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The Analytical Chemistry of Drug Monitoring in Athletes

Larry D. Bowers

United States Anti-Doping Agency, Colorado Springs, Colorado 80906; email: lbowers@usada.org

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Key Words

anabolic steroids, doping, recombinant erythropoietin

Abstract

The detection and deterrence of the abuse of performance-enhancing drugs in sport are important to maintaining a level playing field among athletes and to decreasing the risk to athletes' health. The World Anti-Doping Program consists of six documents, three of which play a role in analytical development: *The World Anti-Doping Code*, *The List of Prohibited Substances and Methods*, and *The International Standard for Laboratories*. Among the classes of prohibited substances, three have given rise to the most recent analytical developments in the field: anabolic agents; peptide and protein hormones; and methods to increase oxygen delivery to the tissues, including recombinant erythropoietin. Methods for anabolic agents, including designer steroids, have been enhanced through the use of liquid chromatography/tandem mass spectrometry and gas chromatography/combustion/isotope-ratio mass spectrometry. Protein and peptide identification and quantification have benefited from advances in liquid chromatography/tandem mass spectrometry. Incorporation of techniques such as flow cytometry and isoelectric focusing have supported the detection of blood doping.

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1. INTRODUCTION

Recognition of the problem of performance-enhancing drugs in the modern era of sport occurred in the 1920s, when the International Amateur Athletics Federation (IAAF) banned the use of stimulants, despite the lack of a test to detect their use. In the 1960s, the Union Cycliste Internationale and the Fédération Internationale de Football Association were among the first international sports federations to introduce tests for doping substances into their respective world championships. The International Olympic Committee (IOC) formed a medical commission in 1967 to deal with the perceived increase in the use of drugs and other performance-enhancing substances that not only affected the evenness of the playing field but also exposed athletes to health risks.

In 1982, the IOC came to an agreement with the IAAF, whose medical commission had established standards for accrediting antidoping laboratories, to assume the role of laboratory accreditor. In the mid-1980s, the IOC recruited a number of laboratories worldwide to carry out drug testing. The initial IOC laboratory-accreditation process began in 1985. At the 1988 Olympic Games in Seoul, Korea, Ben Johnson of Canada became the first Olympic competitor to be sanctioned for doping when his gold medal was rescinded. With the advent of molecular biology techniques, a new generation of recombinant proteins such as erythropoietin (EPO) and growth hormone (GH) have become available for therapeutic use—but also abuse—by athletes. More recently, the sequencing of the human genome has identified gene therapy targets, and the understanding of the structure and function of receptors and proteins identified by gene sequencing has ushered in an era of very specific therapeutic agents. An example is myostatin, a protein responsible for regulating muscle growth. Selective inhibitors of myostatin binding to its receptor have potential for treating muscle-wasting diseases, but also they can also encourage abuse. The analytical chemistry associated with deterrence and detection of performance-enhancing substances is also advancing at a rapid pace.

2. THE WORLD ANTI-DOPING AGENCY

The World Anti-Doping Agency (WADA) was created in 1999 to promote, coordinate, and monitor the fight against doping. In contrast to earlier antidoping efforts, WADA is an independent agency. The sports movement, including the IOC, and various governments fund WADA equally and are represented equally on the WADA Foundation board. WADA coordinated the development and implementation of the World Anti-Doping Program, which was approved at an international congress in Helsinki in 2002. It consists of *The World Anti-Doping Code* (Code) (1), *The Prohibited List: International Standard* (List) (2), *The International Standard for Laboratories* (ISL) (3), *The International Standard for the Protection of Privacy and Personal Information* (4), *The International Standard for Testing* (5), and *The International Standard for Therapeutic Use Exemptions* (6). The Code and several of the standards named in the ISL were modified and approved at a second international congress in Madrid in 2007. The newest version of the Code goes into effect in January 2009. Because governmental bodies cannot sign treaties with nongovernmental entities, 107 governments have ratified a United Nations Educational, Scientific, and Cultural Organization (UNESCO) international convention, effective as of February 2007, to adopt the essential elements of the Code into national laws (7).

The Code sets out the antidoping rules and principles, establishes the role of education and research in the antidoping field, defines the roles and responsibilities of various stakeholders, and provides guidelines for implementation, modification, and compliance for signatories to the Code. The Code also provides that the athlete whose sample has an adverse analytical finding has a right to a hearing to address whether a doping violation occurred and the appropriate consequences. In

the United States, the Ted Stevens Olympic and Amateur Sports Act provides that matters related to an athlete's eligibility are to be heard by the American Arbitration Association. The protocols of the U.S. Anti-Doping Agency establish that arbitrators appointed to hear doping cases must be members of both the American Arbitration Association and the Court of Arbitration for Sport. This is a good illustration of how national law interacts with the rules and procedures of sport and the need for the UNESCO convention.

The antidoping rules, as established by the Code, are incorporated into the rules of each sport. The athlete agrees to the antidoping rules as a condition of competition in the same way that he/she agrees to play the game of basketball, for example, on a court of prescribed dimensions and with a basket of determined diameter and height. Violations of the antidoping rules are handled in much the same way as professional misconduct is handled in the legal or medical profession.

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2.1. *The Prohibited List: International Standard*

The Code requires that the list of prohibited substances and methods be reviewed and revised each year. Compounds are placed on the List if they meet two of the following three criteria:

1. The substance or method, alone or in combination with other substances or methods, has the potential to enhance or enhances sport performance;
2. the use of the substance or method represents an actual or potential health risk; and
3. the use of the substance or method violates the spirit of sport.

In addition, a substance or method that has the potential to mask the use of other prohibited substances may be added to the List. The List is reviewed by a committee of international experts, circulated to stakeholders for commentary, and approved by the WADA Health, Medicine, and Research Committee as well as the Foundation Board.

The classes of prohibited substances and methods are summarized in **Table 1**. Within the List, there are classes of compounds that are closed and classes that are open. A closed class means that laboratories must screen for only the compounds named in the List. An open class means that laboratories must screen for compounds that have similar chemical structures or biological activity to those named in the List. One difference between the WADA List and the lists of some professional sports is that the professional leagues use closed lists. In the case of a closed list, a sanction for using a newly discovered designer drug cannot be pursued.

