Progress towards an alternative screening approach for exogenous anabolic steroids in sport

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Abstract

Anabolic androgenic steroids (AAS) are misused by athletes to improve performance in sports. Most of them are extensively metabolized and excreted in urine as phase II metabolites. Some recently reported long-term phase II metabolites are not detectable using the conventional screening conditions. Thus, the objectives of this thesis were the development of an alternative screening method and the reevaluation of AAS metabolism to discover new metabolites that could expand detection time.

A LC-MS/MS screening method for the simultaneous detection of phase I and phase II intact urinary metabolites was developed. The inclusion of long-term phase II metabolites improved the detection capabilities compared to current methods. Moreover, the sulfate fraction of three AAS was studied resulting in the detection of several unreported metabolites and revealed new insights in AAS metabolism. Some of these sulfates improve the detection capabilities of AAS. The direct detection of AAS conjugates is a well-received option in routine doping controls.

Resum

Els esteroides anabolitzants androgènics (EAA) són utilitzats pels atletes per millorar el seu rendiment esportiu. La major part són àmpliament metabolitzats i excretats en l'orina com a metabòlits de fase II. Alguns metabòlits de fase II descrits recentment són de llarga durada i no es detecten utilitzant els mètodes de cribratge convencionals. Per tant, els objectius d'aquesta tesi van ser, d'una banda, el desenvolupament d'un mètode de cribratge alternatiu i, de l'altra, la revaluació del metabolisme d'EAA per descobrir nous metabòlits que podrien ampliar el temps de detecció.

Es va desenvolupar un mètode de cribratge d'CL-EM/EM per a la detecció simultània de metabòlits urinaris intactes de fase I i fase II. La inclusió de metabòlits de fase II de llarga durada va millorar la capacitat de detecció en comparació dels mètodes actuals. A més, es va estudiar la fracció sulfat de tres EAA que va resultar en la detecció de diversos metabòlits descrits mai abans i va revelar nous coneixements en el metabolisme d'EAA. Alguns d'aquests sulfats milloren les capacitats de detecció dels EAA. La detecció directa dels conjugats d'EAA és una opció ben rebuda en els controls antidopatge de rutina.

Preface

Anabolic androgenic steroids (AAS) are included in the list of forbidden substances in sports by the World Anti-Doping Agency (WADA) due to their performing-enhancing properties and adverse health effects. They are the most prominent group of prohibited substances detected in doping controls, reflecting the wide misuse of these compounds among athletes.

Anti-doping laboratories have to develop analytical tools to detect whether or not an athlete has been using AAS. These substances suffer extensive metabolism, so their misuse is monitored through the analysis of the metabolites excreted in urine. For this reason, the anti-doping scientific community has put a lot of effort in studying the metabolism of AAS in order to identify the best markers to detect them.

The conventional routine methods employ gas and liquid chromatography-mass spectrometry (GC-MS and LC-MS) and rely on the detection of the phase I metabolites described in the past. Although this approach has been the gold-standard method for the last decades and it is still used in all laboratories, it also has some limitations.

The potentialities of LC-MS opened up broad possibilities in the detection of AAS. Among others, it presents several advantages regarding sample preparation and analysis enabling the detection of metabolites which are not detectable using the conventional approaches.

The aim of this thesis was to investigate the capacity of LC-MS/MS to develop an alternative method for the simultaneous detection of intact phase I and phase II AAS metabolites and also to continue the reevaluation of AAS metabolism with the aim to discover new metabolites interesting for doping control analysis.

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List of abbreviations

3STAN	3’-hydroxy-stanozolol
4Cl-MTD	4-chlorometandienone
4STAN	4β-hydroxy-stanozolol
16STAN	16β-hydroxy-stanozolol
AAF	Adverse Analytical Finding
AAS	Anabolic Androgenic Steroids
ACN	Acetonitrile
BOLD-S	Boldenone sulfate
CE	Collision energy
CI	Chemical ionization
CID	Collision induced dissociation
CLO	Clostebol
Code	World Anti-Doping Code
CV	Cone voltage
E.Coli	*Escherichia coli*
EI	Electron ionization
EIC	Extracted ion chromatogram
EPO	Erythropoietin
ESI	Electrospray ionization
eSTAN-*N*-G	epistanozolol-*N*-glucuronide
FIFA	International Federation of Association Football
GC	Gas Chromatography
GC-MS	Gas chromatography-mass spectrometry
H.pomatia	*Helix pomatia*
HRMS	High Resolution Mass Spectrometry

IAAF	International Association of Athletics Federations
IOC	International Olympic Committee
IRMS	Isotope Ratio Mass Spectrometry
IS	Internal standard
ISL	International Standard for Laboratories
LC	Liquid Chromatography
LC-MS/MS	Liquid Chromatography tandem Mass Spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
ME	Matrix effect
MeOH	Methanol
MM	Molecular mass
MRPL	Minimum Required Performance Levels
MS	Mass Spectrometry
MSTFA	N-methyl-N-trimethylsilyltrifluoroacetamide
m/z	mass to charge ratio
NH_4I	ammonium iodide
NL	Neutral loss
NMR	Nuclear Magnetic Resonance Spectroscopy
PAPS	3’-phosphoadenosine-5’-phosphosulfate
P. vulgate	*Patella vulgate*
PrecIS	Precursor Ion Scan
Q1/Q3	Quadrupole
q2	Collision cell
RT	Retention time
SARMs	Selective androgen receptor modulators
SIM	Selected ion monitoring

S/N	Signal to noise ratio
SO_3	Sulfo moiety
SPE	Solid-phase extraction
SPME	Solid-phase micro-extraction
SRM	Selected Reaction Monitoring
STAN	Stanozolol
STAN-*N*-G	Stanozolol-*N*-glucuronide
SULT	Sulfotransferases
TIC	Total ion chromatogram
TMS	trimethylsilyl
TMSI	iodotrimethylsilane
TOF	Time-of-flight
TUEs	Therapeutic Use Exemptions
UCI	International Cycling Union
UGT	Uridine diphosphoglucuronosyl-transferases
UHPLC	Ultra-high performance liquid chromatography
USADA	US Anti-Doping Agency
V_{DC}	Direct current voltage
V_{RF}	Radio frequency voltage
WADA	World Anti-Doping Agency
XIC	Extracted ion chromatogram

Thesis structure

This thesis is structured in 9 chapters.

Chapter 1 includes the introduction, covering the main aspects of doping control analysis, anabolic androgenic steroids, analytical strategies and the need of continuously revising the metabolism of these substances.

In Chapter 2, the justification and objectives of the thesis are presented.

Chapters 3, 4, 5 and 6 comprise the adaptation of four scientific papers representing the experimental and results of this thesis. These chapters have been divided into two major parts depending on their content:

- Part I (Chapter 3) contains the necessary steps for the development, optimization and validation of an LC-MS/MS screening method for the direct detection of intact phase I and phase II anabolic steroid metabolites in routine doping control analysis.

- Part II (Chapter 4, 5 and 6) is devoted to the study of new sulfate metabolites of exogenous anabolic steroids including Clostebol, 4-chlorometandienone and Stanozolol.

Chapter 7 includes a general discussion of the results and suggestion for future work.

Chapter 8 includes the main conclusions extracted from the thesis results.

Three annexes have been added at the end (chapter 9). Annex I includes the supplementary material, Annex II comprises the list of publications included into this thesis and Annex III contains other publications by the same author; two scientific articles and one book chapter.

1. INTRODUCTION

1. INTRODUCTION

1.1 Doping in sports

1.1.1 History of doping

The motivation to use performance enhancing substances arises from the desire to win at all costs, firstly for the pride and recognition and secondly for the financial rewards. According to some ancient reports the use of substances in sport to enhance the performance of athletes is a practice that began many years BC [1]. For example, the intake of hallucinogenic mushrooms was a common practice among a number of athletes attending the first Olympic competitions held between 776 BC and 393 AD [2,3]. This type of practice was extended to other non-human sports such as horseracing [4]. As knowledge about the mode of action of pharmaceutical drugs has evolved from the 19th century until today, the use of performance enhancing substances (doping) by athletes has also become more sophisticated [5,6].

Around the late 19th century, sporting authorities realized that it would be necessary to take measures in order to try and eradicate this behavior from sports. In 1928, the International Athletic Federation (IAAF) became one of the first sporting organizations to officially ban the use of doping (stimulating agents) [6]. However, most of the restrictions implemented at that time remained ineffective due to the lack of testing possibilities in sporting competitions [5]. A turning point occurred following the tragic

death of a cyclist in competition during the 1960 Olympic Games held in Rome, where a post-mortem test evidenced the presence of amphetamines. In 1966, the International Cycling Union (UCI) and International Federation of Association Football (FIFA) implemented the first drugs tests at their World Championships, and a year later the International Olympic Committee (IOC) established its own Medical Commission and set up its first list of prohibited substances [6]. Since then, different groups of substances have been continuously included into the list. The anabolic androgenic steroids (AAS), which are the substances studied in this thesis, were introduced as a banned class in 1974 [7].

The first doping tests at the Olympics were taken during the Winter Games in Grenoble and the Summer Games in Mexico in 1968 [6]. By the 1970s the majority of sporting associations had implemented a drug testing programme [6]. Since this time, several athletes have been caught doping in major competitions. The case of the runner Ben Johnson, winner of the 100 m athletics competition, at the 1988 Seoul Olympics is one notable example. Another high-profile doping scandal erupted during the 1998 Tour de France where a senior member of the Festina team was caught with several prohibited drugs, including growth hormones, steroids, amphetamines, erythropoietin (EPO) and narcotics [6]. This was an indicator that more drastic measures were needed to deter this type of activity, in particular, the need for an independent organization that would coordinate anti-doping activities worldwide. For this reason, approximately one year later, the World Anti-Doping Agency (WADA) was established [6] (Figure 1.1).

Figure 1.1: Brief story of doping and its prohibition.

One of the WADA's most significant accomplishments was the implementation of an international set of anti-doping rules, the World Anti-Doping Code (Code) [8], which is followed and accepted worldwide by most sporting and anti-doping organizations. The Code is the core document that harmonizes anti-doping policies, rules and regulations within sport organizations and among public authorities around the world. It works in conjunction with five International Standards which aim to foster consistency among anti-doping organizations in various areas: testing; laboratories; Therapeutic Use Exemptions (TUEs); the List of Prohibited Substances and Methods; and the protection of privacy and personal information. According to the Code, doping is defined as the violation of one or more of the anti-doping rules [8]. The most frequent violations are *"presence of a prohibited substance or its metabolites or markers in an athlete's sample"* and *"use or attempted use by an athlete of a prohibited substance or a prohibited method"*. Additionally, other situations that include *(i) evading, refusing or failing to submit to sample collection, (ii) tampering or attempted tampering with any part of doping control, (iii) possession of a prohibited substance or a prohibited method,*

(iv) trafficking or attempted trafficking in any prohibited substance or method and *(v) administration, assisting, encouraging, aiding, abetting, covering up or any other type of complicity involving an anti-doping rule violation* are also considered as doping.

The sanctions for infractions under the Code will vary depending on the seriousness and extent of the doping activity. Typical bans from sporting activity vary from six months to several years. In exceptional circumstances, where it is found that the doping activity has been practiced for many years and has infringed most of the anti-doping rules, the athlete can be sanctioned for life. A recent example is the high profile Lance Armstrong case, where the athlete has been stripped by the US Anti-Doping Agency (USADA) of all of his titles won since 1997 and banned from attending any professional competition for life, following the testimonies of team colleagues and members of his staff.

1.1.2 WADA Prohibited List

WADA's List of Prohibited Substances [9] is an annually published document that details all the substances and methods that are prohibited from being used by athletes. A substance or method shall be included if WADA determines that the substance or method meets any two of the following three criteria: (1) the substance or method has the potential to enhance or enhances sport performance as evidenced by medical or scientific data, pharmacological effect or experience; (2) the substance or method represents an actual or potential health risk to the athlete as evidenced by medical or

scientific data, pharmacological effect, or experience; and/or (3) the substance or method violates the spirit of sport as defined in the Code [8].

