conjugated with glucuronic acid and boldenone 17β-sulphate was the major metabolite in the sulphate conjugate group fraction. In addition to hydroxylation at C-16, hydroxylation at C-6 also occurs, and three isomers of a 6,16-dihydroxy metabolite have been identified in equine urine from the non-conjugated fraction.

Trenbolone also appeared to be stabilised to metabolism by the trienone system and the major metabolites resulted from oxidation and subsequent reduction of the 17-hydroxy group with epimerisation at C-17 and hydroxylation at C-16 (Houghton et al. unpublished results) with the metabolites being distributed between the glucuronic acid and sulphate conjugate groups. The metabolism of trenbolone has been studied in man (de Boer et al. 1991) and the cow (Pottier et al. 1981). In the three species, horse, man and cow, the major metabolic pathway is epimerisation at C-17; metabolism in the cow was similar to that in the horse in that it also showed hydroxylation at C-16.

The radioimmunoassay screening procedure was eventually replaced by a GC–MS method (Jondorf and MacDougall 1977; Jondorf 1977; Teale and Houghton 1991) with the ability to monitor a number of steroids in a single analysis. This was necessary in order to extend the coverage to a range of anabolic steroids (testosterone, nandrolone, 1-dehydrotestosterone and trenbolone), to address the complex

metabolism of these steroids in the horse and to allow for the detection of the C_{18} neutral steroids in extracts of urine from the entire male horse. This procedure involved enzyme hydrolysis of the urine with *Escherichia coli* (*E.coli*), extraction of the urine on a C_{18} Sep-Pak cartridge, elution of the aglycones with ether, elution of the sulphate conjugated steroids with ethyl acetate:methanol:sulphuric acid and their subsequent solvolysis. The extracts were derivatised to form methoxime/TBDMS derivatives. These derivatives show major ions for the loss of the *t*-butyl group and are ideal for screening in the SIM mode. Analysis of the derivatised extracts by GC–MS thus provided a method to monitor the distribution of steroids between the free plus glucuronic acid and sulphate conjugate group fractions.

GC–MS confirmatory analysis methods (Dumasia and Houghton 1986; Houghton et al. 1986a) were developed based upon the identification of the major metabolites, and this combination of GC–MS screening and confirmatory analysis provided an effective approach for the control of the veterinary anabolic agents for intramuscular administration, the proprietary preparations of nandrolone, testosterone, boldenone and trenbolone. Attention was then applied to some of the orally active anabolic agents.

Schoene et al. (1994) made a detailed study of the equine metabolism of 17α-methyltestosterone. After oral administration of a 1:1 mixture of the steroid and its $^{2}H_{3}$-analogue to two thoroughbred horses, urines were collected for 72 h. Analysis of metabolites revealed that the main Phase I metabolic processes were complete or partial reduction of the A-ring and the 3-oxo group, 15- and 16-hydroxylation, 17-epimerisation and hydroxylation at least two other sites, postulated as C-6 and C-11. Both glucuronidation and sulphation of metabolites were found to be common features. A stereochemical examination of the metabolites of of 17α-methyltestosterone has been reported (McKinney et al. 2007). Reference standards were either synthesised or purchased and the results showed that reduction of the 4-ene-3-keto function gave rise to metabolites with both 5α,3β- and 5β,3α-stereochemistries and hydroxylation at C-16 with both 16α- and 16β-stereochemistries. The authors stated that phase II metabolism was mainly sulphation with some glucuronidation.

Subsequent to the study of Schoene, considerable effort has been devoted to the study of the metabolism of orally active anabolic agents in the horse. The major metabolites or major metabolic processes of the steroids studied are summarised in Table 6.

In general, the in vivo Phase 1 metabolic processes are similar to those identified for the C_{18}/C_{19} steroids: reduction of the 4-en-3-one group, epimerisation at C-17 and hydroxylation at various sites including C-6 and C-16. In addition, Gourdie and Beresford (1995) have observed the 3-hydroxylation of ethylestrenol to yield norethandrolone prior to its metabolism to 17α-ethyl estrane-3,17-diol isomers.

Also of particular interest is the observation by McKinney et al. (2001a) of the hydroxylation in the 17α-ethyl side-chain of norethandrolone to yield a number of 20-hydroxylated metabolites and 21-oic acids. This oxidation of the 17α-alkyl side chain has also been observed following oral administration of 17α-methyltestosterone to horses (Dumasia 2003). Urine samples were hydrolysed using the enzyme

Table 6 In vivo metabolism of some orally active anabolic steroids in the horse

Steroid	Major metabolites/metabolic routes	References
Ethylestrenol[a]	Norethandrolone	Gourdie and Beresford (1995)
	19-Nor-17α-pregnane-3,17β-diol	Kim et al. (1996)
17α-Methyltestosterone	Reduction of the 4-en-3-one group	Stanley et al. (1997a)
	Hydroxylation at C-6/C-16/C-15	
	Epimerisation at C-17	
Fluoxymesterone[b]	Hydroxylation at C-16	Stanley et al. (1997b)
Oxymetholone[c]	Mestanolone	Tang et al. (2000)
	5α-Androstane-17α-methyl-3β,17β-diol	
	5α-Androstane-17α-methyl-3α,17β-diol	
Mestanolone[d]	5α-Androstane-17α-methyl-3β,17β-diol	Tang et al. (2000)
	5α-Androstane-17α-methyl-3α,17β-diol	
Danazol[e]	2-(Hydroxymethyl)ethisterone	Tang et al. (2001)
	6-Hydroxyethisterone	
	Ethisterone	
Normethandrone[f]	Reduction of the 4-en-3-one group	
	Fox et al. (2001)	
	Epimerisation at C-17	
	Hydroxylation at C-6/C-16	
Methandienone[g]	Reduction of the 4-ene	McKinney et al. (2001a)
	Hydroxylation at C6/C16	
	Reduction of the 1-en-3-one group (slow)	
Norethandrolone[h]	19-Nor-17α-pregnane-3,17β-diols	McKinney et al. (2001b)
	19-Nor-17α-pregnane-3,16,17β-triols	
	19-Nor-17α-pregnane-3,17β,20-triols	
	19-Nor-17α-pregn-4-en-3-one-17β,20-diols	
	19-Nor-17α-pregnane-3,17β-diol-21-oic acids	
	19-Nor-17α-pregn-4-en-3-one-17β-ol-21-oic acid	
Methenolone[i]		
Acetate	Deacetylation	Ho et al. (2005)
	Epimerisation at C-17	
	Hydroxylation at C-16	
	Oxidation at C-17	
17α-Methyltestosterone	Reduction of the 4-en-3-one group	Dumasia (2003)
	Hydroxylation at C-6/C-16	
	Epimerisation at C-17	
	Hydroxylation at C-20	
Clostebol[j]	4-Chlorotestosterone	
Acetate	4-Chloroandrost-4-ene-3α,17β-diol	Leung et al. (2005a, b)
	4-Chloroandrostane-3α,17β-diol	
Mesterolone[k]	1α-Methyl-5α-androstan-3α-ol-17-one	Leung et al. (2005b)
	1α-Methyl-5α-androstan-3β-ol-17-one	
	1α-Methyl-5α-androstane-3β,17β-diol	
	1α-Methyl-5α-androstane-3,17α-diol	
	1α-Methyl-5α-androstane-3,16-diol-17-one	
17α-Methyltestosterone	17α-Methyl-5α-androstane-3β,17β-diol	Yamada et al. (2007a)

(*continued*)

Table 6 (continued)

Steroid	Major metabolites/metabolic routes	References
Mestanolone[l]	17α-Hydroxymethyl-5α-androstane-3β,17β-diol 17α-Methyl-5α-androstane-3β,16β,17β-triol 17α-Methyl-5α-androstane-3β,16α,17β-triol	

[a]Ethylestrenol: 17β-Hydroxy-17α-ethylestr-4-ene
[b]Fluoxymesterone: 9α-Fluoro-11β,17β-dihydroxy-17α-methylandrost-4en-3-one
[c]Oxymetholone: 17β-Hydroxy-2-(hydroxymethylene)-17α-methylandrostan-3-one
[d]Mestanolone: 17β-Hydroxy-17α-methylandrostan-3-one
[e]Danazol: 17α-Pregna-2,4-dien-20-ynol[2,3-d]isoxazol-17β-ol
[f]Normethandrone: 17β-Hydroxy-17α-methylestr-4-en-3-one
[g]Methandienone: 17β-Hydroxy-17α-methylandrosta-1,4-dien-3-one
[h]Norethandrolone: 17β-Hydroxy-17α-ethylestr-4-en-3-one
[i]Methenolone: 17β-Hydroxy-1α-methyl-5α-androst-1-en-3-one
[j]Clostebol: 4-Chlorotestosterone
[k]Mesterolone: 17β-Hydroxy-1α-methyl-5α-androstan-3-one
[l]Mestanolone: 17β-Hydroxy-17α-methylandrostan-3-one

preparation *E. coli* (glucuronidase), the steroid metabolites extracted by solid phase extraction and the extracts derivatised (methyloxime/TMS ether) prior to GC–MS analysis. Some isobaric $C_{20}O_3$ and $C_{20}O_4$ metabolites were detected showing an initial fragment ion by GC–MS analysis corresponding to loss of the radical of mass 103u (CH_2OTMS)**.** This loss is characteristic of a metabolite arising by hydroxylation of the 17α-methyl group.

The group from the Hong Kong Jockey Club Racing laboratory has performed a series of studies for a number of steroids comparing the in vitro metabolism with horse liver microsomes with the in vivo metabolism following oral administration to horses. With danazol (Tang et al. 2001), both the in vitro and in vivo metabolism were complex but comparable. Of the twelve metabolites of danazol identified in the in vitro incubation study, nine were identified in the in vivo study. Other steroids studied by this approach are oxymetholone and mestanolone (Tang et al. 2000), methenolone acetate (Ho et al. 2005), clostebol acetate (Leung et al., 2005a, b), mesterolone (Leung et al. 2005a), turinabol (4-chloro-17α-methyl-17β-hydroxy-1,4-androstadien-3-one) (Ho et al. 2007a) and mesterolone (1α-methyl-5α-androstan-17β-ol-3-one) (Ho et al. 2007b).

In addition to studying the metabolites of 17α-methyltestosterone and mestanolone and detecting their common metabolites (Yamada et al. 2007a; see Table 6), the same group have shown that these metabolites are also common to the metabolism of methandienone, methandriol and oxymetholone in the horse (Yamada et al. 2008a). Yamada et al. (2008b) have also studied the metabolism of fluoxymesterone in the horse and identified the main metabolite as 9α-fluoro-17,17-dimethyl-18-norandrostane-4,13diene-11β-ol-3-one by GC–MS. The metabolite was synthesised and characterised by GC–MS of the TMS derivative and proton and ^{13}C NMR.

This application of GC–MS screening procedures for the separated free plus glucuronic acid and sulphate conjugate group fractions proved particularly effective in resolving the complex urinary steroid profile of the entire male horse and its application resulted in the detection of urinary 19-norandrost-4-ene-3,17-dione isolated by solid phase extraction from the sulphate conjugate group fraction (E. Houghton et al., unpublished result). The finding of a 3,17-dione in the sulphate conjugate group fraction as opposed to the free steroid fraction was an interesting result that led to considerable debate as to a possible precursor. Initial conclusions favoured an "enol-sulphate" and considerable effort was devoted to its detection using liquid chromatography–mass spectrometry but without any success.

The behaviour of this precursor in urine was studied under acid (pH 3) and alkaline conditions and clear evidence was obtained that the 19-nortestosterone detected in extracts of "normal" urine from entire male horses following solvolysis is not being derived from the 17β-sulphate conjugate, even though the 19-nortestosterone is isolated from the sulphate conjugate fraction (Houghton et al. 2007). However, following administration of 19-NT-based proprietary anabolic steroids to all horses, the urinary 19-nortestosterone arising from the administration is excreted primarily as the17β-sulphate conjugate.

The effect of pH on the urinary precursors to 19-norandrost-4-ene-3,17-dione and 19-nortestosterone has been studied in more detail. On the basis of these results it was concluded that the precursors to 19-norandrost-4-ene-3,17-dione and 19-nortestosterone, present in the urine prior to the hydrolysis steps, have the same basic structure except for the functionality at the 17-position. Preparative HPLC and HPLC fractionation have been used to separate the precursors from the high amounts of oestrogenic sulphates present in "normal" urine from the entire male horse. The purified fractions, containing the intact precursors, were subjected to analysis by LC–MS (negative ion mode), LC–MS/MS (negative ion mode) and, following derivatisation (methyl/methoxime/trimethylsilyl derivative), GC–MS to identify the precursors (Houghton et al. 2007). The precursors for 19-norandrost-4-ene-3, 17-dione and 19-nortestosterone isolated from acid hydrolysed urinary extracts were identified as the 19-carboxylic acid of androst-4-ene-3, 17-dione (3,17-dioxo-4-androsten-19-oic acid) and the 19-carboxylic acid of testosterone (3-oxo-17-hydroxy-4-androsten-19-oic acid). The results show that 19-norandrost-4-ene-3, 17-dione and 19-nortestosterone detected in urinary extracts can arise as artefacts by decarboxylation of these 19-carboxylic acids as a result of acid hydrolysis.

For further details on the analysis of steroids in connection with doping control in equestrian and human sport see Kicman et al. (2009).

9 LC–MS Analysis in Equine Drug Testing and Metabolism

The commercial development of atmospheric pressure ionisation (API) in the mid 1980s was undoubtedly, at that time, the most significant development in LC–MS and provided the analyst with expectations of a truly robust, practical and sensitive

LC–MS interface. In drug analysis the technique was rapidly accepted as the industry standard by the pharmaceutical industry in support of drug discovery and development and now API-LC–MS impinges upon many scientific disciplines.

Acceptance of API-LC–MS in the field of drugs in sport was much slower than in the pharmaceutical industry, primarily due to two reasons: the high degree of skill and expertise that existed in sporting laboratories in GC–MS, based around the robust, sensitive and low cost bench-top systems and, secondly, the comparative cost of these systems when compared to LC–MS systems. GC–MS was first applied as a confirmatory analysis method in sporting laboratories in the mid-1970s and, as a result, significant skill was developed in this area. The technique also evolved as the primary tool for metabolite identification in sports applications. This expertise in GC–MS applications to drug analysis also resulted in its widespread application as a generic screening procedure, initially for anabolic steroids and then more generally for basic and acidic drugs. However, LC–MS is now increasingly being used within sports laboratories to complement GC–MS applications. Developments in API technology, MS/MS and data acquisition and data processing in the form of multiple reaction monitoring and data dependent scanning in specific time windows have facilitated this transition. These processes allow for screening of multiple analytes in a single LC run at high sensitivity and specificity.

The development of high resolution accurate mass LC–MS instruments offers the potential to extend this technology even further as a screening tool, particularly if sample data can be compared to a large database of substances with their corresponding accurate masses. It is possible to envisage an LCMS screening test that can cover over 1,000 different substances, rivalling previous applications of GC–MS technology. Shorter LC retention times offer the additional advantage of higher sample throughput.

Early applications of API-LC–MS to steroid analysis in sport addressed the comparative metabolism of methandrostenelone (methandienone) (Edlund et al. 1989) and stanozolol (Muck and Henion 1990) in man and the horse and the target analytes of choice for screening. Using a heated pneumatic nebuliser with corona discharge ionisation on a triple quadrupole instrument and precursor and product ion scans (Edlund et al. 1989) identified 17-epimethandrostenolone as the analyte of choice for screening in the horse. Quantitative applications were developed using [17-methyl-$^{2}H_{3}$]-methandrostenolone as internal standard and the method detection limit was in the pg mL^{-1} range.

Similar LC–MS conditions were used to study the metabolism of stanozolol in man and the horse (Muck and Henion 1990). The authors confirmed the presence of 3′-hydroxystanozolol and 4β-hydroxystanozolol in human urine following oral administration using collision induced dissociation (CID) of protonated molecular ion species $(M + H)^{+}$ and interpretation of the product ion spectra. Strong evidence was obtained for a number of other mono- and di-hydroxylated metabolites and the metabolic profile was in good agreement to that proposed using GC–MS analysis (Schanzer et al. 1990). Hydroxy metabolites of stanozolol were also detected in equine urine (Houghton and Teale, unpublished data).

More recent, McKinney et al. 2004 have studied the metabolism of stanozolol in the horse following intramuscular administration. Using positive ion electrospray ionisation on an ion trap LC–MS, McKinney et al. showed the urinary excretion of stanozolol, 16β-hydroxystanozolol and two other monohydroxylated metabolites tentatively identified as 16α-hydroxystanozolol and 15-hydroxystanozolol. The LC–MS method had a limit of detection around 100 pg mL^{-1} and stanozolol and its metabolites were shown to be extensively conjugated with both glucuronic acid and sulphate.

As with GC–MS, LC–MS has the capability for multi-residue analysis in the steroid field. This has been demonstrated in the development of an LC–MS method to detect 15 parent anabolic steroids added to equine urine at levels of 5–100 ng mL^{-1} (Yu et al. 2005). The steroids were isolated from urine by solid phase extraction and analysed by LC–MS in the positive ion electrospray mode using multiple reaction monitoring. The method specificity, sensitivity, precision and recovery were evaluated and all the steroids were detected consistently. The method was successfully used to analyse methenolone acetate in post-administration urine samples. Following oral administration of methenolone acetate to a horse, the developed method was used to confirm methenolone in urine and also to identify the 17-epimer of methenolone.

The same group have extended this approach to analyse urine extracts for a number of anabolic steroids, corticosteroids and acidic drugs (Ho et al. 2006). Urine samples were extracted using a strong cation exchange cartridge, C8-SCX. The acid/neutral fraction eluted from the cartridge was base washed and the organic extract, containing anabolic and corticosteroid residues, was analysed by LC–MS in the positive ion electrospray mode using multiple reaction monitoring (MRM). The approach allowed for the analysis of a total of forty anabolic steroids and corticosteroids. The base wash was acidified (pH 6) and the acidic drugs isolated by liquid/liquid extraction. A second LC–MS analysis was performed for screening for the acidic drugs and the mass spectrometer was operated in the negative ion electrospray mode again using MRM covering fifty acidic drugs. A broad-based screening method for acidic drugs in protein precipitated plasma and neutral drugs in equine urine has been developed using direct-injection LC–LC hybrid MS/MS methods (Stanley et al. 2007). The majority of the drugs both in plasma and urine were detected in samples fortified at the 10 ng mL^{-1} level. Direct injection of diluted urine and analysis by LC/ESI-MS has also been used for the quantification of hydrocortisone in the horse (Vonparti et al. 2008).

A multi-residue method has also been developed for 21 glucocorticosteroids in equine plasma (Luo et al. 2005). The steroids were added to plasma and the plasma extracted with methyl *tert*-butyl ether and the extracts analysed by positive ion electrospray. The limit of detection of the assay ranged between 50 and 100 pg mL^{-1} and the limit of quantification for most of the analytes was 1 ng mL^{-1}. The method has been successfully applied to screening plasma samples from racehorses in competition.

The application of LC–MS has been extended to the analysis of anabolic/androgenic steroids in plasma and a method has been developed for the detection,

quantification and confirmation of eight anabolic steroids (testosterone, normethandrolone, nandrolone, boldenone, methandrostenolone, tetrahydrogestrinone, trenbolone and stanozolol) (Guan et al. 2005) in equine plasma using ESI in the positive ion mode. Limits of detection and quantification were in the range 25–50 pg mL^{-1}. The group have also used LC–MS methods to study the pharmacokinetics of boldenone and stanozolol in the horse and for the quantification of anabolic and androgenic steroids in race horses and non-race horses (Soma et al. 2007, 2008).

The application of LC–MS to the analysis of intact steroid conjugates in the horse was demonstrated by Dumasia et al. (1996) in the development of a quantitative method for the determination of testosterone sulphate in equine urine. 16,16,17-[$^{2}H_{3}$]-Testosterone sulphate was synthesised as internal standard and the urinary extracts (SPE) were analysed in the negative ion electrospray mode. The calibration line was established over the range 0–400 ng mL^{-1} and the method was used to determine urinary testosterone sulphate levels following administration of the proprietary testosterone ester preparation, Durateston. More recently, Grace et al. (2008) have developed a sensitive, quantitative LC–MS method to determine the normal levels of nandrolone sulphate and boldenone sulphate in urine from the male horse. The method takes advantage of the selective hydrolysis of the aryl sulphates of oestrone and oestradiol by *H. pomatia*. The aryl sulphates are present in high concentrations in normal urine from the male horse and are ideal substrates for the aryl sulphatases of *H. pomatia*, whereas these enzymes show little or no activity for the alkyl sulphates of nandrolone and boldenone. The selective removal of the interfering aryl sulphates allowed for the development of a sensitive assay for nandrolone sulphate (LOQ 1 ng mL^{-1}) and boldenone sulphate (0.5 ng mL^{-1}).

In general, product ion spectra produced under LC–MS electrospray conditions can be difficult to interpret and this applies particularly to steroids. However, Guan et al. (2006) have studied the fragmentation of nine anabolic steroids using a triple quadrupole mass spectrometer, Fourier transform ion cyclotron resonance (FT-ICR) and a linear ion trap instrument. Using a combination of different approaches the authors noted that small differences in chemical structure could result in significantly different fragmentation pathways. Fragmentation pathways are proposed which are helpful for the characterisation of new steroids.

10 The Future

Doping control laboratories around the world have carried out an enormous amount of research and development in drug detection over the last 40 years. This relatively small community of laboratories has also been willing to share the results of that research and so it is possible today to deploy a screening protocol that can detect over a thousand different substances.

However, the continual development of new drugs for legitimate therapeutical application by the pharmaceutical industry and the threat from “illegal” drugs

designed and manufactured to aid sporting performance mean the challenges to doping control laboratories will continue to change.

In particular, the increasing use of peptides and proteins in drug development and the development of gene therapies will require a change to the current doping control techniques that were largely designed to detect the presence of "small" molecules (with molecular weights up to around 500) and their metabolites. For example, Laidler et al. (1998) developed a method to discriminate between human, equine, porcine and bovine growth hormones (GHs) using peptide-mass mapping by matrix-assisted laser desorption/ionisation (MALDI) time-of-flight (TOF) mass spectrometry following tryptic digestion. Legal and sporting authorities had recognised that GHs may be abused to improve sporting performance and that development of methods for their control in sport was necessary. The results of the study showed the peptide mass maps were sufficient to differentiate between the GHs from the four species. Erythropoeitin (EPO) is a substance that has already provided considerable adverse publicity both in the field of human sports and thoroughbred horseracing. EPO stimulates erythrocyte production, allowing for better oxygen uptake and thus is of value in endurance sports. Significant success has been achieved in the development of methods for its detection in both humans and horses using proteomics approaches. Tryptic digestion and LC–MS/MS analysis have been used to provide a method for the confirmatory analysis of recombinant human erythropoietin (rhEPO) and darbepoetin (DPO) (Guan et al. 2007) and to differentiate between the two proteins (Guan et al. 2008). Two specific proteolytic peptides were used to distinguish between to two protein based drugs, confirmatory analysis being based upon chromatographic data and major product ions. Sensitivity was in the range of 0.2 ng mL^{-1} and this was the first LC–MS method with sufficient sensitivity to provide unequivocal confirmatory data for these drugs.

It is likely that a number of major changes to the laboratory approach to drug screening will occur to address the new challenge from biological threats.

Firstly the choice of matrix is likely to change. For example, urine is unlikely to be a good matrix to monitor direct genetic manipulation (so-called gene doping). HFL's research into the challenge of gene doping suggests that monitoring changes to blood protein patterns may provide a solution. This will require knowledge of proteomics and the LC–MS technology and scientific skills to implement such an approach. Other approaches being considered to address this challenge include transcriptomics and genomics.

This approach suggests another likely shift in the screening process. Historically laboratories have relied on direct detection of the doping agent or its chemically related metabolite molecule(s). Biomarkers of the doping agent's effect may become analytical targets in future. An example of biomarker use already exists in doping control where monitoring suppression of the natural corticosteroid hydrocortisone can suggest administration of synthetic corticosteroids such as dexamethasone. Also, You et al. (2007) have identified ethyl glucuronide and ethyl sulphate as biomarkers of alcohol administration to horses and developed a rapid, sensitive LC–MS method for the screening, quantification and confirmatory analysis of these analytes. This principle may be expanded to look for changes in blood protein

patterns that indicate the administration of any one of a particular group of doping agents. For example, it may be possible to identify and monitor biomarkers for growth promoting agents, regardless of whether these are anabolic steroids or peptide hormones.

In any case, the new biological threats do not mean that the established small molecule doping agents will disappear. The doping control laboratory will still need to monitor abuse of these agents. The inevitable result is a more complicated screening procedure that is more expensive, takes longer and produces large amounts of data for assessment, most of which will be negative.

The data assessment challenge in doping control is to filter the majority of negative data points sufficiently to allow the expert scientist to concentrate their interpretive skills on the relatively small number of samples that are truly suspicious. HFL has worked closely with a specialist software company to develop automated data assessment software that uses database library searching and pattern recognition to assess the data from full scan EI GC–MS test results. This has successfully reduced the amount of GC–MS data that requires human expert assessment. This approach is likely to be extended in future to other screening test data.

The era of reliance on post-competition urine tests for doping control in horseracing will change to accommodate more flexible sampling strategies. There will be a greater use of out of competition testing, more sample matrix types will be taken and even the location of the analysis may change. Changes in technology and in attitude mean that research into near-subject testing is now increasing the practical options in this area. Use of biosensors and even portable mass spectrometers mean that the laboratory of the future may be able to travel to the horse as well as providing expert centralised testing.

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Androgenic Anabolic Steroid Abuse and the Cardiovascular System

Paul Vanberg and Dan Atar

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Abstract Abuse of anabolic androgenic steroids (AAS) has been linked to a variety of different cardiovascular side effects. In case reports, acute myocardial infarction is the most common event presented, but other adverse cardiovascular effects such as left ventricular hypertrophy, reduced left ventricular function, arterial thrombosis, pulmonary embolism and several cases of sudden cardiac death have also been reported. However, to date there are no prospective, randomized, interventional studies on the long-term cardiovascular effects of abuse of AAS. In this review we have studied the relevant literature regarding several risk factors for cardiovascular disease where the effects of AAS have been scrutinized:

(1) Echocardiographic studies show that supraphysiologic doses of AAS lead to both morphologic and functional changes of the heart. These include a tendency to produce myocardial hypertrophy (Fig. 3), a possible increase of heart chamber diameters, unequivocal alterations of diastolic function and ventricular relaxation,

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and most likely a subclinically compromised left ventricular contractile function. (2) AAS induce a mild, but transient increase of blood pressure. However, the clinical significance of this effect remains modest. (3) Furthermore, AAS confer an enhanced pro-thrombotic state, most prominently through an activation of platelet aggregability. The concomitant effects on the humoral coagulation cascade are more complex and include activation of both pro-coagulatory and fibrinolytic pathways. (4) Users of AAS often demonstrate unfavorable measurements of vascular reactivity involving endothelial-dependent or endothelial-independent vasodilatation. A degree of reversibility seems to be consistent, though. (5) There is a comprehensive body of evidence documenting that AAS induce various alterations of lipid metabolism. The most prominent changes are concomitant elevations of LDL and decreases of HDL, effects that increase the risk of coronary artery disease. And finally, (6) the use of AAS appears to confer an increased risk of life-threatening arrhythmia leading to sudden death, although the underlying mechanisms are still far from being elucidated. Taken together, various lines of evidence involving a variety of pathophysiologic mechanisms suggest an increased risk for cardiovascular disease in users of anabolic androgenic steroids.

Keywords Anabolic androgenic steroids • Cardiovascular side-effects • Left ventricle hypertrophy • Hypertension • Relaxation abnormalities • Vascular reactivity • Coronary calcification • Blood platelet function • Lipid alterations • Myocardial infarction • Arrhythmia • Sudden death

1 Introduction

Over the last two decades there has been increased public focus on possible detrimental cardiac effects of the abuse of anabolic androgenic steroids (AAS). This awareness has, at least in part, been fuelled by an increasing number of case reports in the literature, many of them describing the occurrence of serious cardiovascular events more or less obviously linked to AAS abuse, some of them even with deadly outcome. The majority of described events have happened to bodybuilding AAS abusers, though not confined to competing athletes in this sport. Abuse of AAS has been associated with a range of different cardiovascular side effects (Fig. 1), starting with a case report of left ventricular hypertrophy in 1986 (McKillop et al. 1986) and one of a myocardial infarction in 1988 (McNutt et al. 1988), affecting a bodybuilder and a weightlifter, respectively, both of them very young. In 1988 there was also a report of a young bodybuilder with an ischemic cerebrovascular event and signs of cardiomyopathy (Mochizuki and Richter 1988). Several reports followed, and by 2008 there were around 40 altogether. Acute myocardial infarction is the most common event presented (Appleby et al. 1994; Capezzuto et al. 1989; Ferenchick and Adelman 1992; Fisher et al. 1996, Güneş et al. 2004; Halvorsen et al. 2004; Hourigan et al. 1998; Huie 1994; Kennedy 1993;

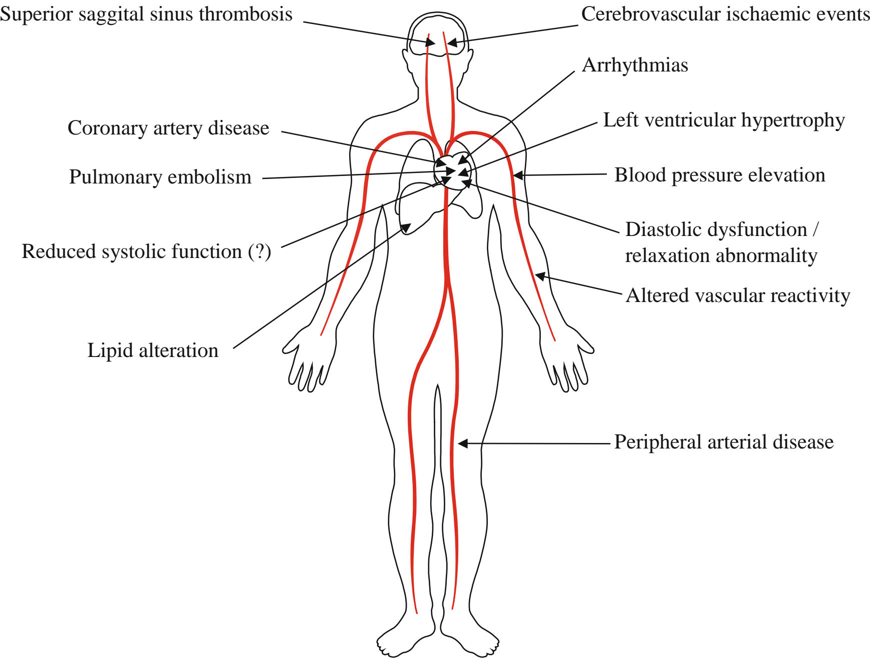

Fig. 1 Potential harmful cardiovascular effects of abuse of AAS

Lyngberg 1991; Tischer et al. 2003; Wysoczanski et al. 2008), but there are also several reports of left ventricular hypertrophy (Figs. 2 and 3) (Campbell et al. 1993; Dickerman et al. 1995, 1997a, c; Hausmann et al. 1998; Kennedy and Lawrence 1993; Mark et al. 2005; McKillop et al. 1986; Stevens et al. 2002; Tischer et al. 2003), reduced left ventricle function (Clark and Schofield 2005; Ferrera et al. 1997; Mark et al. 2005; McCarthy et al. 2000), cerebrovascular incidents (Frankle et al. 1988; Laroche 1990; Mochizuki and Richter 1988; Santamarina et al. 2008), atrial fibrillation (Lau et al. 2007; Sullivan et al. 1999), arterial thrombosis (Alvarado et al. 2001; Falkenberg et al. 1997; Laroche 1990; McCarthy et al. 2000), pulmonary embolism (Gaede and Montine 1992; Liljeqvist et al. 2008), valve disease (Medras et al. 2005; Stevens et al. 2002), even one of a cardiac transplant (Menkis et al. 1991), and, sadly, several ones of sudden cardiac death (Di Paolo et al. 2007; Dickerman et al. 1995; Fineschi et al. 2001, 2007; Hausmann et al. 1998, Kennedy and Lawrence 1993; Luke et al. 1990). Interestingly, a repeated finding has been myocardial infarct without significant atherosclerotic coronary artery disease (Ferenchick and Adelman 1992; Fineschi et al. 2001; McNutt et al. 1988). On the other hand, severe premature coronary artery disease has also been observed (Fisher et al. 1996; Güneş et al. 2004; Huie 1994; Lyngberg 1991; Menkis et al. 1991; Ment and Ludman 2002; Mewis et al. 1996; Nieminen et al. 1996).