2.2. *The International Standard for Laboratories*

The Code mandates that antidoping analysis be performed in a WADA-recognized laboratory. WADA recognition is based on four factors:

1. The sponsoring country of the laboratory must be a signatory to the UNESCO convention;
2. the laboratory must have International Organization for Standardization/International Electrotechnical Commission (ISO/IEC) 17025 accreditation through a national accrediting body that is a signatory to the International Laboratory Accreditation Cooperation Arrangement;
3. the laboratory must be successfully assessed against the ISL through a national accrediting body; and
4. the laboratory must successfully participate in the WADA External Quality Assessment Scheme (EQAS), which includes both blind and double-blind samples.

Further, the Code defines doping as detection by the laboratory of any amount of a prohibited substance, its metabolites, or its markers in an athlete's sample. Only those substances

Table 1 Summary of the 2009 World Anti-Doping Agency *Prohibited List: International Standard*

Class	Class name	Selected examples[a]	Type
S1.1.a	Exogenous anabolic androgenic steroids	Bolasterone, boldenone, clostebol, fluoxy-methandienone, methyltrienolone, methyltestosterone, mibolerone, nandrolone; norbolethone, oxandrolone, oxymesterone, stanozolol, 1-testosterone, tetrahydrogestrinone,	Open
S1.1.b	Endogenous anabolic androgenic steroids	Androstenediol; androstenedione; dihydrotestosterone; testosterone;	Closed
S1.2	Other anabolic agents	Clenbuterol, selective androgen receptor modulators, tibolone, zeranol	Open
S2	Hormones and related substances	Erythropoietin, growth hormone, insulin, adrenocorticotrophin; insulin-like growth factor 1, chorionic gonadotropin	Open
S3	β2-agonists	Salbutamol, salmeterol, and terbutaline	Open
S4	Hormone antagonists and modulators	Aminoglutethimide, tamoxifen, clomiphene, myostatin inhibitors	Open
S5	Diuretics and masking agents	Diuretics, probenecid, plasma expanders (e.g., intravenous administration of albumin, dextran, hydroxyethyl starch and mannitol)	Open
S6.a	Nonspecified stimulants	Amphetamine, amphetaminil, benzphetamine, benzylpiperazine, bromantan, carphedon, clobenzorex, cocaine, dimethylamphetamine, ethylamphetamine, fenproporex, mesocarb, D-methamphetamine, methylenedioxyamphetamine, methylenedioxymethamphetamine, p-methylamphetamine, modafinil, prolintane	Closed
S6.b	Specified stimulants	Ephedrine, fencamfamin, levmetamphetamine, methylephedrine, methylphenidate, parahydroxyamphetamine, pemoline, selegiline	Open
S7	Narcotics	Buprenorphine, heroin, fentanyl, hydromorphone, meperidine, morphine, oxycodone, oxymorphone, pentazocine	Closed
S8	Cannabinoids		Closed
S9	Glucocorticosteroids	Methylprednisone, prednisone, budesonide, triamcinolone acetonide	Open
M1	Enhancement of oxygen transfer	Blood transfusions	Open
M2	Chemical and physical manipulation	Tampering, urine substitution, urine alteration	Open
M3	Gene doping	–	Open
P1	Alcohol	–	Closed
P2	β-blockers	–	Open

[a]For a complete list of examples of prohibited substances and methods, see Reference 2.

naturally found in the body that have a reporting threshold established in the List are exceptions to the rule regarding detection of any amount. Thus, for most doping violations, the laboratory must identify, but not quantify, the prohibited substance, its metabolite(s), or its marker(s). Identification of multiple metabolites of a prohibited substance increases the evidentiary value of the result. Prior to 2004, only urine was analyzed for prohibited substances and methods; since then, however, both blood and urine have been tested. WADA has chosen to set performance criteria for detection of prohibited substances and methods, and thus the methods used in each

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laboratory are developed in house. Validation of the methods is reviewed during the ISO/IEC 17025 assessment.

In addition to the ISL, WADA promulgates technical documents on various topics such as documentation packages, identification criteria for gas and liquid chromatography/mass spectrometry techniques, analysis of endogenous steroid profiles, and reporting of results from the isoelectric focusing analysis of EPO. The GC/MS identification technical document has compared favorably with other GC/MS identification criteria (8). The technical documents are required to be incorporated into the policies and procedures of the WADA-recognized laboratories.

The WADA EQAS involves distribution of both blind and double-blind urine samples to the laboratories (9). The blind program involves approximately five samples per quarter, and not all laboratories receive the same five samples. Because the laboratories frequently monitor more than one metabolite, and because reference compounds for many metabolites do not exist, the urine samples are frequently collected after the administration of a prohibited substance to a volunteer who has given informed consent. The WADA EQAS also involves an educational component for new methods or doping substances. For both parts of the program, the laboratories receive feedback on their performance.

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3. ANALYTICAL CHALLENGES AND ADVANCES

As has occurred in many fields of scientific endeavor, the development of reliable analytical tools for antidoping has preceded advances in detection, measurement, and interpretation. The first testing at an Olympic Games occurred in Mexico City in 1968. At the 1972 Munich Olympics, GC/MS was used for confirmation of stimulants. In 1972, a radioimmunoassay, a technique invented in 1960, was used to perform initial testing of samples for the 12 orally active anabolic steroids then available (10). Lawson & Brooks (11) demonstrated that GC/MS with single-ion monitoring could be used for detection of nanogram-per-milliliter concentrations of the urinary metabolites of the anabolic steroid dianabol. The first official use of the radioimmunoassay screen and GC/MS confirmation was carried out at the Commonwealth Games in 1974. The first use of the combined techniques at the Olympic Games occurred in Montreal in 1976 (12). The introduction of the fused silica capillary GC column and robust benchtop mass analyzers in the early 1980s facilitated extensive study of steroid profiles in urine and allowed faster analysis of the increasing numbers of anabolic steroids. The application of this sensitive technique, which can identify trace amounts of anabolic steroids, resulted in a large number of last-minute withdrawals from competition and in sanctions for anabolic steroid use at the 1983 Caracas Pan American Games. The metabolism of synthetic anabolic steroids became better understood, primarily as a result of research in the antidoping laboratories (13). Improvements in the sensitivity of GC coupled to combustion/isotope-ratio mass spectrometry (GC/C/IRMS) has enabled discrimination of pharmaceutical testosterone from natural testosterone on the basis of ^{13}C depletion (14). Similarly, the development of high-performance liquid chromatography (HPLC) coupled to a tandem mass spectrometer (MS/MS) has allowed the detection of polar small molecules (15) and peptides and proteins (16, 17). In addition, electrophoresis, immunoassay, and flow cytometry have begun to play a greater role. Thus, the analytical techniques available for testing in antidoping laboratories have broadened dramatically in the past decade.