The list includes more than 200 compounds divided into classes, namely: S0 - non-approved substances; S1 - anabolic agents; S2 - peptide hormones, growth factors, related substances and mimetics; S3 - beta-2-agonists; S4 - hormone and metabolic modulators; and S5 - diuretics and masking agents. With respect to the prohibited methods, these are: M1 - manipulation of blood and blood components; M2 - chemical and physical manipulation; and M3 - gene doping. These doping classes and methods are prohibited at all times (in- and out- of competition). Other classes of compounds which are only prohibited in competition are: S6 - stimulants; S7 - narcotics; S8 - cannabinoids; and S9 – glucocorticoids. Further, there are also another two groups of substances that are only prohibited for certain sports: P1 - alcohol; and P2 - beta-blockers (Figure 1.2).

For each group of substances, the list indicates representative examples but other compounds with similar chemical structure or biological activity are also included. In some particular cases, drugs are only forbidden when administered by some routes. For instance, glucocorticoids are only prohibited when administered by oral, intravenous, intramuscular or rectal routes.

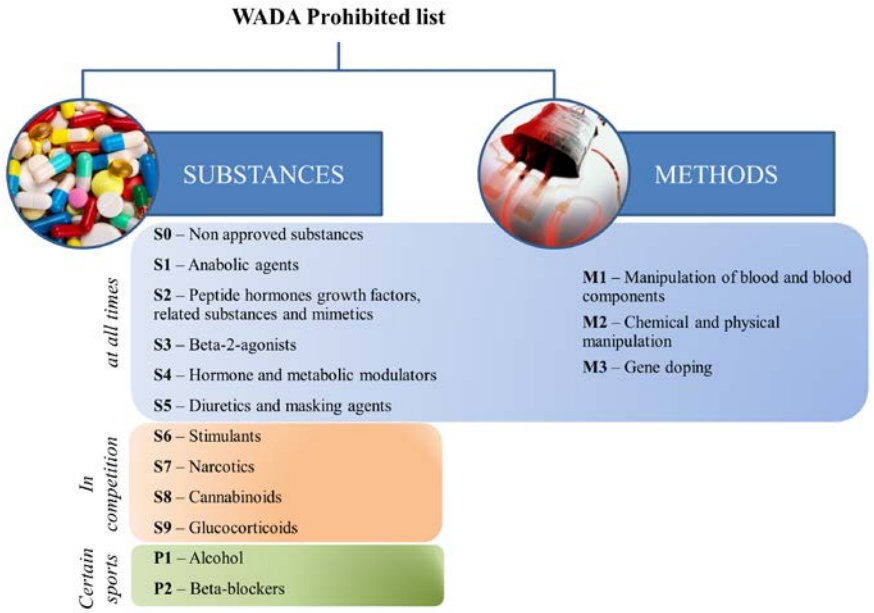

Figure 1.2: Substances and methods prohibited by WADA.

As mentioned before, AAS are the substances studied in this thesis and they are included in the S1class. This class comprises a variety of compounds divided into three separate groups: endogenous, exogenous and other anabolic agents (e.g. clenbuterol, nonsteroidal selective androgen receptor modulators (SARMs)).

1.1.3 General analytical strategies

In order to reliably detect the substances prohibited in sport as defined by WADA's List of Prohibited substances and methods, anti-doping laboratories must achieve and maintain accreditation by WADA to perform the analysis of doping controls for sports. WADA accreditation is based upon compliance with two international standards, ISO 17025 and International Standard for

Laboratories (ISL) [10]. Additionally, WADA publishes specific technical recommendations, addressing particular operational areas of the accredited laboratories, in Technical Documents [11]. Implementation of the technical recommendations described in them is mandatory.

Doping control consists of the analysis of samples collected from an athlete to look for the presence of markers of banned substances or methods in biological fluids. Therefore, anti-doping laboratories have to develop analytical methodologies to ensure detection of all prohibited substances and methods. For most of the analysis, urine is the specimen of choice for a number of reasons. Its collection is non-invasive (although witnessed collection may be considered as some intrusion of privacy), sufficient volume is generally available, drugs are present in relatively higher concentrations than in blood and, since hydrophilic metabolites are also excreted in urine, the detection time window can be enlarged [12]. On the other hand, blood samples are also collected to determine blood parameters and to monitor the use of specific doping agents (e.g. human growth hormone) [13] and its implementation for more general analysis is possible in the near future. The use of less invasive blood collection techniques such as dried blood stops have also been investigated [14,15].

The analytical strategies used in doping control need to take into account several requirements: high sensitivity and selectivity (due to the complex matrices used such as urine and blood), detection of compounds with a wide range of physicochemical properties and

molecular weights, limited sample volumes and fast analysis time due to the short reporting times. In terms of sensitivity, all accredited doping control laboratories should reach the minimum required performance levels (MRPL) established by WADA in order to ensure the homogeneity of the reported results [16]. MRPL values are established taking into account the metabolism, stability, pharmacokinetics and pharmacodynamics of the substances. Thus, substances with long-term doping effect, such as the AAS, have lower MRPL values (MRPL= 5 ng/mL, 2 ng/mL for some exceptions) than substances with immediate effect like stimulants (MRPL= 100ng/mL). The limit of detection of analytical methods for a particular substance must not be higher than 50% of the MRPL. Thus, the MRPL is the concentration of a prohibited substance, metabolite or marker that doping control laboratories shall be able to routinely detect and identify. However, the MRPL is not a threshold nor it is a limit of detection. Hence, the presence of a prohibited substance or its metabolites in a sample, defined as an adverse analytical finding (AAF), may result from concentrations below the established MRPL values.

Doping control includes two different types of procedures, screening and confirmation for the detection of a doping agent (Figure 1.3). The initial testing procedures or screening methods are applied to all samples and provide an indication of the presence or absence of a doping agent. They should be able to detect a wide number of compounds and/or metabolites with similar physicochemical properties [17-20] at the limits of detection established by WADA [16]. The screening methods must be fast

and robust, sensitive and specific, identifying all suspect samples and minimizing the false suspects that will require a confirmatory analysis.

On the contrary, the confirmatory methodologies are applied to a single sample considered suspicious after the screening tests. A specific methodology, optimized for the suspected doping agent, is followed in order to obtain the ultimate confirmation of the presence of the prohibited substance in the athlete's sample.

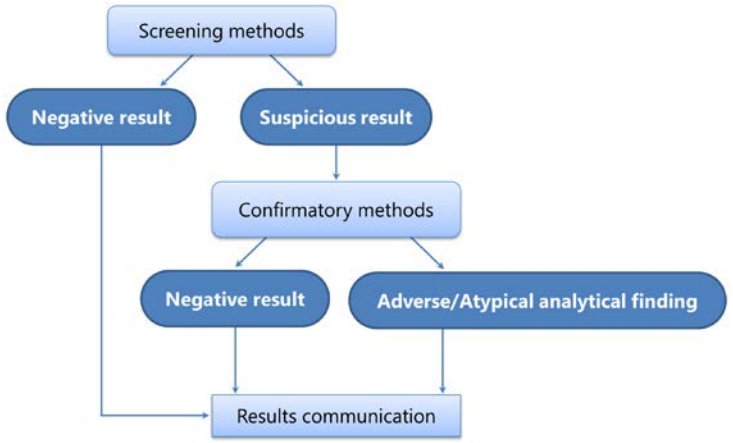

Figure 1.3: Scheme of the doping control analytical process.

For most of the substances of the WADA Prohibited list the mere detection of their presence (non-threshold substances) can be considered evidence for their abuse and the sample is submitted to further confirmatory tests by (tandem) mass spectrometry (MS(/MS)) coupled to either gas chromatography (GC) or liquid chromatography (LC). In this case, the analyte's retention time and the relative abundances of three diagnostic ions (MS analysis) or

two diagnostic ion transitions (MS/MS analysis) are compared to reference material. The analysis is considered as AAF if the identification criteria established in the WADA technical document [21] are fulfilled.

However, other substances of the prohibited list require quantitative measurements either because they are considered as doping agents only above a certain concentration (threshold substances: e.g., salbutamol, formoterol or ephedrines) [22] or due to their endogenous origin (e.g. 19-norandrosterone, glycerol) [23]. Thus, a sample is considered suspicious when the concentrations of the measured analytes exceed a pre-established threshold. For the confirmation of threshold substances, after the unequivocal identification of the analyte by the criteria mentioned in the previous paragraph, the quantification of the substance is needed. The sample is considered as AAF if the mean concentration of three aliquots of the sample is above the decision limit established for each substance. The decision limit has been established for each subtance based on the threshold concentration and the maximum uncertainty accepted for the quantitative method [22].

For the endogenous compounds, neither GC- nor LC-MS(/MS) techniques are able to differentiate endogenous substances from the exogenous analogues [24] and, for this reason, various strategies have been explored. In the particular case of testosterone and related endogenous steroids, the concentrations and ratios of testosterone and some of its metabolites are measured during the screening step. Subsequently, they are compared with the values previously

obtained for the same athlete using a Bayesian model, and if values fall out of the “normal” reference ranges established for the particular athlete, this may be an indication of consumption of endogenous steroids. The final test is to apply isotope ratio mass spectrometry (GC/C/IRMS) to distinguish between endogenous origin and administration of synthetic steroid analogues [25].

Screening and confirmation procedures have to be continuously updated and extended based on WADA requirements to incorporate new doping agents, new markers or metabolites (e.g. novel metabolites excreted for longer periods of time).

1.1.4 Adverse Analytical Findings

Since 2003, WADA annually publishes a report that contains statistical data based on the results obtained by all WADA accredited anti-doping laboratories around the world. According to these reports, anabolic agents (S1) are the most commonly reported group of prohibited substances detected in the doping controls (Table 1.1) [26].

Table 1.1: Summary of substances identified as AAFs in each drug class published in the Anti-Doping Testing Figures Report 2014 [26].

Substance group	Occurrences	% of all ADAMS reported findings
S1. Anabolic Agents	1479	48
S6. Stimulants	474	15
S5. Diuretics and Other Masking Agents	389	13
S9. Glucocorticoids	252	8
S4. Hormone and Metabolic Modulators	145	5
S3. Beta-2 Agonists	122	4
S2. Peptide Hormones, Growth Factors and Related Substances	91	3
S8. Cannabinoids	73	2
S7. Narcotics	26	0.8
P2. Beta-Blockers	25	0.8
M2. Chemical and Physical Manipulation	3	0.1
P1.Alcohol	0	0
M1. Enhancement of Oxigen Trasnfer	0	0

Throughout the years, clenbuterol and the AAS such as testosterone and stanozolol (STAN), were the most frequently detected compounds among the anabolic agents. Nevertheless, in the last version of the document released in 2014 [26], the cases of AAF linked to clenbuterol abuse were the most frequent with 251 occurrences (Table 1.2). However, in the previous annual reports, AAF related to testosterone misuse (described as T/E>4 in the reports, Table 1.2) used to be the most common having very high percentages of occurrence within their class (e.g. 59.6% in 2013 [27] and 55.5% in 2012 [28]).

Table 1.2: Occurrences of AAFs related to a specific anabolic agent published in Anti-Doping Testing Figures Report 2014 [26].

S1.1 Anabolic agents	Occurrences	% within drug class
stanozolol	239	20
19-norandrosterone	195	16
metandienone	123	10
metenolone	84	7
drostanolone	81	7
dehydrochloromethyl-testosterone	76	6
the GC/C/IRMS results are consistent with the exogenous origin of a prohibited substance(s)	72	6
trenbolone	57	5
T/E ratio > 4	56	5
boldenone	53	4
mesterolone	31	3
oxandrolone	28	2
methyltestosterone	14	1
methasterone	12	1
testosterone	10	1
S1.2 Other Anabolic agents	**Occurrences**	**% within drug class**
clenbuterol	251	87
selective androgen receptor modulators (SARMs)	15	5
zilpaterol	13	5
tibolone	7	2
zeranol	1	0.3

It has to be remarked that these findings should not be confused with adjudicated or sanctioned anti-doping rule violations as they summarize both AAF and atypical findings reported by doping control laboratories. Thus, for endogenous compounds, the findings may occur due to the detection of concentrations outside normal reference ranges and not necessary for a doping offense.