A lot of attention has been generated by a Swedish autopsy study (Thiblin et al. 2000) where 34 male users of AAS were medico-legally investigated. Twenty of

Fig. 2 Example of severe left ventricular hypertrophy, transverse section at mid-cavity level (not from Thiblin's study)

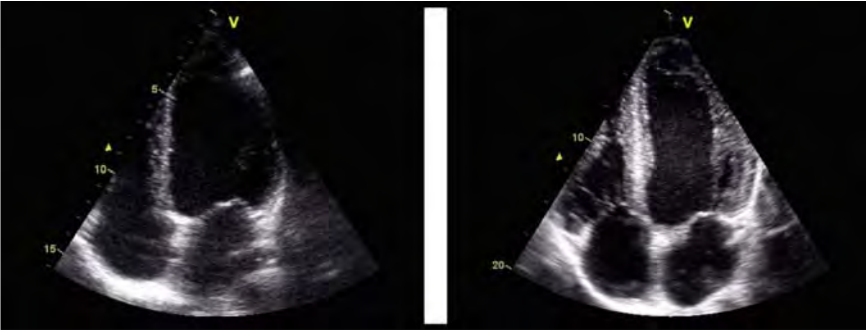

Fig. 3 Echocardiographic image of a drug-free 25-year-old powerlifter (*left*) with normal left ventricular wall thickness and of an AAS-using 37-year-old bodybuilder (*right*) with concentric left ventricular hypertrophy

them were either victims of homicide or had committed suicide, while the other deaths were classified as accidental or indeterminate. Chronic cardiac pathological changes were observed in 12 cases, specifically left ventricular hypertrophy (Fig. 2), cardiac fibrosis and coronary artery disease.

Concomitantly, a number of animal studies have been conducted over the years to shed light on the mechanisms possibly involved in the link between AAS abuse and cardiovascular pathology and/or mortality. The studies that looked at cardiovascular consequences of testosterone or their synthetic derivatives invariably show unfavourable effects. Myocardial hypertrophy (Kinson et al. 1991; Koenig et al. 1982; Marsh et al. 1998; Moore et al. 1978; Morano et al. 1990; Pesola 1988; Tagarakis et al. 2000; Takala et al. 1992), reduced ejection fraction (Rämö et al. 1987), increased myocardial stiffness (Trifunovic et al. 1995), left ventricular remodeling (Woodiwiss et al. 2000), depressed cardiac contractile function (Liang et al. 1993), impaired exercise-induced microvascular adaption (Tagarakis et al. 2000), reduced endothelial function (Cunha et al. 2005), destruction of mitochondria and myofibrils (Appell et al. 1983; Behrendt and Boffin 1977), disruption of morphological integrity of myocardial cells (Melchert et al. 1992) and apoptotic cell death in ventricular myocytes (Zaugg et al. 2001) are among the findings.

Despite this heightened awareness and an increasing knowledge base, to date there are no prospective, interventional studies on the long-term cardiovascular effects of abuse of AAS. In fact, due to ethical considerations, such a study will never be performed, and our present knowledge is largely based on case reports and observational studies. A small number of prospective studies have been published, but they are all done over a short period of time, and the doses of AAS used were relatively low, and do not reflect the real-life practices in the bodybuilding discipline.

As to the almost overwhelmingly high number of case reports and the severity of events described in them, one must – on the other hand – take into consideration the large population (Yesalis et al. 1993) using these drugs, and accept the fact that it still remains to be proven that the associations described are more than chance observations. Hence, from a solely scientific point of view, one must acknowledge the fact that there is only *indirect* evidence pointing towards harmful effects and possibly life-threatening consequences of AAS abuse, including higher cardiovascular morbidity or mortality in bodybuilders using AAS.

In this article we will review the relevant literature regarding several risk factors for cardiovascular disease where the effects of AAS have been scrutinized. In many aspects, the use of supraphysiologic doses of AAS has shown a probable or certain impact on those risk factors. In addition, and of particular interest, we will review those studies that utilized the investigational modality of echocardiography, in order to find abnormalities in the hearts of the individuals using AAS. In this respect a substantial amount of data has been collected, where both cardiac structure as well as function have been investigated.

Reviewing the entire scientific literature covering such a broad topic as the one described here, the authors cannot refrain from applying a certain selection process to the studies that will be highlighted. This should, however, not restrict our conclusions drawn at the end of each section, which we believe reflect the current state of knowledge in the field.

2 Echocardiographic Studies

Cardiac size, i.e. chamber dimensions and wall thickness, as well as cardiac function and hemodynamics, have been amenable to noninvasive assessment since the introduction of echocardiographic techniques in the 1960s. Applying these diagnostic modalities to the study of the effects of AAS on cardiovascular structure and function has been a cornerstone of the growing knowledge base on cardiovascular (CV) side effects of AAS. Hence a substantial part of the chapter on CV effects of AAS will focus on those findings derived from diagnostic ultrasound applications. The first studies on the effects of AAS on the parameters mentioned above were done in the latter half of the 1980s. As technology has developed in a rapid fashion, particularly in the last decade, the echocardiographic methods have increased considerably in sensitivity and specificity. This has given researchers the opportunity to evaluate the heart's changes in different pathologic conditions and at various settings with a greatly improved precision compared to the early echocardiographic studies. While this statement is true for most if not all clinical and investigational applications of echocardiography, this is of course also the case regarding the appraisal of effects of AAS on cardiac structure and function.

Nonetheless, and perhaps somewhat surprisingly, the number of these studies (Table 1) is still rather few, given the long period of time since the first investigation was published in 1985 (Salke et al. 1985). The controversy concerning the side effects of AAS, especially within the bodybuilding milieu, has no doubt been partially fuelled by the conflicting results found in echocardiographic studies in the 1980s and 1990s. As is the case in evaluating all kinds of effects of AAS, the unavoidable study limitations pertinent to research in this particular population always make interpretation of findings debatable. Ethical concerns clearly make prospective, placebo-controlled double-blind studies with "real-life" dosages of steroids impossible to design and execute. In the studies that nonetheless *have* been carried out, the participants often use different types of steroids and different dosages, and the cycles of drug-use vary considerably. In addition, periods of abstinence are not alike, training routines differ, and there is also widespread use of so-called recreational drugs among users of AAS, which can influence the results. Another weakness of the studies is of course the limited number of study participants. Almost all of the studies are conducted on 15 or less AAS users. As previously mentioned, in the few truly randomized studies the drug dosages were low, and the study lengths short.

Nevertheless, and despite all these drawbacks, the echocardiographic studies viewed as a whole have given us an important and comprehensive body of evidence, and a solid basis for further investigations and discussion.

In general, most studies on the impact of AAS on echocardiographic parameters have been done in comparison to strength athletes without AAS use or to sedentary controls, or both. Interpretation of the studies has been made difficult by the everlasting controversy in the literature regarding whether resistance training in itself leads to structural changes in the myocardium, exceeding the degree of

Table 1 Human echocardiographic studies investigating the effects of AAS on cardiac structure and function

Author	Journal	Year	Study design	AAS (*n*)	Control +Exercise (*n*)	Control (*n*)	Follow-up	Adverse effects of AAS	Main findings
Salke	Med Sci Sports Exerc	1985	CS	15	15	15	–	–	
Pearson	Am J Cardiol	1986	CS	5	11	10	–	+	DD, ↑LVmass
Urhausen	Eur J Appl Physiol Occup Physiol	1989	CS	14	7	–	–	+	LVH, ↑IVRT (DD)
Zuliani	Int J Sports Med	1989	P	6	8	–	6 weeks	–	
De Piccoli	Int J Sports Med	1991	P	14	14	14	8 weeks	+	LVH, ↑LVmass, ↑IVRT (DD)
Thompson	J Am Coll Cardiol.	1992	CS	12	11	–	–	–	
Sachtleben	Med Sci Sports Exerc	1993	P	11	13	–	8 weeks	+	LVH, ↑LVmass, ↑LVDd, ↓VO_2max
Palatini	J Clin Pharmacol	1996	CS/P	10	14	–	11 weeks	–	
Yeater	Br J Sports Med	1996	CS	8	27	8*	–	+	↑$LVmass_I$
Dickerman	Clin J Sports Med	1997	CS	8	8	–	–	+	LVH, ↓LVDd (related to BMI)
Di Bello	Med Sci Sports Exerc	1998	CS	10	10	10	–	+	LVH, ↑LVmass, ↓CVI
Dickerman	Cardiology	1998	CS	10	7	–	–	–	
Karila	Int J Sports Med	2003	CS	20	–	15	–	+	↑LVmass, LVH
Hartgens	Int J Sports Med	2003	P	(1) 17 (2) 8	15 8	– –	8–16 weeks	–	
Climstein	J Sci Med Sport	2003	CS	23	23	–	–	+	Abnormal waveforms (cardiokymography)
Urhausen	Heart	2004	CS	17+15	15	–	–	+	↑$LVmass_I$, LVH, relaxation abnormality
Nottin	Am J Cardiol	2006	CS	6	9	16	–	+	↑LVmass, ↑LVDd, DD
Chung	Clin Endocrinol (Oxf)	2007	P			10	4 weeks	–	

(continued)

Table 1 (continued)

Author	Journal	Year	Study design	AAS (*n*)	Control +Exercise (*n*)	Control (*n*)	Follow-up	Adverse effects of AAS	Main findings
				10+ 10					
Krieg	Int J Sports Med	2007	CS	14	11	15	–	+	↑$LVmass_I$, LVH, DD
D'Andrea	Br J Sports Med	2007	CS	20	25	25	–	+	LVH, DD, systolic dysfunction
Kasikcioglu	Int J Cardiol	2008	CS	12	14	15	–	+	↑LVmass and ↑$LVmass_I$, Relaxation abnormality in left and right ventricles

* runners
CS=Cross-sectional, P=prospective
LV=Left ventricle
LVH=Left ventricle hypertrophy
IVRT=Isovolumic relaxation time
CVI=Cyclic variation index
VO_2max=Maximum oxygen consumption
LVDd=Diastolic left ventricle diameter
LV=Left ventricle
DD=Diastolic dysfunction

increase in body dimensions. Several studies have reported a slight concentric hypertrophy in strength athletes (Colan et al. 1985; D'Andrea et al. 2002; Dickerman et al. 1998; Pearson et al. 1986) not considered to use AAS. In most cases in these studies this was based on information provided by the subjects themselves, and could thus not be objectified or controlled. In contrast, other studies with careful control for AAS intake could not demonstrate increases in left ventricular mass above and beyond the proportions of the increases in body dimensions (De Piccoli et al. 1991; Urhausen and Kindermann 1999; Urhausen et al. 1989). In a study (Pelliccia et al. 1991) of 947 elite, highly trained subjects, only 1.7% had hypertrophy of the myocardium (wall thickness greater than or equal to 13 mm). These individuals were either rowers or canoeists, and one cyclist. In a follow-up study (Pelliccia et al. 1993), 100 highly trained athletes participating in weight and power lifting, wrestling, bobsledding and weight-throwing competitions were studied with echocardiography. None of them had wall thickness of the left ventricle which exceeded the generally accepted limits of normal (8–12 mm). On the other hand, the calculated left ventricle mass index was slightly, but significantly greater than in a control group of 26 sedentary healthy subjects. Taken together, much of the evidence is difficult to reconcile, and further scrutiny of the key studies is therefore mandatory in order to reach an understanding of the major issues in AAS research. The following studies have been chosen for their important contribution to the knowledge base.

In an observational, cross-sectional study, published in 1985, 15 bodybuilders abusing AAS were compared to 15 drug-free bodybuilders and the same number of inactive individuals who had never used AAS (Salke et al. 1985). The main finding of this study was a significant increase in the thickness of the left posterior wall and the interventricular septum of the left ventricle in both weight-training groups as compared to controls. There were no differences between the weight-training athletes with and without AAS when comparing the echocardiographic parameters of wall thickness and chamber size. There was, however, a larger degree of disproportionate septal hypertrophy in the AAS group, compared to the drug-free athletes, though not statistically significant. Cardiac dilatation was not observed, and there were no differences in fractional shortening, indicating no difference in ventricular contractility. Finally, there were no electrocardiographic differences between the three groups. The authors concluded that their study revealed no echocardiographic evidence that anabolic steroids potentiate the myocardial response to weight training.

Later, some studies have come to similar conclusions. On the other hand, the majority of these studies actually pinpointed differences between AAS-using and drug-free strength athletes quite clearly, yet never overwhelmingly.

One of those other negative studies (Zuliani et al. 1989) was a prospective study that also aimed to look at blood lipids. The authors could not find any echocardiographic differences between two groups of bodybuilders, a total of 15 persons, one using both AAS and growth hormone (GH), and the other being drug-free. The drug-using group had been free of steroids and GH for at least 8 weeks before entering the study. They self-administered GH and anabolic steroids of different

kinds, apart from one individual who restricted himself to GH use only. After six weeks of medication, there were no significant changes in chamber dimensions, wall thickness, or fractional shortening between the two groups. Apart from the effect of six weeks of AAS use, this study does of course not tell us anything about long-term functional or structural myocardial effects, which, alas, is typical of all prospective studies performed in populations using anabolic steroids.

In a cross-sectional study from 1992, 12 weightlifters on AAS were compared to 11 who claimed never to have used these drugs (Thompson et al. 1992). To be admitted to the study, the drug users were required to have used the drugs for at least three cycles of 6–10 weeks within the past year. Long-term use was not a requirement. All subjects had urine testing done. Interestingly, the detection of steroids in urine did not always match the drug intake as reported by the subjects. Only 66% of 29 declared drugs were actually detected, whereas 58% of the 12 users provided urine samples containing an unreported drug. One of the subjects denied steroid use, but drug testing still revealed traces of nandrolone. This illustrates one of many methodological problems in doing studies on this kind of population. As to the echocardiographic findings, no differences between the two groups were found regarding chamber size, wall thickness or myocardial mass, neither were there any differences in systolic and diastolic function, assessed by ejection fraction, rate of wall thickening and transmitral flow, respectively.

In another mainly negative study (Palatini et al. 1996), the authors did not find any significant echocardiographic differences between ten bodybuilders on AAS and 14 who had not taken drugs. The study on the drug users was done both at the end of a period of AAS intake, and at the end of a period of withdrawal. Only interventricular septal thickness was slightly greater in AAS users than nonusers, but the difference did not attain the level of statistical significance. Left ventricular mass indexed for height or body surface area was only marginally increased in AAS users. No differences were found between the AAS users at the end of a drug cycle (mean 8.4 weeks) and at the end of a withdrawal phase (mean 11 weeks). The *E*/*A* ratio (ratio of passive to active filling of the left ventricle, from the left atrium) assessed by Doppler measurement of left ventricle filling rate was a little higher in nonusers than in users, but did not reach statistical significance.

In a small study (Dickerman et al. 1998) published in 1998, four elite resistance-trained athletes using steroids were found to have significant left ventricular hypertrophy; one of them had a left ventricular wall thickness of 16 mm, which at that time was the largest wall thickness ever reported in a resistance-trained athlete. The investigators went on to look retrospectively at the echocardiographic data from their previous study of 16 bodybuilders (Dickerman et al. 1997c), eight of them on AAS, and eight drug-free. In the latter group, 43% had left ventricle wall thickness beyond the normal range (here defined as 11 mm), whereas all eight AAS users had increased wall thickness. In addition, one of the drug-free subjects and three in the AAS group were beyond the critical value of 13 mm, as defined in Pelliccia's large survey from 1991. None of the subjects demonstrated diastolic dysfunction, detected by mitral inflow velocity patterns. Based on these findings, the authors dispute previous publications stating that left ventricular wall thickness

does not occur in resistance-trained athletes without AAS use, and that the short bursts of arterial hypertension that occur with weightlifting cannot stimulate left ventricular wall thickening. They conclude that wall thickness equal to or above 13 mm can be found in athletes both with and without AAS use. They believe the drugs may increase left ventricular wall thicknesses indirectly through their ability to increase strength, thus allowing a greater overall pressure response with weightlifting.

In a thorough and often cited study from 2003, 25 strength athletes using AAS were compared to 23 drug-free strength athletes (Hartgens et al. 2003). The majority were bodybuilders, a few were power lifters. The investigation was designed and carried out as two separate studies. In Study 1, 17 drug users, who intended to start self-administration, were compared to 15 nonusers. The users had all taken AAS previously, with an average of 4.6 years use before entering the study. They were expected to be free of AAS use for at least 3 months. The subjects were examined at the start of the study, and after eight weeks of self-administration of AAS. A few (seven) were also examined after 12–16 weeks of AAS intake. In Study 2, 16 well-trained recreational bodybuilders were recruited. They were randomized to i.m. injections of either nandrolone decanoate 200 mg/week or placebo. In both groups two subjects had previous experience with AAS, while the remainders had never used AAS. The injections were given in a double-blind fashion. Echocardiographic measurements were performed at baseline and after an eight-week study period. In Study 1, no significant alterations could be detected in the echocardiographic assessment of heart morphology and function after eight weeks. In addition, there were no differences between short-term (eight weeks) and long-term use (12–16 weeks). In Study 2, eight weeks of nandrolone decanoate did not induce significant alterations in echocardiographic measurements of heart morphology nor in parameters reflecting systolic and diastolic function. There were, however, enlargements of the left ventricle and left ventricular wall thickness in a minority of the users in both groups, but considered to be within the limits of physical adaptation to vigorous and demanding training regimens. The authors concluded that steroid regimens of a single- and polydrug regimen as described in this study, as a short-term AAS administration, did not lead to detectable echocardiographic alterations. However, since detrimental cardiac effects of short-term AAS treatment with supraphysiologic doses have been described in animal studies, the authors of this study believed that echocardiographic evaluation may provide incomplete assessment of the actual cardiac condition in AAS users since the method is not sensitive enough to detect alterations at the cellular level. Therefore they stated that no conclusions could be drawn regarding the cardiotoxic effects of long-term AAS abuse.

Later studies have to a certain extent proved the authors right. The introduction of tissue Doppler velocity imaging has given researchers an echocardiographic modality which is more sensitive than traditional echocardiography and standard Doppler measurement of blood flow. The most recent studies have utilized this method and have been able to detect pathological changes not identifiable with conventional echocardiography. We will look closer into these studies later on in this chapter.

As previously mentioned, there are very few prospective, randomized studies looking at cardiac structure and function. Recently, Chung et al. conducted a double-blind, placebo-controlled study on 30 healthy young men, randomized into three groups of ten subjects (Chung et al. 2007). They received four weekly intramuscular injections of 200 mg testosterone esters (Sustanon), 200 mg nandrolone decanoate (Deca-Durabolin), or placebo. None of the participants were athletes. In this study the subjects went through a complete echocardiographic assessment, including the more sensitive method of myocardial tissue velocity measurement with the derived methods of peak systolic strain and strain rates. Bioimpedance measurements of cardiac output and systemic vascular resistance were also performed. Only four weeks of treatment and follow-up was probably the main reason why the study came out completely negative regarding cardiac function, though minor changes within the normal range were observed solely within the testosterone group, among the most interesting being a minor reduction of left ventricle diastolic septal velocity and a slight increase in left ventricle filling pressure.

As previously indicated, several studies, even the older ones, have demonstrated significant increases in left ventricular wall thickness and slight impairment of diastolic function among strength athletes using AAS. In a study by Pearson et al. (1986), 16 weightlifters were examined and compared to ten age-matched control subjects. There was no difference in wall thickness, when corrected for body surface area. Weightlifters had significantly higher LV mass and LV mass index than controls, but there was no difference in Doppler indexes of diastolic filling, indicating that the concentric hypertrophy observed was consistent with a physiologic response. However, five of the weightlifters were AAS users, and in this subset of the group, the Doppler indexes of diastolic filling were significantly reduced. Pathological changes in diastolic filling were also demonstrated in a study by Urhausen et al. (1989). In this cross-sectional study, 14 bodybuilders using AAS were compared to seven who did not use these drugs. The isovolumic relaxation time (the time from aortic valve closure to mitral valve opening) was prolonged in the AAS group, indicating a minor impairment of diastolic function. In addition, the AAS users had lower left ventricle diameter, in spite of having almost identical total heart volume and left ventricle mass as the nonusers. The ratio of left ventricle mass to diameter was increased in the user group.

In another study, by De Piccoli et al. (1991), quite similar changes were demonstrated. In this prospective study, echocardiographic parameters were measured in 14 bodybuilders after eight weeks of self-administration of AAS. The findings were compared to 14 bodybuilders who had never taken steroids, and to a group of 14 sedentary individuals. In addition, the AAS users were examined following nine weeks of drug suspension. Once again, a study of very modest size revealed structural myocardial changes in the user group, although not impressive. Increased wall thickness (ventricular septum) and an increase in left ventricular mass were apparent in the steroid-user group. In the same group there was also a significant increase in the left ventricle end systolic diameter and end diastolic volume. There were, as in previous studies, no changes in systolic function (as judged by ejection fraction). In accordance with Pearson's and Urhausen's

findings, there were signs of diastolic impairment in the AAS group, shown as significantly longer isovolumic relaxation time. There were no significant differences in blood pressures in the three groups, which indicate that mechanisms other than hypertension lie behind the aforementioned changes, for example a direct effect of the steroids. Interestingly, at the nine-week post-steroid examination, the changes were still there, which urged the investigators to presume that such a period is not enough to restore the morphologic and functional characteristics of the heart.

Looking at 11 weight-training males eight weeks after a period of self-administration of AAS, and again at the top of their next cycle, and comparing them to 13 weight-training males who had never used AAS, Sachtleben et al. (1993) found significant morphological changes, but no signs of systolic impairment in either group. In this longitudinal study the users on cycle had larger ventricular mass and increased interventricular septal thickness as compared to measurements off cycle. These findings were also significant compared to the control group, in addition to the left posterior wall thickness being increased. Another interesting finding was a significantly lower maximum oxygen consumption (VO_2 max) in the two user groups compared to the control group. VO_2 max was assessed using a Quinton treadmill, where the subjects walked until a defined maximum speed was reached, depending upon heart rate response. Then the grade was increased by 2% every minute until exhaustion. Expired air was collected and gas analyzes conducted, enabling calculation of VO_2 max. The finding of lower VO_2 max was in spite of the users having reported more frequent aerobic training sessions, though they were shorter in duration. The results in this study indicate that the myocardium, during resistance training, is thickened by approximately six weeks of AAS use (peak cycles occurring at 5–9 weeks).

In another cross-sectional study (Yeater et al. 1996) comparing 24 highly trained resistance athletes, among them a group of eight AAS users, to a group of recreational lifters and a group of eight cross-country runners, the VO_2 max did not differ between the groups of resistance athletes, as was the case in the study by Sachtleben et al. The cross-country runners had, as would be expected, significantly higher VO_2 max than the other groups. As for the echocardiographic assessments, the AAS users had significantly greater left ventricular mass index than the recreational lifters, but not compared to the highly trained lifters without steroids or the runners. Wall thickness was larger in the steroid group, but did not reach statistical significance. Diastolic functions, assessed by transmitral filling indices, were not compromised.

The finding of increased left ventricular mass and increased wall thicknesses in AAS users has been confirmed in other studies as well, among them studies by Di Bello et al. and Karila et al. In the former (Di Bello et al. 1999), ten male weightlifters using AAS were compared to ten without, and to ten healthy sedentary controls. In addition to conventional echocardiography, the participants were examined with ultrasonic videodensitometry, a method able to detect alterations in ultrasonic textural myocardial parameters. The steroid users had a significantly lower cyclic variation index for the septum and posterior wall compared to both nonusers and healthy subjects. The authors' interpretation of this finding was that the changes could probably represent the onset of a specific cardiomyopathy.

Despite an increase in left ventricular mass among the nonusers, they showed normal cyclic variation, indicating a normal ultrasonic myocardial texture. In Karila et al. (2003)'s cross-sectional study of 20 AAS users, there was a significant association between left ventricular mass and the mean daily AAS dose. There was no correlation with body dimensions, except in the control group of 15 sedentary men. Of the 20 users, four were also injecting growth hormone, and it appeared that the combination treatment resulted in a larger degree of hypertrophy than in the AAS-only group. There was no difference in diastolic filling indices.

As to the reversibility of adverse cardiovascular effects after chronic abuse of AAS, not many studies have addressed this question. In Palatini's study the subjects were examined 11 weeks (mean) after withdrawal without finding any changes (Palatini et al. 1996). But the slight changes in myocardial structure noted after a cycle of steroids of eight weeks did not reach significance anyway. In Urhausen's study from 2004 reversibility was addressed specifically (Urhausen et al. 2004). Thirty-two bodybuilders or powerlifters were examined, among them 17 currently on AAS and 15 who had not been taking the drugs for at least a year (mean 43 months). They were compared to 15 AAS-free weightlifters from the national team. Both users and ex-users had higher left ventricular mass related to fat-free body mass than the AAS-free weightlifters. Wall thicknesses were also smaller in the group of weightlifters, but there was no difference between the users and ex-users. Left ventricular wall thickness was correlated with a point score estimating AAS abuse in users. As almost all other studies have concluded, no significant changes in systolic function were noted. However, the maximum late transmitral Doppler flow velocity was higher in users and ex-users than in weightlifters, indicating a slight alteration of diastolic function. The authors concluded that several years after the discontinuation of AAS abuse, strength athletes still show a slight concentric left ventricular hypertrophy in comparison with AAS-free strength athletes.

Urhausen and Kindermans group later published another study (Krieg et al. 2007) where the objective was to clarify whether AAS abuse also induced alterations in left ventricle function, as opposed to mere anatomical changes. Fourteen male body-builders with a substantial intake of AAS were examined with echocardiography and cardiac tissue Doppler imaging. They were compared to 11 steroid-free strength athletes and to 15 sedentary control subjects. As in their previous study, there was an increase of left ventricular muscle mass index in the user group. There were no differences in transmitral filling indices using Doppler measurements of blood flow. However, using tissue Doppler imaging in the mitral annulus, there were significantly reduced values in the user group compared to the other groups. These findings are considered to be a clear sign of compromised diastolic function in the AAS group. There were no differences in systolic parameters. Tissue Doppler imaging, as alluded to above, is known to be more sensitive to changes in diastolic function than the traditional transmitral flow measurements, most probably explaining why several previous studies have come out negative in this respect.

In a study from 2006, Nottin et al. (2006) sought to evaluate the effects of regular AAS administration in bodybuilders using pulsed tissue Doppler imaging (TDI) to evaluate left ventricular relaxation properties. They examined 15 bodybuilders, six

of whom were currently on AAS, and nine of whom had never taken the drugs. The steroid users were required to have used AAS for the past two years. All the strength-trained athletes had been training for 5–12 h weekly for 10 or more years. Sixteen age-matched sedentary males served as controls. Although there were no differences in wall thicknesses between the groups, the drug-using bodybuilders exhibited larger end-diastolic diameters, volumes and masses than their drug-free counterparts. Most strikingly, though, were the changes noted in pulsed TDI measurements. The AAS users had smaller peak Em (peak passive tissue velocity at the level of the mitral annulus) and smaller peak Em/Am (Am being the active contribution of filling) than both of the other groups. This indicates that the AAS users exhibited depressed left ventricular diastolic function, characterized by a decrease in the contribution of left ventricle passive filling to left ventricle filling. There were also alterations of transmitral flow velocity properties, indicating reduced diastolic function. This has also been noted in several previous studies, although not uniformly. Apart from being a more sensitive method than flow velocity measurements, pulsed TDI also has the advantage of being relatively preload-independent, and is generally accepted as being a good index of left ventricular filling properties. In accordance with previous studies, no changes of systolic function were noted in this study either. Using pulsed TDI, there were no differences in the peak E/Em, which indicates normal left ventricular filling pressures as well. As no significant hypertrophy was detected among the AAS users, these findings indicate that an alteration of intrinsic left ventricle relaxation per se is responsible for the depressed diastolic function. This study was also the first to assess diastolic function with TDI in this population.

The only study so far which has utilized TDI *and* the derived method of strain rate imaging to evaluate cardiac function in strength athletes with chronic AAS abuse was published by D'Andrea et al. (2007). In this study the authors included 20 bodybuilders who had taken AAS for at least five years, 25 steroid-free bodybuilders and the same number of age-matched healthy sedentary controls (D'Andrea et al. 2007). Both users and nonusers showed increased wall thickness and stroke volume as compared to controls, whereas left ventricular ejection fraction, end-diastolic diameter and transmitral Doppler indexes were comparable between the three groups. TDI showed significantly lower Em/Am ratios in users at the level of both the basal interventricular septum and the left ventricular lateral wall, which is in accordance with the findings of Nottin et al. In addition, and perhaps most notably, the users had a significant reduction in peak systolic strain and strain rate in the middle interventricular septal wall as well as in the left ventricular lateral free wall. The strain rate imaging technique, assessing longitudinal deformation within the myocardium, gives quantitative information of local myocardial function, and it is not influenced by global cardiac displacement and any tethering effects. As such, it is superior to other techniques assessing regional velocity profiles. The findings in this study point to an early impairment of left ventricular myocardial contractile function in AAS users, and hence constitute a watershed point with regard to picking up such subtle systolic impairments, although Di Bello and co-workers (Di Bello et al. 1999) actually also had claimed to

find the same functional changes by a completely different technique, the ultrasonic myocardial backscatter/videodensitometric measurement. The study by D'Andrea et al. further revealed that the number of weeks of AAS use per year and the weekly dosage of AAS were independent determinants of impaired strain rate. Adding to the negative cardiac effects of AAS was the fact that impaired left ventricular strain in AAS users was associated with a reduced performance during physical effort, as measured by bicycle ergometric testing.

And finally, adding yet another piece to the somewhat blurred, yet comprehensive and nonetheless increasingly recognizable, puzzle of AAS and the heart, Kasikcioglu et al. very recently published another study addressing diastolic function. Twelve bodybuilders on AAS were compared to 14 nonusers, using conventional echocardiography and TDI. Again the latter method revealed impaired relaxation properties, and in this study not only in the left ventricle, but also in the right, which had not been investigated by TDI before (Kasikcioglu et al. 2008).

2.1 Summary

In conclusion, the echocardiographic studies on AAS using subjects viewed as a whole have produced conflicting results over time, partly due to changes in – and improvement of – investigational modalities, partly due to insufficient and in some cases frankly debatable study designs.

Yet it is safe to state that AAS use does lead to both morphologic and functional changes in the heart. These changes encompass (a) a tendency to produce a myocardial, predominantly septal hypertrophy, partly as a consequence of resistance training and AAS, (b) a possible increase of heart chamber diameters, although not as manifest as seen in endurance athletes, (c) unequivocal alterations of diastolic function and ventricular relaxation, particularly as assessed by modern tools, and (d) a subclinically compromised left ventricular contractile function, detectable only by the most sensitive modern investigational modalities.

As far as prognosis is concerned, the clinical significance of these AAS-induced changes remains to be established, even though many of the phenomenological changes (such as impaired relaxation of the left ventricle) are indicators of diseased hearts prone to deterioration over time.

3 Hypertension

The frequently reported findings of severe myocardial hypertrophy in case reports of abusers of anabolic androgenic steroids (AAS), have led to the suspicion that the drugs may induce arterial hypertension, which could explain these structural changes in the heart. In population studies, arterial hypertension is the most prevalent reason for myocardial hypertrophy (Mancia et al. 2007). Studies of rats

and mice have with some frequency reported hypertensive effects of AAS, the first one as early as in 1940 (Grollman et al. 1940). Several possible mechanisms have been suggested, one of them being through an elevation of 11-deoxycorticosterone (DOC), which is caused by testosterone reducing 11β-hydroxylase activity, thus reducing the conversion of DOC to cortisol (Brownie et al. 1978; Gallant et al. 1992). Another contributing mechanism could be by enhanced reactivity of vessels to norepinephrine, which in animal studies has been shown following the administration of testosterone (Ammar et al. 2004; Baker et al. 1978; Bhargava et al. 1967; Greenberg et al. 1974). It has also been reported that testosterone raises plasma renin activity, and that expression of renin mRNA in the adrenal gland, kidney and brain is androgen-dependent (Katz and Roper 1977; Wagner et al. 1990). Aldosterone production is stimulated by testosterone hemisuccinate, according to a study performed in bovine adrenal tissue, which could be another possible contributing mechanism leading to hypertension (Carroll and Goodfriend 1984). In a recent study on rats, the administration of stanozolol for eight weeks caused a significant increase in arterial blood pressure, possibly through increases in cardiac output and peripheral resistance (Beutel et al. 2005).