Another analytical challenge arises from the fact that in antidoping testing, some individuals being tested actively attempt to avoid detection. For example, after the development of a urine test for recombinant EPO, it was apparent from the isoelectric focusing patterns observed over time that some athletes changed from standard dosing regimens to "microdosing" to beat the test. Advisors with scientific or medical expertise have counseled athletes to provide minimum

volumes of urine in the hopes that the volume is insufficient to allow confirmation. They have also advised athletes on drug dosages and kinetics to avoid detection. Athletes have also received advice about the newest undetectable drugs, with varied success. It was also discovered that some cyclists were placing "rice grains" containing proteases in their penile urethras and introducing them into the samples during observed collection in order to destroy any EPO in the urine samples (18). Without the assistance of these unethical scientists and health professionals, testing requirements would not be increasingly rigorous. The fact that there are active attempts to mask drug use means that the selection of analytical approaches to testing schemes must take so-called masking into account. The majority of antidoping tests rely on the combination of GC or HPLC and MS.

Due to space limitations, it is not possible to review the analytical chemistry of all of the classes of prohibited compounds. Typical procedures to detect the presence of β2-agonists (19, 20), hormone antagonists and modulators (21–24), diuretics and masking agents (25–29), stimulants (30–33), narcotics (31, 34), cannabinoids, glucocorticosteroids (35, 36), and β-blockers (31) have been published by the WADA–recognized laboratories. I focus here on three prohibited substance classes: anabolic agents, protein and peptide hormones, and oxygen-transport enhancement.

3.1. Anabolic Agents

The so-called golden age of anabolic steroids occurred in the 1950s and 1960s, when numerous pharmaceutical companies invested in trying to separate androgenic and anabolic effects of synthetic analogs of testosterone (see **Figure 1**). The pharmaceutical companies were also interested in developing orally active anabolic steroids, as orally administered testosterone is inactivated in its first pass through the liver. Although they met with some success in enhancing anabolic activity relative to androgenic activity, these companies did not develop a steroid with only one activity.

The appeal of steroids for athletes is twofold: They lead to increased strength and to enhanced recovery. From the 1950s through the 1970s, most athletes who took steroids were involved in sports that required strength or muscle growth, including bodybuilding, weightlifting, throwing events in track and field, and American football. From the 1980s through the present, steroids have also been used to enhance recovery. Athletes can train harder, longer, and more frequently without their bodies breaking down. Thus, steroids have been used in the sprint disciplines of track and field, cycling, and baseball. Stephens (37) has published a personal account of the effects of anabolic steroids, GH, and EPO on his performance as an amateur cyclist.

Since the 1980s, GC/MS has been the technique of choice to detect anabolic steroids. This general method of analysis has been described in detail elsewhere (19, 38). Many improvements to this technique have been described at one of the 25 annual Cologne Workshops on Dope Analysis, from which Sportverlag Strauß has published 16 volumes of peer-reviewed proceedings. A schematic diagram of the procedure for detection of anabolic steroids is shown in **Figure 2**. As a result of Phase II metabolism, anabolic steroids present in the urine sample are conjugated with either glucuronate or sulfate. The initial step in the original steroid procedure was to enrich the conjugated steroids and remove polar, low-molecular-weight inhibitors of glucuronidase activity from the urine. The enzyme β-glucuronidase from either *Escherichia coli* or *Helix pomatia* was used to free the steroid aglycone. The *E. coli* enzyme was generally preferred because the *H. pomatia* preparations may cause oxidation of 3β-hydroxy-5-ene steroids to 3-keto-4-ene, 4,6-diene, and 6-oxy steroids. The addition of an antioxidant such as ascorbate has recently been recommended to resolve this issue (39). The aryl sulfatase activity in *H. pomatia* preparations has the beneficial effect of cleaving sulfate conjugates, but the enzyme greatly favors cleavage of 3-sulfates over 17-sulfates.

Schänzer and colleagues (40) showed that if the hydrolysis process is monitored, the enzymatic hydrolysis step can be performed directly on the urine. The cleavage of androsterone glucuronide