This thesis will examine current methodologies used for the doping control testing of exogenous AAS, their limitations and further improvements which could improve their detection capabilities.

1.2 Anabolic Androgenic Steroids

1.2.1 General aspects

The AAS are the major subcategory of the anabolic agents prohibited in sports. They are included in the list of forbidden substances due to their performance enhancing properties and adverse health effects [29]. From a chemical point of view, the AAS can be considered as synthetic derivatives of the hormone testosterone whose general structure is based on the perhydrocyclopentanephenantrene nucleus (Figure 1.4).

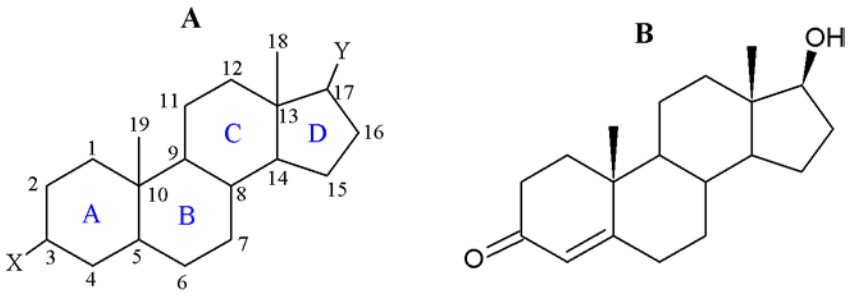

Figure 1.4: General structure for anabolic steroids showing the numbering of the carbon atoms and the rings in the molecule (A). Structure of testosterone (B).

There are two groups of AAS; the endogenous and the exogenous AAS. The endogenous AAS refer to the substances which are naturally produced by the body. Among them, the androgen testosterone is one of the most important steroids as it has a crucial role in the development of male reproductive tissues and promotes secondary sexual characteristics such as the muscle increase [30].

Contrary to the endogenous, the exogenous AAS are synthetic versions of the AAS nucleus (Figure 1.4) that are not naturally produced by the human body. They are the result of modifications in the testosterone chemical structure such as addition/removal of double bonds, reduction of the keto group, substitution using heteroatoms, halogens or, addition of functional groups like hydrocarbon chains and heterocyclic rings in the testosterone molecule. Under this category, one should not consider the esterified or other preparations of endogenous AAS that, once inside the body, will liberate the free form of the naturally produced AAS.

Exogenous AAS have been developed with the objective of circumventing several limitations of the drug's use (e.g. poor testosterone bioavailability related to oral administration) and to increase the dissociation between the androgenic and the anabolic effect (discussed in section 1.2.3).

In the doping control field, the detection of any trace of an exogenous AAS or of one of its metabolites is sufficient to report an AAF. Some of the common exogenous AAS (clostebol (CLO), 4-chlorometandienone (4Cl-MTD) and STAN) are shown in Figure 1.5 and discussed in the following sections as model compounds with regards to general pharmacological aspects.

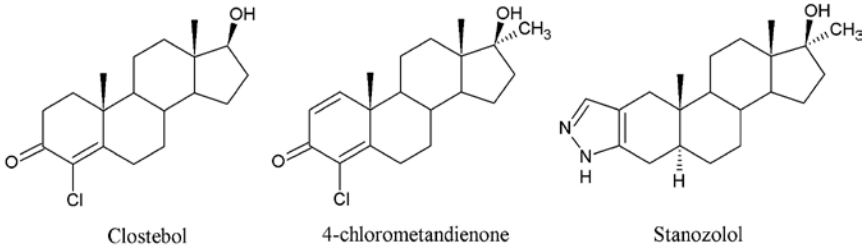

Figure 1.5. Structures of some common AAS.

1.2.2 AAS Metabolism

Steroids are extensively modified to facilitate its elimination from the body. In this process a large number of steroid-metabolizing enzymes are involved. The main site of androgen metabolism is the liver, which is particularly rich in steroid catabolic enzymes, but some metabolic reactions also occur in the kidney, the skin or other tissues [29]. In these tissues, AAS follow a typical metabolic pathway that consists of various phase I and phase II metabolic reactions in order to convert the steroid substrate into a less toxic, less active (although some of the intermediate metabolites remain biologically active) and more polar form. In man, the bulk of steroid metabolites appears in urine. Notably, the percentage of metabolism depends on each drug. Apart from the chemical nature of the substance, there are high inter-individual and inter-ethnic differences in drug metabolism.

1.2.2.1 Phase I

During phase I metabolism of AAS, a complex net of metabolites are produced through the action of numerous enzymes. These

metabolites are the result of consecutive biotranformations in the backbone structure of the AAS that can either be reversible or non-reversible. Some of the most frequently studied metabolic reactions of AAS are summarized in Figure 1.6. Several oxidation and reduction reactions, performed by a variety of dehydrogenases and reductases, can occur mainly at positions C3, C5 and C17 resulting in the α- or β- isomers of the steroids by the gain or loss of two protons. In addition, AAS metabolites can be stereoselectively mono- or poly-hydroxylated in several potential sites of the steroid backbone providing a large number of possible metabolites. Other reactions include 1,2- or 6,7-dehydrogenation.

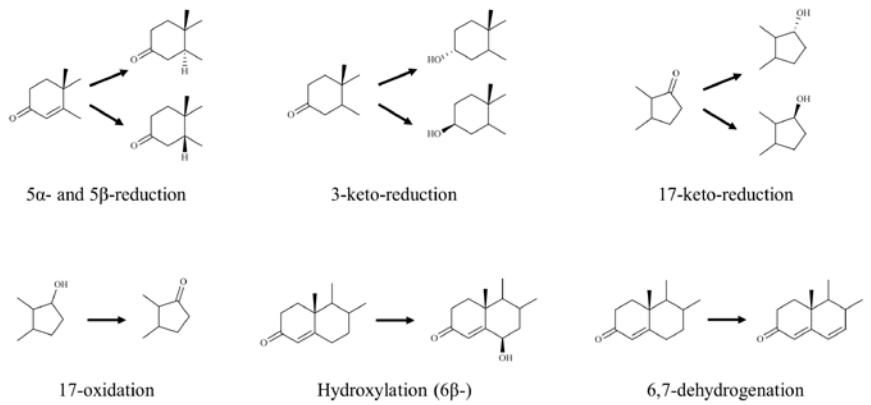

Figure 1.6: Common phase I metabolic reactions of AAS.

1.2.2.2 Phase II

After phase I metabolism, phase II reactions result in the formation of more polar and hydrophilic metabolites. Phase II reactions conjugate the AAS or their phase I metabolites with very polar molecules. The two main reactions are glucuronidation and

sulfation [31-33] (Figure 1.7). Other phase II conjugates have also been described, such as disulfates, diglucuronides, bis-conjugates (glucuronide/sulfate) or conjugates with N-acetylglucosamine or gluthatione [34-38].

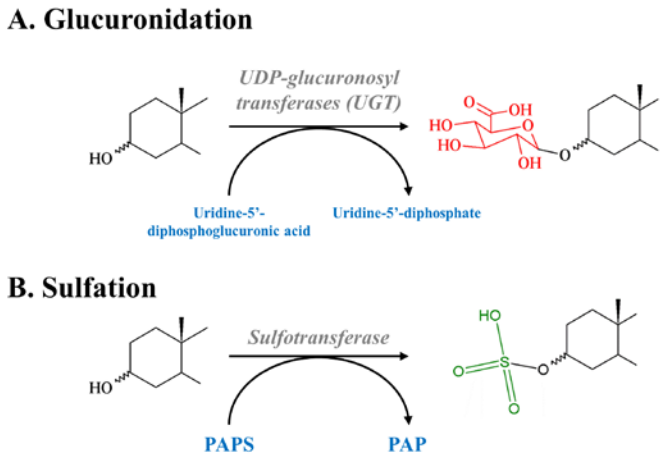

Figure 1.7: Common phase II reactions of AAS. (A) Conjugation with glucuronic acid and (B) conjugation with sulfate.

Glucuronidation, considered the most predominant pathway in human metabolism [39], is catalyzed by uridine diphosphoglucuronosyl-transferases (UGTs) and uses uridine-5'-diphosphoglucuronic acid as the co-substrate leading to the attachment of the highly polar glucuronic acid moiety to the steroid structure (Figure 1.7 A).

Sulfation also constitutes an important pathway in the modulation of the pharmacological activity. The reaction is catalyzed by sulfotransferases (SULT) enzymes, which transfer the sulfo moiety (SO_3) from a co-substrate, 3'-phosphoadenosine-5'-phosphosulfate (PAPS), to the specific substrate (Figure 1.7 B).

Regarding the configuration of phase II metabolites, 3α-hydroxy steroids are usually conjugated with glucuronic acid whereas 3βhydroxy steroids are mainly excreted as sulfates. Stereoselectivity has been observed in the glucuronidation of some diastereomeric compounds (5α- or 5β- configuration) [40]. The hydroxyl group in 17β-position is also conjugated with glucuronic acid or sulfate [31].

Remarkably, some of the urinary products do not come from actual metabolic reactions but the urinary degradation of "real" metabolites. As an example, several degradation products have been described from the 17β-sulfates of tertiary 17β-hydroxy steroids [31,41]. In a first step the sulfate group is removed generating a stabilized cation. From this cation several eliminations or substitutions can be performed generating different compounds (Figure 1.8).

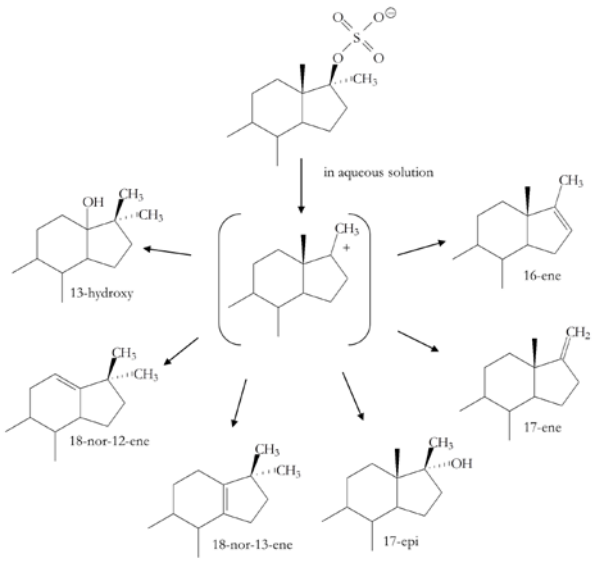

Figure 1.8: Degradation of the 17β-sulfates of tertiary 17β-hydroxy steroids in aqueous solution [31].

Although these compounds cannot be considered as metabolites, they have the same usefulness for doping control analysis as the real metabolites because they are urinary markers for steroid misuse. For this reason, and for simplicity during this thesis, these compounds are also referred to as steroid metabolites. Finally, not all the AAS and their metabolites are excreted as conjugates. Thus, we can distinguish between unconjugated "free" metabolites and conjugated excreted metabolites [31].

1.2.2.3 Metabolism of specific AAS

A metabolic overview of three representatives AAS; CLO, 4Cl-MTD and STAN is presented in the following sections. These compounds are also the focus of the present thesis.

Clostebol

CLO (4-androsten-4-chloro-17β-ol-3-one, Figure 1.9, **1**) is the 4-chloro derivative of testosterone. Metabolites oxidized in C-17 (**2**) and reduced to bis (**3**) and tetrahydro products in the A-ring (**4-6**) were initially described as the predominant metabolites [31,42,43].