References to hypertensive effects during self-administration of AAS are common, and indeed Volume II of *The Underground Steroid Handbook* notes hypertension to be a transient effect of AAS that resolves with completion of the cycle and leaves no residual effects (Duchaine 1989). On the other hand, the clinical studies addressing possible hypertensive effects among strength athletes using AAS have not been able to convincingly confirm these in humans. A few studies have, however, registered a slight elevation of blood pressure compared to strength athletes without AAS or healthy controls, but the main body of evidence tends to lean in the other direction. Amongst the human studies addressing this question, almost all have done so as part of a more comprehensive, broader study looking at other aspects of AAS abuse, for instance the effects on lipids, or as part of an echocardiographic study.

Two human studies performed in the seventies looked at several physiologic and hemodynamic effects of methandienone, among them blood pressure response. In the study by Freed et al. (1975), 13 male weightlifters took either 10 or 25 mg daily, or placebo, for six weeks in a double-blind crossover trial. In the steroid group the systolic blood pressure increased slightly, but significantly, while the diastolic blood pressure remained unchanged. This was of course a small study and there were also withdrawals. In Holma's study 16 well trained athletes participated (Holma 1977). They were tested before and after two months of a daily oral dose of 15 mg methandienone. Several hemodynamic parameters were measured. Even though the total blood volume increased by 15%, there were no significant changes in resting values of cardiac index, peripheral resistance or mean arterial pressure during methandienone treatment.

In a later cross-sectional study (Lenders et al. 1988), 45 amateur bodybuilders had their serum lipoproteins, blood pressure and liver function examined, following self-administration of different types of AAS over a mean period of 1.7 years (range 0.2–13.0). Twenty of them were studied at the end of a course, 42 after

discontinuation of AAS for a mean of five months and 16 after a discontinuation for at least two months and at the end of a nine-week course of AAS. A group of 13 AAS-free bodybuilders served as a control group. All steroid groups showed a slight, but significant, increase of systolic blood pressure, in the range of 3–7 mmHg. This was also the case in the discontinuation group. There were no changes in diastolic blood pressure. In another study of 18 competitive bodybuilders self-administrating AAS, though mainly focusing on the lipid effects, there were no differences in systolic or diastolic blood pressure before and after a 16-week course of AAS, or when compared to a group of non-AAS-using bodybuilders (Kleiner et al. 1989).

There is very little data on blood pressure response to exercise in AAS users. This question was addressed in a study by Riebe et al. (1992) where both rest and exercise blood pressure were measured in nine weightlifters using AAS, and compared to ten individuals not using the drugs, and to ten sedentary controls. The subjects performed a treadmill test until exhaustion as well as a submaximal weightlifting exercise. The AAS users had significantly higher resting blood pressure, though not hypertensive values. They also had higher exercise maximal systolic pressure, exercise diastolic pressure and mean arterial pressure. But there was no significant difference between the groups for the change in systolic, diastolic or mean arterial pressure from rest to maximal exercise. When adjusted for body weight, there were no differences between the groups for either rest or exercise blood pressure values. However, statistical limitations in the study urge the authors to doubt that this alone can explain the differences. When adjusted for biceps size, the higher diastolic blood pressure during exercise in the AAS group still remained significant, which the authors believe implies a possible signal of subsequent hypertension in this group.

An often cited study in this context is the one by Kuipers and Hartgens' group, from Maastricht. In this study 26 male bodybuilders participated (Kuipers et al. 1991). They all had at least three years experience of strength training. They were divided into three groups; one group (SAD) self-administered anabolic steroids for 8–10 weeks, the second group received i.m. injections of 100 mg nandrolone decanoate weekly or placebo over a period of eight weeks, in a double-blind fashion. The third group received the same treatment, but as a double-blind cross-over study, with weekly injections for two periods of eight weeks, separated by a 12-week washout period. Body composition, lipid profile, liver function and blood pressure were studied, the latter noninvasively. In the SAD group the systolic blood pressure increased from 122.3 to 134.0 mmHg (all mean), but this was not a significant difference. However, the diastolic values increased significantly, from 73.6 to 86.0 mmHg. Six weeks after discontinuation, the diastolic blood pressures had returned to pre-treatment values. In the other two groups there were no differences found in either systolic or diastolic blood pressures. As could be expected, the steroid dosages in the SAD group were considerably higher than in the other two groups, and it is therefore suggested that the effect on blood pressure is dose-dependent. The authors believe that a possible explanation for the increase

in blood pressure is through an increase in blood volume, a mechanism which has been suspected by other investigators as well (Riebe et al. 1992; Wilson 1988).

In another study (Hartgens et al. 1996) a few years later, the same group investigated several of the same parameters after at least three months of drug withdrawal in 16 long-term AAS users compared to 12 nonusers, all male body-builders. The drug users had taken i.m. and oral steroids for an average of 3.2 (0.7–6.0) years prior to the withdrawal period. The average systolic and diastolic blood pressures were normal and there was no difference between the two groups. Based on these findings, the authors suggested that blood pressure is within the normal range after an AAS-free period of three months, independent of the number of AAS courses used.

The echocardiographic studies on AAS abuse have been discussed previously. However, most investigators also registered blood pressure in all participants and these results need further mention. Beginning with De Piccoli's study, in which 14 bodybuilders on steroids were compared to the same number of drug-free body-builders and sedentary controls, there was no difference in systolic or diastolic blood pressure (De Piccoli et al. 1991). The drug users were also examined after a period of drug withdrawal (mean nine weeks) without any changes in blood pressure being noted. Even though the blood pressures were normal, the investigators interestingly still found signs of impaired diastolic function and an increase in left ventricular mass, and one could therefore assume that these changes were not secondary to hypertensive blood pressures, but rather a direct effect of the drugs taken. In another echocardiographic study by Thompson et al. (1992), 12 weigh-tlifters using AAS were compared to 11 who had not used these drugs. For admission to the study, the users were required to have taken the drugs for at least three cycles within the past year, and the mean length of drug use was 3.3 years. Again, there were no differences in systolic or diastolic blood pressure between the groups.

In the study by Yeater et al. (1996), weightlifters with or without AAS were compared to runners; echocardiographic and other cardiorespiratory variables as well as lipids and body composition were measured. There was no difference in resting blood pressure or blood pressure at the end of a treadmill test between the weightlifter groups. The runners had lower diastolic pressure during recovery from exercise, though all groups had normal values. It should be noted that the subjects were young, with an average age of 21 (all groups), and the steroid users had taken the drugs for a minimum of six months.

There are almost no data on 24-h blood pressure monitoring in the AAS population. However, in one study by Palatini et al. (1996) the participants under-went a 24-h noninvasive blood pressure measurement, though this study primarily was another small echocardiographic study. Ten bodybuilders with an average age of 28 years, who had self-administerered AAS for an average length of five years (range 2–10 years) were compared to 14 nonusing bodybuilders. The steroid users were examined at the end of a treatment period and after a withdrawal period of 8–16 weeks (mean 11 weeks). There was no difference in resting blood pressure between the groups, and systolic and diastolic blood pressures were within normal

limits. Average 24-h, daytime and night-time systolic and diastolic blood pressures did not differ in the three groups of AAS users during treatment, users after withdrawal or control subjects. However, the difference between daytime and night-time blood pressure was greater in control subjects than in AAS users, both at the end of the treatment cycle and after the period of withdrawal. Another interesting finding in this study was that the extent of the blood pressure decrease at night was inversely related to the interventricular septal thickness and the concentric remodeling of the left ventricle in the steroid group. This study suggests that weightlifting per se does not alter the 24-h blood pressure rhythm, but the addition of AAS does. An abnormal 24-h rhythm of blood pressure has been observed in many subjects with secondary hypertension and in some patients with essential hypertension, and target organ damage appears to be more common in nondippers (Persons in whom there is an absence of the normal nocturnal fall in blood pressure) (Boggia et al. 2007; O'Brien et al. 1988; Palatini et al. 1992; Verdecchia 2000). The data from this study must nonetheless be treated with caution, as this was a small study, and to our knowledge altered 24-h blood pressure rhythm in AAS users has not been confirmed in any later studies. Another reservation is that the main reason for the difference mentioned seems to be that the nonusers had a slightly higher systolic pressure in daytime, and therefore a higher daytime pulse pressure.

In the echocardiographic study by Karila et al. (2003), there were no differences in blood pressure values between the control group and a group of 20 strength athletes, mainly bodybuilders, who were long-term users of AAS. Four of the steroid users had also taken growth hormone (GH) and notably their average diastolic blood pressure was higher (89 mmHg vs. 77 mmHg in the control group and 76 mmHg in the AAS group without GH) than the two other groups, but statistic significance was not reached, possibly due to the low subject numbers.

In the prospective echocardiographic study by Hartgens et al. (2003), where two groups of AAS users were compared to nonusing strength athletes, there were no changes in systolic or diastolic blood pressure. One AAS group self-administered the drugs for 8–16 weeks, and the other received 200 mg of nandrolone decanoate once a week for eight weeks in a double-blind fashion. The authors concluded that one of the main findings in their study was that short-term administration of AAS did not affect blood pressure.

In a cross-sectional study addressing reversibility of cardiac effects of AAS abuse, Urhausen et al. (2004) examined their subjects with echocardiography and cycle ergometry including measurements of blood pressure at rest and during exercise. Fifteen former AAS users (at least 12 months withdrawal, mean 43 months) were compared to 17 current users and 15 AAS free weightlifters. Systolic blood pressure was higher in users, measuring an average of 140 mmHg at rest vs. 130 mmHg for ex-users and 125 mmHg for the nonusers. This difference persisted as a trend for exercising blood pressure. There was no difference in diastolic blood pressure. The authors suggested that the increases in blood pressure with AAS are rather small and transient.

In a double-blind, randomized, placebo-controlled study by Chung et al. (2007) the effects of weekly injections of testosterone, nandrolone or placebo on cardiovascular parameters were investigated, using state-of-the-art echocardiographic methods. There were ten individuals in each group, all of them healthy young men, but none were involved in strength athletics. They received the treatment for four weeks only. There was no effect on systolic, diastolic or mean blood pressure, or on systemic vascular resistance, assessed by thoracic electrical bioimpedance.

Blood pressure as well as rate pressure product (RPP) response to AAS was investigated by Grace et al. (2003) in 16 users compared to 16 nonusers, all subjects being amateur bodybuilders in their mid-twenties. They all had a minimum of three years of weight-training experience. The drug users had taken various AAS for a mean length of 4.3 years in rather large dosages. The subjects' blood pressures were measured noninvasively and the rate pressure product was calculated as (HR $\times$ SBP/100). The recordings were obtained before a steroid cycle, at the end of the cycle and 6–8 weeks post-cycle. The control group was assessed within the same time intervals. Significant increases were found for both diastolic (79–87 mmHg) and mean arterial blood pressure in the AAS group, but not for systolic blood pressure. Even when adjusted for biceps size, diastolic blood pressure was still significantly greater in this group. There was also a significant increase in resting heart rate in the AAS group, with a subsequent increase in RPP. All mentioned parameters returned to pre-cycle baseline levels within 6–8 weeks following drug cessation. The data from this study indicate that AAS acutely influences blood pressure, especially diastolic blood pressure, though it does not seem that the drugs cause hypertension, and the rise in blood pressure appears to be transient. However, the authors suggest that these findings provide a contra-indication to AAS use, especially in borderline hypertensives.

3.1 Summary

Hypertension as a risk factor for cardiovascular disease is well established (Lewington et al. 2002). Although hypertensive effects of supraphysiologic doses of AAS are often referred to in the bodybuilding community and among self-administering AAS users, the scientific support for this view is scarce. Animal studies have to some extent shown hypertensive effects of AAS, and have given rise to a variety of theories as to the underlying mechanisms. The human data, however, are less convincing, and the overall evidence base leans to the opposite. One must keep in mind that the studies are small and most of them are cross-sectional in design. Some of the studies show a slight increase in systolic blood pressure, some show a slight increase in diastolic blood pressure and many do not show any significant pressure changes at all. The studies assessing reversibility demonstrate that when a pressure increase has been found, it is transient and that full normalization is achieved only a few months after cessation of the drugs.

Hence, taken together, it is likely that the administration of supraphysiologic doses of AAS induces a mild, but transient increase of blood pressure; however the clinical significance of this effect remains modest.

4 Vascular Reactivity and Vascular Function

The frequently reported observations of adverse cardiovascular events among abusers of anabolic androgenic steroids (AAS) have stimulated an interest in possible underlying mechanisms beyond those attributed to established atherosclerosis. It remains a notable fact that coronary angiography and/or autopsies of deceased users of AAS have often shown patent coronary arteries, in spite of the subjects having suffered a myocardial infarction or sudden death. One proposed explanation is steroid-induced vasospasm, perhaps superimposed on or triggered by pro-atherogenic state. It has been shown that androgens can promote monocyte adhesion to endothelial cells (McCrohon et al. 1999) and macrophage lipid loading (McCrohon et al. 2000). Androgens have also been associated with impaired arterial reactivity in females taking high-dose androgenic steroids (McCredie et al. 1998), and, conversely, endothelial function is enhanced in androgen-deprived older men (Herman et al. 1997, Sader et al. 2003; Zitzmann et al. 2002). Endothelial dysfunction has been demonstrated to be an early finding in both experimental and clinical studies of atherogenesis, preceding plaque formation and the occurrence of clinical events (Celermajer et al. 1992; Ross 1993).

There are only a few studies addressing vasoreactivity in the population of AAS abusers. Sader et al. (2001) examined 20 male bodybuilders, ten of them using AAS and ten who denied ever having used the drugs, and compared them to a group of ten age-matched non-bodybuilding controls. The drug users' mean age was 37 years and the mean duration of steroid abuse was 6.6 years. The investigators measured carotid intima-media thickness (IMT) and arterial reactivity using high-resolution ultrasound. For the latter they studied the right brachial and radial artery, measuring the artery diameter at rest, during reactive hyperemia (leading to flow-mediated dilatation, FMD) and after administration of sublingual nitroglycerine (GTN), the two measurements enabling assessment of endothelial-dependent and endothelial-independent vasodilatation. The AAS users had slightly, but not significantly larger vessels than the bodybuilder controls. Despite this, FMD responses were similar in both groups of bodybuilders. The responses tended to be lower than in the non-bodybuilding controls, but not significantly so. The GTN responses, assessing endothelial-independent vasodilatation, were also not significantly different between the bodybuilder groups, but significantly lower compared to the control group of non-bodybuilders. Carotid IMT measurements were similar in both bodybuilding groups, but significantly higher than in the control group. The authors suggest that their findings might be explained by a defect in smooth muscle capacity, secondary to increased water retention and/or increased vascular muscle mass impairing smooth muscle dilator responses. But a main point still seems to be

that AAS does not further impair these responses compared to the bodybuilder group not using the drugs.

In another study of flow-mediated dilatation, Ebenbichler et al. (2001b) examined 20 male bodybuilders in different phases of their training and AAS cycle. FMD was determined in the same way as in the previously mentioned study. The subjects were examined in an eight-week training phase without steroids, then in a "build-up" phase, where AAS was taken for 8–12 weeks, and then after up to eight weeks following cessation of the drugs, in the "competition phase". Six athletes not using AAS served as control group. The percent change in brachial artery diameter after reactive hyperemia was diminished in bodybuilders in all phases and the reduction was most pronounced in the competition phase, the differences compared to the control group being significant. Nonendothelial vasodilatation, measured as response to sublingual GTN, was also reduced, though not significantly. Two mechanisms are proposed as explanation of these findings. One could be by endothelial dysfunction through the low HDL-cholesterol found in the steroid user group, the other by a direct effect of AAS on vascular function, which the authors speculate is the most likely mechanism.

In a recent study by Lane et al. (2006), vascular reactivity was assessed by using pulse wave analysis with GTN and salbutamol, to determine endothelial-independent and endothelial-dependent vasodilatation, respectively. The pulse wave analysis noninvasively assessed arterial stiffness and central aortic pressure. Twenty-eight bodybuilders took part in the study; ten who were current users of AAS were compared to eight regular users who had abstained from using substances for the previous three-month period, and to ten who had denied any current or previous use of AAS. Ten healthy sedentary individuals served as control group. Subjects in all four groups had comparable brachial artery blood pressures and resting pulse rates. The administration of salbutamol altered the central arterial waveform and reduced the augmentation index (AIx) in the four groups, reflecting endothelial-dependent dilatation, but there were no significant differences in the degree of change between the groups. Likewise, the GTN administration reduced the AIx in all four groups, but here the percentage reduction of AIx *was* significantly less in the current AAS users compared to the other three groups. The study therefore suggests that there is no difference in endothelial function between the four groups, but that there is reduced endothelial-independent vasodilatation in the group of current AAS users. There were no differences in AIx alterations between the other three groups, suggesting recovery of vasomotor function following the cessation of AAS.

It has been shown that alteration in elastic properties of the aorta can predict cardiovascular risk (Gosling and Budge 2003). Kasikcioglu et al. (2007) therefore examined 14 male bodybuilders using AAS and compared them to 27 male wrestlers, using a standard Doppler echocardiography unit, and measuring the ascending aorta with two-dimensional guided M-mode tracings. Using the systolic and diastolic aortic diameter, pulse pressure and diastolic blood pressure, the investigators calculated aortic strain, aortic distensibility and aortic stiffness index. Their main finding was that the aortic distensibility in athletes using AAS

was significantly lower than in nonusers. This is in contrast to what has been found in endurance athletes (Kasikcioglu et al. 2005), where aortic distensibility was increased.

In summary, there is conflicting evidence regarding vasoreactivity in users of AAS. Nevertheless, based on the studies mentioned before, the steroid-using groups most often tend to demonstrate unfavorable measurements, whether it is involving endothelial-dependent or endothelial-independent vasodilatation. A degree of reversibility seems to be consistent, though. The inconsistent results are likely to arise from the obvious study limitations which are unavoidable in such populations of abusers of AAS. There is a diversity of compounds abused, and a variety of duration and route of administration. Adding to the difficulties of interpreting the results are the highly prevalent simultaneous abuse of other drugs, such as e.g. ephedrine, growth hormone and insulin. Despite these drawbacks, the accumulated evidence base supports the notion of a deleterious effect of AAS abuse on vascular function.

5 Coronary Calcification

Coronary artery calcium content (expressed as a so-called "calcium score") has been shown to correlate with the total plaque burden in the coronary arteries and hence to be a more direct indicator of endothelial damage than other risk factors, such as low HDL-cholesterol (Brown et al. 2001; Kondos et al. 2003; Rumberger et al. 1995). This applies to all populations hitherto studied.

As regards the population of abusers of anabolic androgenic steroids (AAS), a study published in 2006 by Santora and colleagues measured the coronary artery calcium score of 14 professional bodybuilders with electron beam tomography (Santora et al. 2006). The age of the participants in the study ranged from 28 to 55 years and the mean number of years of AAS use in the group was 12.6 years. None of them had a history of cardiovascular disease. Seven of the 14 men had coronary artery calcium accumulation, i.e. an increased score. According to previous large population studies (Hoff et al. 2001; Mitchell et al. 2001), where percentile tables could be established that provide expected coronary calcium scores for different age groups, the likely number to have such a finding in this age group would have been three (as opposed to seven). As anticipated, all participants had significantly lower HDL-cholesterol than the typical population. None of them had any clinical signs of coronary artery disease. Ejection fraction, measured with echocardiography, was within the normal range for this age group.

The authors concluded that this study for the first time indicates that the abuse of AAS contributes directly to the early development of coronary atherosclerosis (Fig. 4), as determined by noninvasive measures of coronary artery calcium. Whether the pathogenesis is indirectly due to the unfavorable effect upon lipid profile, or a direct toxic or inflammatory effect of the steroids on the endothelium, remains unknown.

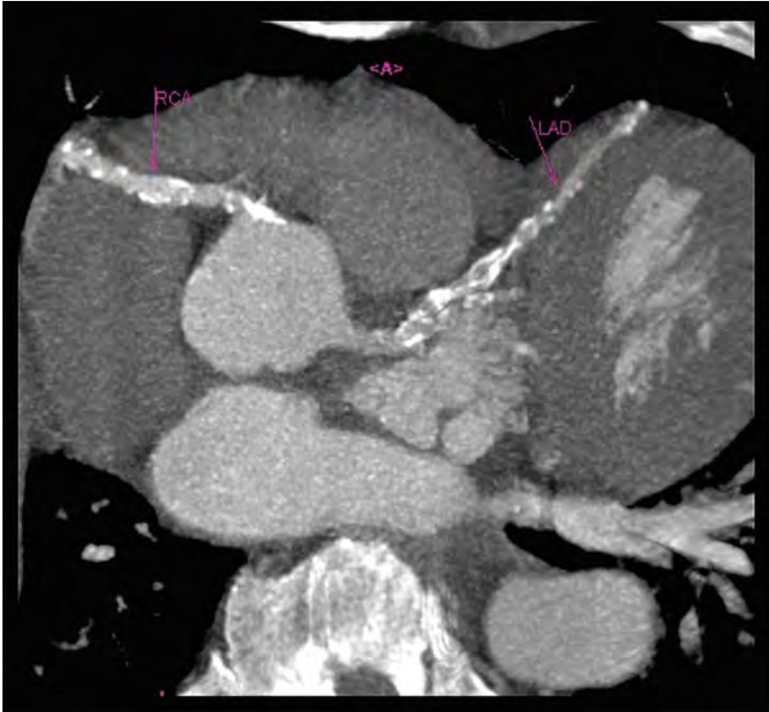

Fig. 4 Multi-detector computed tomography showing extensive calcification of the coronary arteries in a 48-year-old bodybuilder abusing AAS

With the rapid technological development of multi-detector computed tomography equipment, one might expect further and larger studies in the future, addressing the likely association of long-term AAS abuse and coronary atherosclerosis.

6 Blood Platelet Function and Haemostasis

Case reports of anabolic androgenic steroid (AAS) abusers presenting with manifestations of thromboembolic disease are not rare, at least not when arterial in nature. Of the venous types, both deep venous thrombosis and pulmonary embolism have been described (Gaede and Montine 1992; Liljeqvist et al. 2008). Four cases of superior sagittal sinus thrombosis were reported during androgen treatment for hypoplastic anemia (Chu et al. 2001; Shiozawa et al. 1982). At least two additional cases have been reported, affecting a 22-year-old man (Sahraian et al. 2004), and a 31-year-old man (Jaillard et al. 1994), both bodybuilders using AAS. As for the arterial bed, there is an abundance of reports of cerebrovascular events, myocardial infarctions and peripheral arterial thromboses in this population, and even one of a

ventricular thrombosis (McCarthy et al. 2000). However, to date there is no direct scientific evidence that AAS are thrombogenic in humans. Blood platelets together with the fibrinolytic system have a pivotal role in the pathogenesis of arterial thrombosis (Fuster et al. 1985). In support of this notion is the strong evidence from large-scale randomized trials that platelet inhibition significantly decreases the death and infarction rate in patients with coronary artery disease and cerebrovascular disease (Budaj et al. 2002, Cairns et al. 1985; Collins et al. 1994; Diener et al. 2008; Lewis et al. 1983). Reports of venous thromboembolic manifestations in AAS abusers are not as common as the ones of arterial thrombosis. A possible prothrombotic effect through the aromatization of testosterone to estradiol has been suggested (Liljeqvist et al. 2008), as estrogen-based therapies have been shown to increase the risk of venous thromboembolism (Lowe 2002). There are also experimental reports showing alterations at different stages of the hemostatic and fibrinolytic system by AAS administration, as well as effects on platelet function, which might explain what seems to be an increased risk of thromboembolic disease in this population (Ferenchick 1991). Some of these findings have been confirmed in human studies.

Androgen-mediated enhancement of platelet aggregability and augmenting of thrombosis have been shown in experimental animal models of thrombosis, in studies done in the 1970s and 1980s (Emms and Lewis 1985; Johnson et al. 1975; Johnson et al. 1977; Uzunova et al. 1976; Uzunova et al. 1977). Platelet aggregability is largely dependent on arachidonic acid metabolism (Fig. 5), and it has been

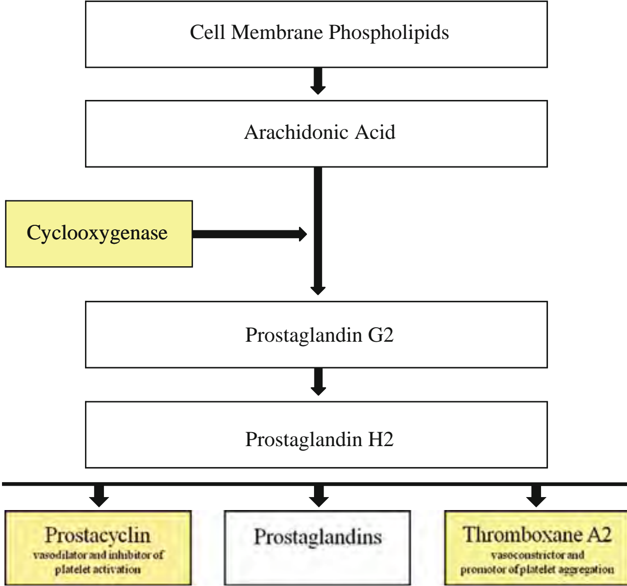

Fig. 5 Cyclooxygenase and substrates associated with thromoboxane A2 and prostacyclin synthesis. AAS may enhance platelet aggregation through their effects on prostacyclin and thromboxane A2 (*boxes highlighted in yellow*)

shown that large doses of androgens affect both the platelet (Pilo et al. 1981) and the vascular cyclooxygenase activity (Greenberg et al. 1974). A decrease in the production of prostacyclin PgI_2, a potent inhibitor of platelet aggregation, has been found in rat smooth muscle cells in culture after administration of testosterone (Nakao et al. 1981; Rosenblum et al. 1987). Furthermore, potentiation of platelet aggregation in vitro was demonstrated when incubating human platelets with testosterone, causing intensified response to several different aggregating agents (Pilo et al. 1981). Interestingly, significant bleeding in hypogonadal men has been linked to hypoactive platelets (Pawlowitzki et al. 1986).

In a report from 1992, Ferenchick et al. (1992) studied blood platelet stimulation thresholds in 28 male weightlifters, 24 of them using different kinds of AAS. Non-significant trends toward increased platelet counts and increased platelet aggregation to adenosine diphosphate in the AAS group were noted. Unfortunately the study did not have the power to detect potential between-group differences, as the control group ended up with only four subjects, as ironically eight of the 12 declared nonusers had unexpected findings of positive urine androgen assays. In the same study, subgroup analysis by age showed that AAS users older than 22 years had significantly lower aggregometric collagen threshold levels than their younger counterparts, but, referring to additional studies by the same group, the authors believe that age alone cannot explain this finding.

Ajayi et al. (1995) investigated testosterone as a regulator of the expression of human platelet thromboxane A2 receptors (TXA2 is a metabolite of arachidonic acid, and a potent vasoconstrictor and platelet aggregator). The authors recruited 16 healthy men who in a double-blind, placebo-controlled study were given testosterone cypionate 200 mg i.m. twice, two weeks apart, or saline placebo. As a result, in the treatment group there was a significant increase in platelet TXA2 receptor density and an increase in the maximum platelet aggregation response, though the latter did not increase in proportion with the receptor density increase. The researchers concluded that testosterone confers prothrombotic effects on the platelets.

As for the hemostatic and fibrinolytic system, the picture is less clear. Studies of AAS indicate an effect on these systems, but the results have in some respects been contradictory. The literature gives support to the view that AAS has some hypercoagulable effects, but the extent of it is unclear, and to what degree concomitant apparent AAS-induced fibrinolytic, i.e. protective, mechanism is operational is uncertain. Early studies of stanozolol (Barbosa et al. 1971; Small et al. 1982; Verheijen et al. 1984) gave rise to expectations that the drug had therapeutic potential as an activator of the fibrinolytic system, as elevated levels of both plasminogen activator and plasminogen were found following i.m. injections of the drug. Several 17α-alkylated androgens have been shown to increase plasminogen activator activity and serum levels of plasminogen, protein C and antithrombin III (Kluft et al. 1984; Small et al. 1984). Stanozolol also increased the levels of antithrombin III and protein C in male and female patients suffering from congenital insufficiency syndrome, but the clinical effects were disappointing (Broekmans et al. 1987; Winter et al. 1984). Neither did stanozolol have any preventive effect

against postoperative deep vein thrombosis following abdominal surgery (Blamey et al. 1984). However, stanozolol has been shown to increase fibrinolytic activity by reducing the plasma levels and activity of PAI-1 (plasminogen activator inhibitor), yet clinical utilization has hitherto not been justified. In a male contraception study, the hemostatic effects of i.m. injections of testosterone enanthate were investigated. The subjects, 32 healthy men, received the treatment weekly for 52 weeks (Anderson et al. 1995). A slight increase in antithrombin III and prothrombin fragments F1+2 was noted, as well as a decrease in protein C levels, free protein S and plasminogen activator inhibitor. The authors summarized that these changes indicate an increase in coagulatory activity, but that the decrease of PAI-1 might outweigh the prothrombotic effects.

Studies of hemostasis in AAS abusers are almost nonexistent. Ansell et al. (1993) found indications of an activated fibrinolytic state, but no signs of a hypercoagulable state when investigating 16 bodybuilders, 11 of them during the influence of a variety of AAS. Those with paired samples ($n=6$) showed a significant increase in protein C antigen and free protein S antigen during steroid use. An increase in euglobulin lysis time indicated an activation of the fibrinolytic mechanism in the steroid-using group. All other parameters apart from elevated platelet count (in the steroid group) turned out negative, among them fibrinogen, antithrombin III activity, protein C activity, plasminogen activity, D-dimer and factor VII and VIII activity. However, this study has been criticized because the subjects' self-reporting of AAS was not confirmed by urine assays, as other studies have demonstrated a remarkable discrepancy between self-reported use of AAS and documented use by urine analyses.

In a larger study of AAS and hemostasis, Ferenchick et al. (1995) investigated 32 weightlifters, 23 users of AAS and 17 nonusers. This was the first, and to our knowledge the only study to describe the effects of AAS on humoral coagulation utilizing steroid-free weightlifters as controls and confirming self-reports with urine assays. Markers of clotting and fibrinolytic activity, namely thrombin/antithrombin III (TAT) complexes, prothrombin fragments1+2 and D-dimer were all increased in the user group, while endothelial-based fibrinolytic components, tissue plasminogen activator antigen (t-Pa Ag) and plasminogen activator inhibitor (PAI-1) interestingly were more likely to be higher in the nonuser group. Inhibitors of the clotting system, antithrombin III activity, protein C and S activity were significantly higher in users vs. nonusers. The authors concluded that some AAS-using weightlifters have an accelerated activation of their hemostatic systems evidenced by increased generation of both thrombin and plasmin, and that these changes could reflect a thrombotic diathesis that may contribute to vascular occlusion in AAS-using athletes. They also pointed out that the predictive value of these coagulation abnormalities, in terms of risk of thrombosis to individual athletes or the population as a whole, remains to be studied. Ironically, this statement still is applicable, more than 10 years later, which underscores the difficulties of elucidating clear evidence as to thrombosis and hemostasis in these cohorts.

