Chapter 13
The Assay of Endogenous and Exogenous Anabolic Androgenic Steroids

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Introduction

According to the regulations of the World Anti-Doping Agency (WADA) [1], anabolic androgenic steroids are classified as prohibited substances in sports. They are covered in the section "S1. Anabolic Agents 1. Anabolic Androgenic Steroids (AAS)". This section is further subdivided into "a. Exogenous AAS" and "b. Endogenous AAS". AAS represent the class of substances most frequently detected in human sports doping control analyses for many years [2]. The analysis for AAS in doping control is generally performed using urine specimen as matrix.

Most of the AAS are extensively metabolized in the human body and only small amounts of the parent substances are excreted in the urine. The most important metabolic phase-I reactions include oxidation or reduction in positions C-3 and C-17. Furthermore, Δ4 steroids in general are substrates for 5α reduction. Additional double bonds in ring A or B or additional substituents in position C-4 or C-6 push the reduction toward 5β orientation. The introduction of hydroxy functions and their subsequent oxidation especially in positions C-4, C-6, and C-16 is also observed and found most abundantly in steroids like metandienone, dehydrochloromethyltestosterone, stanozolol, and other steroids with additional condensed heterocycles on the A-ring [3–10]. Phase-II metabolism of AAS in humans includes mainly 3α-glucuronidation, while sulfatation mainly occurs at 3β-hydroxy functions. On 17-hydroxy functions, sulfatation as well as glucuronidation occurs equally.

In case of 17-alkylated steroids, 17-epimerization occurs by hydrolysis of the phase-II metabolite, 17-sulfate. As a by-product of this reaction, 17,17-dimethyl-18-nor-13-ene analogs are also detectable [11].

Endogenous AAS represent a special group of compounds. In a strict sense, the relevant steroids are not truly endogenous but are obtained by partial synthesis from plant sterols [12]. The number of compounds with potential of abuse is comparably restricted. Testosterone, biologically the most important anabolic androgenic hormone, is still

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likely to be the molecule with the highest potential of abuse. Dehydroepiandrosterone (DHEA), androst-4-ene-3,17-dione, and androst-5-ene-3,17-dione constitute so-called pro-hormones which after application are metabolized to testosterone. On principle, this mimics the physiological situation and the conversion generally follows the biological pathway. As with xenobiotic steroids, mostly oxidations or reductions at positions 3 and 17 occur where the direction is controlled by substrate concentration. Consequently, also androstenediols may serve as pro-hormones.

Finally, the reactions mentioned above result in the formation of testosterone where the physiological yields may differ. In case of the application of Δ5 compounds, these are converted to the corresponding Δ4 isomers beforehand.

Boldenone, nortestosterone, and their pro-hormones could be classified as either endogenous or exogenous AAS. They may be synthesized physiologically in very small concentrations. Most likely, these steroids are by-products of the steroid metabolism. In spite of their anabolic and androgenic effects, they can hardly be said to truly represent natural hormones.

Phase-I and phase-II metabolism generally proceed as described above. The reduction of the double bond at position 4 is the initial and also rate-limiting step [13]. This step often is accompanied by the loss of biological activity. The most abundant metabolites following application of testosterone or testosterone pro-hormones are 3α-hydroxy-5α-androstan-17-one (androsterone), 3α-hydroxy-5β-androstan-17-one (etiocholanolone), and the corresponding 17β-diols.

Evidently, classical chemical methodology is incapable of discriminating synthetic hormones from the biosynthesized congeners. However, stable isotope analysis at the natural abundance level represents a powerful way to meet this goal [14–19]. There seem to exist very few sources of raw sterols that serve as educts for partial synthesis of pharmaceutical steroid preparations [12]. Mostly, yams (*Dioscorea* sp.) and soy (*Glycine max*) are used. Both plants feature what is known as the C-3 photosynthetic pathway [20, 21]. This results in a relatively strong depletion of ^{13}C versus atmospheric CO_2. By contrast, humans typically feed on a large variety of foodstuff including seafood and so-called C-4 plants, such as corn (*Zea mays*) and sorghum (*Sorghum* sp.). The $^{13}C/^{12}C$ ratio of this dietary mixture is slightly but significantly larger than that of purely C-3 plant-borne steroids. Based on these distinct isotope signatures, a discrimination of synthetic and natural hormones can be achieved. The signature also propagates into urinary metabolites of steroid hormones. This is advantageous as, for example, testosterone itself is typically found in urine in only small concentrations.

In order to compensate for baseline variations and uncertainties of the calibration, the $^{13}C/^{12}C$ ratios of AAS and corresponding metabolites are not evaluated directly. Instead, the $^{13}C/^{12}C$ ratios of target compounds are compared to those of steroids from androgen-independent pathways.