The exact configuration of the A-ring reduced metabolites is as yet undetermined. The main metabolite excreted is 4-chloro-androst-4-en-3α-ol-17-one (**3**). A further abundant hydroxyl metabolite, hydroxylated at C-16 and fully A-ring-reduced was also described [31] but configuration has not been confirmed (**7**).

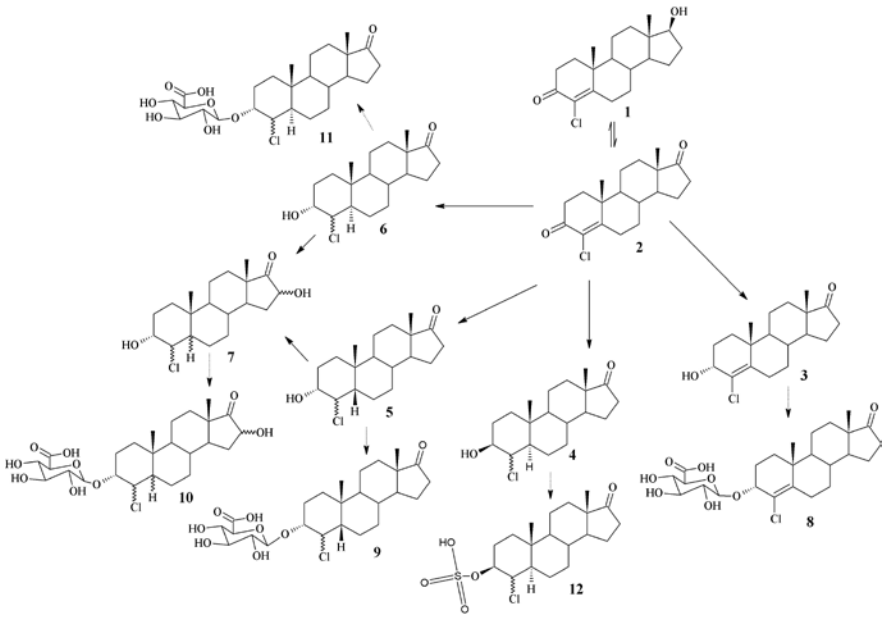

Figure 1.9: Scheme of CLO (1) metabolism and potential chemical structures for the main described metabolites.

These metabolites are all excreted as glucuronides (**8-11**). Besides, a 17-keto tetrahydro metabolite (**4**) excreted as a sulfate (**12**) was already suggested in these early metabolic studies [31]. Also, a sulfate metabolite with the same molecular mass (MM) was recently reported for Chinese population [44]. The structure proposed for that sulfate metabolite was 4ζ-chloro-5ζ-androst-3ζ-ol-17-one 3ζ-sulfate, based only on mass spectrometric data obtained after liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF) analysis. Some other metabolites without the C17 oxidation have also been described [44] (not shown in Figure 1.9).

4-chlorometandienone

The metabolism of 4Cl-MTD (4-chloro-17β-hydroxy,17α-methylandrosta-1,4-dien-3-one, Figure 1.10, compound **(1)**), also known as dehydrochloromethyltestosterone or oral-turinabol, has been widely investigated and the main metabolites described are presented in Figure 1.10 [45-50]. The first studies revealed the presence of the parent drug (**1**) together with three major hydroxylated metabolites: 6β-hydroxy-4Cl-MTD (**2**), 16β-hydroxy-4Cl-MTD (**3**) and 6β,16-dihydroxy-4Cl-MTD (**4**) in post-administration urine [45,46]. Later, 6β,12-dihydroxy-4Cl-MTD (**5**) and minor amounts of epi4Cl-MTD (**6**) were also identified [47]. Afterwards, a more in depth study of 4Cl-MTD metabolism in human urine revealed an important metabolite detectable in urine up to 14 days after administration that was characterized as 4-chloro-3α,6β,17β-trihydroxy-17α-methyl-androst-1-en-16-one (**7**) [48]. Thereafter, the existence of 18-nor-17-hydroxymethyl metabolite of 4Cl-MTD was demonstrated [49]. This metabolite, 4-chloro-18-nor-17β-hydroxymethyl,17α-methylandrosta-1,4,13-trien-3-one (**8**), was found to be as valuable as metabolite **7**, both being detectable 22 days after administration by GC-MS/MS.

Finally, six long-term metabolites were identified as 4-chloro-17α-methyl-5β-androstan-3α,16,17β-triol (**9**), 4-chloro-18-nor-17β-hydroxymethyl,17α-methylandrosta-4,13-dien-3α-ol (**10**) and its 17-epimer (**epi10**), 4-chloro-18-nor-17β-hydroxymethyl,17α-methyl-5β-androsta-1,13-dien-3α-ol (**11**), 4-chloro-18-nor-17β-hydroxymethyl,17α-methyl-5β-androst-13-en-3α-ol (**12**), its 17-

epimer (**epi12**). Metabolite **12** and, to a lesser extent, its epimer (**epi12**) showed to be superior regarding retrospectivity to the other known 4Cl-MTD metabolites, such as metabolites **7** and **8**, in most of the cases [50].

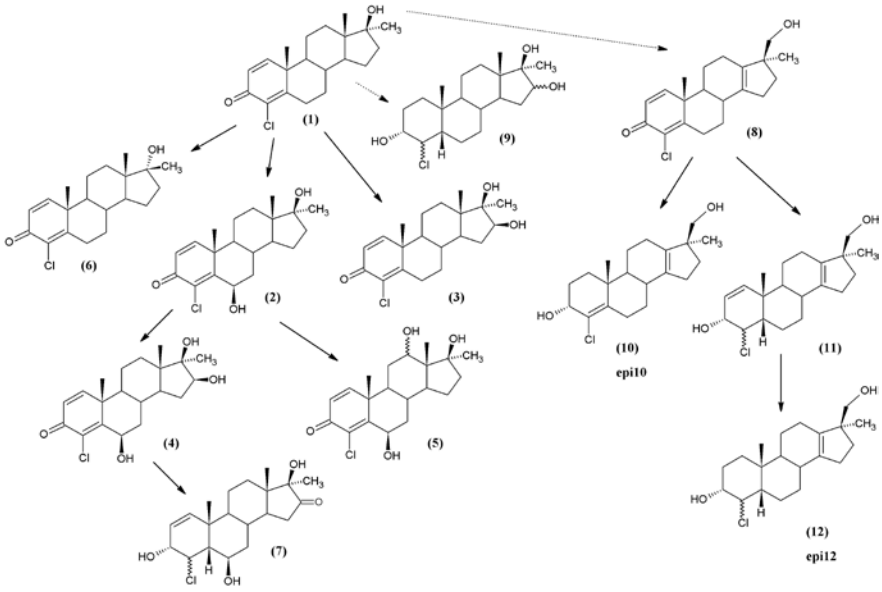

Figure 1.10: Scheme of metabolism of 4Cl-MTD based on literature; (**1**): 4Cl-MTD, (**2**): 6β-hydroxy-4Cl-MTD, (**3**): 16β-hydroxy-4Cl-MTD, (**4**): 6β,16-dihydroxy-4Cl-MTD, (**5**): 6β,12-dihydroxy-4Cl-MTD, (**6**): epi-4Cl-MTD, (**7**): 4-chloro-3α,6β,17β-trihydroxy-17α-methyl-androst-1-en-16-one, (**8**): 4-chloro-18-nor-17β-hydroxymethyl,17α-methylandrosta-1,4,13-trien-3-one, (**9**): 4-chloro-17α-methyl-5β-androstan-3α,16,17β-triol, (**10**): 4-chloro-18-nor-17β-hydroxymethyl,17α-methylandrosta-4,13-dien-3α-ol, (**epi10**): 4-chloro-18-nor-17α-hydroxymethyl,17β-methylandrosta-4,13-dien-3α-ol, (**11**): 4-chloro-18-nor-17β-hydroxymethyl,17α-methyl-5β-androsta-1,13-dien-3α-ol, (**12**): 4-chloro-18-nor-17β-hydroxymethyl,17α-methyl-5β-androst-13-en-3α-ol, (**epi12**): 4-chloro-18-nor-17α-hydroxymethyl,17β-methyl-5β-androst-13-en-3α-ol.

Up to now, studies have only focused on glucuronoconjugated metabolites obtained after hydrolysis with β-glucuronidase enzymes and the detection of the released phase I metabolites by GC-MS and GC-MS/MS. Sulfate metabolites have not been systematically

studied. However, formation of epimers in C17 and 18-nor-17,17-dimethyl metabolites is due to the formation of a sulfate and subsequent decomposition in urine.

Stanozolol

STAN (17β-hydroxy-17α-methyl-5α-androst-2-eno(3,2-c)-pyrazole, Figure 1.11, compound **1**) differs considerably from CLO and 4Cl-MTD in its metabolic reactions due to its pronounced structural difference resulting from the pirazol residue condensed to the A-ring of the steroidal core.

The major phase I metabolites initially observed and identified in human urine were 3'-hydroxy-stanozolol (3STAN, **2**), 4β-hydroxy-stanozolol (4STAN, **3**) and 16β-hydroxy-stanozolol (16STAN, **4**) [51] as illustrated in Figure 1.11. Unchanged STAN was also detected. Over the years, other metabolites (e.g. epimerized analogues in C17 (**5**), mono and dihydroxylated metabolites…) were detected and characterized [31,52]. Most of these metabolites were detected in urine after hydrolysis with β-glucuronidase enzymes, therefore they are mainly excreted conjugated with glucuronic acid (**6-8)** [53,54]. The *N*-glucuronidation, which occurs to a major extent in rat [55], was also expected in humans. More recently, two additional metabolic products metabolized by *N*-conjugation, epistanozolol-*N*-glucuronide (eSTAN-*N*-G, **9**) and stanozolol-*N*-glucuronide (STAN-*N*-G, **10**) were described and detected in urine [56]. These metabolites are not hydrolysable using β-glucuronidase enzymes and their detection was achieved by using LC-MS/MS and detecting the intact conjugates.

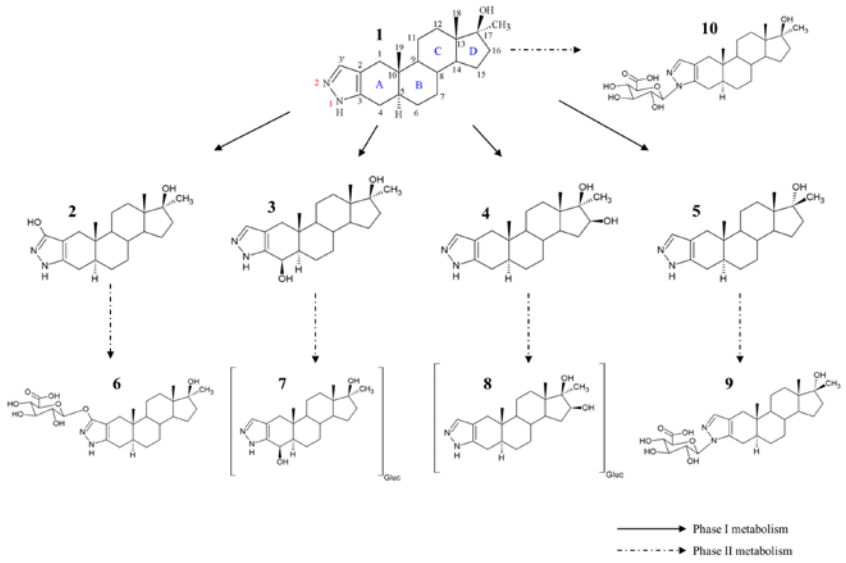

Figure 1.11: Scheme of the metabolism of STAN involving phase I and phase II metabolism based on literature; 1: STAN, 2: 3'-hydroxy-stanzolol (3STAN), 3: 4β-hydroxy-stanozolol (4STAN), 4: 16β-hydroxy-stanozolol (16STAN), 5: epistanozolol (eSTAN), 6: 3'-hydroxy-stanzolol glucuronide (3STAN-G), 7: 4β-hydroxy-stanozolol glucuronide (4STAN-G), 8: 16β-hydroxy-stanozolol glucuronide (16STAN-G), 9: epistanozolol-N-glucuronide (eSTAN-N-G) and 10: stanozolol-N-glucuronide (STAN-N-G).