In a more recent study, Kahn et al. (2006) evaluated the short-term effects of oxandrolone, taken 10 mg twice daily for 14 days, on both platelet function and the

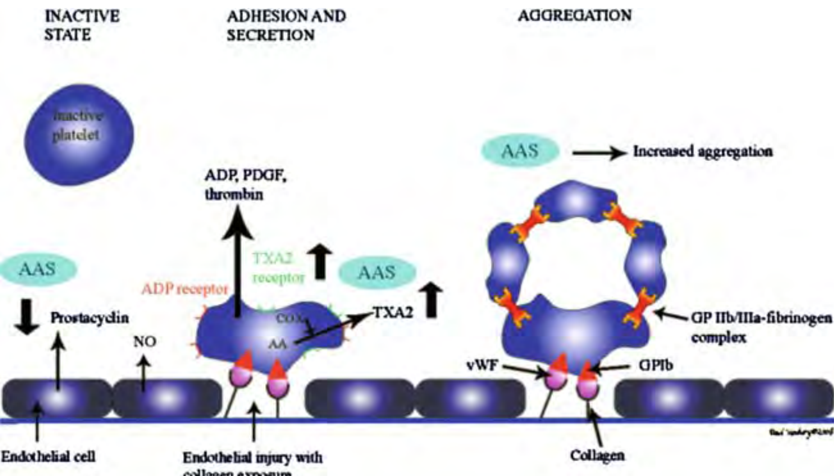

Fig. 6 The illustration shows different pathways of blood platelet activation, adhesion and aggregation. AAS appear to increase the platelet aggregation response. Experimental and human studies have indicated that AAS increase platelet production of TXA2 and increase both platelet and vascular TXA2 receptor density. The drugs may also decrease the production of PGI_2. ADP= Adenosine diphosphate; COX=Cyclooxygenase; NO=Nitric oxide; AA=Arachidonic acid; PDGF=Platelet derived growth factor; PGI_2=Prostacyclin; TXA2=Thromboxane A2; vWF= von Willebrand factor; GP=Glycoprotein

hemostatic/fibrinolytic system. The platelet part of the study turned out negative, as the effect on ADP-induced platelet aggregation was not significant. However, the authors believe this was because of a concomitant increase in factor V and X, causing inhibition of thromboxane A2 synthesis in platelets. Oxandrolone induced a highly significant increase in plasminogen and a significant decrease in PAI-1, indicating activation of the fibrinolytic system. The levels of coagulation factors II and V increased significantly, while fibrinogen and factors VII, VIII and X did not change or only did so nonsignificantly. The authors summarize the findings to be evidence of a simultaneous stimulation of both pro-coagulatory and fibrinolytic activity, resulting in a balanced effect on the hemostatic system by oxandrolone.

Another mechanism possibly contributing to thrombogenesis is the AAS-induced increase in the level of erythropoietin causing elevated levels of hemoglobin, a very common finding in AAS users (Shahidi 1973; Teruel et al. 1995). According to the Framingham data, increased hematocrit values are correlated with an increase in the cardiovascular risk and total mortality (Gagnon et al. 1994). Combined with an increase in platelet aggregability, it is likely that the elevated hematocrit in AAS users will contribute to the increased risk of thrombosis.

Taken together, the abuse of AAS does confer an enhanced pro-thrombotic state, most prominently through an activation of platelet aggregability (Fig. 6). The concomitant effects on the humoral coagulation cascade are more complex and include both pro-coagulatory and fibrinolytic pathways of activation.

7 Lipids

The most studied cardiovascular effects of anabolic androgenic steroids (AAS) are probably the effects on lipoproteins. The last 25 years have yielded numerous reports from human studies on the subject, and for once the results to a large extent concur, at least concerning readily measurable parameters. The long-term consequences of the alterations of lipid metabolism that these drugs cause are unfortunately still largely unknown.

In the general population, epidemiological studies demonstrate a strong relationship between levels of low-density lipoprotein (LDL) cholesterol and the incidence of atherosclerotic cardiovascular disease (Stamler et al. 1986). Furthermore, there is a large body of evidence which supports a central role for lowering levels of LDL in the prevention of cardiovascular events (Downs et al. 1998, HPS Collaborative Group 2002; Pedersen et al. 1994; Shepherd et al. 1995). Epidemiological studies also show a strong relationship between low levels of high-density lipoprotein (HDL) cholesterol and risk for cardiovascular events (Castelli and Anderson 1986; Gordon et al. 1977). Multivariate analyzes have shown that changes in HDL levels are an independent predictor of atheroma burden (Jacobs et al. 1990). The combined evidence derived from placebo-controlled trials have revealed that the greatest benefit of statin therapy in terms of absolute risk reduction was observed in patients with the lowest baseline levels of HDL (Downs et al. 1998; HPS Collaborative Group 2002; Pedersen et al. 1994). In a recent post-hoc analysis, Nicholls et al. (2007) provide evidence that increases in HDL levels are correlated with beneficial effects of statins on the progression of coronary artery disease.

Atherothrombosis is a complex disease in which cholesterol deposition, inflammation and thrombus formation play a major role. LDL exerts its pathological effects by infiltrating the arterial endothelium into the intima, where matrix proteins including proteoglycans, collagen and fibronectin are attracted. Another important feature of lipoprotein transport is related to the effect of HDL, which promotes reverse cholesterol transport from the arterial wall, specifically from lipid-laden macrophages. The HDL subfractions may play a role in this beneficial effect, with HDL_2 being the most important for reverse lipid transport (Fuster et al. 2005). Interestingly, despite its protective effects, patients with high HDL plasma levels still can present an acute coronary syndrome, probably related to elevations in HDL_3 rather than in HDL_2.

AAS clearly induce an extreme atherogenic alteration on lipid levels. A solid body of evidence has been published on the subject. The drugs cause a marked depression in HDL, especially on subfraction HDL_2. The levels of LDL are also negatively affected, though not as pronounced as the effects on HDL are. Though it is far from proven that these alterations transform into coronary artery disease in the population at debate, the association cannot be denied.

The first report of the effect of AAS on plasma lipid values came in 1980, when it was shown that oxandrolone reduced HDL and apolipoproteins AII and AII_1 in hyperlipidemic subjects (Cheung et al. 1980). The findings were confirmed in

normolipidemic subjects two years later (Taggart et al. 1982). Numerous reports (Bonetti et al. 2008; Frölich et al. 1989; Glazer 1991; Hartgens et al. 1996; Hurley et al. 1984; Kuipers et al. 1991; Palatini et al. 1996; Sachtleben et al. 1997; Thompson et al. 1989; Urhausen et al. 2003; Yeater et al. 1996) with similar findings surfaced over the next two decades, several of them specifically addressing strength athletes abusing AAS. A frequent finding in these studies is also the increase of LDL levels. Most of the studies were prospective cohort studies or cross-sectional studies and the steroids were self-administered, in supraphysiologic doses and as combinations of orally and injected preparations. Many of the studies did not control for diet, exercise, or the type, purity, and dose of AAS used. Of course this precludes the findings from delivering the desired scientific attainments in a purist sense, but the comprehensiveness of the available data nevertheless allows for conclusions. Some of the studies were done with therapeutic doses and were placebo-controlled. A head-to-head comparison of oral AAS vs. injected testosterone has also been done (Thompson et al. 1989).

Although slightly dated, the comprehensive review by Glazer (1991) gives a fine overview of the impact of AAS on HDL and LDL, the underlying mechanisms behind the changes and their possible clinical significance. Only a modest amount of new knowledge has surfaced in subsequent years. Fifteen studies are referred to by Glazer, and the average HDL reduction was 52% (range, 39–63%) for the prospective cohort studies and 51% (range, 41–70%) for the cross-sectional studies. Reductions of this magnitude are far greater than those produced by other pharmacologic or nonpharmacologic agents including smoking and obesity. As for the subfractions of HDL, the most pronounced effect is on HDL_2, which has been shown to be reduced by 78% (range, 71–89%), while the average reduction of HDL_3 was 35%.

AAS are believed to exert their influence on lipoproteins by the induction of hepatic triglyceride lipase (HTGL) synthesis in the liver. Localized to the luminal surface of hepatic endothelium, HTGL is thought to catabolize HDL via its phospholipase activity and remove HDL from plasma (Applebaum-Bowden et al. 1987; Ehnholm et al. 1975; Haffner et al. 1983; Kantor et al. 1985; Lenders et al. 1988; Taggart et al. 1982). Studies of AAS and HTGL activity have shown increases of HTGL in the range of 143–232% during AAS use (Glazer 1991).

The orally taken 17α-alkylated substances (such as stanozolol, oxymetholone and methandienone) exert much stronger effects than other AAS (Friedl et al. 1990; Thompson et al. 1989). Generally, nonaromatizable androgens have more pronounced effects on HTGL induction and plasma HDL (Friedl et al. 1990; Melchert and Welder 1995; Zmuda et al. 1993). In the prospective crossover study by Thompson et al., 11 weightlifters were given either oral stanozolol (6 mg/day) or intramuscular testosterone enanthate (200 mg/week) for six weeks. Stanozolol reduced HDL and the HDL_2 subfraction by 33 and 71% respectively. In contrast, testosterone decreased HDL by only 9%. Moreover, stanozolol increased the HTGL activity by 123% while the effect of testosterone (on the enzyme) was not significant. A similar effect on HDL has also been demonstrated with other 17α-alkylated steroids (Cheung et al. 1980; Friedl et al. 1990).

There appears to be minimal or no dose relationship in oral AAS-induced HDL depression (Glazer 1991) and maximal or near maximal HDL level decrement results from even therapeutic dosages of oral AAS. The injectable testosterones on the other hand seem to affect HDL in a more dose-related manner. In a study on healthy young men, Bhasin et al. (2001) demonstrated that testosterone enanthate dosages (and testosterone concentrations) were negatively correlated to plasma levels of HDL. In another study by Kouri et al. using i.m. injections of escalating dosages of testosterone cypionate, maximum HDL depression was reached at 300 mg/week and this depression remained unchanged with 600 mg/week for two more weeks. Singh et al. demonstrated that i.m. testosterone enanthate 600 mg/week reduced HDL levels, whereas lower doses did not exert any effect on lipoprotein profiles (Singh et al. 2002). The use of therapeutic or supraphysiologic doses of nandrolone decanoate does not seem to affect lipoproteins in a detrimental way (Friedl et al. 1990; Glazer and Suchman 1994; Kuipers et al. 1991).

The AAS-induced HDL depression can be observed within a few days of starting with the drugs and studies suggest that the HDL level reaches its nadir within a few weeks (Glazer 1991; Thompson et al. 1989). In a study by Hartgens et al. (2004), 19 strength athletes self-administering supraphysiologic doses of AAS had no further decline of HDL, HDL_2 and HDL_3 after eight weeks. After cessation of AAS, the lipoprotein levels seem to recover completely after one to three months (Alén et al. 1985; Hartgens et al. 2004; Kuipers et al. 1991). Notably, in the recent study by Hartgens et al., the authors demonstrated that recovery strongly depended on the duration of the AAS course. In their study, one group self-administered supraphysiologic dosages of AAS for an average of 14 weeks. Their serum concentrations of HDL, apolipoprotein-A1 (Apo-A1) and lipoprotein(a) (Lp(a)) were not normalized six weeks after cessation, while in another group of so-called short-term users (AAS use for eight weeks) the same parameters had returned to baseline levels. Urhausen et al. (2003) investigated the reversibility of AAS effects in 15 long-term (average 26 weeks per year for nine years) users of supraphysiologic doses 12–43 months after cessation. Seventeen current users served as control group. In all of the ex-users HDL concentrations had returned to normal values, with the exception of one, which was believed to be genetically caused.

As for LDL, AAS will generally induce an elevation of serum values, in much the same pattern as they do on HDL, though not to such an extreme level (Applebaum-Bowden et al. 1987; Haffner et al. 1983; Hurley et al. 1984; Kantor et al. 1985; Kleiner et al. 1989; Lenders et al. 1988; Thompson et al. 1989; Webb et al. 1984). In Glazer's review the degree of increase was estimated at 36%. Injections of testosterone up to a dosage of 600 mg/week do not seem to alter LDL levels (Kouri et al. 1996; Singh et al. 2002; Zmuda et al. 1993). Total cholesterol and triglycerides were generally not significantly changed in the studies previously referred to.

The nonfasting Apo-B/Apo-A1 ratio has recently been assessed as superior to any of the cholesterol ratios for estimation of the risk of acute myocardial infarction in all ethnic groups (McQueen et al. 2008). While Apo-A1 is the major HDL apolipoprotein, it is not surprising that AAS severely decreases its level, in Glazer's review by 33–41%, calculated from five studies. At the same time an increase in

Apo-B levels of the same magnitude was also found. In the recent study by Hartgens et al., the group of self-administering AAS abusers was found to have a large and highly significant decrease of Apo-A1 and a concomitant increase of Apo-B. These changes were apparent after eight weeks' treatment, but did not increase further at 14 weeks. As for HDL, the 17α-alkylated drugs rather than testosterone esters are responsible for inducing more profound effects on the apolipoproteins (Friedl et al. 1990; Singh et al. 2002; Thompson et al. 1989).

Lipoprotein (a) (Lp(a)) is a risk factor for both atherothrombotic and purely thrombotic events, and it increases global cardiovascular risk, especially when LDL cholesterol is concomitantly elevated (Koschinsky 2005; Kostner et al. 1981; Kronenberg et al. 1999; White et al. 1996). Several reports (Cohen et al. 1996; Crook et al. 1992; Zmunda et al. 1996) have suggested that AAS may favorably lower Lp(a) concentrations, most recently by Hartgens et al. In this study (Hartgens et al. 2004), the investigators found Lp(a) to be highly significantly reduced after 8 and 14 weeks of supraphysiologic dosages of AAS. However, if or to what degree this apparent reduction translates into cardiovascular protection remains unknown.

Finally, another marker of atherosclerotic disease is homocysteine, a byproduct of methionine metabolism (Nygård et al. 1997; Tonstad et al. 1996). Homocysteine has also been shown to impair vascular endothelial function through impairment of nitric oxide production (van Guldener and Stehouwer 2000). Two studies have identified hyperhomocysteinemia in bodybuilders using supraphysiologic doses of various types of AAS (Ebenbichler et al. 2001a; Graham et al. 2006). In a previous study (Zmuda et al. 1997), short-term administration of testosterone enanthate did not affect fasting homocysteine levels in weight lifters. In the study by Graham et al., consisting of ten current users of AAS and control groups, three of the participants died during the study. Interestingly they all had cardiovascular disease as cause of death and their homocysteine levels were – ironically – markedly elevated, and above the mean average of the user group as a whole.

Based on current knowledge of the lipid effects of AAS, how large is the increased risk of developing coronary heart disease (CHD)? Obviously, there are no studies that can answer this most pivotal question. The Framingham data showed that patients with HDL levels less than 1.0 mmol L^{-1} had a fourfold increase in risk of CHD compared to those with levels of 1.03–1.27 mmol L^{-1}, when total cholesterol levels were normal. Based on the described changes in HDL in the AAS-abusing population and the Framingham data, Glazer in his review puts forward an estimate of risk of CHD to be increased three- to six-fold. However, as he comments, this estimate is complicated by several factors, among them the fact that the studied steroid abusers are younger than the patients who have delivered the data on lipid levels and risk evaluation. Another important feature is that most AAS users take the drugs intermittently, in cycles of 6–16 weeks, with abstinence periods in between, often long enough to reverse the detrimental lipid effects.

To summarize, there is a comprehensive body of evidence documenting the various alterations of lipid metabolism induced by AAS. The most prominent changes are concomitant elevations of LDL and decreases of HDL, the combined effects of which are deleterious. This should be taken into account when counseling

abusers, advising health authorities, health care providers and prevention stakeholders, and informing the general public.

8 Arrhythmias and Sudden Death

It is a tragic fact that sudden death is not an unknown end stage after long-term abuse of AAS. The incidence is of course impossible to estimate, but there are several published case reports describing devastatingly fatal events (Di Paolo et al. 2007; Dickerman et al. 1995; Fineschi et al. 2001, 2007; Hausmann et al. 1998; Kennedy and Lawrence 1993; Luke et al. 1990). Indeed, most abusers of AAS are familiar with the risk of sudden death, at least in the sense that they have knowledge of someone in their close or distant milieu who abruptly and tragically lost his life. The etiology behind sudden death in this population is unknown, and will unfortunately most likely continue to be so. It would be impossible to conduct a study to shed light on incidence and underlying mechanisms. It is however not a controversial assumption that a malignant arrhythmia is the underlying cause of death. Whether these arrhythmias are directly triggered by AAS or whether they occur as indirect or secondary consequences of AAS, as for instance through left ventricular hypertrophy or cardiomyopathy, is unclear. To further complicate the issue, there is the widespread use of other drugs among abusers of AAS, both recreational substances as stimulants, marijuana, cocaine and alcohol, the concomitant use of masking agents such as diuretics, and the use of anti-estrogens, thyroid hormones and growth hormone (Furlanello et al. 2003). Insulin injections carry the risk of death due to hypoglycemia. Among stimulants known to have potential arrhythmic properties are ephedrine, caffeine and unequivocally amphetamine, all of which are highly prevalent substances among bodybuilders using AAS. Another important consideration to bear in mind is that inherited arrhythmogenic diseases, such as long QT syndrome and other ion channel diseases and arrhythmogenic right ventricular cardiomyopathy (a genetic disease characterized by fatty infiltration in the right ventricle, causing increased risk of arrhythmia and sudden death) do also exist in the growing population of abusers of AAS. Hard physical exercise combined with illicit drugs of various kinds can very well trigger a malignant arrhythmia in these particularly predisposed individuals.

There are very few scientific reports on arrhythmias and AAS. A handful of case reports have been published, but hardly any work has been done in the field of the pathophysiology of arrhythmias in this particular population, at least not human electrophysiologic studies.

A case report by Sullivan et al. (1999) presents a 22-year-old male bodybuilder who was admitted to hospital with symptomatic rapid atrial fibrillation. He had consumed large doses of AAS, in the form of intramuscular injections. He had no past cardiac medical history or any other condition known to cause atrial fibrillation and did not use tobacco, alcohol or any drugs other than AAS. Echocardiography showed an area of septal hypokinesis, posterior and septal wall thickness at the upper limit of normal, and preserved left ventricular function. The patient was

discharged from hospital after two days, after having spontaneously converted to sinus rhythm. Twenty-four hours Holter monitoring three weeks later showed normal sinus rhythm, and the patient had at that time lost 9 kg following steroid cessation. Afterwards he was lost to follow-up, which regrettably is often the case with AAS abusers.

A similar story was quite recently presented in a case report by Lau et al. A 36-year-old bodybuilder was referred to hospital with persistent atrial fibrillation (Lau et al. 2007). He had no medical history, was a nonsmoker and had no cardiovascular risk factors apart from taking intramuscular injections of testosterone enanthate and stanozolol in supraphysiologic doses. Echocardiography showed normal left ventricular size and function and a normal left atrial diameter. The patient was treated with DC cardioversion, but reverted back to atrial fibrillation after 15 days. He was then treated with flecainide 100 mg twice daily and metoprolol 150 mg twice daily. A second cardioversion was successful, though two attempts were needed, finally with 360 J. His left atrium had by this time increased considerably, but the left ventricle parameters had remained unaltered. In the mean time, the patient had lost 16 kg following his own decision to cease the administration of AAS. A year later, he was still in sinus rhythm and without exogenous steroids or antiarrhythmic medication. Despite any other known condition predisposing this patient to arrhythmia, and though his symptomatic onset of atrial fibrillation correlated with the initiation of AAS abuse, the association between AAS and atrial fibrillation must be reviewed as an association only, and there is no evidence of causal relationship.

In a report of four weightlifters abusing AAS, Nieminen et al. (1996) describe serious cardiac side effects of the drugs, one of them being ventricular tachycardia (VT). The weightlifter who experienced this arrhythmia was 29 years old and had taken AAS during several periods over the past eight years. At a routine exercise test at 250 W workload, a monomorphic VT with a frequency of 230/min was recorded and exercise was stopped. After one minute of rest, the rhythm degenerated into a polymorphic VT and then to ventricular fibrillation causing unconsciousness. Cardioversion was performed and the patient regained sinus rhythm and consciousness. There was no sign of myocardial infarction. Echocardiography showed a dilated left ventricle with marked wall thickening. Angiography revealed no stenoses in the coronary arteries, but ventriculography showed a dilated left ventricle with Am ejection fraction of 40%. Programmed electrophysiologic stimulation could not induce VT. Endomyocardial biopsy revealed focal fibrosis of the left ventricle and fat degeneration, and there was no sign of granulocyte infiltration. Five months later, after cessation of AAS, the left ventricular dimension was normalized and the hypertrophy had decreased slightly. There were no further episodes of VT during follow-up. Although the association between AAS and this patient's symptoms and findings seems rather convincing, his drug regimen also included clenbuterol, growth hormone and human chorionic gonadotropin. As so often in this setting, this contributes to clouding the picture.

Two other case reports describe the occurrence of ventricular tachycardia in AAS-abusing bodybuilders (Appleby et al. 1994; Mewis et al. 1996). However, in

both cases the subjects also had coronary artery disease, believed to be the underlying cause of the arrhythmia. The subject described in Mewis' report had reduced function of left ventricle, assessed by echocardiography. Intriguingly, other than long-term use of stanozolol, this individual had no classic coronary risk factors.

In an electrophysiologic study by Stolt et al. 1999, 15 male powerlifters using large doses of AAS were compared to 30 male orienteering runners from the Finnish national training program and to 15 sedentary young men. The investigators measured QT interval and QT dispersion (the difference between the longest and the shortest QT interval) in a 12-lead resting electrocardiogram. Echocardiographic assessment of the left ventricle (LV) mass, wall thickness and chamber dimension was also performed. The dispersion of the QT intervals between the electrocardiographic leads is an indirect measure of the heterogeneity of ventricular depolarization, and several studies suggest that an increase in QT dispersion is associated with increased risk of arrhythmic events (Higham and Campbell 1994; Mänttäri et al. 1997; Pye et al. 1994). Left ventricular hypertrophy is associated with increased QT dispersion and increased mortality in hypertensive patients (Mayet et al. 1996; Perkiömäki et al. 1996). The athlete group was considered to be a model of physiologic adaptive left ventricular hypertrophy, while the AAS group were expected to have features of pathologic hypertrophy as well. As for the echocardiographic findings, the LV mass was largest in the AAS group, but when related to body surface area, the endurance athletes had 9% greater LV mass than the AAS group. The septum diameter was identical in these two groups, but the posterior wall was thicker in the AAS group than in the endurance athlete group. Both groups had significantly higher values than the sedentary control group, for LV mass as well as LV mass index and wall thicknesses. The main finding, however, was that the endurance athletes had the longest QT intervals and the AAS group of power athletes had the shortest, and the latter group also had the greatest amount of QT dispersion, while the endurance athletes had the least. Even when adjusted for heart rate, the QT interval differences were highly significant. The QT interval did not correlate with LV mass, but it did so significantly with E/A ratio, a Doppler index of altered diastolic function. The AAS group had significantly lower E/A ratio than the endurance athletes. The authors believe the prolonged QT interval seen in the endurance athlete group mainly is a result of enhanced vagal activity. It did not seem that increased LV mass played any role in prolonging the QT interval. In fact, the AAS group, despite their increased LV mass, had shorter QT interval and increased QT dispersion. The authors believe this to be caused by LV hypertrophy with altered myocardial structure. Their findings would suggest that the use of AAS together with resistance training leads to an increased risk of malignant arrhythmias.

In the absence of further human studies on the topic, it appears appropriate to refer to some suggested mechanisms of arrhythmia from studies of rats exposed to AAS. In a study by Pereira-Junior et al. (2006), treatment of rats with supraphysiologic doses of nandrolone decanoate for eight weeks showed impaired tonic cardiac autonomic regulation, which the authors suggest may provide a key mechanism for anabolic steroid-induced arrhythmia and sudden cardiac death. Another

nandrolone study, done in vivo on male rats by Phillis et al. (2007), administered a high dose of the drug acutely, and potentiated ischemia-induced arrhythmia and thereby decreased the proportion of rats surviving the ischemia. Though the study does not identify the mechanism behind this finding, the authors speculate that nandrolone may cause the release of intracellular calcium in a similar way to that shown in skeletal muscle, and thereby explain the observed proarrhythmic effects. They also refer to previous studies where other anabolic steroids have been shown to inhibit the reuptake of catecholamines into extraneuronal tissues, thereby increasing catecholamine concentrations at receptor sites. In a recent experimental study by Rocha et al. (2007), in which rats were treated with supraphysiologic doses of nandrolone decanoate, the investigators for the first time show that the combination of exercise and AAS causes an increase in the heart collagen concentration associated with the activation of the cardiac renin–angiotensin system. These pathophysiologic changes undoubtedly provide a patho-anatomic substrate that may explain the increased propensity to the generation and continuation of malignant cardiac arrhythmias.

Taken together, the use of AAS appears to confer an increased risk of life-threatening arrhythmia leading to sudden death, albeit the underlying mechanisms are still far from being elucidated.

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Side Effects of Anabolic Androgenic Steroids: Pathological Findings and Structure–Activity Relationships

Andreas Büttner and Detlef Thieme

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Abstract Side effects of anabolic steroids with relevance in forensic medicine are mainly due to life-threatening health risks with potential fatal outcome and cases of uncertain limitations of criminal liability after steroid administration. Both problems are typically associated with long-term abuse and excessive overdose of anabolic steroids. Side effects may be due to direct genomic or nongenomic activities (myotrophic, hepatotoxic), can result from down-regulation of endogenous biosynthesis (antiandrogenic) or be indirect consequence of steroid biotransformation (estrogenic).

Logically, there are no systematic clinical studies available and the number of causally determined fatalities is fairly limited. The following compilation reviews typical abundant observations in cases where nonnatural deaths (mostly liver failure

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and sudden cardiac death) were concurrent with steroid abuse. Moreover, frequent associations between structural characteristics and typical side effects are summarized.

Keywords Side effects • Heart • Liver • Arteriosclerosis • Psychiatry

1 Methodological Limitations

The evaluation of side effects of anabolic androgenic steroid (AAS) abuse contains several methodological problems. Firstly, the exorbitant dosages, which are up to 40 times higher than typical medical applications, prohibit any ethically justified clinical studies. Observations of side effects of therapeutic applications are only partially valid due to their comparatively low dosages. Moreover, the therapeutic relevance of steroids is reduced to a few testosterone analogs for treatment of, e.g., male hypogonadism, renal failure associated with anemia, cancer associated protein wasting diseases, burns, AIDS and hereditary angioedema.

Reliable empirical data from bodybuilding are virtually unavailable, because self-reports of administered dosages and corresponding side effects are certainly biased.

Finally, observations of pathological findings in autopsy cases are less frequent and therefore not statistically significant. Moreover, the causality between steroid abuses, the cluster of pathological findings and a presumptive cause of death needs to be challenged, because steroid effects (including side effects, health risks and hazards) do not coincide with their analytical identification at the time of death. Therefore, these observations do reflect a general steroid-associated elevation of health risks in the bodybuilder cohort but typically cannot clarify the individual situation.

2 Mechanisms of Action and Substance Specificity

Genomic steroid action consists of direct regulation of gene transcription by ligand–receptor interaction or indirect modulation of coactivators. Biochemical effects (including side effects) of steroids may be categorized according to the various receptor types, i.e., androgen (hAR), estrogen, glucocorticoid (hGR), progesterone (hPR) and mineralcorticoid receptors. In addition, nongenomic effects of steroids are well described, e.g. the direct activation of ionotropic $GABA_A$ receptors by neurosteroids.

Both mechanisms are characterized by a high specificity of receptor–ligand binding, requiring structural congruence of steroids and corresponding receptors.

Different steroid receptor types are structurally related; the sequence homology of the ligand binding domains of hAR is relatively high (e.g. 55% with hPR, 51% with hGR; Gao et al. 2005) which suggests the potential of cross-reaction in particular after administration of high doses of steroids. Extra to this high homology of relevant

receptors, the steroidal ligands are characterized by a considerable structural similarity and may often be biotransformed into each other. Therefore, a strict differentiation of steroids into functional subgroups is neither expected nor observed.

All relevant endogenous steroid hormones are synthesized and biotransformed within the biochemical pathway shown in Fig. 1 (compare Kicman 2009). Consequently, biological phenomena can result from direct substance supplementation (e.g. abuse of anabolic steroids) or indirect effects associated with inhibition (suppression of gonadotropic hormones) or induction (formation of estrogens from testosterone) of endogenous biosynthesis. The availability of steroidogenic enzymes in target tissues (e.g. 5α-steroid-reductase in prostate or skin) is hence of primary importance for the regio-specificity of steroidal effects.

Regulation of steroid biochemistry is a complex process and subject to positive enzymatic amplification and negative feedback mechanisms. Any administration of a steroid – especially in amounts far beyond endogenous levels – will compromise the balance of its endogenous synthesis. The administration of testosterone leads to a rapid down-regulation of gonadotropic hormones (e.g. luteinising hormone), followed by termination of endogenous production of steroids and atrophy of steroidogenic organs (testis). This may result in a suppression of other endogenous steroids (e.g. glucocorticoids or neurosteroids) produced within the same biochemical pathway.

Specific correlations between steroid structure and corresponding side effects are based on exemplary animal or clinical studies in therapeutic dose ranges and remain vague.

With respect to endogenous steroids, androgenic side effects may clearly be attributed to reduction of a 4-double bond to 5α-dihydrotestosterone (DHT) by 5α-steroid-reductase and estrogenic effects are due to aromatisation of the steroid A-ring by steroid-aromatase (CYP19). However, a simplified extension of these principles to synthetic steroids is not generally valid.

Steroid side effects may either result from direct receptor agonism (whether or not in combination with metabolic activation) or may be due to a suppression of steroid biosynthesis. Factors determining the efficiency of steroid action are therefore the structural specificity as well as the availability of activating enzymes in the respective target cells (e.g. skin, brain region).

As a simplified review, main side effects may be associated with compounds frequently abused in sports and bodybuilding and typical structural characteristics, i.e.:

- Anabolic side effects of testosterone and analogs
- Enhanced androgenic effects, often associated with 5α-dihydrogenation of steroids
- Estrogenic side effects, depending on susceptibility to A-ring aromatisation
- Antiandrogenic effects based on a suppression of the hypothalamus–pituitary–adrenal/gonadal (HPA/HPG) axes, without clear structural determination
- Hepatotoxicity, clearly correlated with 17α-alkylation and the related inhibition of metabolic deactivation of steroids by oxidation of the 17β-hydroxy group.

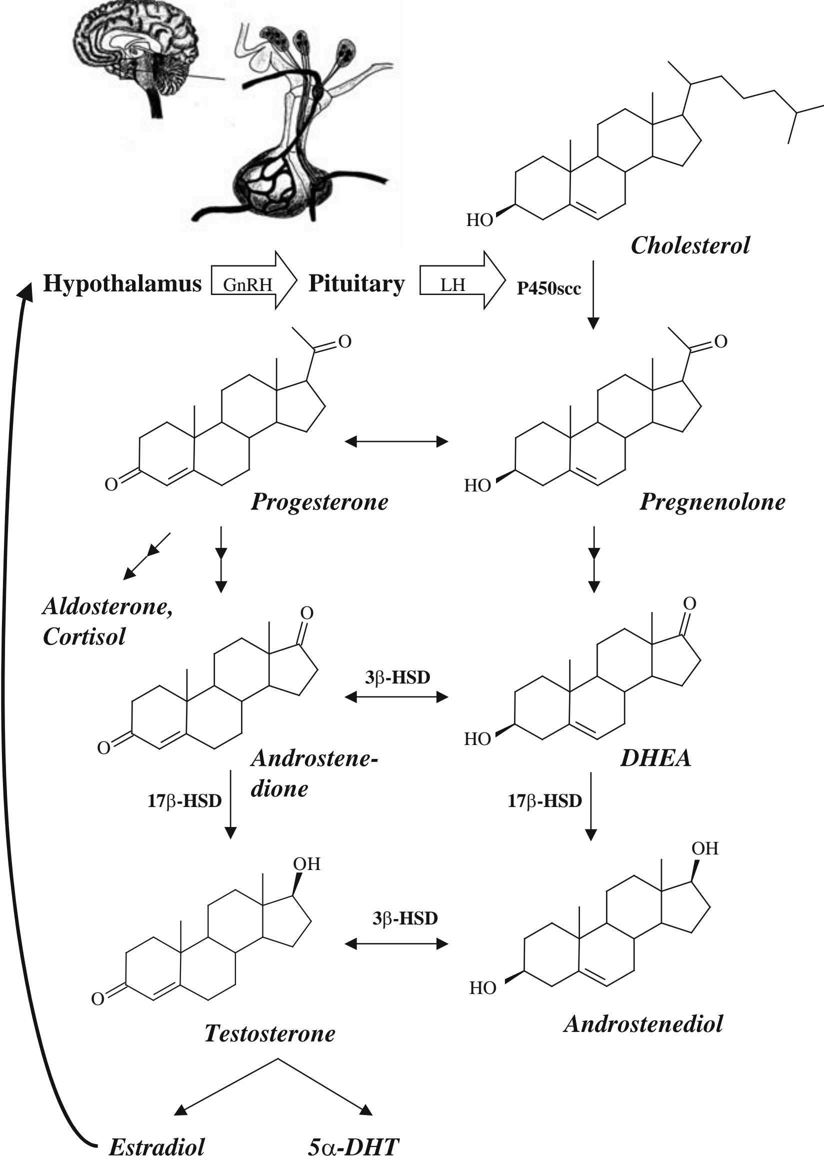

Fig. 1 Biochemical synthesis and metabolism of steroids

- Psychiatric effects of neurosteroids (characterized by 3α-hydroxylation and 5α-conformation of the A-ring) synthesized locally in the brain. The availability, induction or blocking of steroidogenic enzymes in relevant brain regions seems to be more relevant than peripheral steroid levels.