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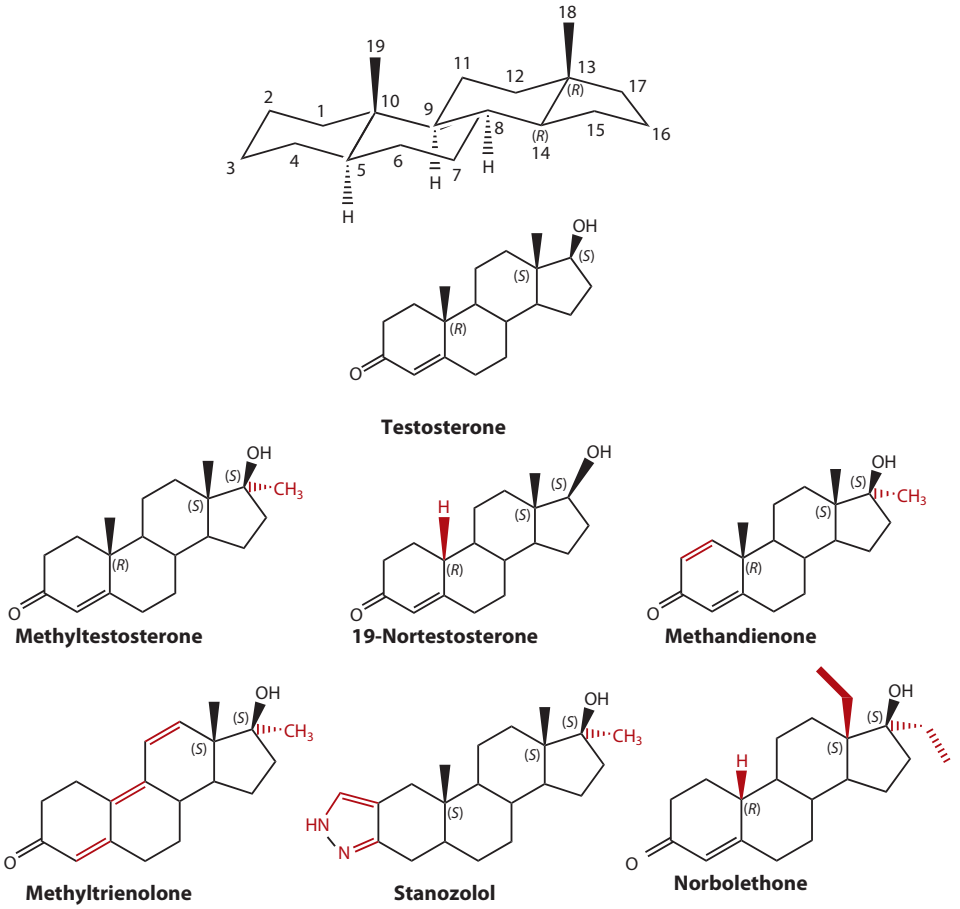

Figure 1

Anabolic steroid structures.

is the slowest among the steroids of interest. If deuterated androsterone glucuronide is added to the urine and if the hydrolysis yield is compared to an equimolar amount of deuterated etiocholanolone, the presence of enzyme inhibition in an individual sample can be detected, and that sample can be run through a more thorough sample-cleanup procedure. Using this direct hydrolysis approach, Mareck et al. (41) showed that only ~0.4% of urine samples contain β-glucuronidase inhibitors.

The nonpolar steroids are then extracted into a nonpolar solvent such as *t*-butyl methyl ether (TBME) or *n*-pentane. The polarizability of TBME allows a broader polarity range of steroid metabolites to be extracted. Pentane, however, can be used to eliminate interferences in the procedure. For example, when the sample is extracted with TBME, a polar metabolite of vitamin E elutes close to norandrosterone, a metabolite of the anabolic steroid nandrolone. The use of pentane extraction effectively eliminates this interference. Pentane extraction cannot be used for the analysis of more polar steroids such as stanozolol or fluoxymesterone.

The steroids are derivatized using a mixture of N-methyl-N-(trimethlysilyl)trifluoroacetamide (MSTFA), ammonium iodide, and ethanethiol. The trimethyl iodosilane formed in this mixture facilitates enolization of ketosteroids and the formation of trimethylsilyl derivatives. For example,

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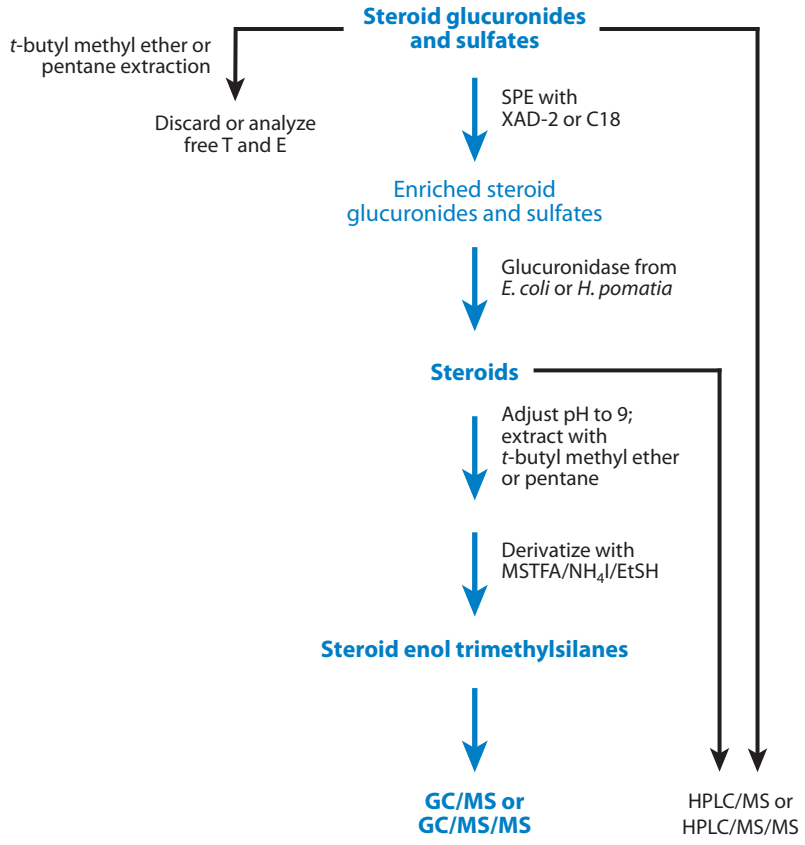

Figure 2

Schematic diagram of initial and confirmatory anabolic steroid tests. Abbreviations: EtSH, ethanethiol; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; MSTFA, N-methyl-N-(trimethlysilyl)trifluoroacetamide; NH_4I, ammonium iodide; SPE: solid-phase extraction; T and E, testosterone and epitestosterone.

testosterone forms androst-2,4-diene-3,17β-bis-trimethylsiloxane. The presence of large amounts of therapeutic drug metabolites in the urine can consume the derivatizing reagent, leading to incomplete reaction of the steroids of interest. This effect can be easily detected by monitoring for the presence on the mono-trimethylsilyl derivatives of androsterone and etiocholanolone. In those samples where derivatization is incomplete, addition of more derivatizing reagent normally ensures complete reaction.