Sulfate metabolites of STAN have not been studied. However, formation of the epimers in C17 (e.g. eSTAN-*N*-G, **9**) indicates the formation of a sulfate which decomposes in urine to yield several degradation products (Figure 1.8), being the 17-epimer the most abundant one.

1.2.2.4 Genetic polymorphism

Genetic polymorphisms can affect the metabolism of AAS and this has implications for doping control [57]. For example, the deletion polymorphism of UGT2B17 gene is associated with significantly lower glucuronidation rates of testosterone, which diminishes the

excretion of testosterone glucuronide but does not seem to affect epitestosterone glucuronide [58]. The UGT2B17 del/del genotype appears to be much more common in Asian than Caucasian population, and this inter-ethnic variation in metabolism has implications for the detection of testosterone administration [59]. Polymorphisms in SULT have also been described and differ by ethnicity [57]. Further, differences in plasma concentrations of endogenous steroid sulfates have been described between ethnic groups [60].

1.2.3 Effects and side effects

AAS exert their effects in many parts of the body, including reproductive tissues, muscle, bone, hair follicles, in the skin, the livers and kidneys, and the haematopoietic, immune and central nervous systems [30]. The effects of the AAS can be divided into anabolic and androgenic effects. The anabolic effects are strongly associated with protein building in skeletal muscle and bone [61]. On the other hand, the androgenic effects can be generally considered as those associated with masculinization; enlarging of the larynx causing a deepening of the voice, the growth of terminal hair (in the pubic, axillary and facial regions), increase of sebaceous gland activity (can lead to acne) and increased aggression [29].

Athletes are obviously interested in the anabolic effects rather than the androgenic effects [29]. Although a lot of effort has been made to develop synthetic molecules that would enhance the anabolic and decrease the androgenic activity of the AAS, the complete

dissociation of the two effects has not been achieved. In this sense, the development of nonsteroidal SARMs may offer better dissociation of biological effects.

It has to be mentioned that the therapeutic use of AAS under the attention of a specialist can be regarded as a safe pharmacological manipulation. However, their abuse may have several risks on the health status of a person that are mainly dependent on the sex, the dose, the duration of administration, whether hepatoxic 17α-alkylated steroids are being administered and the susceptibility of individuals themselves to androgen exposure. Among others, the main adverse effects of AAS abuse include bone effects (stunting of linear growth in children), breast effects (atrophy in women and gynecomastia in men), cardiovascular effects (risk of thrombotic events, cardiac damage or sudden cardiac death) and central nervous system effects (irritability, increased libido and hostility, destructive and self-destructive impulses) [7,29,62,63].

1.3 Analytical strategies for screening of exogenous AAS misuse

Since the Winter Olympic Montreal Games of 1976 when doping control laboratories officially started the analysis for the detection of exogenous AAS [64], the analytical strategies to screen for both, endogenous and exogenous AAS have continuously improved. These two types of AAS have important features and quite different issues with regards to the detection and so are dealt separately. For endogenous AAS, several experiments have to be carried out to

establish the exogenous origin of an endogenous steroid. Nevertheless, the target compounds of this thesis are the exogenous AAS. Importantly, to screen for exogenous AAS, quantification is not needed and identification of the parent compound and/or its metabolites in the urine samples is sufficient to demonstrate the administration of the drug [10]. For some of the steroids, more than one metabolite is used as makers to cover different excretion times (see section 1.4).

The analyses for the detection of AAS misuse are commonly performed in urine. Nevertheless, urine is a complex matrix that contains an enormous number of heterogeneous substances which can interfere with the analysis and hamper the detection of the desired analytes often present in traces. For this type of matrix, the coupling of powerful chromatographic techniques, such as GC or LC that can adequately separate the measured compounds from the matrix interferences, coupled with sensitive detectors, such as those of MS or MS/MS is necessary (hyphenated techniques). In fact, the use of MS based approaches, when suitable, are compulsory for doping control analysis.

The coupling of these chromatographic techniques with MS can also provide several benefits in terms of selectivity, sensitivity and identification power of the analysis. For example, when GC-MS(/MS) or LC-MS/MS are used, the baseline separation is not normally necessary due to the selectivity offered by the MS. Only for those analytes sharing the same mass and fragmentation, i.e. stereoisomers, a chromatographic separation would be fundamental.

This selectivity, based on the unique ionization or fragmentation pattern of the analytes under specific mass spectrometric conditions, is one of greatest benefits of the MS over other types of molecular detection.

The development of GC-MS(/MS) or LC-MS(/MS) methods for the detection of AAS is focused on: i) the sample preparation and ii) the analytical methodology to separate and detect the target analytes. The following subsections will cover these steps.

1.3.1 Sample preparation

The conventional routine analysis of AAS in doping control laboratories is performed by the indirect measurement of free and glucuronoconjugated metabolites of the AAS in urine samples. The generic sample preparation consists of an enzymatic hydrolysis of the urine samples and extraction based on liquid-liquid extraction (LLE) or solid-phase extraction (SPE) (Figure 1.12).

Figure 1.12: Sample preparation procedure for urine samples.

The initial step includes the hydrolysis of conjugated substances to release the respective phase I metabolite and enable its extraction from the urine sample (and detection by GC-MS) [65]. Since the hydrolysis is performed with β-glucuronidase derived from *Escherichia coli* (*E. coli*), the main focus is on the glucuronide conjugated fraction. This step presents some limitations because it

has been shown that the percentage of hydrolysis of each glucuronide may depend on various factors such as the affinity of the β-glucuronidase for the glucuronide [65-67], as well as the incubation time, temperature and the pH conditions [65,66]. Also the differences between different β-glucuronidase preparations [68-70] or the presence of enzyme inhibitors in the urine matrix [66] may play an important role on the efficiency of the enzymatic hydrolysis. Apart from that, some AAS glucuronides are totally resistant to enzymatic hydrolysis (e.g. eSTAN-*N*-G) [56].

In some cases extracts from *Helix pomatia* (*H. pomatia*), which contain both β-glucuronidase and arylsulfatase enzymes are used to cleave the sulfate conjugates. However, some sulfate metabolites are not efficiently hydrolyzed and problems associated with the production of artifacts and conversion between steroids have been described for *H. pomatia* extracts [71], which have made the use of *E. coli* mandatory for endogenous steroids. The alternative solvolysis (e.g. methanolysis), although more efficient, might lead to the degradation of certain substances [71,72].

Thereafter, extraction of the AAS from the urine matrix is generally performed by LLE using a non-polar organic solvent such as diethylether or *tert*-butyl methyl ether. The neutral AAS are favorable partitioned in this organic solvent. After separation of the organic phase, the solvent is evaporated.

The dry extracts are then reconstituted (in water/methanol or water/acetonitrile) in case LC-MS/MS analysis is performed. For

GC-MS(/MS) analysis a derivatization step is needed to improve the chromatographic properties, volatility and thermostability of the substances. A common applied derivatization is based on trimethylsiliyation of AAS [73-75]. To convert both hydroxyl and keto functions of AAS to their trimethylsilyl (TMS) ether and enol derivatives respectively, a derivatization mixture consisting of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), ethanethiol and ammonium iodide (NH_4I) is used. Derivatization of hydroxyl functions can be performed by adding MSTFA. However, for derivatization of the more stable keto functions a catalyst such as iodotrimethylsilane (TMSI) is required. Since NH_4I is more stable than TMSI, TMSI is generated *in situ* by the reaction of MSTFA and NH_4I. To inhibit the formation of iodine, an anti-oxidant such as ethanethiol is added to the derivatization mixture. This derivatization step requires anhydrous conditions to assure complete efficiency since both reagents and the formed derivatives are sensitive to water [7,76].

Apart from the aforementioned limitations of the hydrolysis step, the derivatization process may result in more than one reaction product for some steroids [77] or in chemical rearrangement of others [78]. All these variables can have a great impact on the analysis and might lead to the underestimation of the real urinary concentration of different glucuronides. Moreover, this procedure has failed to detect some AAS metabolites due to derivatization problems (e.g. trenbolone, its metabolites and related steroids) [77]. It is for this reason that LC-MS technology was also implemented.

To overcome these limitations, some methods using direct injection or dilute-and-shoot procedures combined with LC-MS analysis have been developed [56,79-82]. The driving forces behind this trend are the improved turnaround times and the substantially reduction in sample preparation time and reagents costs. However, the main drawback is the limited sensitivity due to the absence of a pre-concentration step. Taking into account the progressive increase in instruments sensitivity, it is expected that its application will be possible for substances with low required detection limits (such as the AAS) or limited ionization efficiency in a near future.

1.3.2 Instrumental analysis

The introduction of GC-MS for detection of AAS and their metabolites in urine was an important progress for the doping control. As a matter of fact, GC-MS(/MS) methods have been the gold standard methods for the last decades and they are used in all doping control laboratories [73,74,83]. However, due to certain limitations presented by this technique, LC-MS/MS has grown in importance [84].

Nowadays, GC-MS(/MS) and LC-MS/MS are the technologies of choice for the detection of AAS in doping control laboratories. LC-MS/MS offers the benefit over GC-MS(/MS) that sample preparation can be reduced [76] (Table 1.3). Indeed, GC-MS(/MS) often requires additional steps during sample preparation, including hydrolysis to detect the conjugate metabolites and derivatization of compounds to improve their chromatographic properties [7].

Moreover, some compounds are thermolabile, non-volatile and have marginal GC chromatographic properties (e.g. STAN), even after derivatization [71,72,85-87]. For these compounds, LC-MS/MS analysis can be applied, which does not require derivatization and enables the direct analysis of phase II metabolites [76].

Table 1.3: Overview of GC-MS and LC-MS techniques and their advantages and disadvantages for the screening of AAS.

Parameter	GC-MS	GC-MS/MS	LC-MS/MS	LC-HRMS
Analytes	**Volatile, thermally stable, non-polar, small compounds** ✓ Detection of saturated AAS feasible (e.g. androstanediols) ✗ Improve GC properties: derivatization ✗ Hydrolysis (deconjugation) needed		**Non-volatile, thermally unstable, polar, small/large compounds** ✓ Direct detection of AAS conjugates ✓ Detection of AAS such as trenbolone	
Sample preparation	**Hydrolysis & LLE/SPE & derivatization**		**Hydrolysis & LLE/SPE** **LLE/SPE** ✓ dilute-and-shoot is possible	
Column	**Apolar**		**Apolar**	
Mobile phase	**Gas** (He, H_2,..)		**Polar solvent** (H_2O, MeOH, acetonitrile)	
Ionization	**EI** ✓ Fragmentation: additional structural information ✗ Less abundant molecular ion	**EI** (*see GC-MS*) **CI** ✓ More abundant molecular ion ✗ Less structural information	**ESI** ✓ Abundant molecular ion ✗ Less structural information ✗ Ionizable groups needed ✗ More prone to matrix effects	
Screening AAS	**SIM** ✓ More sensitive compared to full scan ✗ Targeted detection	**MRM** ✓ More sensitive compared to SIM ✗ Targeted detection	**MRM** ✓ More sensitive compared to SIM ✗ Targeted detection	**Full scan** ✓ Retrospective data analysis feasible
Identification analytes	**RT, characteristic *m/z***	**RT, characteristic ion transitions**	**RT, characteristic ion transitions**	**RT, exact mass, fragmentation**
Resolution	**1 amu**	**1 amu**	**1 amu**	**0,001-0,0001 amu** ✓ reduced background
Cost	**€**	**€€**	**€€€**	**€€€-€€€€**

Other benefits of the methods based on LC-MS/MS instrumentation are generally associated to increased sensitivity, shorter analytical

times and simpler sample preparation procedures than GC-MS/(MS) [72,85,86]. If dilute-and-shoot methods or methods consisting of a simple extraction are used, problems related to hydrolysis are also circumvented. However, GC-MS remains a valuable technique especially for saturated AAS (e.g. 5α-/5β-androstane-3α,17β-diols), which present ionization difficulties by LC-MS.