3 Anabolic Effects

3.1 Correlation of Steroid Structure and Anabolic Effects

The inactive intracellular receptor proteins are located in the cytosol. After formation of a receptor–ligand complex with suitable steroids and dimerisation, the receptor–ligand complex migrates into the cell nucleus and binds to a specific sequence of the DNA, leading to transcriptional activation of protein synthesis. Moreover, anticatabolic effects based on glucocorticoid receptor inhibition may contribute to anabolic effects.

The process is controlled by the specificity of ligand binding and DNA binding domain of the steroid receptors. Owing to the high similarity of the ligand binding domains of all receptors, steroid ligands cross-react with different receptors.

17β-Hydroxylation and an A-ring carrying a 3-keto or equivalent substitutions are thought to be the essential structural characteristics of anabolic effects and therefore affected by its biotransformation (Fig. 2). The time-limiting metabolic reaction of testosterone degradation is an A-ring reduction by 5α/β-hydrogenation. Further metabolic degradation leading to (partial) deactivation of anabolic steroids are oxidation of 3- and 17α/β-hydroxysteroids by various hydroxysteroid dehydrogenase (HSD) isoforms. Moreover, anabolic steroids are partially deactivated by aromatisation of the A-ring to yield estrogens.

Therefore, a protection of the respective groups contributes to the efficacy of anabolic effects and modifies the spectrum of potential side effects. The most potent measure to prolong its activity consists of alkylation in position 17α protecting the 17β-hydroxy group and enhances bioavailability and anabolic effects in particular after oral administration.

Typical modifications to prevent aromatisation and/or saturation of the A-ring are alkylation in positions 1 or 2, chlorination in position 4, insertion of the 2-oxa group (oxandrolone), introduction of a 1–2 double bond, reduction of the 4–5 double bond to yield 5α-dihydrosteroids and removal of the C19-methyl group.

Fig. 2 Modifications of biological effects of steroids and their bioavailability may be achieved by structural modifications, particularly at positions 1, 2, 4 and 17 or removal of the angular C19. The β-conformation of the 17-hydroxy-group is essential for anabolic effects of steroids while 3-keto groups are considered to be supportive but not essential and may be replaced by a 2–3 double bond (e.g., desoxymethyltestosterone)

Generally, androgenic anabolic efficacy of steroids is governed by their receptor binding and susceptibility to biotransformation. DHT, metandienone and oxymetholone are supposed to be strong anabolic agents. Remarkably, the anabolic effects of desoxymethyltestosterone (1.6 times superior to testosterone), stanozolol or furazabol are significant in spite of lacking 3-keto groups.

3.2 *Pathological Findings*

The use of AAS in athletes is widespread, both for performance improvement and for augmenting muscular development and strength. However, AAS use is not limited to elite athletes and seems to be more extensive among recreational and amateur athletes (Catlin and Hatton 1991; Hartgens and Kuipers 2004). Strength athletes mainly use AAS to increase muscle mass and strength. Weightlifters and power-lifters strive primarily for strength, whereas bodybuilders train for muscle mass and body dimensions (Fig. 3).

The long-term side effects are related to structure of steroids, dosage, frequency of use, age at initiation and concurrent illicit drug use and have not been fully elucidated (Yesalis and Bahrke 1995). Nevertheless, there is a broad spectrum of possible adverse effects caused by supraphysiological AAS levels which are summarized in Table 1 (Catlin and Hatton 1991; Eklöf et al. 2003; Evans 2004; Graham and Kennedy 1990; Hartgens and Kuipers 2004; Haupt and Rovere 1984; Hickson et al. 1989; Karila 2003; LaBree 1991; Maravelias et al. 2005; Melnik et al. 2007; Modlinski et al. 2006; Narducci et al. 1990; Yesalis and Bahrke 1995). Of these, some are reversible and often constitute cosmetic problems only. However, others are irreversible and may lead to serious harm. Of these, the best documented effects are those on the cardiovascular system, serum lipids, liver and the reproductive system.

Although life-threatening side effects seem to be rare, nearly 100% of persons using AAS experience subjective side effects as a result of AAS use (Evans 2004; Parkinson and Evans 2006). However, there are numerous case studies and reports which reported serious adverse effects and deaths. Obviously, even many well designed studies do not reflect the AAS problem in real life. Many studies lack a control group and do not account for individual, exercise and environmental

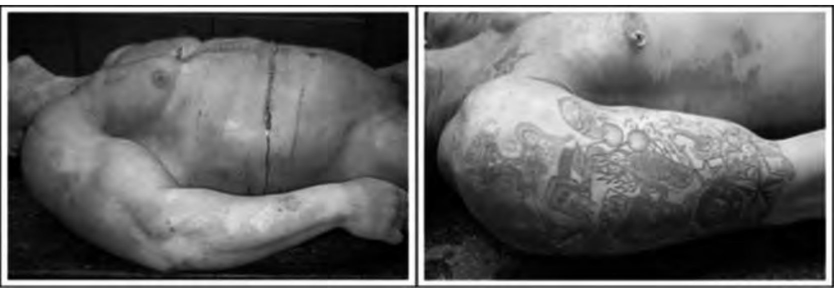

Fig. 3 External aspect of a professional bodybuilder (*left*) and of a recreational AAS user

Table 1 Possible adverse effects of AAS abuse

Cardiovascular system	*Psychic/behavioral disturbances*
Disturbed lipid metabolism	Mood swings
Elevated blood pressure, hypertension	Aggressiveness
Cardiac arrhythmias	Depressive symptoms
Myocardial hypertrophy	Manic symptoms
Cardiomyopathy	Sleep disturbances
Thrombosis	Withdrawal, dependence
Early myocardial infarction	Psychosis
Sudden cardiac death	
Endocrine/reproductive system	*Kidney*
Decrease of libido	Pollakisuria
Decrease of fertility	Increase of serum creatinine
Decreased LH and FSH	Kidney stones
Altered glucose metabolism	
Male-specific side effects	*Skin*
Testicular atrophy	Acne
Erectile dysfunction, impotence	Urticaria
Subfertility	Striae
Prostatic hypertrophy	Alopecia
Impaired spermatogenesis	
Gynecomastia	
Female-specific side effects	*Gastrointestinal tract*
Hirsutism, virilization	Queasiness
Voice deepening	Emesis
Menstrual irregularities	Diarrhoe
Clitoral enlargement	Hematemesis
Reduced breast size	
Liver	*Musculoskeletal*
Cholestasis, jaundice	Tendon damage
Peliosis hepatis	Bone pain
Neoplasia	Premature epiphyseal closure (adolescents)
Gall bladder stones	
Injection-related	*Various*
Hematoma	Edema
Infection	Fever, shivers
Fibrosis	Anaphylactic shock
Neuro-vascular injury	
Hepatitis B or C and HIV infection	

variables. Furthermore, the covert nature of their misuse may explain the limited and conflicting data concerning their side effects. Therefore, adverse effects might be much more severe than reported in the literature.

In a prospective study, a Finnish cohort of former elite power-lifters with presumed, but not verified, earlier use of AAS exhibited a 4.6-fold higher mortality compared to a control population, with myocardial infarction (Fig. 4) as the main cause of death (Pärssinen et al. 2000). A Swedish study suggested that AAS abuse is associated with an increased risk of premature death, especially among persons with additional substance abuse and/or psychiatric disease (Petersson et al. 2006a).

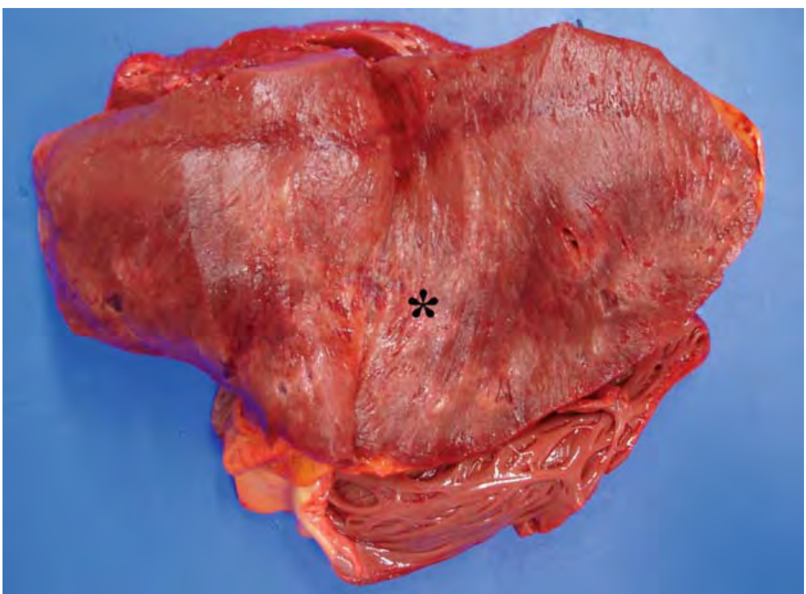

Fig. 4 Old myocardial infarction in a 30-year old AAS abuser

After long-term AAS abuse (sudden) cardiac death is the most common manifestation of AAS toxicity (Di Paolo et al. 2007; Dickerman et al. 1995; Fineschi et al. 2007; Hausmann et al. 1998; Luke et al. 1990; own observation).

Upon autopsy as well as in living persons, a dose-dependent left-ventricular hypertrophy has been observed (De Piccoli et al. 1991; Dickerman et al. 1997; Karila 2003; Karila et al. 2003; Krieg et al. 2007; McKillop et al. 1986; Nottin et al. 2006; Sachtleben et al. 1993; Sader et al. 2001; Thiblin et al. 2000; Urhausen et al. 2004; Yeater et al. 1996; own observation). Similar findings have been described for the cardiac ventricular septum (Sader et al. 2001). According to an echocardiographic study this concentric left-ventricular myocardial hypertrophy can still be demonstrated a long time after cessation of AAS abuse (Urhausen et al. 2004).

Other cardiac complications of AAS abuse include myocardial fibroses and myocardial necroses (Halvorsen et al. 2004; Luke et al. 1990; Sullivan et al. 1998; own observation), as well as early coronary sclerosis (Santora et al. 2006; own observation, Fig. 5).

As the cause of these alterations, coronary spasm, increased atherogenesis, impairment of coagulation and fibrinolyis as well as direct cardiotoxic (arrhythmogenic) effects of AAS have been proposed.

The effects of AAS on blood pressure are still discussed controversially (Hartgens and Kuipers 2004; Sullivan et al. 1998). In some studies the development of hypertension could not be demonstrated after the chronic intake of AAS (Hartgens et al. 2003; Karila et al. 2003; Kuipers et al. 1991). In contrast, other studies

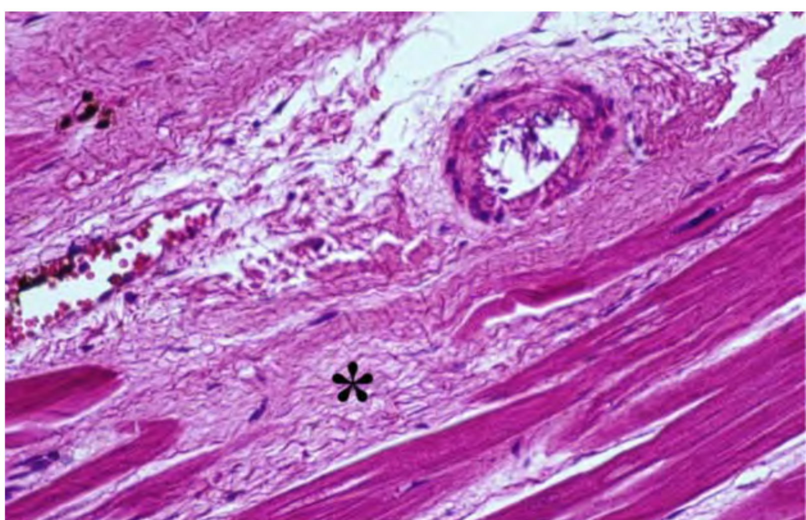

Fig. 5 Microphotograph of myocardial fibrosis in an AAS abuser

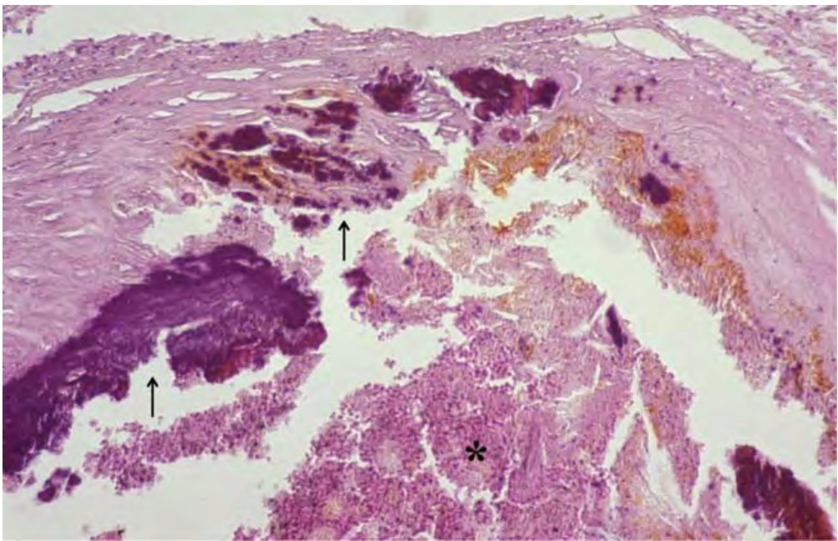

Fig. 6 Marked coronary artery sclerosis

observed the occurrence of hypertension after long-term AAS abuse (Grace et al. 2003; Rockhold 1993).

Besides morphological alterations, functional changes of the vascular system have been described after AAS abuse. These consisted of an increased aortic stiffness (Kasikcioglu et al. 2007) and an impaired vascular reactivity (D'Ascenzo et al. 2007; Ebenbichler et al. 2001; Lane et al. 2006). Although the alterations of

impaired vascular reactivity seem to be partially reversible after abstinence, consumers are at risk for vasospasm with subsequent myocardial infarction (Fig. 4). In an own autopsy case (Fig. 6), a 41-year old man died due to sudden cardiac death. The heart weighted 482 g and showed a severe coronary artery sclerosis. Toxicological analyses revealed high levels of metandienone.

4 Bioconversion of Steroids to Characteristic Androgens and Estrogens

4.1 Conversion to Androgens

Although androgenic and anabolic effects are mediated through the same receptor (hAR), the balance between both effects of steroids varies significantly and seems to be associated with certain structural characteristics. DHT in particular exhibits a hAR binding which is 2–10 times superior to testosterone. Therefore, conversion of T to DHT in androgen-responsive tissues (prostate) amplifies the androgenic effects considerably.

Since 5α-DHT is a potent androgenic substance, 5α-reduction of steroid A-rings is supposed to be a characteristic structural feature of androgens. Similarly, synthetic steroids with original 5α structure, e.g. mesterolone or methenolone, are potent androgenic compounds. Steroids with A-ring modifications stabilizing the A-ring conformation, e.g. stanozolol, oxandrolone or oxabolone, appear to be comparatively less androgenic. Another promising approach to partial separation of androgenic and anabolic effects is the removal of 19-methyl to yield 19-norsteroids. Nandrolone (19-nortestosterone) exhibits reduced androgenic effects but retains anabolic activity comparable to testosterone (Gao et al. 2005).

The ratio of anabolic (myotrophic) to androgenic effects of nandrolone is significantly higher than the corresponding index of testosterone, although both steroids are biotransformed by analog enzymes and pathways (Fig. 7). This is assumed to be due to the fact that 5α-dehydro-19-nortestosterone (relative to nortestosterone) binds with weaker affinity to the hAR, opposite to DHT which exhibits a higher affinity than testosterone.

Consequently, adrenergic effects of steroids are inconsistent and depend on the particular chemical structure. The administration of metandienone, nandrolone decanoate or testosterone cypionate stimulated male sexual behavior after gonadectomy, while stanozolol, oxymetholone or methyltestosterone showed no effect (Clark and Harrold 1997; Clark et al. 1997).

In females the administration of AAS will induce masculinization. Acne, reduction of libido and voice deepening was reported in the first weeks of abuse. After long-term AAS abuse menstrual irregularities, enlargement of the clitoris and reduction of breast size usually develops (Evans 2004; Hartgens and Kuipers 2004; Wu 1997). In contrast to males, some changes, e.g. voice deepening, are not fully reversible (Maravelias et al. 2005).

Fig. 7 Partial dissociation of myotrophic and androgenic effects by biotransformation of testosterone (T) and nandrolone (NT) to corresponding 5α-dehydro metabolites result in opposite effects. This is due to the fact that DHT has 2–10-fold androgen-receptor affinity (compared to T) while receptor binding of DHNT is weaker than NT. Therefore, bioconversion in androgen-responsive tissues may alter its activity in both directions

4.2 Conversion to Estrogens

Endogenic estrogens are biosynthesized by aromatisation of suitable steroid precursors, e.g. testosterone to estradiol. Similarly to AAS, the biological effects of estrogens are mainly governed by stereospecificity, i.e. 17β-estradiol is the most potent estrogen. Hence the estrogenic side effects are dependent on susceptibility of steroid A-rings to aromatisation (i.e. 19-hydroxylation, followed by cleavage of the C19 and subsequent enolisation of the 3-keto group; see Kicmann 2009). Structural modifications like reduction of 4–5 double bond (mesterolone or methenolone), A-ring condensation (e.g. stanozolol, Fig. 8), methylene substitution in position 2 (e.g. oxymetholone) or additional double bonds in the A-ring (trenbolone or tetrahydrogestrinone) significantly impede the conversion to estrogens and corresponding side effects.

In spite of lacking aromatisation, certain steroids (oxymetholone) exhibit significant estrogenic side effects, which are thought to be due to the cross-reaction of respective substances at high dosages with estrogen or progesterone receptors.

On the other hand, estrogenic effects of synthetic anabolic steroids may be increased with respect to testosterone, e.g. by 17α-alkylation. Aiming at a stabilization of the 17β-hydroxy group to prolong the anabolic effect, the 17α-alkyl group prevents a deactivation of 17β-estradiol to the (less potent) 17-keto or 17α-OH compounds (Fig. 8). Therefore, 17α- alkylated steroids – insofar as not A-ring protected – often cause comparatively stronger estrogenic effects (e.g. methyltestosterone, metandienone).

At supratherapeutic doses of AAS, the peripheral conversion of androgens to estrogens results in gynecomastia. Besides the pain and cosmetic implications of gynecomastia, surgical correction may be necessary (Hartgens and Kuipers 2004).

Estrogenic side effects may be reduced by administration of antiestrogens, which either suppress the aromatisation of steroids or selectively block the estrogen receptor. Due to the high potency of estrogenic side effects, these so-called antiestrogens are frequently abused amongst bodybuilders and are prohibited in sports.

stanozolol

methyltestosterone

17α-methylestradiol

Fig. 8 Synthetic modification of steroids may suppress aromatisation and resulting side effects by A-ring stabilization (e.g., stanozolol, top). Alternatively, the presence of a 17α-methyl group prevents the deactivation of 17α-methylestradiol by oxidation of the 17β-hydroxy group, resulting in comparatively elevated estrogenic effects of methyltestosterone

For the prevention of gynecomastia, the self-administration of estrogen-receptor blocking substances such as tamoxifen is widespread (Hartgens and Kuipers 2004).

4.3 Suppression of Endogenous Steroid Biosynthesis and Pathological Effects on the Endocrine and Reproductive System

The negative feedback of elevated concentrations of testosterone, DHT or estrogens to the hypothalamus and pituitary leads to a suppression of endogenous production of gonadotropic hormones and endogenous steroids (Fig. 1), associated with morphological effects on endocrine systems (testis), psyche and sexual behavior.

According to animal experiments dealing with the influence of steroids on male sexual behavior (Clark and Harrold 1997; Clark et al. 1997), there was a significant suppression after high-dose administration of 17α-alkylated steroids (stanozolol, oxymetholone or methyltestosterone), whereas the application of metandienone, nandrolone decanoate or testosterone cypionate had little influence on expression of mounts, intromissions or ejaculations of intact male rats.

According to self reports of bodybuilders, boldenone, trenbolone and nanrolone were considered as the most unpleasant steroids due to their suppression of libido (Bachmann and Sinner 2007).

AAS suppress the HPG axis. As a consequence the exogenous intake of AAS will decrease the endogenous production of testosterone and gonadotropins

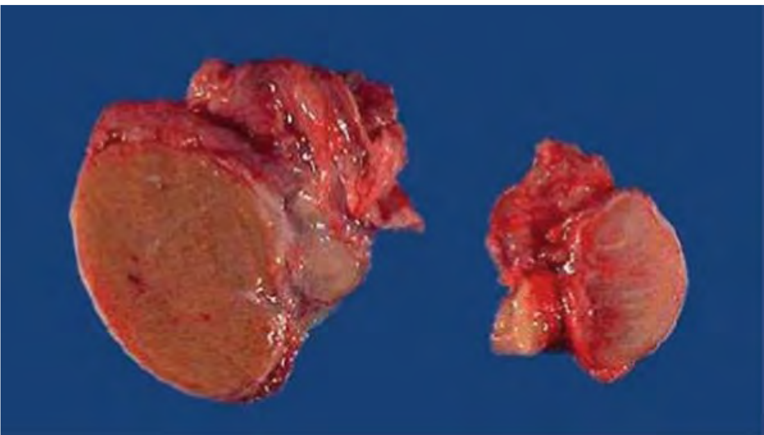

Fig. 9 Testicular atrophy in a 30-year old AAS abuser (*right*) compared to normal size (*left*)

(luteinising hormone – LH and follicle-stimulating hormone – FSH). The resulting side effects are gender-specific (Evans 2004; Hartgens and Kuipers 2004; Wu 1997; own observation). In males this suppression leads to a testicular atrophy (Fig. 9), a decreased spermatogenesis with a reduction of sperm count and motility, erectile dysfunction, impotence and a decrease of libido. These side effects are dose- and time-dependent and fully reversible months after abstinence (Hartgens and Kuipers 2004; Yesalis and Bahrke 1995). For erectile dysfunction the coadministration of sildenafil, tadalafil or vardenafil which might increase the risk of sudden cardiac death (own observation) is regularly reported.

The effects of AAS on the blood glucose level may include peripheral insulin resistance, hyperinsulism, hyperglycemia, and a reduced reaction to glucagons (Cohen and Hickman 1987; Graham and Kennedy 1990).

A reduction of thyroid hormones has also been observed after AAS use (Shahidi 2001).

4.4 *Metabolic Suppression and Hepatotoxic Effects*

There is sufficient evidence that hepatotoxic effects of steroids are associated with 17α-alkylation of the molecules (Applebaum-Bowden et al. 1987; Pey et al. 2003; Socas et al. 2005; Stimac et al. 2002). Both biochemical malfunctions such as effects on lipid profiles (increase of alkaline phosphatase, lactate dehydrogenase, conjugated bilirubin (Hall and Hall 2005)), high density lipoprotein (HDL)-C reduction or pathological increase of serum transaminases as well as morphological indicators of hepatocellular and intrahepatic cholestasis, adenomas, hepatic failure, hepatocellular hyperplasia, and general hepatic damage are significantly associated with 17α-alkylation of steroids. Interestingly, there seems to be a characteristic structural correlation to hepatotoxicity, extra to the obvious fact that 17α-steroids

are mainly taken orally at relatively high dosages, which potentially damage liver cells due to the high steroid load (first pass effects). However, other substances applied orally at high concentrations are not characteristically hepatotoxic (e.g. methenolone acetate). On the other hand, stanozolol is reported to cause liver damage regardless of its application pathway (intramuscular or oral).

The significant association of hepatotoxicity and 17α-alkylation of steroids is likely due to its conjugation. D-ring glucuronides of estradiol and ethinylestradiol (Vore et al. 1983a,b) were found to cause dose-dependent reversible cholestasis (Vore et al. 1983a,b). Alternative injection of estradiols conjugated at position 3, [3-(β-D-glucuronide)] or position 17 [17β-(β-D-glucuronide)] to rats demonstrated that hepatotoxicity is clearly restricted to D-ring glucuronidation (Slikker et al. 1983; Vore et al. 1983a,b). Moreover, the development of cholestasis was observed after administration of glucuronides of testosterone or DHT, suggesting that A-ring modifications are less significant than conformation of the 17-glucuronide. A general decrease in toxicity was observed after A-ring saturation and reduction of 3-keto group to 3-hydroxy-steroids, but there is no correlation between liver toxicity and primary pharmacological (i.e. anabolic, estrogenic or progestational) effects (DeLorimier et al. 1965). 17β-Glucuronides were found to be specifically and strongly bound to a site in the canalicular membrane (Changchit et al. 1990). This structural and stereo specificity is thought to be due to the similarity of 17β-conjugates of steroids to bile acids, which are acidic 17β-substituted steroids. Steroid-induced cholestasis and hepatotoxicity may therefore initially be attributed to a competition between steroid-17β-(β-D-glucuronides) and bile acids for recognition at receptor sites or the decrease of permeability of hepatocytes (Vore et al. 1983b).

17α-Alkylation of steroids hugely diminishes the variety of metabolic pathways, because the formation of 17-keto metabolites or 17-epimers is blocked. In the case of testosterone, these metabolites represent the majority of urinary metabolites (see Fig. 9, Kicman 2009). After 17α-alkylation, the remaining portion of critical 17β-glucuronides is therefore potentially higher leading to an elevated risk of hepatotoxic effects. On the other hand, the formation of 17β-glucuronides maybe suppressed by alkylation.

4.4.1 Pathological Findings in the Liver

The alterations after AAS abuse are broad and include cholestatic jaundice (Chitturi and Farrell 2001; Ishak and Zimmerman 1987; own observations), peliosis hepatis (Ishak and Zimmerman 1987; Soe et al. 1992), focal nodular hyperplasia and adenoma (Ishak and Zimmerman 1987; Socas et al. 2005; Soe et al. 1992; own observation), as well as hepatocellular carcinoma (Ishak and Zimmerman 1987; Soe et al. 1992).

17α-Alkylated steroids as well as nonalkylated anabolic steroids are responsible for the development of hepatic adenoma (Fig. 10) and carcinoma (Socas et al. 2005;

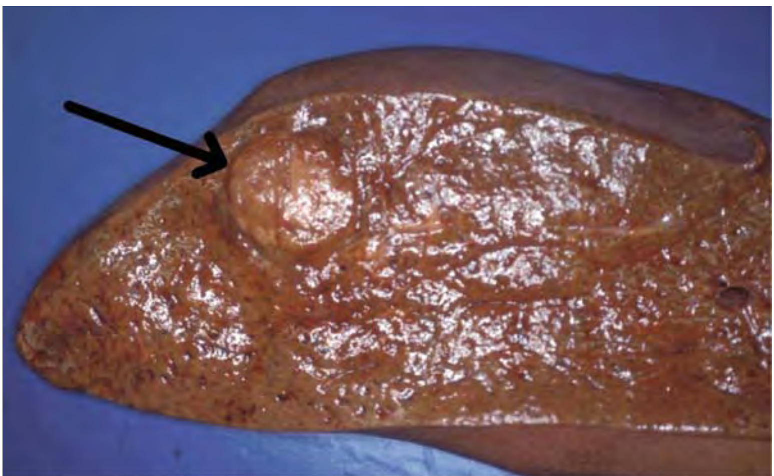

Fig. 10 Adenoma of the liver in an AAS abuser

Soe et al. 1992). Some authors recommend surgical removal of adenomas since those persons are at high risk for malignant progression and tumor hemorrhage with subsequent hepatic rupture (Socas et al. 2005). The risk of hepatic hemorrhage with hepatic failure is also high in peliosis hepatis characterized by the formation of multiple blood-filled cysts within the liver.

The mechanism of action is most likely from a direct toxic effect (Modlinski and Fields 2006). However, the incidence of the above mentioned changes after AAS abuse is still unclear.

4.4.2 Lipid Metabolism

Although many reports demonstrated premature arteriosclerosis after AAS abuse, there is so far no direct evidence for the causation of arteriosclerosis by AAS (Melchert and Welder 1995). However, the chronic abuse of AAS has been shown to induce profound alterations in serum lipid concentrations. There is a decrease of the vasoprotective HDL level (Ebenbichler et al. 2001; Fröhlich et al. 1989; Glazer 1991; Hartgens et al. 2004; Hislop et al. 2001; Karila 2003; Sader et al. 2001) and an elevation of the vasoaggressive low-density lipoprotein (LDL) (Fröhlich et al. 1989; Glazer 1991; Hurley et al. 1984; Webb et al. 1984), resulting in a decreased HDL/LDL ratio. These alterations of lipid parameters seem to be especially associated with stanozolol (Applebaum-Bowden et al. 1987; Sloane and Lee 2007) and oxymetholone (Pavlatos et al. 2001).

Other laboratory parameters of lipid metabolism that might be impaired are a decrease in lipoprotein (a) (Cohen et al. 1996; Fröhlich et al. 1989; Hartgens et al. 2004; Hislop et al. 2001; Lenders et al. 1988; Lippi et al. 1997; Zmuda et al. 1996)

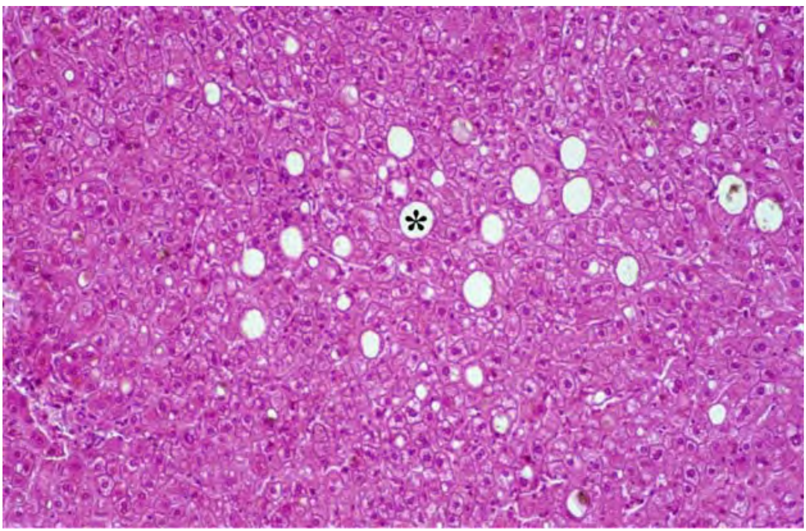

Fig. 11 Lipid droplets in the liver of an AAS abuser

and apolipoprotein A-I and A-II (Fröhlich et al. 1989; Zuliani et al. 1989) and an elevation of the apolipoprotein B level (Hartgens et al. 2004; Zuliani et al. 1989).

This lipid profile has been demonstrated to persist up to five months after cessation of AAS abuse (Thiblin et al. 2000). However, based on the observations that AAS may induce an atherogenic lipid profile, they are considered as a potent cardiovascular risk factor (Ebenbichler et al. 2001; Glazer 1991). Therefore, the abuse of AAS is associated with the development of coronary artery disease and an increased risk of both acute vascular occlusion and arrhythmic sudden death (Thiblin et al. 2000).