Once the sample is derivatized, it is usually analyzed by capillary GC/MS. Given the low concentrations of steroid metabolites in the urine and the need to search for over 100 steroid metabolites, laboratories generally use selected-ion monitoring (SIM). Results are reviewed manually, comparing each retention time and ratio of characteristic mass ions from the sample to those of a urine sample containing each metabolite. Chemometric methods have been proposed to identify patterns of steroid excretion in the urine (42).

3.1.1. Exogenous anabolic agents. Most work in antidoping in the late 1980s and early 1990s aimed to identify the metabolites of anabolic steroids that gave the longest detection window

(40, 43–48). The determination of the metabolite structures allowed synthesis of reference materials that improved detection. Efforts to identify metabolites that provide a longer detection window in the urine are still under way (49). The electron ionization fragmentation patterns of several steroid glucuronides have been reported (50, 51).

In recent years, several designer steroids based on the literature from the "golden age" of steroids have emerged. Designer steroids such as norbolethone (52), tetrahydrogestrinone (THG) (53), and desoxymethyltestosterone (54) appeared either in confiscated powders or in urine samples from both male and female athletes. No toxicity or teratogenicity studies had been performed on these anabolic steroids before they were given to the athletes. The compounds were made and distributed to take advantage of blind spots in the SIM GC/MS procedures. The recent development of time-of-flight (TOF) mass spectrometers allows the acquisition of complete mass spectra and may allow detection of steroids that otherwise would not have had ions programmed into the SIM acquisition program. In addition, many of the WADA-recognized laboratories have implemented HPLC/MS/MS procedures for detection of methyltrienolone, stanozolol (55), THG, and trenbolone. Steroid fragmentation patterns for even-electron ions such as those formed in LC/MS/MS have been reported (56). Recently, direct measurement of the glucuronide conjugates of a number of anabolic steroid metabolites has been reported (57). This interlaboratory validation study incorporated glucuronide conjugates of deuterium-labeled steroid metabolites (58).

There are also substances such as clenbuterol (19, 20, 59), zeranol, tibolone, zilpaterol, and selective androgen-receptor modulators (24, 60–65) that do not have steroid structures but that have been shown to increase muscle growth. These substances are routinely monitored via either GC/MS or LC/MS/MS.

3.1.2. Endogenous anabolic steroids. The urinary testosterone to epitestosterone (T/E) ratio was the first test to be used to determine abuse of a natural (or endogenous) substance, such as testosterone, to enhance performance in sport. Donike and coworkers (66) observed an unusual distribution of testosterone concentrations in samples collected at the Moscow Olympics in 1980. Because the testosterone concentration varies with the specific gravity of the urine, Donike et al. proposed the use of the urinary T/E ratio based on the observation that epitestosterone was excreted in the urine in a relatively constant amount and as such constituted a way to compensate for the variability in testosterone concentration. Subsequently, a number of other groups (19, 67, 68) confirmed that the median value for the population reference range is approximately 1:1 and that more than 98% of the population has a value less than 4:1 (see **Figure 3**).

Because urine is not a sterile biological fluid, and because urine samples are frequently transported from the collection site to the laboratory under ambient conditions, microbial growth has been observed to alter the steroid profile. Bacterial deconjugation of androsterone and etiocholanolone glucuronide, followed by oxidation of the 3-hydroxyl group, leads to the formation of free 5α- and 5β-androstane-3,17-dione. WADA-accredited laboratories monitor the samples for the formation of 5α- and/or 5β-androstanedione, an indicator of microbial growth. The appearance of unconjugated testosterone in the urine has rarely been observed and is hypothesized to result from bacterial conversion of 5-androstene-3β,17β-diol sulfate. As a result, laboratories monitor the amount of free testosterone extracted from the urine prior to β-glucuronidase treatment relative to the amount of testosterone glucuronide (see **Figure 2**).

Evaluation of other steroids in the urine can provide additional information about the use of prohibited substances. Measurement of androsterone, etiocholanolone, 11β-hydroxyandrosterone and -etiocholanolone, 5α- and 5β-androstane-3α,17β-diols, dihydrotestosterone, dehydroepiandrosterone, epitestosterone, and testosterone (collectively referred to as the steroid profile) can be useful in identifying the abuse of a number of steroids (41). The natural response of

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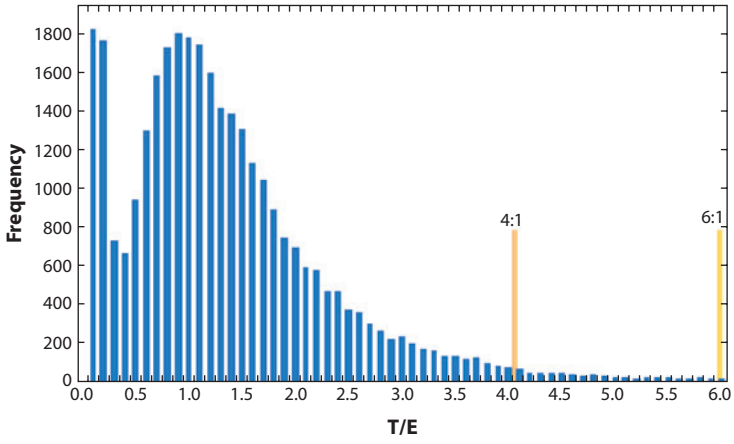

Figure 3

The distribution of testosterone to epitestosterone (T/E) values for approximately 32,000 male athletes. Reprinted from Reference 67 with permission. Copyright 2008, Elsevier.

the body to pharmaceutical steroids is to suppress the production of steroids. Thus, the alteration of concentrations and ratios of steroids in the steroid profile can be very helpful in identifying samples that contain synthetic anabolic steroids.