Sample Preparation

The methods routinely used in steroid screening mainly focus on those metabolites that are excreted in the unconjugated form or as glucuronides with the urine. Common procedures include deconjugation using the β-glucuronidase enzyme

derived from *Escherichia coli*. Less frequently, β-glucuronidase/arylsulfatase from *Helix pomatia* is used. Extraction of the aglycons from the matrix and concentration of the analytes is performed by liquid–liquid extraction or solid-phase extraction. To cover the significant diversity of steroids by a single method and to provide high specificity and sensitivity, mass spectrometric methods are utilized for the detection. The final residues are either redissolved in buffer solutions for LC–MS(/MS) analysis or silylated for GC–MS(/MS) analyses. As proposed by Donike et al. [22], most of the laboratories use *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) as reagent for derivatization. The formation of per-TMS derivatives utilizing trimethyliodosilane as catalyst has proven to drastically improve the sensitivity for most of the steroids in gas chromatography–mass spectrometry (GC–MS) based assays [23, 24]. Several compounds, most of them deuterated, composing the internal standard allow to control the critical steps of sample preparation and to determine the amount of metabolite excreted [25]. The first screening analysis, which covers a wide variety of AAS (metabolites), is followed by the analysis of a second aliquot of the same specimen in case of a suspicious result in screening (confirmation).

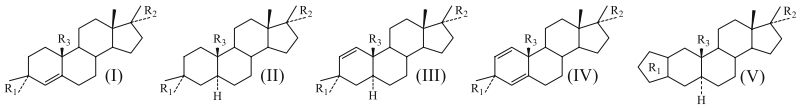
Generally, the sample preparation for $^{13}C/^{12}C$ analysis follows the standard protocol for urinary steroids. However, the instrumental methodology requires complete conversion of the analytes to CO_2 before mass spectrometric analysis. No structural information thus can be obtained from the resulting signals. Consequently, coelutions may significantly blur the isotopic signals. Therefore, HPLC is incorporated as an additional purification step following hydrolysis [26, 27]. The obtained fractions are analyzed separately.

The mandatory conversion to CO_2 is performed online by catalytic combustion over CuO at 800–1,000°C. This conflicts with the common derivatization of steroids by silylating agents such as MSTFA. Therefore, urinary steroids are typically analyzed either in free form [27] or as acetates [14, 16, 17, 28]. Analysis of derivatized compounds requires correction of the resulting isotope ratios for the added carbons. However, the derivatization procedure may be accompanied by isotope effects which cannot be eliminated mathematically [29].

Analysis for Exogenous AAS

Steroids Explicitly Listed on WADA List

The list of prohibited substances [1] explicitly names a wide variety of exogenous AAS. Their structures are illustrated in Fig. 13.1. Detection of the misuse of these substances is preferably done using GC–MS complemented by liquid chromatography–tandem mass spectrometry (LC–MS/MS). To improve the selectivity and sensitivity, traditional GC–MS methods are accompanied by high-resolution mass spectrometry (HRMS) and/or MS/MS techniques. Special focus is given on the main and the long-term metabolites. The laboratories are obliged to test for the parent substance or metabolites with a minimum sensitivity of 10 ng/mL for anabolic agents in general and with 2 ng/mL for the metabolites