In an own autopsy case, a 31-year-old male died due to metabolic failure (Fig. 11). The heart weighted 536 g. Toxicological analyses revealed high levels of steroid esters (testosterone, nandrolone, boldenone) as well as stanozolol, clenbuterol, and tetrahydrocannabinol.

5 Neurosteroids and Psychiatric Effects

5.1 Biosynthesis of Neurosteroids

The influence of steroids on mood, behavior and libido is undisputed and proven by a multitude of clinical observations. Typical changes in behavior and mood are significantly correlated to variations in steroid biochemistry (e.g. menstrual cycle, age-related steroid reduction). Biochemical malfunctions may cause typical

Fig. 12 Neuronal biosynthesis of neurosteroids, e.g., 3α,5α-tetrahydrodesoxycorticosterone (3α,5α-THDOC) from endogenous progesterone (PROG). The biochemical pathway is controlled by conventional enzymes of steroid biochmistry, i.e., 3α-hydroxysteroid dehydrogenase (3α-HSD) and 5α-reductase

psychiatric symptoms (adrenal hypoactivity associated with sleeplessness and inability to concentrate), and inhibition of steroid secretion and/or blocking of their effects proved to be an adequate measure for treatment of depressions (Dubrovsky 2005; Giammanco et al. 2005; Nelson and Chiavegatto 2001).

The best examined mechanism of steroid-related neuroactivity is the $GABA_A$ modulatory effect of neurosteroids (Belelli and Lambert 2005; Herd et al. 2007; Hosie et al. 2006). It consists of a positive modulation of the GABAergic effects at low dosages and GABA-mimetic activity at elevated concentrations, leading to a general suppression of excitatory neurotransmissions. This is consistent with a central inhibitory activity of these neurosteroids, which are known to express hypnotic, antidepressive or anxiolytic effects. The steroid ganaxolone 3α-methyl-progesterone, for instance, was approved as a hypnotic due to its GABA mimetic potential.

These GABA modulatory neurosteroids comprises certain metabolites of endogenous steroids which are mainly characterized by 3α-hydroxylation and the 5α-conformation of the saturated A-ring and often the presence of a 20-carbonyl group. The 3α orientation of the hydroxy group seems to be of particular importance for neuroactivity of steroids (Fig. 12). 3β-Alkylation significantly improves

the pharmacological effect of respective compounds (e.g. ganaxolone) by suppression of metabolic degradation (similar to 17α-alkylation of steroids to prolong their anabolic effects). Typical examples are 3α,5α-tetrahydroprogesterone (allopregnanolone) and 3α,5α-tetrahydrodesoxycortisosterone (THDOC).

These neuroactive steroids do not act as endocrine hormones. Steroids are definitely capable of easily passing the blood–brain barrier and may diffuse into the central nervous system after endogenous synthesis in the gonads or adrenal glands as well as after abuse of synthetic steroids. Nevertheless, there is evidence that neurosteroids act as paracrine receptor modulators and their specific neuronal response is mainly governed by biotransformation in the brain. The regio-selectivity of neuronal effects appears to be controlled by the availability of enzymes in the brain.

However, the main pharmacodynamic mode of neuroactivity action is central inhibition and hence not eligible to explain enhanced aggressiveness as a potential side effect of steroid abuse. Conclusive alternative pathways are

- Inhibition of the GABA mimetic effects of neurosteroids by antagonism,
- Suppression of biosynthesis of GABA mimetic steroids,
- Influence of glucocorticoid suppression as a consequence of steroid supplementation (e.g. trenbolone),
- Effects of high levels of estrogen biotransformation products from anabolic steroids,
- Indirect effects on serotonin or dopamine neurotransmission.

Another group of neuroactive steroids with potential GABA antagonistic effects includes estrogens or androgen sulfoconjugates (Gibbs et al. 2006; Schumacher et al. 2008). Typical examples of the latter group are sulfates of pregnenolone and dehydroepiandrosterone. Polar conjugates are no longer capable of migrating through the blood–brain barrier and are hence synthesized locally. Brain concentrations of steroid conjugates were found to be independent of blood levels and remained unchanged after adrenalectomy or gonadectomy. Generally, these compounds are considered to be GABA antagonists with opposite biological effects. Structural characteristics of these compounds are less significant.

5.2 Behavioral–Psychiatric Side Effects

The general development of aggressiveness after steroid abuse is epidemiologically not proven. The evaluation of this presumptive correlation in recent literature remains controversial. Cases of positive association of steroid abuse and aggressiveness (Daly et al. 2003; Kouri et al. 1995; Perry et al. 2003; Pope et al. 2000a,b; Su et al. 1993) are described as well as the absence of significant effects (Anderson et al. 1992; Yates et al. 1999), which may be due to the existence of opposed

pharmacodynamic mechanisms, involving similar steroids acting as either agonists or antagonists. Moreover, suppression of biosynthesis and biotransformation of steroids is of paramount importance for neuroactivity of AAS.

So far, there is no obvious structure–activity correlation to explain or predict the potential of anabolic steroids as candidates for elicitation of aggressive behavior.

The enzymatic biosynthesis of neurosteroids is a prerequisite for neuroactivity of steroids and may logically be affected by inhibition of enzymes. Application of enzyme blockers (e.g. finasteride to inhibit 5α-reductase) significantly reduces central nervous effects of neurosteroids (Reddy 2004).

Similarly, neuroactive effects are significantly reduced by competitive antagonism with structurally related compounds like 17-phenyl-5α-androst-16ene-3α-ol (Kelley et al. 2007; Mennerick et al. 2004).

Self reports of enhanced aggression connected with the abuse of anabolic steroids is often devoted to particular compounds like fluoxymesterone or trenbolone. According to animal studies, enhanced aggression was correlated with the administration of testosterone and methyltestosterone, while stanozolol was even found to inhibit aggression (Breuer et al. 2001; McGinnis et al. 2002).

Moreover, there are assumptions that the balance between estrogen and androgen receptor mediated signaling is essential for relevance of steroids in aggression enhancement (Clark and Henderson 2003).

However, there is clear evidence that changes of behavior and aggression enhancement do not necessarily develop coincidentally with steroid abuse. These effects seem to parallel the suppression of endogenous biosynthesis of steroids rather than levels of exogenous steroids. Administration of testosterone propionate was demonstrated to cause reduction of concentration of GABA inhibitory allopregnenolone in the brain of treated mice, corresponding to enhancement of aggression (Pinna et al. 2005). Elevated aggression amongst AAS abusers was correlated with attenuated concentrations of endogenous testosterone and behavioral effects of AAS were shown to be secondary to endogenous steroid levels (Thiblin and Petersson 2005; Daly et al. 2003).

Testosterone levels have been associated with aggressive behavior (Bahrke and Yesalis 2004; Brower 2002; Copeland et al. 2000; Trenton and Currier 2005; Uzych 1992). Moreover, violent behavior and concomitant criminality associated with AAS abuse has been reported in several cases (Choi et al. 1990; Eklöf et al. 2003; Hall et al. 2005; Klötz et al. 2006, 2007; Pope et al. 2000a,b; Pope and Katz 1994; Thiblin et al. 2000; Trenton and Currier 2005; Yesalis and Bahrke 1995). A confounding factor in those cases is that high risk behavior may also be the primary problem of those who abuse AAS (Middleman and DuRant 1996).

Other behavioral changes include irritability, depressive and manic symptoms as well as psychosis (Hall et al. 2005; Talih et al. 2007; Thiblin et al. 1999). In addition, it has been suggested that depressive symptoms during AAS use may convey an increased risk of suicide (Brower et al. 1989; Thiblin et al. 2000).

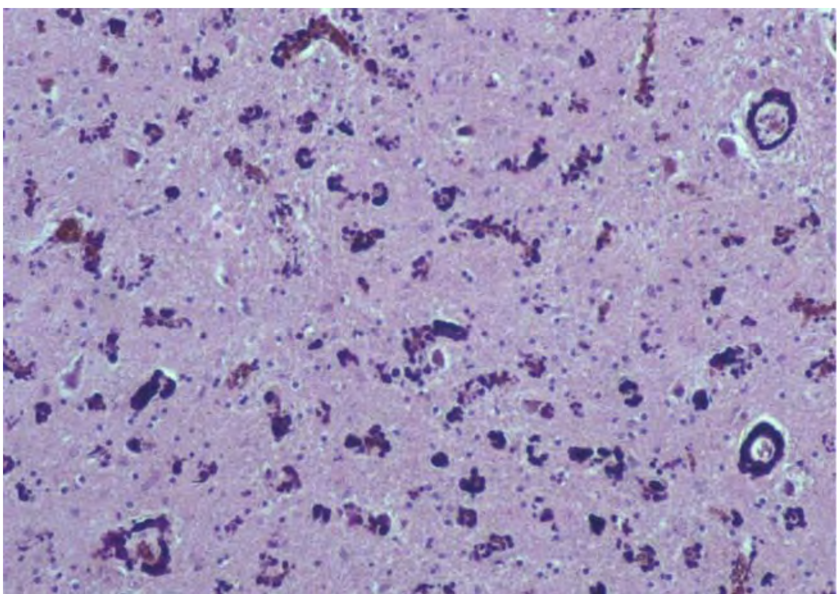

Fig. 13 Microphotograph of striato-pallido-dentate calcinosis (Fahr's disease) in an AAS abuser

Another emerging problem is the concomitant use of other drugs. Significant associations between the use of anabolic steroids and the use of cannabis, amphetamines, cocaine, cigarettes, and alcohol have been reported (Arvary and Pope 2000; DuRant et al. 1993; Kanayama et al. 2003; Petersson et al. 2006a,b; Simon et al. 2006; own observation). Especially in adolescents, the protocol of injecting steroids represents an increased level of commitment to illicit drug use, which often leads anabolic steroid users to engage in behavior similar to that of other drug abusers (DuRant et al. 1993). Furthermore it has been demonstrated that AAS have the potential for physical and psychic addiction, with the occurrence of withdrawal symptoms after cessation of AAS abuse (Arvary and Pope 2000; Bahrke and Yesalis 2004; Brower 2002; Copeland et al. 2000; Lukas 1996; Talih et al. 2007; Trenton and Currier 2005). Therefore, AAS may serve as "gateway" to other illicit drug abuse with substantial associated morbidity and even mortality (Arvary and Pope 2000; Kanayama et al. 2003).

Moreover, morphological alterations of the CNS are observed. Besides cardiovascular complications, AAS have been associated with the occurrence of stroke (Akhter et al. 1994; Chu et al. 2001; Frankle et al. 1988; Laroche 1990; Mochizuki and Richter 1988; Pálfi et al. 1997). Striato-pallido-dentate calcinosis (Fahr's disease) has been described in an AAS abuser which could be associated with AAS-induced hypercalcemia (Büttner et al. 2001; Sahraian et al. 2004) (Fig. 13).

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Gene Doping

Hassan M. E. Azzazy

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Abstract Gene doping abuses the legitimate approach of gene therapy. While gene therapy aims to correct genetic disorders by introducing a foreign gene to replace an existing faulty one or by manipulating existing gene(s) to achieve a therapeutic benefit, gene doping employs the same concepts to bestow performance advantages

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on athletes over their competitors. Recent developments in genetic engineering have contributed significantly to the progress of gene therapy research and currently numerous clinical trials are underway. Some athletes and their staff are probably watching this progress closely. Any gene that plays a role in muscle development, oxygen delivery to tissues, neuromuscular coordination, or even pain control is considered a candidate for gene dopers. Unfortunately, detecting gene doping is technically very difficult because the transgenic proteins expressed by the introduced genes are similar to their endogenous counterparts. Researchers today are racing the clock because assuring the continued integrity of sports competition depends on their ability to develop effective detection strategies in preparation for the 2012 Olympics, which may mark the appearance of genetically modified athletes.

Keywords Erythropoietin • Gene doping • Gene delivery • Insulin-like growth factor-1 • Hypoxia-inducible factors • Myostatin

Abbreviations

ACE	Angiotensin-converting enzyme
cDNA	Complementary DNA
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
GH	Growth hormone
GHRH	Growth hormone releasing hormone
GTP	Guanosine triphosphate
HIFs	Hypoxia-inducible factors
IGF-1	Insulin-like growth factor-1
IL-6	Interleukin-6
INF-γ	Interferon gamma
ND	Not detected
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
pDNA	Plasmid DNA
PEIs	Polyethylenimines
PEPCK-C	Cytosolic phosphoenol pyruvate carboxykinase
PLGA	Poly(D,L-lactic-co-glycolic acid)
PPAR-δ	Peroxisome proliferator-activated receptor delta
RBCs	Red blood cells
rAAV	Recombinant adeno-associated virus
rEPO	Recombinant erythropoietin
RG	Resomer grade
SB	Sleeping beauty

SELDI-TOF	Surface enhanced laser desorption/ionization time-of-flight mass spectrometry
siRNA	Short interfering RNA
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
WADA	World Anti-Doping Agency
X-SCID	X-linked severe combined immunodeficiency

1 Introduction

In most cases, doping involves exploitation of therapeutic agents to gain performance advantages over competitors. This is the case with gene doping which has branched out from legitimate gene therapy research and clinical trials originally designed to correct genetic disorders. Gene doping is based on the introduction and subsequent expression of a transgene, or modulating the activity of an existing gene to achieve an additional physiological advantage (Unal and Ozer Unal 2004; DeFrancesco 2004; Azzazy et al. 2005; Haisma and de Hon 2006; Azzazy and Mansour 2007; Baoutina et al. 2007). According to the 2008 list of prohibited substances published by the World Anti-Doping Agency (WADA), gene doping is defined as "nontherapeutic use of cells, genes, genetic elements, or modulation of gene expression, having the capacity to enhance athletic performance" (WADA 2009).

Erythropoietin (EPO), insulin-like growth factor-1 (IGF-1), growth hormone (GH), hypoxia-inducible factors (HIFs), peroxisome proliferator-activated receptor delta (PPAR-δ), and myostatin genes are perceived as prime candidates for gene doping (DeFrancesco 2004; Azzazy et al. 2005; Baoutina et al. 2007). Each one of these genes has been linked to a specific performance enhancement such as increased endurance or muscle mass. Specifically, the administration of EPO gene results in increased oxygen delivery to muscles, while silencing the myostatin gene results in increased muscle development. Gene doping holds the potential to make the "super athlete" at the cost of sports ethics and athletes' health.

In addition to the notion that the promises of gene doping may be too great a temptation for some athletes, there are several indicators that justify the expectations that gene doping could soon be a reality. Since the initial approval of gene therapy trials in the early 1990s, over 1,000 clinical trials have been approved worldwide with minimal side effects, excluding one fatality on account of autoimmune response (Baoutina et al. 2007). There have also been several successful trials for "gene doping" in animal models. Table 1 presents examples of successful studies of gene therapy and doping trials in animal models. The outcome of such studies is the main cause for concern over the imminent threat of gene doping. As with anabolic steroids, some athletes would try whatever they can get to obtain performance enhancement, regardless of the risks, and there is little doubt that some

Table 1 Selected gene therapy and doping studies

Target gene	Study	Animal model	Results	References
EPO	Gene delivery using an adeno-virus	Cynomolgus macaques ($n=9$)	Successful expression of fully functional EPO protein (serum EPO levels were measured by ELISA) Side effects: – Severe immune response towards endogenous and new EPO – High increase in blood viscosity hampering normal blood flow and heart function	Chenuaud et al. (2004)
EPO	Hydrodynamic limb vein delivery of plasmid DNA into skeletal muscle	Rat anemia model ($n=10$)	Long-term EPO expression for more than 450 days. The expression was also proportional to the dose of injected plasmid DNA. Serum EPO levels were measured using ELISA methods	Sebestyen et al. (2007)
VEGF	Plasmid injection into ischemic limb muscle	Patients with chronic critical leg ischemia (24 limbs in 21 patients)	– Distal flow improved in 79% of limbs – 75% of ischemic ulcers healed or markedly improved	Shyu et al. (2003)
PPAR-δ	Gene injection into mice zygotes	Mice ($n=4$, for each of the control and transgenic groups)	– Running time improved by 67% while the distance improved by 92% (as determined by running of mice on a treadmill; exhaustion endpoint was when the mice could not avoid repetitive electric shocks) – Resistance to obesity even in lack of exercise and on fat-rich diet	Wang et al. (2004)
IGF-1	Gene delivery using an adeno-associated viral vector	Rats ($n=24$)	20–30% increase in muscle strength and mass and an increase in endurance. IGF-1 expression level was measured using reverse-transcription PCR. Contractile properties of flexor hallucis longus muscle were measured in situ	Lee et al. (2004)

Human GH	Gene delivery using adeno-associated virus serotype 2 via ductal delivery to salivary glands	Male Balb/c mice ($n=5$)	A notable increase in salivary human GH levels of $1,084 \pm 102$ pg mL^{-1} and serum levels of 151.5 ± 17.3 pg mL^{-1} 4 weeks post gene administration. Serum and saliva hGH levels were determined using a chemiluminescence immunoassay kit	Voutetakis et al. (2005)
PEPCK-C	Ex vivo gene transfer	Mice ($n=9$)	– PEPCK-C over expression – Increased homecage activity – At a speed of 20 m min^{-1}, transgenic mice ran for up to 6 km while the controls ran for only 0.2 km before exhaustion – Lower weight and body fat as compared to controls despite eating 60% more	Hakimi et al. (2007)

Modified from Azzazy et al. (2005). Abbreviations: ELISA: enzyme-linked immunosorbent assay; EPO: erythropoietin; IGF-1: insulin-like growth factor-1; PEPCK-C: cytosolic phosphoenol pyruvate carboxykinase; PPAR-δ: peroxisome proliferator-activated receptor delta; VEGF: vascular endothelial growth factor; GH: growth hormone

athletes would attempt gene doping. Finally, detection of gene doping is a challenging task and currently there are no tests for its detection. The core of the detection challenge lies in the fact that proteins expressed by the introduced transgenes are basically the same as their endogenous counterparts. Nevertheless, there are several promising innovative strategies that are currently being pursued for detection of gene doping.

This chapter presents viral and nonviral gene delivery methods, candidate genes targeted for doping, and an overview of the potential detection strategies.

2 Methods of Gene Delivery

An ideal gene delivery method should carry the transgene across the plasma membrane and into the nucleus of target cells, protect the transgene against degradation by nucleases, and have no toxic effects. The transgene can be introduced into the body either in vivo or ex vivo (Fig. 1). In the case of in vivo gene delivery, the target gene is introduced directly into the human body (either as naked DNA or as a part of a plasmid or viral vectors) using a syringe or a gene gun or chemical carriers such as lipososmes (Niidome and Huang 2002; Baoutina et al. 2008). Ex vivo gene delivery involves gene transfer to host cells in culture (using viral or nonviral methods) and re-intro duction of the transfected cells into the host.

Simple needle injection of naked plasmid DNA directly into a tissue such as muscle leads to cell transfection and low-level gene expression (Wolff and Budker 2005). Specific or nonspecific cell surface DNA receptors have been implicated in the uptake mechanism. However, naked plasmid DNA, being a large and hydrophilic molecule, is kept out of cells by several barriers. In addition, DNA degradation by nucleases reduces the chance that DNA that succeeds in entering the cell will be functional. Therefore, viral and nonviral gene delivery methods have been developed to increase the efficiency of delivering intact and functional DNA into target cells.

2.1 Viral Gene Delivery

In this method, replication-deficient vectors derived from retroviruses, adenoviruses, or lentiviruses are used to deliver the gene of interest. Like Trojan horses, these genetically engineered viruses are introduced into the body where they enter the cells and release their transgene load. The transgene will be expressed using the cells' biochemical machinery. Some of these viruses, such as retroviruses, integrate their genetic materials into a chromosome of a human cell. Other viruses such as adenoviruses introduce the transgene into the nucleus of the cell without integrating the transgene into a chromosome. Viral vectors are efficient gene delivery vehicles as they offer several advantages: large packaging capacity, cell-specific tropism, and/or long-term expression (Jager and Ehrhardt 2007).

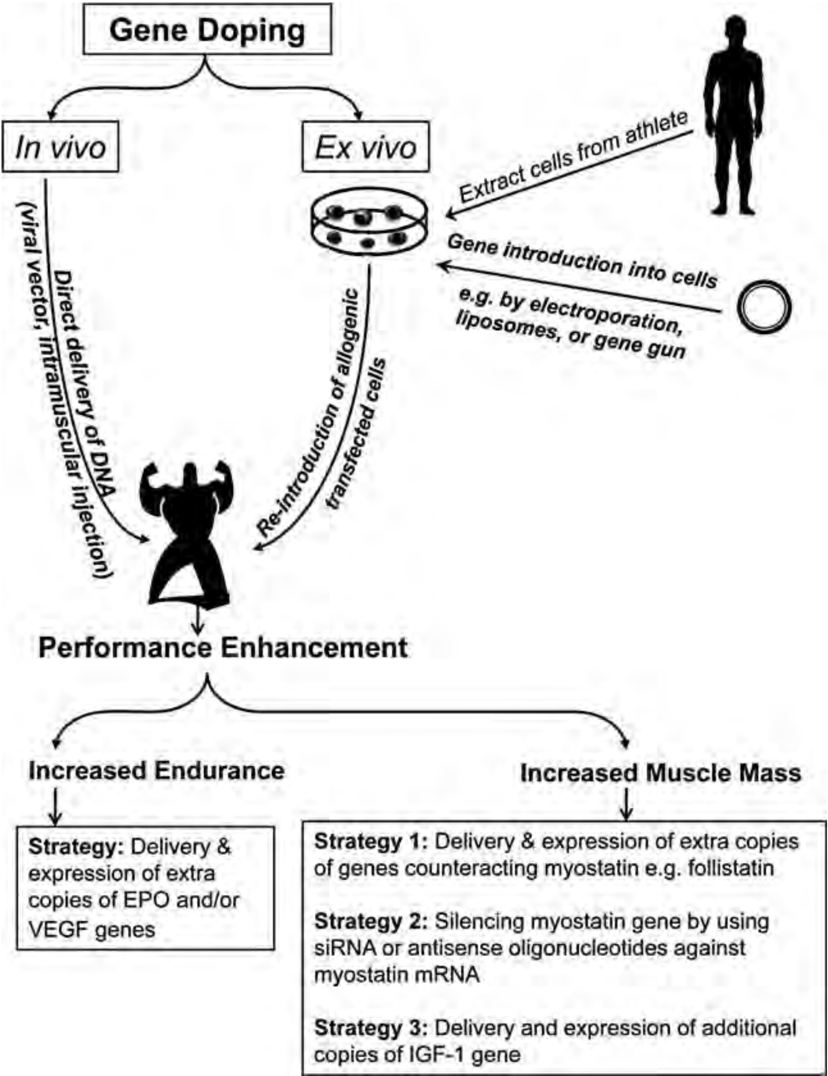

Fig. 1 Diagram illustrating possible gene doping strategies

2.2 Nonviral Gene Delivery

In order to overcome problems of viral gene delivery such as unpredicted endogenous virus recombination, oncogenic effects, and immune response, several physical and chemical gene delivery techniques have been developed for transfer of naked plasmid DNA into target cells/tissues. DNA receptors have been found in various tissues that have been proposed as mediators of DNA uptake into cells; however, their function has not clearly been understood (Siess et al. 2000).

2.2.1 Physical Methods

Electroporation

This technique involves the application of controlled electric fields to facilitate cell membrane permeability and uptake of transgenes. Skin is the ideal target and muscles are also good targets for gene delivery using electroporation. Several factors should be optimized including electrical field strength, electrode shape, and ionic strength of the injection medium. Electroporation can be applied to increase efficiency of in vivo gene transfer after direct injection of naked plasmid DNA. Widera et al. (2000) reported that intramuscular injection of animals with hepatitis B and HIV DNA vaccines, followed by electroporation of the injected muscle, resulted in a substantial increase of DNA delivery into cells, increased expression of the transgenic proteins, and potent immune responses against HIV gag protein and hepatitis B surface antigen.

Gene Gun

This technique involves shooting gold particles coated with DNA through cell membranes thus allowing direct gene delivery into the cytoplasm and the nucleus. A limitation of this technique is the shallow penetration of DNA into tissues. Direct immunization against malaria has been reported using DNA vaccine delivered by gene gun (Yoshida et al. 2000).

Ultrasound

This technique can increase the permeability of cell membrane to plasmid DNA.

Ultrasound application is flexible, safe, and is suitable for clinical use. Amabile et al. (2001) reported high-efficiency gene delivery via therapeutic ultrasound.

Hydrodynamic Injection

Potent gene transfer to internal organs such as liver has been achieved using rapid injection of naked plasmid DNA solution (5 μg plasmid DNA in 1.6 mL saline solution, injected in 5–8 s) in the tail of a 20 g mouse (Suda et al. 2007).

2.2.2 Chemical Methods for Gene Delivery

Chemical DNA carriers have been developed to protect DNA from nucleases and other blood components, target DNA to specific cell types, and/or increase delivery of the DNA to the cytosol or the nucleus.

Lipids

A large number of cationic liposomes such as quaternary ammonium lipids and cationic derivatives of cholesterol and diacylglycerol have been developed. Lipids vary in the number of charges in their hydrophilic head and the detailed structure of their hydrophobic tail. Lipoplexes form when cationic liposomes are mixed with DNA first through rapid electrostatic interaction and then by a slow lipid rearrangement process. The transfection efficiency of liposomes varies according to the chemical structure of the cationic lipid, the charge ratio between DNA and the cationic lipid, and size and structure of lipososmes (Liu et al. 2003). Despite extensive research on cationic liposomes, they are still far from being viable alternatives to the use of viral vectors in gene therapy.

Polymers

Polyethylenimines (PEIs) have been reported as nontoxic and efficient agents for in vivo gene delivery. Linear or branched PEIs can be prepared and small PEI/DNA complexes can be made using PEIs of low molecular weight (Lemkine and Demeneix 2001).

Biodegradable polymers exhibit low toxicity and they do not accumulate in the cells after repeated administration. Also biodegradation of the polymer can be used as a tool to release the plasmid DNA into the cytosol. Poly(D,L-lactic-co-glycolic acid) (PLGA) microparticles were prepared by mixing solutions of different types of PLGA polymers with different Resomer grades (e.g., RG502, RG503, RG504). These biodegradable and biocompatible microparticles can condense plasmid DNA without compromising its structural and functional integrity and subsequently release DNA upon degradation of the polymer matrix. PLGA was also shown to have a protective effect against endonuclease digestion (Díez and Tros de Ilarduya 2006).

Peptides

Anionic pH-sensitive peptides and cationic fusogenic peptides show membrane disrupting activities in endosomes and could enhance translocation of DNA to the cytosol. A bifunctional peptide with both DNA-binding and membrane-disrupting activities increased gene expression in lung tissues following tail-vein injection (Rittner et al. 2002).

On the other hand, attaching nucleus-localizing signal signal peptides to gene carriers such as PEI or directly to DNA has been proposed to facilitate entry of DNA into the nucleus, a problem that is quite serious in nondividing cells (Cartier and Reszka 2002).

2.3 Technical Challenges in Gene Delivery

2.3.1 Durability of Genetic Expression

Long-term effect can be achieved by via multiple rounds of gene doping. Alternatively, durability can be achieved by integrating the transgene into a chromosome so that it remains active for some time. However, integrating gene transfer vectors pose a risk of undesirable side effects including insertional mutagenesis, where integration of the transgene at the wrong site may cause the development of cancerous cells. This approach has been employed to treat babies with X-linked severe combined immunodeficiency (X-SCID) syndrome however, three of the treated babies developed leukemia-like symptoms (Kaiser 2003). The use of stable, nonintegrating vectors is thus preferred to achieve a long-term effect.

The sleeping beauty (SB) transposon system has been proposed as an alternative to achieve sustained expression without risking mutagenesis. This vector, which lacks viral sequences, precisely transfers specific DNA constructs from a donor plasmid into a mammalian chromosome. An enzyme, SB transposase, is required for the excision of the transposon from a donor plasmid and integration into a chromosomal site. SB transposase can be delivered to cells as a gene or mRNA (Hackett et al. 2005).

2.3.2 Immune Response

When viral vectors are used to deliver transgenes they may be recognized by the immune system of the host. Immune response against viral vectors will reduce the efficacy of gene doping and may cause other serious side effects. Enhanced immune response would also complicate repeat administrations of the transgene. Problems also exist for nonviral gene delivery systems. Using cationic liposomes causes rapid induction of proinflammatory cytokines (such as TNF-α, IL-6 and INF-γ) by immune cells stimulated by the unmethylated CpG motifs in the plasmid DNA. However, several approaches have been designed to circumvent inflammatory toxicity including elimination of CpG motifs in plasmid DNA (Yew et al. 2000).

2.3.3 Safety

While viral vectors are the carriers of choice for gene delivery they may pose several problems for the doping athlete including toxicity, immune and inflammatory responses. In addition, issues of targeting and gene expression control are uncertain. Finally, there is a possibility that a viral vector may recover its pathogenic ability once it enters the host cells.

3 Candidates for Gene Doping

Any gene that contributes to production or implementation of motion, oxygen delivery, neuromuscular coordination, muscle growth/repair, or pain relief could be a candidate for gene doping (Fig. 1). EPO, HIFs, IGF-1, PPAR-δ, and myostatin inhibitor genes have been identified as primary targets for doping (DeFrancesco 2004; Azzazy et al. 2005; Azzazy and Mansour 2007; Baoutina et al. 2007). Genes encoding for analgesic peptides such as endorphins and enkephalins may be used as alternatives to the widely used and Banned analgesics. The biochemical, physiological, and expected performance enhancement properties of candidate doping genes and their expressed proteins are summarized in Table 2.

3.1 Erythropoietin

Increasing oxygen delivery to tissues is important to optimize muscular activity and improve endurance. Oxygen delivery to tissues can be increased by training at high altitude or in hypoxic rooms, blood transfusion, and administration of EPO.

The first evidence linking genetic alteration, and EPO, to enhanced athletic performance was demonstrated by a Finnish skier, Eero Mantyranta, who dominated the Olympic Nordic skiing in 1964. Later studies documented that Mantyranta benefited from a natural mutation in his EPO receptor gene that resulted in the generation of a greater number of RBCs and enhanced capacity to deliver oxygen to various tissues including muscles (Sweeney 2004; Azzazy and Mansour 2007).

EPO is a 165-amino acid (34 kDa) glycoprotein synthesized by the kidney in response to low blood oxygenation. EPO stimulates erythropoiesis through actions on erythroid progenitor cells. The carbohydrate content of EPO is about 40%, a feature which contributed greatly to the detection of recombinant EPO (rEPO). rEPO is widely used to treat anemia caused by chronic renal disease. EPO was the first recombinant hematopoietic growth factor produced and has been available commercially as a recombinant protein drug since 1989 (Caldini et al. 2003). Several types of rEPO are commercially available including: epoetin alpha (Eprex, Janssen-Cilag), epoetin beta (Neorecormon, Roche), and darbepoetin alfa (Nespo, Dompè) (Caldini et al. 2003; Pascual et al. 2004). It is estimated that doping using rEPO was being implemented by 3–7% of the best athletes of endurance sports (Wilber 2002). Side effects of EPO include hypertension and thrombotic cardiovascular and coronary events. The Sydney 2000 Olympics marked the beginning of the use of effective methods to detect injected rEPO.

For gene doping, an additional copy of the EPO gene can be delivered to the athlete using a viral vector leading to expression of excess EPO and enhanced oxygen-carrying capacity of the blood. EPO gene transfer to animals including monkeys and mice has been successful (see Table 1).