It has been known since 1993 that the intraindividual variation in T/E ratio is much smaller than the variation within a population observed for T/E ratios (69, 70). This observation led to evaluation of the results of the T/E ratio against a subject-based reference range. In 1993, the IOC Medical Commission required that the subject-based evaluation occur when the athlete's T/E ratio is between 6:1 and 10:1. More recently, techniques such as the reference change limit (71) and Bayesian statistics (68, 72) have been used to evaluate the upper limit of an individual's T/E ratio based on his or her previous and subsequent test results. One situation that can be resolved by subject-based reference ranges is doping by the small subpopulation of individuals who have a naturally low (<0.6) T/E ratio (see **Figure 3**). Although this subpopulation has been recognized in the antidoping community for more than 15 years, the underlying mechanism—a double deletion of the *UGT 2B17* gene—has only recently been reported (73). Administration of testosterone to these individuals did not elevate their urinary T/E ratio above the current population threshold of 4:1 (74). Application of the subject-based reference limit from the Bayesian model allowed detection of doping among these individuals (68).

Distinguishing between steroid metabolites arising from normal biochemical function and those arising from a pharmaceutical preparation is a significant analytical challenge. The fact that steroid metabolites appear in urine at concentrations ranging from a few nanograms per milliliter to a few micrograms per milliliter made the application of IRMS impossible until commercial instrumentation achieved sufficient sensitivity in the late 1990s. The first reported method for detection of testosterone was a cumbersome analytical technique that required 30–40 ml of urine (14). Subsequent developments saw improvement of the sample-cleanup techniques (75, 76). Shackleton and coworkers (77, 78) developed a technique based on measurement of the acetate derivatives of androstanediols and introduced the concept of an endogenous reference compound (ERC) to the antidoping GC/C/IRMS field. The ERC provides an individual-based reference δ value for normal metabolism. Commonly used ERCs include 11-hydroxyandrosterone and 11-hydroxyetiocholanolone (79), 11-ketoetiocholanolone (80), pregnanediol (77), pregnanetriol (77), and 16(5α)-androstenediol (81). Currently, a number of steroids have been monitored to

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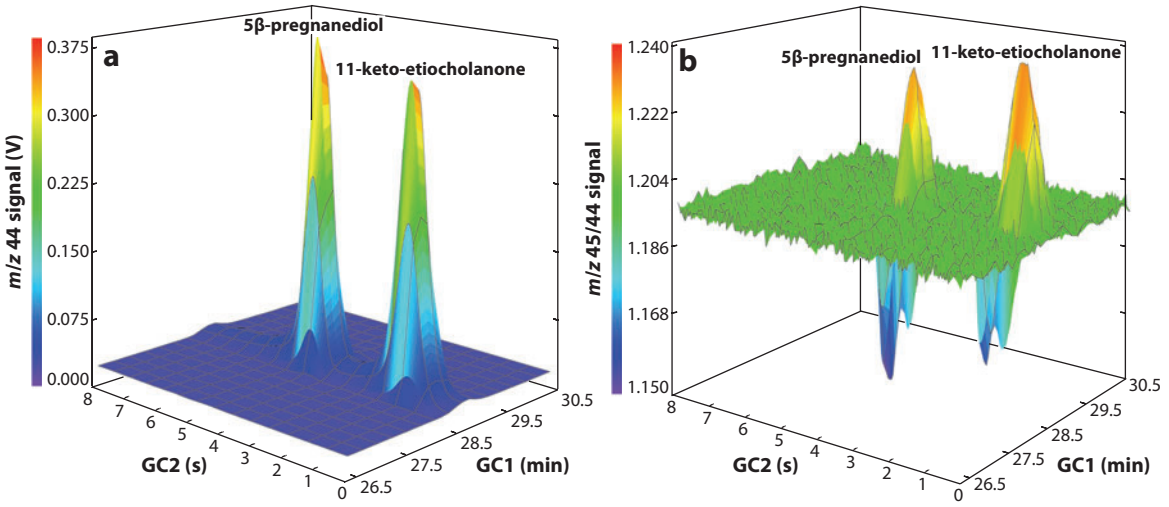

Figure 4

Three-dimensional representation of the tandem gas chromatography/combustion/isotope-ratio mass spectrometry (GC × GC/C/IRMS) separation of 11-keto-etiocholanolone and 5β-pregnanediol. The *m/z* 44 trace (*a*) and the *m/z* 45/44 ratio trace (*b*) show that the peaks are depleted and then enriched in both dimensions. Reprinted from Reference 89 with permission. Copyright 2009, American Chemical Society.

determine the use of exogenous testosterone, including androsterone, etiocholanolone, 5α- and 5β-androstane-3α,7β-diol, and testosterone itself. Methods based on GC/C/IRMS have also been reported for epitestosterone (82), norandrosterone (83, 84), and dehydroepiandrosterone (85, 86).

Recent developments have improved the sensitivity and speed of GC/C/IRMS. The use of programmed-temperature splitless injections increases the amount of sample that can be placed on the column while decreasing the solvent load on the system (87). Sacks and coworkers (88) described a system that decreased the extracolumn band-broadening effects of the combustion interface and associated connecting tubing, which allowed fast GC with peak widths of 250 ms to be interfaced with a C/IRMS system. The peak height increase from the narrower fast GC peaks improved the limit of detection about tenfold, and the run time was decreased two- to threefold. Two-dimensional GC coupled to C/IRMS was also described by the same group (89). A longitudinally cryogenically modulated system was used to focus 4-s slices of the separation in the first dimension onto the second GC column. Steroids that were resolved on the first GC column gave results from the reconstructed peak within 0.34‰ of the true value. Two steroids that were not separated in the first dimension were separated by the second GC column and gave average accuracies of 0.26‰. Efforts continue to further optimize both the hardware and the software approaches to the analysis of three-dimensional data (**Figure 4**).

3.2. Proteins and Peptides

The potential advantage conferred by the use of protein hormones such as GH and insulin has been reviewed elsewhere (90). In this section, I briefly describe recent achievements in detecting human chorionic gonadotropin (hGC), insulin, recombinant human GH, and recombinant EPO and mimetics.