LC-MS/MS and GC-MS(/MS) instruments have a similar configuration consisting of a chromatograph, ion source and detector (MS). During the chromatography, the separation of the analytes is achieved due to differential interactions with the stationary phase and the mobile phase. Therefore the analytes elute at different retention times. For GC and LC screening of AAS, apolar columns are generally applied. These GC columns are capillary columns based on fused silica (such as dimethylpolysiloxane (e.g. J&W Ultra 1 and HP-1MS)). Since the GC column is located in an oven, a temperature program can be applied to optimize the separation of the compounds.

LC-MS/MS methods for the detection of AAS are based on C18 or C8 columns and a mobile phase generally containing water and methanol (MeOH) or, water and acetonitrile (ACN). The application of an apolar column with a polar solvent for LC separation is called “reversed phase” LC. Several mobile phase modifiers can be added to the mobile phase (e.g. formic acid or acetic acid and ammonium formate or ammonium acetate) to optimize the chromatographic behavior and ionization. To ensure

the separation of the compounds, the chromatographic run is operated in gradient mode (the content of organic solvent is modified within a predefined range of time) or isocratic mode (the content of organic solvent is constant throughout the whole chromatographic run). Ultra-high performance liquid chromatography (UHPLC) which permits separations in a relative short analysis time thanks to the small particle and pore size of the stationary phase are also commonly used [88].

After the chromatographic separation of the analytes, they are ionized in the ion source of the mass spectrometer. For GC-MS(/MS), electron ionization (EI) and chemical ionization (CI) are often applied, whereas electrospray ionization (ESI) is commonly used for LC-MS/MS instruments. EI is rather a hard ionization technique, resulting in additional fragmentation of the compounds. This has the advantage that additional structural information can be obtained in the corresponding EI mass spectrum. However, the disadvantage of this "destructive" ionization technique is that less abundant molecular ions can be observed. CI and ESI are soft ionization techniques which advantageously lead to less fragmentation and more abundant molecular ions, but consequently provide less structural information. A more abundant molecular ion is beneficial for the development of selected reaction monitoring (SRM) methods as more diagnostic precursor ions (high mass to charge ratio (*m/z*) at relative high abundance) can be selected. During ESI competitive ionization can occur, which makes the technique more prone to matrix effects (ion enhancement/suppression). Therefore, the determination of matrix

effects is important in LC-MS/MS method validation and the use of stable isotope labeled internal standards is recommended for quantitative LC-MS/MS methods. For the efficient ionization of AAS by ESI and thus LC-MS/MS analysis, ionizable groups such as a conjugated carbonyl function or heteroatoms are required. Consequently, the detection of saturated AAS such as 5α-/5β-androstane-3α,17β-diols is impeded by LC-MS/MS analysis. For the detection of these compounds, GC-MS(/MS) analysis is required.

The ionized analytes are separated in the mass analyzer depending on their *m/z* using an electromagnetic field. At present, several types of mass analyzers of low (quadrupoles (single/triple) and ion traps) or high resolution (time-of-flight, orbitrap, magnetic sectors, and Fourier transform ion-cyclotron resonance) are available. The characteristics of the mass spectrometer (sensitivity, accurate mass, resolution) basically depend on the mass analyzer used. Although the aim of this section is not to discuss in depth all the available analyzers, as several books can be consulted [89], a brief description of the single/triple quadrupole will be made, as it was the main analyzer used in this thesis.

The quadrupole consists of four parallel rods. Each opposing rod pair is electrically connected and a radio frequency voltage (V_{RF}) is applied between the pairs of rods. A positive/negative direct current voltage (V_{DC}) is then superimposed on the V_{RF}. Ions entering the quadrupole undergo oscillating movements between the rods due to these V_{RF} and V_{DC} electric fields. For a given ratio of voltages, only

those ions of a certain *m/z* are stabilized and therefore will pass the quadrupoles and eventually reach the detector. Ions with other *m/z* have unstable trajectories and will collide with the rods (non-resonant ions). Triple quadrupole MS instruments (QqQ) consist of a linear series of three quadrupoles (Figure 1.13).

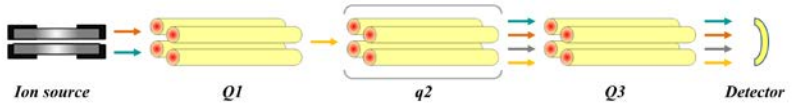

Figure 1.13: Scheme for a triple quadrupole; *Q1*: 1st quadrupole, *q2*: collision cell and *Q3*: 3rd quadrupole.

Q1 (1st quadrupole) and *Q3* (3rd quadrupole) are conventional quadrupoles which act as mass filters whereas the *q2* is employed as a collision cell. *Q1* can select an ion with particular *m/z* or operate in full scan mode. The *q2* is an RF-only quadrupole (non-mass filtering) which uses an inert gas, such as argon, helium, or nitrogen, for collision induced dissociation (CID) of the ions selected in the *Q1*. The subsequent fragments of the ions are passed through to *Q3* where they may be filtered or fully scanned depending on the acquisition mode used (Figure 1.14).

When using GC-(EI)MS, the molecular ion and several fragment ions are observed in the full scan mass spectra of AAS. It should be taken into account that for GC-MS the compounds are derivatized with TMS prior to GC-MS analysis. For example, if both the 3-keto and the 17-hydroxy groups of testosterone are derivatized with TMS a molecular ion of *m/z* 432 (288+(2x72)) will be observed. Besides the molecular ion, the fragment ion *m/z* 73, which

represents the trimethylsilyl radical, is commonly observed in the mass spectra of TMS derivatives. Losses of methyl radicals ([M-15]$^{+}$) and TMSOH ([M-90]$^{+}$) groups are also frequently observed in the GC-MS mass spectra of AAS.

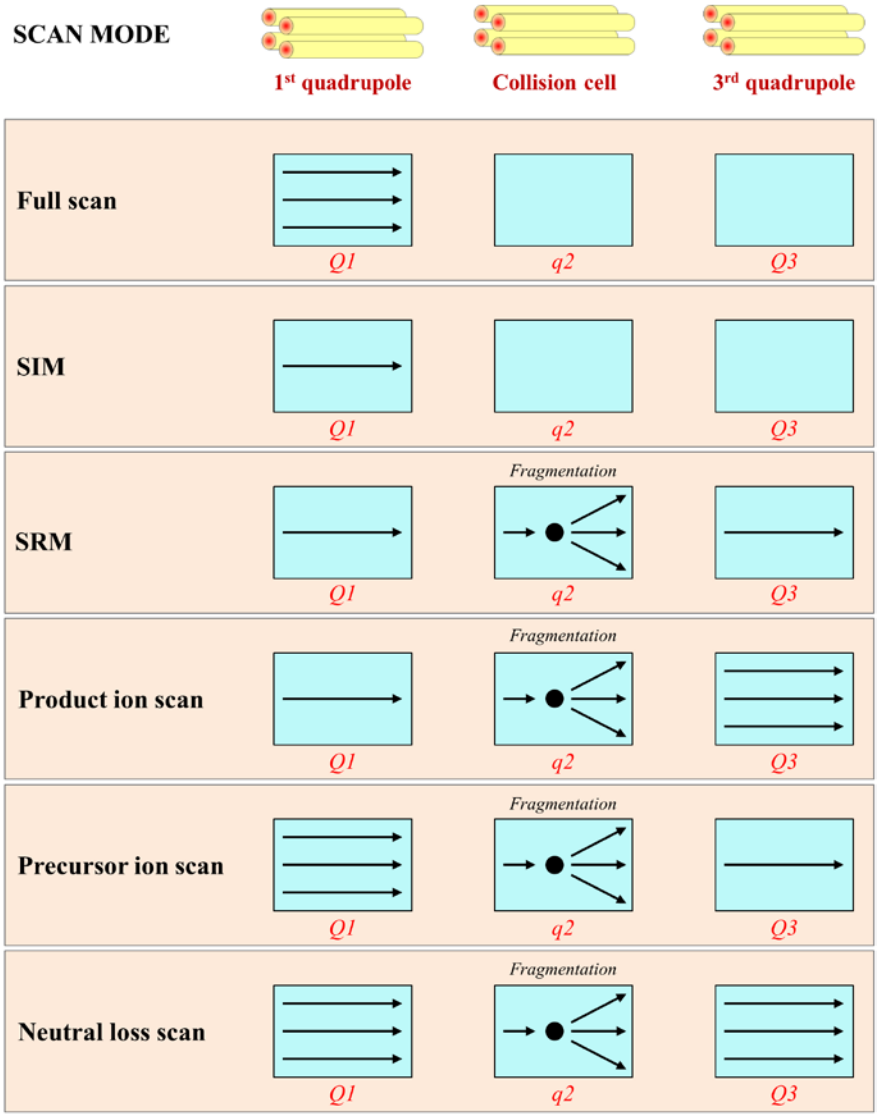

Figure 1.14: Acquisition modes available for a triple quadrupole.

In the LC-(ESI)MS full scan spectra of AAS protonated $[M+H]^+$ and deprotonated $[M-H]^-$ molecules can be observed depending on the ionization mode that is applied. For molecules exhibiting a high proton affinity (basic molecules), $[M+H]^+$ will be the most abundant specie while for molecules which can transfer a proton to the solvent (acidic molecules), $[M-H]^-$ will be more abundant. Other species that can be observed include sodium ($[M+Na]^+$), potassium ($[M+K]^+$), acetate ($[M+OAc]^-$) and ammonium adducts ($[M+NH_4]^+$).

In order to fulfil the higher demands regarding sensitivity, selectivity and sample throughput, MS techniques have experienced an evolution. Initially, by the use of classical MS, full scan analyses evolved to the use of more selective selected ion monitoring (SIM) mode (Figure 1.14). Subsequently, detection limits were further improved by the implementation of tandem MS (MS/MS). This MS/MS analysis involves CID of compounds which results in characteristic MS/MS spectra. With these MS/MS instruments product ion scan mass spectra or selected reaction monitoring (SRM) can be applied (Figure 1.14). Although these improved MS techniques lead to an efficient detection of a range of known compounds, a drawback from the high selectivity is that it does not allow the detection of non-targeted or unknown compounds. These targeted methods require information about compounds and their metabolites, subsequent study of their MS behavior and selection of the most appropriate ions and its retention time.

The application of liquid chromatography-high resolution mass spectrometry instruments (LC-HRMS) can lead to a reduction of background noise by measuring the specific ions with accurate mass. This in turn enables the detection of quite low levels of analytes and requires less sample clean up. Evaluation of HRMS data requires a post-acquisition processing method to select the target substances. However, an advantage of the use of HRMS instruments is that they allow retrospective data analysis as data processing is a post-acquisition rather than a pre-acquisition process. Eventually, the full scan HRMS data can also be combined with fragmentation data to obtain more structure information of unknown compounds.

Taking into account the drawbacks related to targeted screening methods, other strategies have been developed to enable the detection of unknown doping substances. These strategies were applied to AAS and include, among others, open screening strategies. The open screening strategies are based on the finding that related steroids have similar MS/MS fragmentation patters which give rise to common ion fragments [90,91]. An approach for the detection and characterization of unknown AAS has been developed [92].

1.4 Metabolic studies of AAS in doping control

Metabolic studies are essential to determine the best markers after administration of a drug. The study and re-evaluation of the metabolism is a constant ongoing research in the doping control

field and the aim is to identify as many metabolites as possible and to select the most suitable ones. Afterwards, these markers must be included into screening methods in order to ensure their major effectiveness.