Type	R_1	R_2	R_3	Further substituents	Name WADA list
III	HO, H	OH, H	CH_3		1-Androstenediol
III	O	O	CH_3		1-Androstenedione
I	HO, H	OH, H	H		Bolandiol (19-norandrostenediol)
I	O	OH, CH_3	CH_3	7α-CH_3	Bolasterone
IV	O	OH, H	CH_3		Boldenone
IV	O	O	CH_3		Boldione
I	O	OH, CH_3	CH_3	7β-CH_3	Calusterone
I	O	OH, H	CH_3	4-Cl	Clostebol
V	N, O	OH, C≡CH	CH_3	delta-4	Danazol
IV	O	OH, CH_3	CH_3	4-Cl	Dehydrochlormethyltestosterone
II	H -	OH, CH_3	CH_3	delta-2	Desoxymethyltestosterone
II	O	OH, H	CH_3	2α-CH_3	Drostanolone
I	H, H	OH, C_2H_5	H		Ethylestrenol
I	O	OH, CH_3	CH_3	9α-F, 11β-OH	Fluoxymesterone
IV	O	OH, CH_3	CH_3	2-CHO, 11α-OH	Formebolone
V	O, N, N	OH, CH_3	CH_3		Furazabol
I	O	OH, C≡CH	H	18-CH3	Gestrinone
I	O	OH, H	CH_3	4-OH	4-Hydroxytestosterone
II	O	OH, CH_3	CH_3		Mestanolone
II	O	OH, H	CH_3	1α-CH_3	Mesterolone
III	O	OH, H	CH_3	1-CH_3	Metenolone
IV	O	OH, CH_3	CH_3		Metandienone
II	HO, H	OH, CH_3	CH_3	delta-5	Methandriol
II	O	OH, CH_3	CH_3	2α-CH_3	Methasterone
I	O	OH, CH_3	H	delta-9(10)	Methyldienolone
III	O	OH, CH_3	CH_3		Methyl-1-testosterone
I	O	OH, CH_3	H		Methylnortestosterone
I	O	OH, CH_3	H	delta-9,11	Methyltrienolone
I	O	OH, CH_3	CH_3		Methyltestosterone
I	O	OH, CH_3	H	7α-CH_3	Mibolerone
I	O	OH, H	H		Nandrolone
I	O	O	H		19-Norandrostenedione
I	O	OH, C_2H_5	H	18-CH_3	Norbolethone
I	O	OH, H	H	4-Cl	Norclostebol
I	O	OH, CH_3	H	18-CH_3	Norethandrolone
I	O	OH, H	H	4-OH	Oxabolone
II	O	OH, CH_3	CH_3	2-oxa	Oxandrolone
I	O	OH, CH_3	CH_3	4-OH	Oxymesterone
II	O	OH, CH_3	CH_3	2=CHOH	Oxymetholone
V	HN, N	OH, H	CH_3		Prostanozol
IV	O	O, H	CH_3		Quinbolone
V	HN, N	OH, CH_3	CH_3		Stanozolol
III	O	OH, H	CH_3	2-CH_3	Stenbolone
III	O	OH, H	CH_3		1-Testosterone
I	O	OH, C_2H_5	H	delta-9,11, 18-CH_3	Tetrahydrogestrinone
I	O	OH, H	H	delta-9,11	Trenbolone

Fig. 13.1 Chemical structure of exogenous anabolic androgenic steroids

of metandienone (17β-methyl-5β-androst-1-ene-3α,17α-diol), methyltestosterone (17α-methyl-5β-androstane-3α,17β-diol), and stanozolol (3′-hydroxystanozolol) [30]. Criteria for substance identification as proposed by WADA [31] include the retention time and the relative abundances of at least three diagnostic ions or ion transitions compared to reference urines or reference substances. Methods for the synthesis of these reference metabolites are described by Schänzer and Donike [32].

New "Designer Steroids"

Lately, more and more products appeared on the market as "dietary supplements". They contain steroids that had never been marketed as approved drugs, mostly without proper labeling of the contents [33]. Syntheses and few data on pharmacological effects are available dated back mainly to the 1950s or 1960s. Only little knowledge exists about effects and side effects of these steroids in humans. They are only produced for the "supplement market" and are advertised as anabolic steroids or aromatase inhibitors.

The legal status of these supplements is not clear in several countries. With these "new" steroids, it is possible to circumvent the Anabolic Steroid Control Act 2004 [34]. According to this act, anabolic steroids are classified as schedule III controlled drugs and their trade as nutritional supplements is prohibited in the USA. However, these new steroids are not listed in the annex of banned steroids [34]. European legislation classifies these products as nonlicensed pharmaceuticals even if they are marketed as nutritional supplements. In most cases, the labeling of these products consists of nonapproved or fancy names of the steroids. The sources are not known but most likely Chinese pharmaceutical companies are involved. Some of the new steroids detected are advertised in their product lists on the Internet. In sports, these new steroids belong to the prohibited classes of AAS or aromatase inhibitors [1]. These steroids are not mentioned explicitly on the WADA list of prohibited substances but are covered by the wording "…and other substances with a similar chemical structure or similar biological effect(s)." The relevance of such supplements in athletes was proven in 2006 where metabolites of 6α-methylandrostenedione were found in an athlete's urine [35]. Also metabolites of androsta-1,4,6-triene-3,17-dione were already detected in doping control urines [36]. To cover the whole range of these "designer steroids," comprehensive screening tools are required. Applying mass spectrometric techniques like GC–MS or LC–MS/MS offers the possibility of unknown steroid detection [37] by monitoring common fragment ions or losses indicating the principle structure and functional groups. Especially, the precursor ion scanning option of triple–quadrupole mass analyzers is a useful tool for the detection of unknown steroids when focusing on product ions derived from common steroid structures and nuclei [38, 39].