3.2.1. Human chorionic gonadotropin. hCG is a heterodimeric glycoprotein whose α-chain contains 92 amino acids and two N-linked carbohydrate chains and that is shared with several

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other glycoproteins, including luteinizing hormone. The β-chain contains 145 amino acids, two N-linked glycosylation sites, and four O-linked glycosylation sites. In some physiological and pathological conditions, hCG can be hyperglycosylated. Male athletes can use hCG to stimulate natural steroid production after a cycle of synthetic anabolic steroids. Kicman and colleagues (91, 92) discussed the misuse of hCG in sport. Laidler et al. (93) proposed a threshold for urinary hCG in males based on the Serono MAIAclone immunoradiometric assay. The values measured in 1400 men gave a "far outside" value (computed as the 75th quartile plus three times the interquartile range) of 5 IU liter^{-1}. Laidler recommended a decision threshold of 10 IU liter^{-1} for ultrafiltered, nonconcentrated urine samples to ensure that no false positives arise. Cole (94) described the differences in epitopes recognized by various commercial immunoassays for detection of hCG. As a result of these differences, commercial immunoassays measure different combinations of hCG and its metabolites.

hCG was the first LC/MS/MS protein confirmation attempted in urine. Liu & Bowers (16, 95, 96) developed an immunoaffinity extraction scheme followed by a bottom-up identification method based on tryptic peptides T3, T4, and T5 of the reduced pyridylethylated β-chain. This method had a limit of quantification of approximately 20 mIU ml^{-1}. Gam et al. (97) modified this method by quantifying the b_6^+, b_9^+, and y_{11}^+ product ions of the T5 tryptic peptide from the β-chain. Using this approach, Gam et al. achieved a limit of quantification of 5 mIU ml^{-1}. Laidler and coworkers (93, 98) described a MALDI-TOF (matrix-assisted laser desorption/ionization–time of flight) method for identifying and quantifying hCG.

3.2.2. Insulin. Insulin is a heterodimeric peptide of mass 5807 Da composed of a 21-amino-acid A-chain covalently linked by two disulfide bridges to a 30-amino-acid B-chain. Insulin has been reportedly used by athletes as an "insulin clamp" to rapidly replenish glycogen stores in the cell. Insulin has also been reported to have significant anticatabolic activity (90), although well-controlled studies have not been performed. In addition to recombinant human insulin, several short-acting forms of insulin (e.g., Humalog LisPro, Novolog Aspart, and Glulisine Apidra) have been engineered. These nonnative insulins have amino acid sequence alterations to enhance speed of onset of action. These short-acting insulins have been identified in plasma (99) and urine (100) through use of LC/MS/MS.

Native insulin and long-acting insulins (e.g., Lantus Glargine and Detemir) can be measured in serum or plasma (99) but appear in the urine as degradation products. Thomas and coworkers (101) have reported LC/MS/MS methods for the detection of insulin degradation products in urine. Proteolytic cleavage of the B-chain gives rise to metabolites lacking carboxy-terminal amino acids, such as ^{30}alanine for insulin and Detemir and ^{24}phenylalanine–^{32}arginine for Lantus. The unique urinary metabolites of Lantus provide an approach to detect doping.

3.2.3. Recombinant human growth hormone. GH is a pituitary hormone with a broad range of effects on the body. As secreted by the pituitary, GH consists of a number of different isoforms with different molecular weights. The predominant active form has a mass of approximately 22 kDa, but other forms of mass 20 kDa, 17 kDa, and 5 kDa have been detected in the circulation. Recombinant GH consists only of the 22-kDa form, and its administration suppresses secretion of the native forms from the pituitary (102). An attempt to differentiate between natural and pharmaceutical GH using isotopic depletion was unsuccessful (103). Two approaches to detection of GH abuse have been developed: a method that measures the isoforms of GH (104) and a method that measures the biomarkers of GH action (105).

The isoform assay, originally described by Wu et al. (106), measures the 22-kDa isoform with a specific assay and a mixture of other isoforms using a "permissive" assay. The final configuration of

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the assay has recently been described (106a). The 20-kDa isoform is not measured in the permissive assay because one of the recognized epitopes in the sandwich immunoassay recognizes the amino acid sequence that is deleted in the 20-kDa isoform. The full complement of isoforms is released after exercise (107). A sandwich immunoassay that specifically recognize the 20-kDa isoform has been reported but is not in routine use (108).

Research on the biomarkers approach began in 1996, led by Peter Sonksen and an international group of investigators (109). Although the initial studies considered numerous plasma and urine biomarkers of human GH action, the group focused on two markers, insulin-like growth factor 1 (IGF-1) (109) and procollagen type III aminoterminal peptide (P-III-NP), because discriminant analysis showed that variations in these two markers could reflect GH administration (110, 111) and because additional markers added little discriminant power (112). Other groups have independently confirmed the discriminating ability of these two biomarkers (113–116). Commercial immunoassays were used for the measurement of IGF-1 and P-III-NP. In the case of P-III-NP, only two immunoassays were available: Orion Diagnostica and Cisbio International. The epitopes recognized by the antibodies are not known, particularly those for the sheep polyclonal antibody used in the Orion Diagnostica assay. Reference material for calibration of the assays also differs between the two assays, although there is a reasonable correlation between results (117). A number of immunoassays are available for IGF-1, although recent efforts have been directed at quantification of IGF-1 by LC/MS/MS. Because of the inclusion of a biomarker related to collagen growth, there was concern about the effect of bone injury, but studies have shown that this is not the case (118). Nguyen et al. (119) have recently presented data on the potential for subject-based reference ranges for IGF-1 and P-II-NP.

Recently, HPLC/MS/MS quantification of plasma IGF-1 and identification of its several analogs were reported. Nelson et al. (120) reported a mass spectrometric immunoassay method for quantification of IGF-1 in human plasma using rat IGF-1 as an internal standard. The method was linear between approximately 8 and 1000 ng ml^{-1}, covering the population reference range. Bredehöft et al. (121) described an assay for quantification of IGF-1 and identification of pharmaceutical forms of IGF-1, such as des(1–3)IGF-1 and LONG^{TM} R^3IGF-1.

3.2.4. Recombinant erythropoietin and mimetics. EPO is an approximately 34-kD glycoprotein that stimulates the production of red blood cells (RBCs). Like all glycoproteins, there are a number of different isoforms due to differences in the carbohydrate moieties attached to the protein. The recombinant EPO must be expressed in mammalian cells such as Chinese hamster ovary cells, baby hamster kidney cells, and modified human cells in order to have the carbohydrate chains attached. The differences in glycosylation between different species and cells within species give rise to the charge differences between human EPO and recombinant EPO. Various EPO molecules are described by the word epoetin followed by a Greek letter indicating the type of glycosylation, which varies among different cell lines and different purification procedures.