As previously stated, AAS are extensively metabolized (section 1.2.2). Since they are substances employed during training periods with long lasting effects, the most effective way to detect their misuse is to perform comprehensive and in-depth metabolic studies and to select the most adequate markers to improve the retrospectivity of the detection. The most suitable metabolite is not always the most abundant metabolite but the metabolite detectable for the longest period of time after administration, the so called long-term metabolite (Figure 1.15). For some AAS, minor metabolites (from a quantitative point of view) might help expand the detection time in comparison with major metabolites.

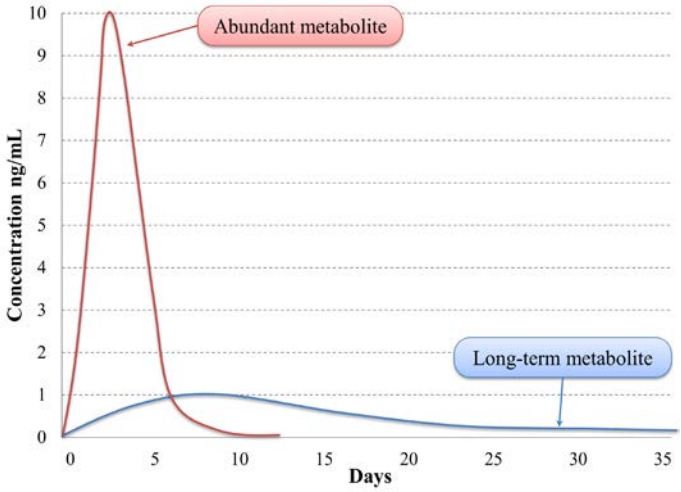

Figure 1.15: Scheme of a compound excretion profile.

1.4.1 Evaluation of AAS metabolism

The vast knowledge of AAS metabolism has been acquired through the decades and it is still a work in progress. Metabolic studies have been traditionally performed by GC-MS using an indirect approach (Figure 1.16A). Later, a new era on the identification of steroid metabolites started with the introduction of LC-MS(/MS) instruments. Since LC-MS(/MS) can be applied to the analysis of unvolatile compounds, it do not require the derivatization of steroids, thus allowing for the detection of steroid phase I metabolites which are not easy to derivatize such as polyhydroxylated metabolites (Figure 1.16A).

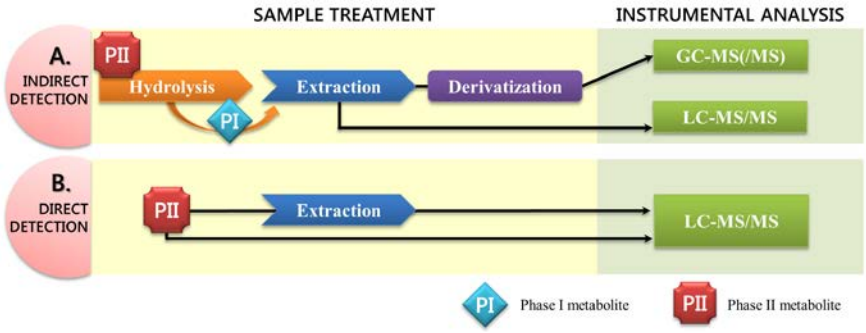

Figure 1.16: Indirect and direct detection approaches used for the evaluation of AAS metabolism.

LC-MS(/MS) offers another great possibility; the direct detection of phase II metabolites (e.g. glucuronide, sulfates, etc.) in the exact same form as they are present in urine (Figure 1.16 B). This approach avoids hydrolysis and (sometimes) extraction and derivatization steps. Furthermore, it allows the development of open methods [neutral loss (NL) and precursor ion (PrecIS) scan methods

(Figure 1.14)] for the detection of metabolites having a common chemical structure [92].

In this section, different approaches for the evaluation of phase I metabolism are reviewed, and later, the study of phase II metabolism is also discussed.

1.4.1.1 Evaluation of phase I metabolism

For the detection of phase I metabolites, several parameters in sample treatment and instrumental analysis have to be considered since the results dramatically depend on the suitability of these strategies.

Sample treatment

The study of phase I metabolites is generally performed after hydrolyzing the urinary phase II metabolites. Hydrolysis procedures are discussed in section 1.4.1.2 (below). Following hydrolysis, phase I steroids have to be isolated from the rest of the complex matrix. In metabolic studies, this extraction step is crucial due to the wide range of polarities of phase I metabolites [31]. Ideally, all metabolites should be extracted while minimizing the co-extraction of interfering substances. The most frequently employed strategies are LLE with *tert*-buthylmethyl ether and SPE with C18 cartridges [93]. When using LLE, alkaline conditions are recommended since interferences are reduced at high pH (9-10) whereas when employing SPE, interferences are usually concentrated. However, the wide variety of solid-phase packing material provides additional benefits for the study of specific AAS [94]. Other options to

improve selectivity are based on solid-phase micro-extraction (SPME) [95] and the combination of several SPE and LLE [96].

As previously mentioned, AAS metabolites must be derivatized to become amendable for GC-MS analysis. Apart from the common protocol (with MSTFA) [97], other derivatization procedures are also useful to elucidate the structure of the metabolites [98]. For LC-MS/MS analysis, derivatization is unnecessary, mainly if AAS have ionizable groups in their structure (e.g. conjugated carbonyl group). However, derivatization can convert poorly-ionizable metabolites into easily-detectable analytes, which in turn increases the overall sensitivity. Also, like in GC-MS, the formation of specific derivatives for each functional group provides valuable structural information [99].

Studies using GC-MS analysis

Knowledge about the MS behavior of AAS phase I metabolites using GC-MS is an essential tool for the identification of new metabolites, as it allows for both; the prediction of characteristic ions of theoretical metabolites and the proposal of potentials structures for unknown metabolites.

The detection of AAS by GC-MS(/MS) after EI and the determination of the fragmentation rules have been extensively investigated for more than 50 years [75,93,100]. Softer ionization methods in GC-MS(/MS) instruments have been successfully tested for the target detection of some AAS [101,102].

Most of the metabolic studies are conducted comparing urine samples collected before and after the administration of the drug to healthy volunteers. Only peaks detected in post-administration samples are considered AAS metabolites if their MS behavior is consistent with an AAS-metabolite structure.

GC-EI-MS analysis allows for the application of several strategies. One possibility is to evaluate the peaks appearing only in the full-scan chromatogram of post-administration urine samples [50,103]. This approach has the main advantage of detecting metabolites generated by unforeseen metabolic biotransformations. However, the relative poor sensitivity of the scanning mode can prevent the detection of minor metabolites. The common procedure is to evaluate extracted ion chromatograms (EICs) corresponding to common ions of AAs or to perform targeted analysis using SIM mode in GC-MS or SRM mode in GC-MS/MS instruments [104]. The use of this approach significantly improves the sensitivity, but it requires an *ex ante* prediction of the metabolism and precludes the determination of unexpected metabolites. Other approaches such as the synthesis of potential metabolites or the prediction of metabolism based on principal-component analysis (PCA) have also been employed [105].

It is expected that, analogously to what is happening in LC-MS/MS (see below), the use of GC coupled to QqQ will add strategies based on PrecIS and NL scan methods in the near future. In addition, HRMS instruments are also an alternative for the detection of AAS

metabolites. Also, accurate masses obtained using these instruments can provide valuable information in metabolic studies.

Studies using LC-MS analysis

Ionization is probably the bottleneck for the AAS LC-MS(/MS) detection. Several AAS exhibit poor ionization due to the low proton affinity of the chemical functions (normally hydroxyl, alkenes and/or keto functions). Only AAS containing a conjugated keto function (e.g. testosterone) or nitrogen (e.g. STAN) shown an abundant $[M+H]^+$ ion in ESI [86]. The formation of adducts is necessary for the ionization of AAS without these functions. Adducts {$[M+NH_4]^+$, $[M+Na]^+$, $[M+Ag]^+$, $[M+H+CH_3OH]^+$, $[M+F]^-$, $[M+HCOO]^-$} have been described for the ionization of AAS [86,106,107].

The CID behavior of AAS has been studied, dividing the AAS into groups based on chemical similarities [108,109]. Thus, several ions or losses are common to specific chemical features. At high collision energy, most AAS show thee common ions at *m/z* 77, 91 and 105 [90].

Similarly to GC-MS, evaluation of full scan chromatograms with nominal single mass analyzers (single quadrupole or ion trap) could be used. However, the low sensitivity and selectivity of this approach limits its use for the detection of only the main metabolites. The use of different analyzers (e.g. TOF, Orbitrap or QqQ) common in LC-MS(/MS) analysis provides alternative strategies for the open detection of unknown metabolites [110].

The use of HRMS improves the detectability of unknown metabolites with strategies based on accurate mass measurements. These strategies usually compare full acquisition chromatograms obtained for a pre- and post-administration sample. The large number of peaks in the compared chromatograms can be processed by; i) extracting the expected ions for each theoretical metabolite with a narrow *m/z* mass window [111,112] or ii) using a dedicated software for the identification of potential metabolites. The extraction of the pre-defined *m/z* has the inherent limitation of not detecting unexpected metabolites. On the contrary, the main advantage of the second option is that every single peak is evaluated as a potential biomarker for the administration of AAS. The main limitation is that the identified biomarker not necessarily needs to be associated with the compound [113].

The great versatility of QqQ instruments allows the development of several scan modes, including PrecIS and NL. Due to the presence of common ions and neutral losses depending on the AAS structure, the setting up of methods based on these modes of operation provides alternatives tools for the study of the metabolism. Thus several methods have been proposed for the detection of AAS depending on the structure [90,109] and their application to urine samples allowed for the discovery of unreported metabolites [52,114-117]. This approach shows a relative high sensitivity and specificity, which makes the determination of differences between negative and positive samples relatively simple. However, part of the AAS structure is normally predefined and therefore, metabolites without that part will not be detected by these methods.

1.4.1.2 Evaluation of phase II metabolism

Phase II metabolic reactions have been normally studied using the indirect approach (with hydrolysis step) (Figure 1.16A). In addition to the factors discussed above, the selection of the hydrolysis conditions is one of the key steps in these studies, so it is treated separately in this section. The other possibility to study phase II metabolism is by performing direct detection of the conjugates (Figure 1.16B). The LC-MS(/MS)-based strategies used are reviewed.

Indirect determination (hydrolysis step)

Several types of phase II conjugates have been described for AAS but only the hydrolysis of glucuronides and sulfates will be considered in this subsection.

Glucuronides are commonly hydrolyzed with β-glucuronidase preparations from various sources [71]. Other hydrolysis based on chemical approaches have also been reported {e.g. use of sodium periodate [118] and acidic hydrolysis [119]}. Among them, hydrolysis with β-glucuronidase from *E. coli* is considered the gold standard [66]. Besides having better efficiency, the enzymatic hydrolysis is also preferred because it avoids undesired effects shown by chemical hydrolysis. However, as it was previously discussed, efficiency may be influenced by several factors and some AAS glucuronides have shown to be partially or totally resistant to enzymatic hydrolysis [56,81].

In the same way, the hydrolysis of sulfate conjugates is also performed by using enzymatic or chemical methods. Different enzymes with sulfatase activity have been reported [71], the preparations from *Patella vulgate* (*P. vulgate)* and *H. pomatia* with both β-glucuronidase and arylsulfatase activities are the most commonly used [72]. Depending on the position of the sulfate moiety and the α/β configuration, some sulfates show partial or total resistance to enzymatic hydrolysis [71,72]. Also, the use of *H. pomatia* can lead to steroid conversion, degradation and artifact formation.