Table 2 Top genes targeted for doping

Gene	Expressed protein	Physiological function of expressed protein	Expected performance enhancement	References
Erythropoietin (EPO)	Glycoprotein hormone	Increases the number of red blood cells and thus increases blood oxygenation	Endurance	DeFrancesco (2004); Lippi et al. (2006)
Insulin-like growth factor-1 (IGF-1)	Protein consisting of 70 amino acids	Increases muscle size and mass	Strength	DeFrancesco (2004)
Human growth hormone (GH)	Protein consisting of 191 amino acids. It has a number of isoforms, the major ones have sizes of 22 and 20 kDa	Increases muscle size and mass	Strength	DeFrancesco (2004)
Peroxisome proliferator-activated receptor-delta (PPAR-δ)	Nuclear hormone receptor protein	Associated with the formation of type I or slow-twitch skeletal muscle and can induce their conversion from type II or fast-twitch fibers, which are determinants of endurance and speed respectively. Also, it is very likely to have a role in body weight control	Speed and endurance	Wang et al. (2004); Grimaldi (2005)
Myostatin	A protein consisting of two subunits, 110 amino acids each	Acts as negative regulator of muscle mass. Its inhibition would lead to limited restriction of muscle growth	Strength	Whittemore et al. (2003)

Cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C)	622 amino acid enzyme (EC 4.1.1.32)	Key regulator of gluconeogenesis. It catalyzes conversion of oxaloacetate into phosphoenol pyruvate using GTP, a rate-limiting step of gluconeogenesis. It also affects the influx of intermediates of the citric acid cycle	Endurance	Hakimi et al. (2007); BRENDA
Vascular endothelial growth factor (VEGF)	Glycosylated disulfide-bonded homodimer protein. Several isoforms exist	Induces production of new blood vessels	Endurance	Shyu et al. (2003); Gaffney and Parisotto (2007)

Modified from Azzazy et al. (2005). GTP: guanosine triphosphate

In 2002, Oxford BioMedica, a British pharmaceutical company, developed Repoxygen as a potential treatment for anemia associated with renal disease and cancer chemotherapy. The drug is administered intramuscularly and consists of a viral vector delivering a modified human EPO gene under the control of an oxygen-sensitive gene switch. The EPO transgene is expressed in response to low oxygen levels and is turned off again when oxygen concentration returns to normal. In 2006, Repoxygen came into the doping spotlight when Thomas Springstein, a German track coach, was accused of giving repoxygen to high-school-age female runners. Repoxygen is prohibited according to the World Anti-Doping Code 2009 Prohibited List.

3.2 Hypoxia-Inducible Factors

HIFs are transcription factors that are naturally released under hypoxic conditions and alter the transcription of genes that control aerobic performance such as genes encoding for EPO and glycolytic enzymes (Lippi and Guidi 2004). The stimulation of HIF release under normal oxygen supply, e.g. by chemical agents, would automatically lead to dramatic improvement in athletic endurance. Of note, HIFs also stimulate genes involved in cell growth and division and may ultimately promote cancer development (Lippi and Guidi 2004). The ability of HIFs to modulate EPO gene expression makes their use fall under the scope of the WADA's definition of gene doping. They may also be categorized within substance doping, since releasing factors for EPO are explicitly prohibited in the substance doping list (WADA 2009).

3.3 Growth Hormone

GH is synthesized and released from the anterior pituitary as multiple isoforms. Production of GH is stimulated by GH-releasing hormone (GHRH), a peptide hormone released by the hypothalamus. Evidence regarding the enhancing effects of GH on muscle strength and cardiac and respiratory functions in trained healthy individuals is lacking or insufficient. In contrast, health risks attributed to GH, including insulin resistance, impaired glucose tolerance, and hazardous effects on the cardiovascular and respiratory systems, are accumulating. However, there is anecdotal evidence that GH is widely abused by athletes. The recent development and introduction of a new GH test into antidoping control may somewhat deter athletes from abusing recombinant GH.

Several studies on GH gene transfer into animals have been reported. In one study, allogenic fibroblasts engineered to produce GH were transplanted into rats and human GH production was successfully regulated by glucocorticoids (Inazawa et al. 2001).

Gene therapy with GHRH has also been applied in several animal species to increase GH production. Intramuscular injection of a plasmid containing GHRH

gene under the control of a muscle-specific promoter resulted in increased levels of GH and IGF-1 for over a year and enhanced anabolic and hematological parameters (Khan et al. 2005).

3.4 Insulin-Like Growth Factor-1

IGF-1 is reported to mediate some of the anabolic actions of GH. Increased IGF-1 can also promote hypertrophy through increased protein synthesis and satellite cell proliferation. Although injection of human recombinant IGF-1 protein is more straightforward than introducing the IGF-1 gene, it is limited, however, by the high concentration needed to produce a significant effect, given its short half-life and rapid clearance. Gene doping with IGF-1 could provide the needed stable high concentrations of IGF-1. This approach would be relatively safe as the effects would be limited to the targeted muscle. Overexpression of IGF-1 combined with resistance training induced greater muscle hypertrophy than that observed with either treatment (Lee et al. 2004). IGF-1 gene transfer has also proven successful in promoting skeletal muscle regeneration after injury and more effective than systemic administration of IGF-1 protein (Schertzer and Lynch 2006). However, IGF-1 administration may lead to profound hypoglycemia, as can insulin. It is also suspected to promote cell cycle progression and inhibit apoptosis, either by triggering other growth factors or by interacting with pathways with established roles in carcinogenesis (Tentori and Graziani 2007).

3.5 Myostatin

Myostatin is a negative regulator of muscle development. Silencing or mutating the myostatin gene or introduction of substances that inhibit myostatin would result in increased muscle development. A child was born in Berlin with a mutation that turns off myostatin gene. At 4.5 years, this boy was reported to have the physique of a mini-bodybuilder (Schuelke et al. 2004). For therapeutic purposes, this mutation could be investigated to treat muscle wasting diseases. For doping purposes, athletes can be injected with a viral vector carrying a silencing gene that blocks myostatin or with small interfering RNA (siRNA) against myostatin (Furalyov et al. 2008). Such treatment would elicit superphysiological muscle development, in terms of number and thickness of cells, that would be difficult to trace.

3.6 Peroxisome Proliferator-Activated Receptor Delta

PPAR-δ, a nuclear receptor, was reported to control energy balance, reduce fat burden and protect against lipotoxicity caused by ectopic lipid deposition. Experiments demonstrated that activation of PPAR-δ reduces weight gain, increases

skeletal muscle metabolic rate and endurance, improves insulin sensitivity and cardiovascular function and suppresses atherogenic inflammation (Reilly and Lee 2008). One study has suggested that polymorphisms in PPAR-δ gene may modify changes in cardio-respiratory fitness and plasma lipid levels in healthy individuals in response to regular exercise (Hautala et al. 2007).

3.7 Vascular Endothelial Growth Factor

VEGF increases the development of new blood vessels and may be advantageous to patients suffering from coronary artery disease (Tiong and Freedman 2004). VEGF gene doping may result in enhanced vasculature and oxygen delivery to peripheral tissues of athletes, thus improving endurance.

3.8 Angiotensin-Converting Enzyme

ACE inhibitors are regularly used for treatment of hypertension. Researchers have reported that the ACE-I allele may contribute to endurance and could be useful for long distance runners. The ACE-D allele, however, is associated with elite sprinting performance (Macarthur and North 2005; Hruskovicová et al. 2006).

3.9 Endorphins

For refractory and severe pain, which is not adequately controlled by conventional therapeutic options, gene therapy is being investigated as a viable alternative for pain management. Athletes suffer from numerous injuries and consume large quantities of antiinflammatory and pain-relieving drugs. Athletic performance would clearly benefit from increasing the pain threshold and alleviating the discomfort of sustained injuries. The introduction of genes encoding endorphins and enkephalins would increase the threshold of pain experienced in a competition as a result of either lactic acid build-up or acute or chronic injury (Fink et al. 2003).

4 Health Risks

Gene doping lures athletes as the ultimate performance enhancement method. The effectiveness of gene doping is evident from several animal studies (Table 1). Despite all the setbacks of gene therapy, over 1,000 gene therapy clinical trials have been approved and new gene delivery vectors, including those based on chromosomal elements, are constantly being developed and optimized. It is

expected that athletes may attempt gene doping without necessarily waiting for full approval of gene delivery trials for therapeutic purposes.

The expected health side effects include lethal immune response among other unexpected complications. Jesse Gelsinger died in 1999 at the age of 18 during a gene therapy clinical trial at University of Pennsylvania (Somia and Verma 2000). Gelsinger suffered from ornithine transcarbamylase deficiency, an X-linked disease, and was injected with an adenovirus carrying the correct gene. He suffered a massive immune response against the viral vector and died. In addition, several patients who received gene therapy for X-linked hemophilia developed leukemia (McCrory 2003). Finally, macaque monkeys which received a single intramuscular injection of a recombinant adeno-associated virus encoding the monkey EPO produced such a high concentration of RBCs that their blood thrombosed. Several monkeys, in the same study, also developed anemia as a result of an immune response to the viral vector used (Zhou et al. 1998).

Health risks may also result from gene overexpression, a common problem in gene therapy and doping, which vary according to the type of introduced gene. Gene doping with IGF-1 or myostatin can lead to excessively large muscles that may overload tendons and bones or cause damaging differential stresses on them. Gene overexpression may cause toxicity due to accumulation of expressed proteins and/or unusual adaptation of the body to the excess amount. Because GH is a potent mitogenic and antiapoptotic agent, its overexpression may be related to development of tumors (Bidlingmaier et al. 2003).

It is customary for athletes to both ignore dosing recommendations and use multiple drugs simultaneously. For example, Barry Bonds, a successful athlete, allegedly used anabolic steroids, GH, insulin, modafinil, clomiphene, and EPO (Gaffney and Parisotto 2007). Similarly, it is highly likely that genes for strength, analgesia, oxygen delivery, and repair may be transferred to the same athlete. Clearly, there will be hazardous side effects and probably fatal interactions.

Contrary to gene therapy that is carried out under strict conditions and approved guidelines, gene doping is likely to be done in secret with limited protective actions and consequently more expected health hazards. Preparation of gene transfer vectors in noncontrolled laboratories may lead to their contamination with chemicals and pyrogens. Virulent viral vectors may be generated inadvertently and these could be harmful to athletes and the general population. While clinical trials also monitor enrolled subjects for virus shedding, no monitoring is expected for gene cheats. Unfortunately, this extensive list of hazards is not expected to deter dopers because the rewards of athletic excellence – financial, social, and psychological – are tempting enough for many athletes to willingly take the risk.

5 Detecting Gene Doping

Currently detection of gene doping represents a priority for sport organizations because of its strong potential to enhance athletic performance, success of recent gene doping trials of experimental animals (Table 1), and progress achieved in over

1,000 clinical trials of gene therapy in humans. Similar to other doping agents, gene doping undermines principles of fair play in sports and also poses a great risk to athletes. The WADA is currently funding several research projects for developing new techniques for detection of gene doping (Table 3).

A conclusive test for detecting gene doping does not exist to date (Azzazy and Mansour 2007; Baoutina et al. 2008), but extensive research is currently underway to investigate some promising strategies. A number of conceptual and practical factors have led to the fact that no tests are currently available to detect gene doping. In addition to the fact that the protein produced by the foreign gene or genetically manipulated cells will be very similar to the endogenous one, most transgenic proteins, particularly muscle enhancing ones, are generated locally in the injected muscle and may not show in blood or urine. The only reliable assay would require a muscle biopsy; such an assay is virtually inapplicable in a sport setting. Nevertheless, a number of promising strategies are currently being investigated.

5.1 Detection of the Transgenic Protein

Minor structural differences between recombinant proteins expressed by the transgenes and their endogenous counterparts may be used as basis for detection. Although the exact cause of this difference is not fully understood, it may be due to different posttranslational modifications of the proteins expressed in different cells (Baoutina et al. 2008). Monkeys doped by the EPO gene at the French National Doping Laboratories produced EPO that has a slightly different glycosylation pattern from the endogenous one (Lasne et al. 2004). The structural difference would also account for a different immune response by the host that could be traced. Transgenic EPO would appear in circulation and perhaps in urine and its analysis would be informative for doping control. However, if dopers use expression vectors with controllable promoters that can be turned on by administration of a specific drug, regular out-of-competition testing for the transgenic proteins may be necessary to monitor any suspicious activities.

5.2 Detection of the Delivery Vector

It is possible to detect the vector at the site of intramuscular injection or tissues in its immediate vicinity weeks and perhaps months after the doping event. However, collection of samples for testing would require information about the precise site of injection and ultimately a muscle biopsy; both are inapplicable in a sport setting (Baoutina et al. 2008). On the other hand, detection of delivery vectors in bodily fluids would require sampling within a relatively short time after the doping event. This calls for regular out-of-competition testing of athletes. Sample collection, storage, and handling should follow standardized validated protocols including

Table 3 List of gene doping studies funded by WADA as of 2008

Institution	Funded Project
Royal Free and University College Medical School London, UK; Universita degli Studi di Pavia, Roma, Italy	[a]Manipulation of muscle mass via the GH/IGF-1 axis.
ARC Seibersdorf Research, Seibersdorf, Austria	[a]Application of microarray technology for the detection of changes in gene expression after doping with recombinant human GH.
University of California in San Diego, California, USA	Microarray detection methods for GH and IGF-1.
Pharmacology Research Unit Institution Municipal Investigacio Medica, Barcelona, Spain	[a]IMAGENE: Non-invasive molecular imaging of gene expression useful for doping control: Pilot study in animals after EPO gene transfer.
Immunochemistry & Biotechnology Section, HFL Ltd, New Market, UK; Nottingham Trent University Nottingham, UK; UCL Medical School, University of London, UK	[a]The application of cellular chemistry and proteomic approaches to the detection of gene doping.
University of British Columbia, Canada	Development of a prototype blood-based test for exogenous EPO activity based on transcriptional profiling.
Garvan Institute of Medical Research, Austria; ANZAC Research Institute, Sydney, Australia	Detection of GH doping by gene expression profiling of peripheral blood cells in humans.
University of Tsukuba, Japan	Detection of hypoxia-inducible gene manipulation.
German Sports University, Germany; Chimera Biotech GmbH, Germany	[a]High sensitive detection of genetically and pharmacological manipulations of the myostatin signal transduction pathway by multiplex immune-PCR fingerprint analysis.
Hopitan St-Antoine, INRA Paris University, France	Methabonomic signature in bike athletes: a pilot study.
German Sport University, Cologne, Germany; Russian Research Institute, German Sport University, Cologne, Germany	Analysis of GH isoform profiles in human plasma using proteomics strategies.
International Centre for Genetic Engineering and Biotechnology (ICGEB), University of Milano, Italy; University of Florence, Italy; Instituto di Biofisica, Pisa, Italy	Molecular Signatures of IGF-1 gene doping after AAV-Mediated gene transfer.
Medical University Clinic Tubingen, Tubingen, Germany	Sensitivity and specificity of a gene doping test detecting transgenic DNA on a single molecule level in peripheral blood probes.
Aarhus University Hospital, Denmark; Ohio University, OH, USA	Proteomic analysis of serum exposed to GH: a future assay for detection of GH doping.
University of Pennsylvania, Philadelphia, PA, USA	Development of tests for detecting myostatin-based doping to enhance athletic performance.
Technical Unviersity of Munich, Munich, Germany; Institute of doping analysis, Krescha, Germany; Institute of Sport, Warsaw, Poland	Comparative gene expression profiling in human buccal epithelium and leukocytes after the abuse of beta-2-agonists and anabolic steroids.
University of Florida, Gainesville, FL; INSERM, Nantes, France	[a]A Pilot study to develop a reliable blood test for the detection of gene doping after intramuscular injection of naked plasmid DNA.

(*continued*)

Table 3 (continued)

Institution	Funded Project
Aker University Hospital, Oslo, Norway	Genetic regulation of epitestosterone glucuronidation. Consequences for evaluation of urinary T/E ratio.
University of Florida, Gainesville, FL USA; CHU Nantes, France	Development of a reliable blood test for the detection of doping after intramuscular injection of recombinant AAV vectors.
University of British Columbia Vancouver, B.C. Canada	Development of a highly-sensitive quantitative assay to detect siRNA- mediated gene doping.
University of California, La Jolla, CA, USA	Genomic, proteomic and informatics analysis of doping.
University of California, San Diego, CA, USA	[a]Pilot Project for a WADA Bioinformatics Core Facility.
University of Pennsylvania, PA, USA	Development of tests for detecting hypoxia-inducible gene doping to enhance athletic performance.
Austrian Research Centers, GmbH-ARC, Seibersdorf, Austria	Quantitative proteomics of rhGH-doping by multiplexed stable isotope labeling and MALDI-TOF/TOF mass spectrometry.
German Sports University, Cologne, Germany	Improvement of a Myostatin Imperacer assay towards a high-sensitive test system for the detection of anabolic manipulations, including gene doping strategies.
Universita degli Sutdi di Firenze Sesto, Italy	Affinity-based biosensing (ABBs) for gene doping detection: an integrated approach.
Australian Sports Commission, Australia; Bispebjerg Hospital, Denmark	The effect of training, altitude exposure and athlete's sex on expression of genes know to change following autologous blood transfusion.
Umea University, Sweden	Detection of autologous blood transfusion by proteomics: screening to find unique biomarkers.
Umea University, Sweden	Skeletal Muscle Proteome Alterations after long term Anabolic Steroid Abuse.
Medical University Clinic, Germany	Sensitivity and specificity of a gene doping test detecting transgenic DNA on a single molecule level in peripheral blood probes

[a]These projects have been completed.

snap freezing of samples in liquid nitrogen. Table 4 presents selected examples, based on animal studies and clinical trials, for distribution of gene therapy vectors in different tissues using delivery routes of relevance to gene doping.

5.3 *Monitoring the Immune Response*

Viral vectors are the most popular methods for gene delivery and are most likely to be used for gene doping. Direct detection of gene doping vectors is difficult as they

Table 4 Selected animal studies and clinical trials showing distribution and persistence of gene therapy vectors in different tissues

Vector	Mode of administration	Sample tested	Study type	Persistence of vector	References
rAAV	Intravascular	Blood	Animal	Days	Gao et al. (2006)
		Urine	Animal and clinical trials	ND/days–weeks	Nathwani et al. (2002); Manno et al. (2006)
		Saliva	Animal	ND/days	Nathwani et al. (2002)
		PBMC	Animal and clinical trials	ND/weeks–months	Hernandez et al. (1999); Manno et al. (2006)
	Intramuscular	Blood	Animal	Weeks	Rip et al. (2005)
		Serum	Animal and clinical trials	Days	Favre et al. (2001); Manno et al. (2003); Rip et al. (2005)
		PBMC	Animal	Weeks–months	Hernandez et al. (1999); Favre et al. (2001)
		Muscle (injection site)	Animal and clinical trials	Months	Favre et al. (2001); Manno et al. (2003); Rip et al. (2005)
pDNA	Intravenous	Blood	Animal	ND/hours–days–weeks	Parker et al. (1999); Koshkina et al. (2003); Quezada et al. (2004)
		Plasma	Animal	Hours[a]	Oh et al. (2001); Zhang et al. (2006)
	Intramuscular	Blood	Animal	ND/hours–days	Parker et al. (1999)
		Plasma	Animal and clinical trial	ND/weeks	Comerota et al. (2002); Thomas et al. (2003)
		Urine	Animal and clinical trial	ND/hours	Comerota et al. (2002); Romero et al. (2004)
		PBMC	Animal	ND/hours	Romero et al. (2004)
		Muscle (injection site)	Animal and clinical trial	Hours–days–weeks	Parker et al. (1999); Thomas et al. (2003); Quezada et al. (2004); Romero et al. (2004)
siRNA	Intravenous	Blood Plasma Muscle	Animal	Hours[a]–days	Judge et al. (2005); Morrissey et al. (2005); Medarova et al. (2007)

Table is modified from Baoutina et al. (2008). rAAV: Recombinant adeno-associated virus; pDNA: plasmid DNA; siRNA: short interfering RNA; PBMC: peripheral blood mononuclear cells; ND: not detected. In all experiments, the delivered vector was detected by PCR

[a]A radio-labeled vector was used and detected by measuring radioactivity

are only measurable shortly after administration and may require tissue sampling. However, test strategies based on assessing the host's immune response to viral vectors may prove rather effective. Nevertheless, there is a possibility that the tested athlete could have been infected by the virus via nondoping routes (such as viral infection or reactivation of latent viral infection). Therefore, detection of antibodies in his/her blood will not provide conclusive evidence of doping. Also, it is possible to genetically engineer the viral vectors to make them less immunogenic (tamed) and minimize the host immune response (Azzazy and Mansour 2007; Baoutina et al. 2008). Prolonged routine screening of athletes for antibodies against different viruses will be necessary to establish their baseline antibody titers against different viruses.

5.4 *Profiling Approaches*

This approach is based on monitoring secondary changes that arise as a result of gene doping. The expression of a transgene will lead to changes in the expression of other genes, their protein products, and ultimately other biochemical metabolites.

5.4.1 Gene Expression Profiling

This approach assesses the expression profile of endogenous genes that may be altered following the expression of a foreign gene (Diamanti-Kandarakis et al. 2005; Azzazy and Mansour 2007; Baoutina et al. 2008). This can be done using DNA microarrays which are used to simultaneously compare mRNA expression patterns of thousands of genes. Briefly, mRNA is extracted from cells of the doping athlete and cells from a normal individual, and then reverse transcribed into complementary DNA (cDNA). A radioactive label or a fluorescent tag is incorporated in the cDNA; a different tag is chosen for each condition, e.g. green tag for cDNA from the doping sample and red for the normal one. The basis of the microarray operation is that the synthesized cDNA binds to complementary oligonucleotides (probes) immobilized on a glass or silicon solid support, commonly referred to as a chip. The chip has numerous spots each containing a particular oligonucleotide sequence (Joos et al. 2003; Chaudhuri 2005). A laser is then used to scan the chip to visualize the fluorescent signal given by the cDNA bound to the complementary probes. The color of the signals obtained from the spots indicates whether a specific gene is up- or down-regulated or remains at normal expression levels in the doping vs. normal condition. The level of gene expression is determined by quantification of the fluorescence signal intensity. The results obtained from the DNA microarray are typically confirmed using real-time PCR (Joos et al. 2003; Chaudhuri 2005).

Based on this approach, particular gene chips could be developed for the suspected targets for gene doping, and used to compare doping vs. nondoping

conditions. The chip would contain probes for the target doping gene as well as the genes associated with its expression. This way the chip would assess the expression of the target gene itself as well as its expression signature. For example, an EPO gene doping chip can be developed based on the knowledge that exposure of erythroid progenitor cells to EPO results in the up-regulation of 54 genes and down-regulation of 36 other genes (Diamanti-Kandarakis et al. 2005). When a reference expression signature is established, the altered expression patterns of these genes along with that of the EPO gene can be used to detect EPO gene doping.

5.4.2 Proteomic Profiling

Investigation of global changes in protein biomarkers upon doping can be done using mass spectrometry-based screening. Surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) combines chromatography and mass spectrometry for protein profiling (Issaq et al. 2002). This technique can provide a rapid protein expression profile from a variety of biological and clinical samples. Chung et al. (2006) investigated exogenous GH administration in 60 healthy subjects who received either placebo or recombinant human GH using protein chip mass spectrometry. A prominent biomarker of 15.1 kDa was detected and identified as hemoglobin alpha-chain. Measurement of this biomarker may be used to detect GH doping (Chung et al. 2006).

Both gene expression and proteomic profiling approaches would require extensive studies to establish reference ranges for the general population and elite athletes. Specific reference ranges should be established with respect to gender, race, sex, and sport.

5.5 DNA Bar Codes

DNA barcoding has initially been proposed to provide a way to track genetically modified agricultural products. A unique short oligonucleotide sequence could be added to each transgene and/or viral vector used for delivery (Azzazy et al. 2005; Diamanti-Kandarakis et al. 2005). Similar to supermarket barcodes that are used to identify different products, the DNA barcode can be used to identify transgenes and their manufacturing companies. A conventional molecular diagnostic test, e.g. PCR, can be employed to detect the presence of the transgene. Constructing a database of barcodes would facilitate tracking the transgenes used for doping. Although such an initiative would greatly facilitate the detection efforts, it would require immense coordination among various sport federations and organizations, doping detection centers, and the involved diagnostic and pharmaceutical corporations. The latter may be the least cooperative since they are likely to

oppose the idea, claiming extra cost and risk of losing commercial secrets and advantages.

6 Conclusions

As gene therapy research and clinical trials advance, the potential of gene doping increases. At the same time, the promise of this new doping method to make the "super athlete" and the increasingly lucrative nature of competitive sport make the situation even more difficult. Novel approaches including ex vivo gene transfer to allogenic stem cells are also expected to advance gene doping to yet another frontier and increase the possibility of its application. The consequences of transferring foreign genes into an athlete's genome cannot be predicted. The human genome is very complex with poorly understood interactions involving the genes themselves and arrays of internal and environmental effectors.

Despite the documented and predicted risks of gene doping, some athletes are willing to ignore safety concerns and do whatever it takes to get that gold medal. There is a little doubt that some athletes would try some form of gene doping. The first genetically modified athletes are expected to compete as early as the London 2012 Olympic Games. This puts increasing pressure on clinical scientists to develop reliable and feasible methods for gene doping detection. Although the concepts of gene doping detection are almost fully developed, there is lengthy research needed to resolve related technical challenges, and optimize and standardize gene detection methods. All developed methods should be adapted to the sport setting with its unique preanalytical variables. As with all laboratory tests, the developed tests should have documented sensitivity, specificity, and validity in order to withstand the legal scrutiny expected when elite athletes test positive for gene doping.

Ultimately, a longitudinal testing approach may be most appropriate for gene doping control, where each athlete becomes his or her own reference. Baseline biochemical, hematological, and perhaps gene expression profile data should be collected for each athlete and monitored over time for any suspicious activities. The WADA is considering a project known as the Athlete Passport to gather individual athlete testing data which, over time, would serve as a baseline reference for each athlete (WADA 2009). It may be possible to expand this concept later to include expression profiles related to suspect doping genes.

Various international sport authorities should organize awareness campaigns to educate athletes about the risks of gene doping. Most athletes as well as their supporting staff may not have enough background to fully comprehend the potential risks imposed by gene doping. Therefore, educational programs would be crucial in minimizing both the risks and abuse of gene doping. An effort to develop and refine our understanding of genetic enhancement ethics should also be considered.

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Science and the Rules Governing Anti-Doping Violations

Larry D. Bowers

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Abstract The fight against the use of performance-enhancing drugs in sports has been in effect for nearly 90 years. The formation of the World Anti-Doping Agency in 1999 was a major event because an independent agency was entrusted with harmonization of the antidoping program. In addition to sports governing bodies, governments have endorsed WADA and its programs by signing a United Nations Education, Science, and Cultural Organization Convention on Doping. The first step in the harmonization process was the development of the World Anti-Doping Program. This program consisted of five documents – the Code, the International Standard for Testing, the International Standard for Laboratories, the Prohibited List, and the International Standard for Therapeutic Use Exemptions – which unified the approach of the international federations and national antidoping agencies in applying antidoping rules. For laboratory testing, the International Standard for Laboratories establishes the performance expectations for and competence of laboratories recognized by WADA, including accreditation under ISO/IEC 17025.

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The antidoping rules are adjudicated by arbitration using the internationally recognized Court of Arbitration for Sport.

Keywords WADA Code • Doping Rules • Doping Rule Violation • Doping Rule Arbitration

The goal of the antidoping movement is to deter the use of performance-enhancing drugs in order to level the playing field, reduce the coercive impact of successful drug abusers on other athletes, and protect the spirit of sport. Perceptual deterrence theory maintains that the more certain and severe the perceived threat of moral, social, or legal sanction, the more likely a behavior can be prevented. Detection of the use of performance-enhancing drugs is critical to initiating the sanctioning process. The scientific aspects of detection have been covered elsewhere in this book. It should be appreciated that laboratory detection of drugs is closely tied to the timing and fidelity of the collection process. Many of the components of collection and laboratory analysis and the consequences of abuse of performance-enhancing drugs are established by the antidoping rules.

The fight against doping by sport organizations began in 1928 with a prohibition by the International Amateur Athletic Federation of the use of stimulants such as strychnine in endurance running. Unfortunately, there were no laboratory tests that could reliably detect and identify the stimulants, and thus the success of the ban was dependent on the athlete's moral and ethical behavior. The International Olympic Committee (IOC) formed a Medical Commission in 1967 to deal with the perceived increase in drugs and other performance-enhancing substances that not only affected the evenness of the playing field but also exposed the athlete to health risks. Anabolic steroids, first used in sport in the early 1950s, were added to the prohibited lists in the mid-1970s. The Subcommission on Doping and Biochemistry in Sport maintained an annual "List of Prohibited Classes of Substances and Prohibited Methods". In 1995, the Medical Commission published the International Olympic Committee Medical Code that contained the "List of Prohibited Classes of Substances and Prohibited Methods", a description of collection procedures, the laboratory accreditation procedures, and a brief overview of the testing procedures in its 64 pages. The IOC published the Olympic Movement Anti-Doping Code (OMADC) in 1999, which included the "List of Prohibited Classes of Substances and Prohibited Methods" in its 47 pages. A significant departure from previous documents was a legal definition of doping and a description of the rights and responsibilities of athletes. Despite the advances, the decentralized application of the antidoping rules by international federations, national Olympic committees, and national sport governing bodies made uniform enforcement of the rules problematic. In some cases, sports organizations' fear of litigation over sanctioning an athlete had more deterrent effect on enforcement than the antidoping rules did on athlete behavior.

In parallel with the evolution of the antidoping rules, advances in analytical technology made the laboratory an increasingly important part of antidoping policy.

In 1966, the Union Cycliste Internationale and Fédération Internationale de Football Association were among the first international sports federations to introduce tests for doping substances into their respective World Championships. The first doping tests at an Olympic Games occurred in Mexico City in 1968. As chemical testing technology evolved, including the development of radioimmunoassay and the introduction of commercial gas chromatography–mass spectrometry instruments, these techniques were incorporated into the few laboratories that were performing antidoping testing. The IAAF recognized the need to harmonize the performance of the laboratories involved in antidoping testing, and established a laboratory accreditation program under its medical commission. In 1982, the IOC came to an agreement with the IAAF to assume the responsibility for laboratory accreditation. In the mid-1980s, the IOC recruited a number of laboratories to carry out an increased volume of drug testing worldwide. The initial IOC laboratory "accreditation" process began in 1985. Professor Manfred Donike, secretary of the IOC Subcommission, began training laboratories that were accepted into the IOC program at the Cologne Workshop on Analysis. By the mid-1990s, sport administrations began to recognize the importance of well-timed, no-advance-notice testing in out-of-competition settings and a comprehensive test distribution plan.

The earliest government interest in antidoping occurred in France and Belgium, which passed antidoping legislation in the mid-1960s. A more generalized interest of governments and inter-governmental agencies in doping in sport began in the 1980s. The Dubin Commission in Canada studied the use of steroids and other performance-enhancing substances by government-supported athletes following Ben Johnson's positive test for anabolic steroids at the Seoul Olympic Games in 1988. The 1990 Australian Senate Drugs in Sport Inquiry found systematic use of performance-enhancing drugs at the government-financed Australian Institute for Sport. This resulted in the government forming one of the first independent national antidoping agencies, the Australian Sport Doping Agency. In the United States, Congress held hearings into the use of anabolic steroids in American football. This resulted in the addition of anabolic steroids to Schedule III of the Controlled Substances Act in 1990. Interestingly, some of the strongest support for regulation of steroids in the United States came from the athletes themselves. The Council of Europe enacted an Anti-Doping Convention in 1989 that established a number of common standards and regulations for the 48 signatories. This is generally regarded as the first multilateral legal standard in antidoping. In addition to monitoring the "List of Classes of Prohibited Substances and Prohibited Methods", the Anti-Doping Convention encouraged the production and distribution of educational materials by the signatories. The recognition that an antidoping program consisted of more than just testing was an important, and previously missing, step for the movement.

The convergence of these antidoping forces occurred in February of 1999 when the IOC convened the First World Conference on Doping in Sport in Lausanne, Switzerland. For the first time, governments and sporting bodies came together to find a solution to doping in sport. The Declaration of Lausanne established the World Anti-Doping Agency (WADA) as the body to promote, coordinate, and

monitor the fight against doping. In contrast to earlier antidoping efforts, WADA is an independent agency. Perhaps more importantly, the WADA antidoping mission was to be supported equally by sport and governmental and inter-governmental agencies. WADA was incorporated as a Swiss foundation, and is governed by a Foundation Board that has equal representation by the sports movement and public authorities. WADA coordinated the development of the World Anti-Doping Program with numerous stakeholders. The Program was implemented in January 2004.