Wide and coworkers (122, 123) first described an electrophoretic method for detecting the isoforms of EPO in serum and urine. Lasne & de Ceaurriz (124) described an isoelectric focusing double-immunoblotting assay with greater sensitivity. After isoelectric focusing, the EPO is detected by transfer to a membrane, followed by reduction with dithiothreitol and reaction with a mouse monoclonal anti-EPO antibody (AE7A5). The AE7A5 antibody is then dissociated from the EPO in the first membrane under acidic conditions and is transferred to a second membrane to decrease the nonspecific binding of the visualization antibody. The second membrane is immunoblotted with a biotin-labeled goat anti–mouse antibody that allows the isoforms of EPO to be visualized using an enzyme-mediated chemiluminescence procedure (125). The method is capable of separating and detecting epoetin α (Epogen, Procrit), epoetin β (NeoRecormon),

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epoetin γ, epoetin δ (Dynepo), epoetin ω, novel erythropoiesis-stimulating protein (darbopoietin) (126–128), methoxy polyethylene glycol–epoetin β (Mircera), and even the subtle differences in glycosylation associated with gene insertion into the muscle (129). Software has been reported to quantify the EPO isoforms (130, 131). The use of discriminant analysis of the patterns to detect the use of recombinant EPO has been described (132).

Several authors have criticized the one-dimensional isoelectric focusing approach (133–137). Most of the criticism has focused on the nonspecificity of the AE7A5 monoclonal antibody used for detection. Unfortunately, these authors significantly modified the Lasne procedure, and therefore it is unclear whether the hypothesized interference actually occurs in the properly conducted method (138–141). Interestingly, comparing the Lasne procedure to a method incorporating a specific monoclonal antibody in an immunoaffinity sample-preparation scheme resulted only in the disappearance of one set of bands far outside of the pI window in which native human and recombinant human EPO are observed (142). These bands, which migrate in a more basic region of the gel, have been identified as zinc α2-glycoprotein (143).

A nonglycosylated protein that appears in the urine after intense exercise was detected through one of the modified methods (134). Its importance in the Lasne method is unclear. Lasne has shown that the EPO glycoforms appear to be processed to a slightly more acidic form during passage through the human kidney (142). In a subject with excess urinary protein due to renal failure, the pI shift in EPO isoforms did not occur. This could explain the EPO isoform pattern associated with proteinuria after intense exercise, but it requires additional investigation.

Some urine samples have been shown to contain enzymatic activity (possibly arising from bacteria or viral contaminants in the urine) that alters the isoform pattern. In routine testing, the laboratories add novel erythropoiesis-stimulating protein to a suspicious sample to determine whether the suspicious urine sample contains enzymatic activity. Belalcazar et al. (144) have studied the issue of urine samples containing enzymatic activity that changes the isoform pattern. The authors simulated this activity by adding arylsulfate or sialidase to a urine sample; they also inhibited the changes by adding inhibitors of arylsulfatase and sialidase activity to actual urine samples exhibiting enzymatic activity. In addition, the enzymatic activity apparently affected only the recombinant proteins and thus could only result in a false negative result.

Kohler et al. (145) reported a one-dimensional SDS-PAGE (sodium dodecyl sulfate– polyacrylamide gel electrophoresis) technique that employed both an immunoaffinity purification step as well as selective antibody-based chemiluminescent detection. Khan and coworkers (133) reported a two-dimensional SDS-PAGE method for separating EPO isoforms. Although either technique may supplement the isoelectric focusing immunoblotting technique after further study, neither approach is routinely used.

A technique that can provide supplemental identification information such as MS is highly desirable. Because the carbohydrate portion of the EPO molecule distinguishes native EPO from pharmaceutical EPO, there have been attempts to characterize the glycosylation of EPO after separation by various techniques (146–148). MALDI-MS combined with a high-resolution TOF mass analyzer was used to detect purified intact EPO in the low femtomolar range (25–50 fmol) (149). Unfortunately, the speed of analysis and sensitivity do not currently allow routine analysis of urine samples.

3.3. Enhancement of Oxygen Transport: Allogenic (Homologous) Blood Transfusion

Increasing both the rate of oxygen delivery to the muscle and oxygen's efficiency in producing energy is the most effective way to improve performance. Approaches to increase oxygen transport

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and conversion into cellular energy include increases in RBC mass via transfusions or EPO, increases in vasculature, and enhanced mitochondrial efficiency.

Increasing RBC mass through transfusion of typed and cross-matched units of blood from another individual is an effective means of blood doping. Nelson and coworkers (150, 151) developed a flow cytometric assay for the presence of minor RBC surface markers such as Kell. An individual is either positive (an expressor) or negative (a nonexpressor) for the surface marker. The presence of two populations of RBCs exhibiting both the presence and the absence of the surface marker is an indication that an allogeneic blood transfusion has occurred. Giraud et al. (152) have described the validation of the flow cytometry method implemented in their laboratory: On the basis of validation studies, they claim a specificity of 100% and a sensitivity of 78%. Arndt & Kumpel (153) recently reviewed this approach and confirmed that by using two samples taken at different times, one can find a change in percentage of the minor population consistent with allogeneic blood transfusion.

In conclusion, great advances have been made in the analytical science that supports antidoping rule violations. The advances have included not only instrumental improvements, but also improvements in the interpretation of the data. Robust statistical approaches have been used to extract greater information from the data collected. With the introduction of blood testing to complement urine testing, new doping strategies that were undetectable in the past can now be detected. Scientists are achieving the dual goals of deterrence and detection of doping by athletes more successfully than in the past. Continued analytical advances hold great promise for allowing this trend to continue.

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DISCLOSURE STATEMENT

The author is an employee of the United States Anti-Doping Agency and is a volunteer serving on committees for the World Anti-Doping Agency.

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