Analysis for Endogenous AAS

The administration of AAS that are capable of being physiologically produced by the human body is also prohibited in sports. Table 13.1 summarizes the structures of the endogenous steroids listed explicitly by WADA. As these compounds and their metabolites also occur naturally in the human body-specific indicators for the detection of the exogenous administration of these steroids are required [40].

Steroid Profiling

For screening purpose, a set of urinary concentrations of several endogenous steroids or metabolites (Table 13.1) is generally determined by the GC–MS method used for

Table 13.1 Endogenous steroids listed explicitly by WADA (monitored in steroid profiling) and additionally monitored steroid profile parameters (indicated with asterisks)

Name	Type	R_1	R_2	R_3	Additional modification
Androst-5-ene-3ξ,17ξ-diol	II	–OH	–OH	$–CH_3$	delta-5
Androst-4-ene-3,17-dione	I	=O	=O	$–CH_3$	
Dihydrotestosterone	II	=O	–OH	$–CH_3$	
Dehydroepiandrosterone (DHEA)	II	–OH	=O	$–CH_3$	delta-5
Testosterone	I	=O	–OH	$–CH_3$	
5α-Androstane-3ξ,17ξ-diol	II	–OH	–OH	$–CH_3$	
5α-Androstane-3,17-dione	II	=O	=O	$–CH_3$	
17-Epi-dihydrotestosterone	II	=O	–OH	$–CH_3$	
Epitestosterone (17α-hydroxyandrost-4-ene-3-one)	I	=O	–OH	$–CH_3$	
Androsterone (3α-hydroxy-5α-androstan-17-one)	II	–OH	=O	$–CH_3$	
Etiocholanolone (3α-hydroxy-5β-androstan-17-one)	II	–OH	=O	$–CH_3$	5β-H
19-Norandrosterone (3α-hydroxy-5α-estran-17-one)	II	–OH	=O	–H	
19-Noretiocholanolone (3α-hydroxy-5β-estran-17-one)	II	–OH	=O	–H	5β-H
5β-Androstane-3ξ,17ξ-diol*	II	–OH	–OH	$–CH_3$	5β-H
5β-Androstane-3,17-dione*	II	=O	=O	$–CH_3$	5β-H
Epiandrosterone (3β-hydroxy-5α-androstan-17-one)*	II	–OH	=O	$–CH_3$	
Boldenone*	IV	=O	–OH	$–CH_3$	
17β-Hydroxy-5β-androst-1-en-3-one*	III	=O	–OH	$–CH_3$	5β-H
11β-Hydroxy-androsterone*	II	–OH	=O	$–CH_3$	11β-OH
11β-Hydroxy-etiocholanolone*	II	–OH	=O	$–CH_3$	11β-OH, 5β-H
5β-Pregnane-3α,20α-diol*	II	–OH		$–CH_3$	17β-CH(OH)CH3
5β-Pregnane-3α,17α,20α-triol*	II	–OH	–OH	$–CH_3$	17β-CH(OH)CH3

"ξ" represents "α" and/or "β" orientation
For allocation of structure types, see Fig. 13.1

the detection of steroid abuse. The method of steroid profiling was first introduced into routine doping control by Donike et al. [41] (T/E ratio). Some ratios of these steroids have been proven to be very stable [42–48]. Especially, the intraindividual variances are quite small. But also population-based reference ranges are suitable for screening purpose. Longitudinal and retrospective evaluation of steroid profiles offers a suitable basis for individual reference ranges.

The most important steroid profile parameters in doping control are the ratios of T/E, And/Etio, And/T, and Adiol/Bdiol. The administration of steroids like testosterone, its precursors like androstenediol, androstenedione, or DHEA, or metabolites, dihydrotestosterone, or epitestosterone are proven to alter one or more of the parameters of the urinary steroid profile [41, 49–55]. Consequently, monitoring the steroid profile parameters allows to screen for potential misuse.

Isotope Ratio Mass Spectrometry

The analysis of $^{13}C/^{12}C$ ratios at natural abundance levels typically is performed in a dedicated instrument known as (gas) isotope ratio mass spectrometer. As the number of applications currently is increasing dramatically, this technology often is recognized as very modern. However, the principal design has not been changed since the 1940s [56]. Basically, it is a magnetic sector field mass spectrometer where the detection is performed simultaneously for few relevant species [57]. What has been developed only recently is a diversity of peripherals allowing for rapid and precise online analysis of isotope ratios of a variety of compounds and materials. GC hyphenation has been introduced in the 1980s [58]. Commercial instrumentation based on the design of Brandt [59] is available since the early 1990s. The development is far from complete and several improvements have been introduced [60–62] recently.

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