In the summer of 2006, WADA began a second extensive international consultative process to revise the World Anti-Doping Code ("Code") and the International Standards. Many of the proposed changes to the Code arose from experiences in the adjudication of antidoping rule violations under the initial version of the Code. The 2003 BALCO scandal in the United States, and in particular the beneficial interactions between law enforcement and the antidoping community, provided additional insight into what constituted a doping offense. The development of antidoping rule violations based on any reliable information, including nonanalytical information, was a major change. In addition, the rigid, harmonized sanctions that characterized the first version of the Code were altered to provide greater flexibility in sanction length as determined by the arbitration decision makers. After three rigorous rounds of consultation, Version 3.0 of the Code was approved at the Third World Conference on Doping in Sport in Madrid in November, 2007. The latest version of the Code went into effect on January 1, 2009. The delay in implementation was caused, in part, by the need to educate the athletes and other stakeholders on the numerous changes in the Code and the International Standards. The Code and accompanying International Standards and Technical Documents now fill nearly 400 pages of text.

1 WADA Anti-Doping Program

The World Anti-Doping Program consists of six documents: the Code; the Prohibited List International Standard ("List"); the International Standard for Laboratories (ISL); the International Standard for Testing; the International Standard for Therapeutic Use Exemptions; and the International Standard for the Protection of Privacy and Personal Information (World Anti-Doping Agency 2009a, b, c, d, e, f). WADA also promulgates Models of Best Practice.

1.1 WADA Code

The Code is the centerpiece of the Anti-Doping Program. The Code itself consists of four parts: Doping Control; Education and Research; Roles and Responsibilities; and Acceptance, Compliance, Modification, and Interpretation. The Code also contains definitions of the key terms in doping. The IOC required that all international federations that wished to participate in the Olympic Games incorporate key

elements of the Code (WADA Code 2009, Article 23.2.2) into their rules prior to the 2006 Turin Winter Olympic Games. This is a significant step forward, since earlier programs suffered from inconsistency amongst sports organizations (vide infra). Athletes accept the rules of their sport, including antidoping rules, as a condition of participation. The Code states:

> These sport-specific rules and procedures aimed at enforcing antidoping rules in a global and harmonized way are distinct from and are, therefore, not intended to be subject to or limited by any national requirements and legal standards applicable to criminal proceedings or employment matters. (. . .) rules represent the consensus of a broad spectrum of stakeholders around the world with an interest in fair sport (WADA Code 2009, p 18).

1.1.1 Definitions of Doping

One of the most difficult tasks for the antidoping movement has been an easy to understand, yet comprehensive, definition of doping. There are a number of reasons for this difficulty. There is a public perception that doping is only associated with increased muscle mass, which is not correct. Stimulants were among the first doping substances because they alter the perception of fatigue, increase focus and stimulation, and can enhance weight loss. Testimony in recent doping cases has revealed that doping agents such as erythropoietin (EPO), steroids, and insulin are used for recovery both during training and at competitions. There is also a misconception that some "over the counter" drugs such as pseudoephedrine are not effective doping agents. In order to understand doping with these agents, one must consider the dosage and the fact that stimulant side effects from metabolites give rise to the desired doping response. An additional contributor is the disconnect between science and the law. Legally trained decision makers must deal with the state of knowledge that exists at the time of the hearing and seem to prefer as little ambiguity as possible. In contrast, scientists rarely deal in absolutes, have a different way of dealing with hypotheses than lawyers, and always seem to prefer additional information before formulating a final opinion. The result can be a decision that is confusing to all parties, and the public.

The first definition of doping is the presence of a prohibited substance or its metabolites or markers in an athlete's sample. For substances that do not appear naturally in the body, such as most pharmaceutical agents, the identification of any amount of the drug or its metabolites is sufficient to result in an adverse analytical finding. The finding of multiple metabolites increases the certainty of the finding. Because of the relatively limited capability of the antidoping organizations to obtain evidence, it is not necessary to prove intent. This is known as a strict liability standard, and the athlete is warned that it is their personal duty to ensure that no prohibited substances enter their body. For substances that are found naturally in the body, such as testosterone, thresholds or ratios that define doping are given in the

Prohibited List. These thresholds are so far outside of normal physiological values that doping and pathological conditions are the only explanations for the results.

The definition of doping has been expanded to include the use or attempted use of a prohibited substance, possession, trafficking or attempted trafficking, and administration. Thus the antidoping organization can use any reliable means to prove a case of doping. The first cases involving a "nonanalytical positive" arose from the Bay Area Laboratory Cooperative (BALCO) scandal. Documents such as doping calendars, ledgers of test results, medical test results, and unexplainable intra-individual variations in test results as well as the testimony of other athletes was used to sanction five athletes (e.g., United States Anti-Doping Agency, Michelle Collins 2004). Kayle Leogrande received a two-year sanction based on an admission of use of EPO to the cycling team soignier (United States Anti-Doping Agency, Kayle Leogrande 2009). While there was corroborating evidence, such as suspicious urine EPO tests and pictures of Mr. Leogrande holding a box of EPO vials, the key factor was the admission. For many years, some athletes complained that they were sanctioned for the actions of their coach, trainer, or physician. With the change in the definition of doping, athlete support personnel can also be held accountable. Remi Korchemny and Trevor Graham, two coaches associated with BALCO, both accepted lifetime bans as a result of their involvement in doping athletes under their supervision.

The importance of no-advance-notice, out-of-competition (OOC) testing in a comprehensive doping control program cannot be underestimated. In the late 1990s, antidoping organizations were able to complete less than 50% of their OOC missions due to the unavailability of the athlete. In 2001, USADA developed a "missed test policy" as part of its protocol. Under this policy, an athlete who was unavailable for three test attempts in an 18-month period could be sanctioned for a doping offense. As part of the protocol, athletes were required to provide their whereabouts information quarterly so that they could be located for testing. The Code includes in its definitions of doping both the refusal to provide a sample or evasion of the doping control officer and unavailability for three missed tests. This ensures that an athlete cannot hide from antidoping agencies in order to dope. The conditions under which a missed test can be a sanctionable event are covered in the International Standard for Testing (WADA International Standard for Testing, 2008, Article 11).

1.1.2 Rights of Athletes

The Code ensures the rights of the athlete to a fair hearing (WADA Code 2009, Article 8). This includes the right to be informed of the antidoping rule violation, the right to be represented by legal counsel, the right to respond to the allegations of an antidoping rule violation, the right to a timely, impartial, and fair hearing, and the right of both parties to present evidence and call and question witnesses. The athlete is also entitled to a timely written reasoned opinion from the arbitration body. The Code does not preclude a sports body from enacting its own procedures

as long as those procedures are consistent with the above principles. In the United States, for example, the Ted Stephens Olympic and Amateur Sports Act requires that the American Arbitration Association (AAA) arbitrate any issue related to eligibility for Olympic sport. The USADA protocol recognizes this legal requirement, but in addition requires that the AAA arbitrators in North America are also members of the CAS. The Code also identifies which parties are permitted to appeal arbitral decisions. All appeals must be made to the CAS.

Doping in sport is analogous to professional misconduct in other fields such as medicine or the law. The burden of proof is also similar to that used in professional misconduct. The burden of proof under the Code is:

> . . . to the comfortable satisfaction of the hearing panel bearing in mind the seriousness of the allegation which is made. This standard of proof in all cases is greater than a mere balance of probability but less than proof beyond a reasonable doubt.

1.1.3 Antidoping Violation Resolutions

Prior to the WADA Program, individual sports had antidoping rule wording and procedures, including the burden of proof and sanctions, which were sport-specific. In addition, the application of the doping rules could differ substantially within a sport. A perfect example of the inconsistent handling of antidoping rule violations can be found in the cases of Sandis Prusis and Pavle Jovanovich, two bobsled athletes, occurring just before the 2002 Salt Lake City Winter Olympic Games. Both athletes claimed to have inadvertently consumed nandrolone or one of its precursors in a contaminated dietary supplement to explain the presence of a prohibited substance in their urine samples. The Federation Internationale de Bobsleigh et de Togogganing (FIBT) gave Prusis a three-month "retroactive" sanction that expired just prior to the Olympic competition. Interestingly, the FIBT apparently never considered any scientific evidence before arriving at their conclusion. The IOC suspended Prusis from the Winter Olympics, but CAS overturned the suspension on the grounds that the IOC did not have jurisdiction over the FIBT unless it had violated Olympic standards (Sandis Prusis and the Lavian Olympic Committee v the IOC 2002). Jovanovic received a nine-month period of ineligibility from the American Arbitration Association, based in part on the FIBT handling of the Prusis case (Jovanovic v USADA 2002). On appeal to CAS on the eve of the Olympics, Jovanovic's suspension was increased to two years, in accordance with the rules (Jovanovic v USADA 2002). Such inconsistent handling of sanctions contributes neither to the deterrence objective nor to the perception of fundamental fairness.

The Code provides for a sanction of two years for a first doping offense (WADA Code 2009, Article 10). There has been a great deal of debate about the fairness of this specified sanction length. The Code now provides for reduction of sanction if the athlete or other party can prove either no fault or negligence or no significant fault or negligence. Athletes may also receive a reduction in sanction for the use

of specified substances (vide infra) under special circumstances. The Code also provides for longer periods of ineligibility if aggravating circumstances apply.

1.2 WADA International Standards

As stated above, WADA has also developed six international standards that govern various processes in the antidoping field. The International Standard for Testing describes the test distribution planning activities required of international federations and antidoping organizations, procedures for collection of samples, the transport of the samples to the laboratory, and the responsibility of the athlete in providing whereabouts information for no-advance-notice out-of-competition testing. The incorporation of both out-of-competition testing as well as in-competition testing is critical to achieving the deterrence objective. The collection procedures address not only urine collection but also blood collection for tests such as blood boosting and growth hormone. A major change in the IST harmonizes the need for athlete availability. If athletes were not at the location they provided, a review process determined if they should incur a missed test. Some antidoping organizations placed calls to the athlete and required that they be available within a certain time period after the call. Other organizations only required that athletes be available for 1 h per day and did not require whereabouts information for any other time. In the new standard, the athlete can only obtain a "missed test" if they are not at the location they specify 1 h per day, although the athletes must provide whereabouts information for the remaining 23 h. Given the sophistication of the cheaters and their advisors, it remains to be seen whether this change will provide the necessary detection potential to achieve deterrence. It will be up to the responsible antidoping organizations to ensure that no-advance-notice testing is thoughtfully implemented.

The International Standard for the Protection of Privacy and Personal Information addresses the responsibilities of antidoping organizations when dealing with athletes' personal whereabouts and medical information, such as that required for therapeutic use exemptions. The International Standard for Therapeutic Use Exemptions defines the process to be used in determining whether an athlete has a medical need to use a prohibited substance. A number of best practice guidelines have been developed for specific disease states like asthma and for including the medical information necessary to support a therapeutic use exemption application.

1.2.1 Prohibited List International Standard

The IOC transferred stewardship of the "List of Classes of Prohibited Substances and Methods" to WADA in 2003. The Code requires an annual review of the Prohibited List, which culminates in its publication in October of each year. A

committee of eleven international experts is responsible for the review. In order for a substance to be included on the Prohibited List, it must fulfill two of the following three criteria (WADA Code 2009, Article 4.3):

1. Medical or other scientific evidence, pharmacological effect or experience that the substance or method, alone or in combination with other substances or methods, has the potential to enhance or enhances sport performance.
2. Medical or other scientific evidence, pharmacological effect or experience that the *Use* of the substance or method, represents an actual or potential health risk to the *Athlete*.
3. WADA's determination that the Use of the substance or method violates the spirit of sport described in the introduction to the Code.

The Code provides that WADA's decision to place a substance on the prohibited list cannot be challenged as part of the defense in a hearing. After the List is proposed, it is circulated to stakeholders for commentary, and approved by the WADA Health, Medicine, and Research Committee and the Foundation Board. The List then goes into effect at the beginning of the following calendar year. The lag time between publication and implementation provides the laboratories time to incorporate new substances into their testing methods and the sports organizations time to educate their constituents about the changes.

As mentioned above, when stewardship of the list was transferred to WADA it was entitled "The List of Prohibited Classes of Substances and Prohibited Methods". This reflected the scientific reality that compounds developed or available in one country or region of the world might not be available or known in another. Each prohibited class contained a list of *examples* of prohibited substances and finished with the phrase "and related compounds." Whether the compound was explicitly listed or not, the doped athlete had potentially already obtained an advantage by taking the substance. An excellent example of the problem is the psychostimulant bromantan, which was developed for the Russian military. At the 1996 Atlanta Olympic Games, the CAS-appointed pharmacology expert testified that bromantan "has the capacity to produce the same central nervous system stimulant effects as other drugs in the category" (Andrei Korneev, International Olympic Committee and Zakhar Gouliev, International Olympic Committee 1996, p 13). The expert also testified that the lack of data made it impossible to predict the quantitative effects of bromantan. Part of the difficulty in obtaining the information was that the appellants were unable to produce the documentation during the time of the hearing since "the entire month of August everybody is on vacation" (Andrei Korneev, International Olympic Committee and Zakhar Gouliev, International Olympic Committee 1996, (ibid, p 16)). The conclusion of the panel was

> "the scientific evidence before us establishes that Bromantan may well be a stimulant within the meaning of the Medical Code but that the evidence before the Court is not sufficient to establish that conclusion to the relevant and high degree of satisfaction necessary to support such a finding." (Andrei Korneev and International Olympic Committee 1996, (ibid, p 23))

Thus, while some critics view this result as a flaw in the system, it also determines what must be established to successfully prove a doping violation for a substance not specifically listed among the examples.

The importance of an “open list” was supported by the cases involving the designer steroid tetrahydrogestrinone (THG). The sole issue in the arbitration was whether or not UK Athletics, with the assistance of USADA, could prove beyond a reasonable doubt (the UK standard in 2003) that THG was chemically or pharmacologically related to gestrinone (UK Athletics Limited v Dwain Chambers 2004). The USADA was able to commission and produce scientific studies that showed that THG had a structure similar to anabolic steroids and that it bound to the anabolic steroid receptor and caused similar intracellular signaling. This scientific data ultimately resulted in doping violations for six athletes. The balance between arguments that a substance must be specifically listed in order to afford the athlete notice that the substance is prohibited and the reality that athletes, advisors, or potentially even ambitious national programs may use a substance that is not listed on the Prohibited List specifically because it is not listed will continue to be debated.

The Code provides for a subgroup of prohibited substances called specified substances. The concept behind this split is to allow the arbitral decision makers some latitude in sanction for substances that might be inadvertently used. The most dangerous prohibited substances, including anabolic agents and hormones, cannot be specified by rule. For stimulants and hormone antagonists and modulators, the List committee must stipulate which prohibited substances, like methamphetamine and cocaine, are not specified. This is a good example of the friction between the legal system and science. For example, pseudoephedrine is not prohibited but it is to be monitored by the laboratories. Norpseudoephedrine (cathine), a minor metabolite of pseudoephedrine, is a potent and toxic stimulant with a reporting threshold of $5\,g\ mL^{-1}$. When large amounts of pseudoephedrine are consumed, the amount of norpseudoephedrine can exceed the threshold leaving a legal conundrum – an adverse analytical finding potentially caused by a nonprohibited substance. Another issue is that some of the specified substances when ingested will appear in the urine as the nonspecified substance amphetamine. These examples suggest that trying to over-simplify the antidoping rules can cause conflicts with well-established scientific principles.

1.2.2 International Standard for Laboratories

The global nature of Olympic sport required a quality laboratory system that could be recognized internationally. In 1999, the OMADC required that all IOC-“accredited” laboratories also be accredited under the International Standard Organization (ISO) Guide 25. During the transition from IOC-“accreditation” to WADA-“accreditation”, the requirement for ISO/IEC 17025:1999 accreditation was maintained. It must be recognized that neither the IOC nor WADA are accrediting bodies under the usual definition of accreditation. The ISL was originally conceived as an

application of the ISO/IEC 17025 standard entitled "General requirements for the competence of testing and calibration laboratories." WADA has worked with the International Laboratory Accreditation Cooperation (ILAC) to ensure that assessors from national accrediting bodies who are signatories to the ILAC convention have proper training to assess the laboratory against both ISO/IEC 17025:2005 and the ISL. Accreditation may be defined as

> "a formal recognition that an organization is competent to perform certain specified tasks."

> Laboratory accreditation bodies assess whether a laboratory meets certain criteria and the decision of an accreditation body to accredit a laboratory is thus *a statement of the competence of the laboratory* in a specific technical area. But such a statement of competence does not mean that the accreditation body guarantees the validity of each and every test result (ILAC 1994).

Thus, while accreditation deals with the overall competence of the laboratory, it does not make any guarantee about the test that resulted in the adverse analytical finding. As a result, the laboratory must be able to document that it performed the test following its validated methods.

Because of the need for harmonization of testing performance and reporting, technical documents were developed under the Code to allow a relatively rapid response to changes in scientific or other knowledge and to deal with specific issues. Technical documents must be incorporated into the laboratory's policies and procedures and supersede any similar information in the ISL. The technical documents currently (1 January 2010) in effect are: Harmonization of the Method for Identification of Recombinant Erythropoietins (i.e. Epoetins) and Analogues (e.g. Darbepoetin and Methoxypolyethylene Glycol-Epoetin Beta) (TD2009EPO): Criteria for Qualitative Assays Incorporating Chromatography and Mass Spectrometry (TD2003IDCR); Laboratory Documentation Packages ((TD2009LDOC); Laboratory Internal Chain of Custody (TD2009LCOC); Minimum Required Performance Levels for Detection of Prohibited Substances (TD2009MRPL); Reporting and Evaluation Guidance for Testosterone, Epitestosterone, T/E ratio, and other Endogenous Steroids (TD2004EAAS); and Reporting Norandrosterone Findings (TD2009NA).

The Code includes the presumption that WADA-accredited laboratories have conducted custodial and analytical testing procedures in accordance with the ISL. The athlete may rebut this presumption, but in doing so he must be able to show that the deviation from the ISL rules could be responsible for the adverse analytical finding. In other words, simply showing a deviation is not sufficient to overturn the evidence of doping. As a result, the ISL and technical documents have taken on another role in which careful scrutiny of the precise wording is used to attempt to rebut the presumption. For example, in the Landis case, a great deal of discussion was devoted to deciding whether the phrase "metabolite(s)" meant one or more metabolites or more than one metabolite. Since the adverse analytical finding in this case was based on only one metabolite, the potential deviation from the ISL and technical documents could have "caused" the adverse analytical finding

(USADA v. Floyd Landis 2007). The presumptions granted the WADA-accredited laboratories have been somewhat controversial.

The ISL also describes the processes for obtaining and maintaining WADA laboratory accreditation. As part of the ISL, WADA has put in place an external quality assessment system (EQAS) that verifies the performance of the laboratories (Ivanova et al. 2007). WADA-accredited laboratories are required to participate in the program. Due to the sophistication of the testing procedures, urine samples collected after administration of the prohibited substance are frequently used in EQAS assessments. The WADA program includes both blind (laboratory knows that it is an EQAS sample, but does not know the content) and double-blind (laboratory does not know that it is an EQAS sample or the compound) in its assessment program. It also distributes educational EQAS samples, which are not graded but provide feedback to the laboratories to improve harmonization.

1.3 WADA Models of Best Practice

To assist in the harmonization of the process, WADA has made available Models of Best Practice. Implementation of these documents is not required for compliance with the Code. They do provide interested stakeholders with a template for complying with various aspects of the Anti-Doping Program. For example, the Guidelines for Implementation of an Effective Whereabouts Program would assist an antidoping organization with developing an athlete's whereabouts program. Other model rules and guidelines include Laboratory Test Reports and Reporting and Management of Elevated *T*/*E* ratios.

2 UNESCO Convention

Governments cannot enter into legal agreements with nongovernmental entities. Thus while the governments were stakeholders in WADA, they could not legally endorse the WADA Program. In 2005, the United Nations Education, Science, and Cultural Organization (UNESCO) passed a Convention against Doping in Sport (United Nations Education Science and Cultural Organization 2005). To date, 109 countries have signed the UNESCO Convention.

3 Arbitration of Doping Rule Violations

Using arbitration as a means of resolving disputes among stakeholders is specified in the Code and is a consensual agreement on the part of all parties. Domestic and international arbitration are considered favorable mechanisms for adjudication of

disputes for a variety of reasons. Included among them is the general belief that arbitration is faster, cheaper, and more efficient adjudication. This is largely because parties to arbitration are not subject to long delays in the standard public courts and are not subject to the same evidentiary and procedural standards as those present in the official public system. Under the United States Federal Arbitration Act, for example, the parties to arbitration are free to conduct the arbitration and to construct the arbitral agreement that specifies procedures in any manner they see fit. As such, the federal rules of evidence, federal rules of civil or criminal procedure, and other court mandated procedures are not necessarily applicable to an arbitration procedure. Further, there is general belief that by minimizing the discovery process and bypassing substantial delays associated with the court process, parties save money. In essence these components are believed to create improved efficiency in adjudicating disputes. In the case of antidoping rules, it is important for the sport competition that disputes involving eligibility be resolved rapidly.

For a global enterprise like Olympic sport, the benefits associated with arbitration are even more notable. To avoid the potentially unending difficulty of obtaining jurisdiction over foreign parties, determining which country's law is most applicable, and additionally enforcing that decision on a sovereign country's citizen, parties in sport have agreed to arbitrate disputes using the CAS. The CAS has a body of rules that control the procedural aspects of the dispute. The CAS rules, in combination with the Code and the rules of the particular sport, allow enforcement of international arbitral decisions. The CAS rules stipulate that the Swiss Federal Tribunal must hear any challenge to a CAS arbitral award. The issue of dealing with innumerable national laws has been addressed. With these rules in place, antidoping rule disputes will be resolved and there is no ambiguity as to where or how any such settlement will be made. After an arbitral decision is made by the contracted-for body, either party may take that decision to any country that has agreed to enforce arbitral decisions and seek enforcement of the arbitral award on the assets of the other party.

Because the arbitration contract is subject to freedom of contract principles, the bargain struck during formation of the arbitration agreement will guide the nature and design of the proceedings. As such, the parties may craft special rules of discovery or special procedural rules tailored to the needs of the parties in settling a dispute. For example, in maritime arbitration, which focuses on disputes related to maritime trade, parties to a dispute will often agree that a panel of maritime experts, and not legal experts, will arbitrate any disagreement. Having an expert in the field adjudicate and decide the finality of disagreements improves the likelihood that the outcome will reflect nuances and attributes of the trade, whereas a general judge may have little or no knowledge, understanding, or experience in questions related to maritime trade. Thus, one considerable benefit of arbitration agreements is the ability to contract for more knowledgeable and familiar decision makers. The Code avails itself of some of the benefits of this approach in selecting arbitrators who are familiar with sport law, but the CAS rules require that members have legal training, which limits the expertise in matters related to the science of doping. Further, the Freedom of Contract that applies to arbitration agreements enables the parties to

construct a procedure that meets the needs and interests of the parties to a contract. For example, the WADA Code and the ISL provide for the content of documentation packages provided by the laboratories. This provision is intended to provide sufficient documentation to allow for formulation of the athlete's defense while at the same time avoiding a time-consuming and expensive discovery process.

An additional benefit of arbitration is gained by the final and binding nature of both the agreement to arbitrate and the decision of the arbitrators. The US courts and the courts of most other countries have strictly enforced arbitration decisions made in accordance with arbitration agreements. The United States Federal Arbitration Act states in Section 2 that such contracts for the arbitration of disputes shall be "final, irrevocable, and enforceable, save upon such grounds as exist at law or in equity for the revocation of any contract." The FAA further provides under Section 10 that there are limited grounds for vacating an arbitral award, including: corruption, fraud or undue mean; partiality; misconduct in the proceedings; or acting in excess of the powers or subject matter referred to arbitration. There is no judicial review of the merits in arbitration. Thus, the avenues for appeal to the court of a particular country are limited. A recent Federal Court suit filed by Landis against the USADA specifically challenged the partiality of the arbitrators and, in particular, the revelation of potential conflicts of interest. The case was settled "with prejudice" on agreement of the parties meaning that Landis cannot dispute further the outcome of the arbitration.

In the case of the Code, the athlete is entitled to a "written, reasoned decision, specifically including an explanation of the reason(s) for any period of ineligibility" (WADA Code; Article 8.1, 2009). Written, published decisions are not required in arbitration agreements. As such, arbitration is not necessarily bound by precedent. Arbitrators do not have to uphold the prior decisions of other arbitrators because they are not formal adjudicators who have made a decision on the basis of the government's established set of rules. Their interpretation of the rules and facts is not subject to the same standards and scrutiny as the courts and therefore is not subject to the deference given to the courts in the hierarchical public judicial system. Thus, decisions or reasons in one case may not be applicable or given deference in another case. Finally, due to freedom of contract principles, the parties may choose their adjudication of a dispute to be subject to practices or standards provided in the industry, and therefore wholly irrespective of federal judicial holdings. CAS has published all of the reasoned decisions as CAS jurisprudence, and in arbitration hearings the lawyers on both sides argue for the consistent applicability of earlier rulings. Thus, precedent does seem to have some weight in CAS arbitrations.

Despite the advantages given above, critics are quick to point out the disadvantages of arbitration. The fact that private adjudication of disputes is given a final and binding status in the courts has been repugnant to some. This is in part because the accountability and certainty of the rules of the game that are established by governmental courts are not necessarily consistent with arbitral decision-making. Some would argue that the assumption that both parties have voluntarily consented to have disputes resolved by arbitration is flawed. US Federal Courts have held that union contracts to arbitrate or employment clauses found within employee manuals

have been considered adequate notice that the employee is agreeing to arbitrate by taking the job. Despite the Code's language distancing itself from employment law, this logic may apply to athletes as well, since they are notified of the rules in advance and agree to the WADA rules as a condition of participation.

A further criticism is the so-called "repeat player affect." The argument here is that the antidoping organizations are likely to have repeated experience with arbitration. This criticism is ameliorated in antidoping arbitration by the publication of the written decisions, providing both parties with a history of decisions by the arbitrators, and by the repeated appearance of the same attorneys as advocates for the athlete, which essentially makes both sides "repeat players".

Some critics argue that the "repeat player effect" combined with selection of arbitrators by the parties may result in issues with arbitrator impartiality. Where the arbitrators are selected by parties to arbitration and paid for their time, there is an incentive to be seen in a positive light in order to obtain future work. Some argue that this interest in future employment will prompt arbitrators to decide consistently and fairly under the applicable rules, as deviation from those rules will be evident and will prevent future appointment as an arbitrator. In addition, the "neutral" arbitrator in sport arbitration is chosen by the two arbitrators selected by the parties and acts as the chair of the panel.

3.1 Court of Arbitration for Sport

The need for a body with the experience and expertise to arbitrate disagreements involving sport organizations became apparent in the early 1980s. Judge Kéba Mbaye, who was then a judge at the International Court of Justice in The Hague and a member of the IOC, chaired an IOC working group that was to create the model for what would become the Court of Arbitration for Sport (CAS). The CAS became operational in June 1984. The CAS has heard a wide variety of cases involving issues such as the nationality of athletes, contracts concerning television rights, sponsorship and licensing, disputes between sporting organizations, and antidoping rule violations. In 1991, the CAS was re-organized to be more independent of the IOC and is now governed by the International Council for Arbitration in Sport (ICAS). The ICAS is responsible for the administrative and financial aspects of CAS, and as such safeguards the independence of the CAS and the rights of all stakeholders in sport. Since 1994, the Code of Sports-related Arbitration has governed the organization and arbitration procedures of the CAS.

The approximately 300 CAS arbitrators are appointed by the ICAS for a renewable term of four years. Qualifications for being a CAS arbitrator include legal training and recognized competence with regard to sport. The CAS arbitrators may be nominated by the IOC, the international federations, or the national Olympic committees, but may also be directly appointed by ICAS "with a view to safeguard the interests of the athletes" (Code of Sports-related Arbitration 1994, Article S14).

In 2003, Larissa Lazutina and Olga Danilova challenged the independence of ICAS/CAS at the Swiss Federal Tribunal in an appeal against a CAS award disqualifying them from the 2002 Olympic Games. The Federal Tribunal ruled that ICAS/CAS was sufficiently independent of all parties using its services to have its decisions considered as true awards, comparable to the decisions of a State tribunal. The Tribunal also observed that CAS served a valuable role in resolving international sports disputes in a rapid and cost-effective manner. It also noted that CAS could be improved.

4 Evidence in Doping Rule Arbitration

The rules of evidence determine if, when, how, and for what purpose evidence may be brought before the decision makers. The rules of evidence address issues such as relevance, privilege, witnesses, expert testimony, and the rules of physical evidence. As mentioned above, the arbitration agreement can substantially affect the rules of evidence. As a general statement, the rules for the introduction of evidence are more relaxed in arbitration than in a court of law. Basically there are two types of witnesses in legal or arbitration proceedings – fact witnesses and expert witnesses. Fact witnesses are individuals who have heard, seen, or touched something of relevance to the case and can testify under oath about it. With the inclusion of factors such as athlete admissions of use of prohibited substances in the definition of doping, fact witnesses are becoming a more frequent part of antidoping arbitration. A laboratory employee could testify as a fact witness regarding specific issues with the chain of custody or analysis. As with any legal proceeding, testimony is offered under the penalties of perjury.

In contrast, expert witnesses are individuals who as a result of their education, training, and/or experience can provide opinions on technical matters beyond the average person's understanding. The expert witness has an obligation to assist the decision maker(s) in understanding the technical issues, and should not be an advocate for either side, regardless of the party paying him or her for their time. In the United States, parties can also hire nontestifying experts to assist them in evaluating a case. The expert opinion of a nontestifying expert is protected from discovery. While the expert witness is given greater latitude than a fact witness in expressing opinions, the expert opinion must be relevant and reliable, as determined by the decision makers. The US Supreme Court established a legal precedent in Daubert (Daubert v Merrell Dow Pharmaceuticals 1993) as to how federal judges are to evaluate the admissibility of an expert opinion. The relevance of the testimony is determined by how closely the testimony fits with the specifics of the case. The reliability of the testimony requires that the expert came to their opinion from application of the scientific method. The Court offered several guidelines for determining whether the scientific method was applied, but did not mandate that the list was a checklist. In order for a theory or technique to be an acceptable basis for testimony, it must be falsifiable, refutable, and testable. The theory or technique

must be subjected to peer review and publication. The potential error rate for the theory or technique should be known and there should be established and maintained standards concerning its operation. Finally, a relevant scientific community must generally accept the theory or technique. While the Daubert standard is not mandated by international arbitration, it does set a standard for introduction of a technical opinion that has been widely accepted.

5 Laboratory Documentation of Adverse Analytical Findings

In general, laboratory results are considered nontestimonial hearsay evidence, and the results may be introduced as a business record. The basis of this decision was that the analyst did not know the identity of the person who provided the sample and had no expectation at the time of the test that the results would be used in court. A recent ruling (U.S. v Hargrove 2008) involving a crime laboratory analysis of seized powders opined that laboratory reports are testimonial hearsay evidence because the analyst could reasonably believe that the report would be used in a trial. Because of the arbitration rules set by the WADA and CAS Codes, it is unlikely that this ruling would affect sport cases. But if it were applicable to sport laboratory testing, one could argue the target testing, confirmation analyses, and retest results could be reasonably expected to be used in the hearing, and thus all laboratory personnel involved in producing the laboratory report could be required to testify.

Documentation for antidoping violations is established by the Laboratory Documentation Packages technical document. The origin of the documents listed was a meeting between defense lawyers and antidoping organizations to find a standardized list of documents that would provide sufficient evidence that the test results were performed according to scientifically robust procedures and would avoid time-consuming discovery requests. The need for a timely hearing arises because an athlete charged with a doping violation is provisionally suspended from competition during the adjudication process. It is important to note that the athlete in person or an expert may attend the analysis of a split portion of the sample, allowing additional scrutiny of the laboratory's procedures.

In conclusion, a great deal of progress has been made since the formation of WADA and other independent antidoping organizations in 2000. The implementation of the Code and the UNESCO Convention has narrowed the inconsistency in the enforcement of doping control rules, established standards for sample collection and analysis, improved laboratory performance, and emphasized the importance of contributions of stakeholders to research and education. Nevertheless, troubling inconsistencies remain. The challenge for the antidoping community in the upcoming years will be to harmonize the application of testing and ensure that all stakeholders are compliant with the WADA Anti-Doping Program. The deterrent effect of the antidoping rules can only take place when the athletes are confident that everyone is treated the same way.

Disclaimer. The views expressed in this article are those of the author and do not represent the United States Anti-Doping Agency in any way.

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