Chemical hydrolysis is achieved by using hot acidic conditions (hydrochloric or sulfuric) and the efficiency is influenced by temperature, time of reaction and acid selected [71]. For most of the steroid sulfates, solvolysis efficiencies are over 60 % [120]. Thus a wide range of sulfoconjugates are hydrolyzed compared to enzymatic hydrolysis. Other chemical hydrolyses include the use of trimethychlorosilane for the hydrolysis of a wide range of steroid androgens [71,121] and methanolysis [72,121]. In summary, unlike glucuronide, there is no universal method for the cleavage of all steroid sulfates.

Once the hydrolysis is completed, extraction and detection of the released steroids is performed as described in the previous section (Section 1.4.1.1).

Direct analysis of phase II metabolites

One of the critical steps for the direct detection of phase II metabolites in urine is their separation from the rest of polar compounds present in the urinary matrix. Besides the clean-up, one of the important goals is to pre-concentrate the analytes in order to reach the required sensitivity. Different treatment approaches have been described for the target detection of the preselected glucuronide {e.g. LLE [122], SPE [123,124] or liquid-phase microextraction (LPME) [122]}. Similar to glucuronides, several strategies have been described to isolate sulfoconjugates from the polar matrix components. Due to their anionic character, sulfates can be retained together with glucuronides in quaternary ammonium SPE cartridges [125]. The steroidal part reduces the total polarity of the metabolite, so steroid sulfates can also be retained in common C18 cartridges [72] or they can be extracted using polar organic solvents (e.g. ethyl acetate) [126,127].

Regarding the ions formed in positive ESI mode, glucuronides show ionization behavior similar to free steroids. Hence, analytes with a conjugated 3-keto function exhibit the $[M+H]^+$ ion [81,122,123], whereas those lacking this feature are preferentially ionize by adduct formation [81,122]. Because adduct formation takes place in the glucuronide moiety even glucuronides without an ionizable center can be detected. In negative mode, besides other minor adducts, all steroid glucuronides exhibit the $[M-H]^-$ ion [81,124].

Depending on the structure of the steroid glucuronide, several common product ions and NL are observed in the CID analysis in

both modes. Since these conjugates are mainly ionized in the glucuronide moiety, all common ions (*m/z* 177, 159, 141 in positive-ionization mode and *m/z* 75, 85 and 113 in negative-ionization mode) relate to the glucuronide. A similar situation is observed with the common NL. Also, in positive mode, the product ion spectra of AAS glucuronides exhibit the ions observed in the CID analysis of the free steroid, which can be helpful for structural elucidation.

Steroid sulfates are almost exclusively ionized in negative mode as $[M-H]^-$ [127] (Figure 1.17). Only those sulfates containing a Δ^4-^{3}CO structure can be also ionized as $[M+H]^+$. Formation of characteristic adducts in each ionization mode has also been observed [120] (Figure 1.17).

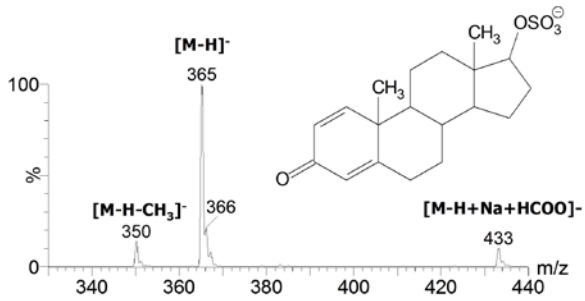

Figure 1.17: MS Scan of boldenone sulfate standard in ESI negative mode.

Generally the CID spectra of the $[M-H]^-$ ion of steroid sulfates show only one abundant product ion at *m/z* 97, corresponding to the HSO_4^- [35,126,128]. For some steroids, specific NLs has been observed (e.g., the NL of a methyl group for boldenone sulfate [127]) (Figure 1.18). In positive mode, the most common fragments

of steroid sulfates are the NL of 80 Da (loss of sulfur trioxide group) and subsequent losses of water from the steroid nucleus [72,120,127,129]. After the loss of the sulfate group, CID fragmentation is the same as for the free steroid.

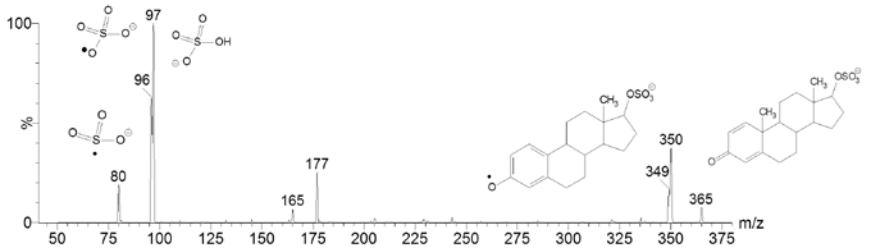

Figure 1.18: Product ion mass spectra of boldenone sulfate in ESI negative mode at 40 eV.

As it has been observed, phase II metabolites have predictable MS behavior, depending on the conjugate and the AAS structure. Thus, the most abundant species in the ionization of a potential metabolite can be predicted based on its structure. This approach can be used for the detection of unknown AAS metabolites when using MS instruments with accurate-mass measurements capabilities (HRMS).

Due to the common fragmentation behavior of the phase II metabolites of AAS, two alternatives strategies can be designed for the detection of unknown phase II metabolites with QqQ instruments: (a) PrecIS and NL scan methods; and (b) targeted methods for the detection of predicted metabolites.

The occurrence of common ions or losses allows the development of PrecIS and NL for QqQ instruments. Thus, PrecIS of *m/z* 113,85 and 75 in negative mode and *m/z* 177, 159 and 141in positive mode

can be used for the direct detection of glucuronides. Also, NL of 176, 194, 211 and 229 Da are also suitable for this task. Only a few methods have been reported using this approach [81,130]. Similarly to glucuronides, sulfates can also find useful open scan methods based on NL and/or PrecIS. In positive mode, a NL scan method can be based on losses of 80 Da and 98 Da. However, this approach is limited to steroid sulfates that are ionizable in positive mode. In negative mode, a PrecIS scan method of *m/z* 97 can be used. This approach showed to be useful in the identification of new biomarkers [130].

Furthermore, theoretical transitions for potential unknown metabolites can also be predicted. Taking into account the common fragmentation of the different conjugates and the expected metabolism, it is straightforward to hypothesize a theoretical transition for every potential metabolite. The application of this strategy resulted in the identification of sulfate metabolites of several AAS [126,127].

1.4.2 New AAS metabolites: from detection to synthesis

The process to study steroid metabolism for doping control purposes would include:

i) detection of new metabolites (Section 1.4.2.1)
ii) the study of the excretion profile of each metabolite to evaluate the excretion time (Section 1.4.2.2), and

iii) confirmation of the postulated structure by synthesis of the authentic material (Section 1.4.2.3). The comparison of the new metabolite with the reference standards is the ultimate goal when identifying new metabolites.

1.4.2.1 Detection of new metabolites

The detection of a new metabolite can be targeted or untargeted (Figure 1.19).

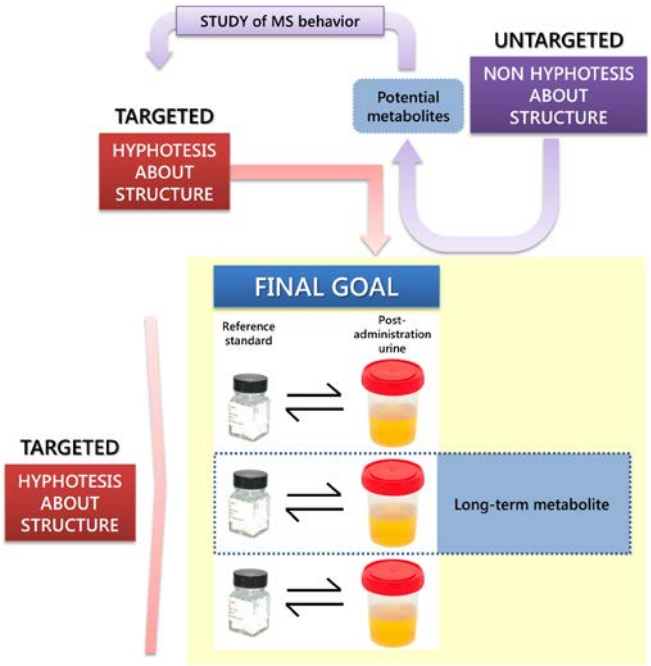

Figure 1.19: Identification of new metabolites considering targeted and untargeted approaches.

On the one hand, by targeted, one refers to those investigations in which the researchers have an *ex ante* hypothesis about the structure of the metabolite. Having the putative structure in mind, one can

develop analytical strategies to identify new metabolites in post-administration urines or; alternatively, use authentic reference materials to elaborate a specific detection method (if they are commercially available or have been synthetized). Many examples of the application of this approach in the doping control field can be found in the literature [131-135]. On the other hand, some research lines try to identify new metabolites without having *a priori* hypothesis about the structure, those are the untargeted which are far more complex and include, in their last step the use of a targeted approach for the final confirmation of the new metabolite.

The elucidation of the metabolism can be performed by using *in vivo* or *in vitro* approaches [136]. Both approaches have advantages and limitations (Table 1.4). Thus, whereas the use of *in vivo* systems with human volunteers is the best option and allows for the detection of the actual metabolites generated by the human organism, it comes up against the ethical aspects regarding administration of AAS. On the other hand, higher concentrations can be used on the metabolic studies using either animal models or *in vitro* systems helping to the detection of AAS. An approach based on the use of a chimeric mouse with humanized liver for metabolic studies have been successfully validated [114] and applied for the determination of several AAS metabolites [52,137]. However, the main disadvantages of these procedures are the small volume of sample obtained and the impossibility to directly extrapolate the results to the function of the human organism.

Table 1.4: Overview of *in vitro* and *in vivo* models used for metabolism studies and their main advantages (+) and disadvantages (-).

Irrespective of both, the use of *in vitro* or *in vivo* experiments and the extraction strategy applied, metabolic studies are based on the comparison between a blank/pre-administration and a post-administration sample as it has been extensively discussed in Section 1.4.1.

1.4.2.2 Excretion samples and evaluation of excretion profiles

After the detection of new metabolites for an AAS, the detection windows (e.g. time that the metabolite is detected in urine) is evaluated by using excretion study samples of, ideally, individuals from different ethnics, different doses, different administration routes and long excretion periods with the aim of determining the most interesting metabolites for doing control purposes. Further, the detection times of the novel metabolites are compared with those of the commonly monitored metabolites to end up establishing the most retrospective metabolite.

1.4.2.3 Confirmation of the structure

After the identification and evaluation of the metabolites, confirmation of the structure of the most interesting metabolite should be performed. The structural information obtained by the

direct analysis of new conjugates is rather limited because most of the product ions are related to the conjugated moiety. For this reason, once the unknown phase II metabolite is detected, performing the hydrolysis of the conjugate and the ulterior analysis of the free steroid by either LC-MS/MS or GC-MS(/MS) is a common procedure for establishing the metabolite structure.

Although some information could be obtained after careful evaluation of the MS spectra obtained from both, the phase II metabolite and its corresponding phase I metabolites (after hydrolysis), the chemical synthesis of the potential metabolite is the ultimate confirmation of the structure of a new metabolite. The MS spectrum must be compared to the one obtained for a standard solution of a reference material in order to unequivocally confirm the suspected structure of the investigated metabolite.

After the synthesis of the reference material, a proper characterization is required. The structural information obtained by the MS techniques is sometimes not sufficient for the unequivocal determination of the standard's structure. Thus, the use of other techniques of molecular characterization is necessary. Currently, the nuclear magnetic resonance spectroscopy (NMR) is one of the most versatile and commonly used techniques for the structure determination of almost any organic molecule, as well as that of many inorganic molecules. Aside from the X-ray crystallography which can uncover the complete molecular structure of some crystalline materials, NMR is the most direct and general tool for

identifying the structure of both pure compounds and mixtures as either solids or liquids.

After the complete characterization of the reference material and comparison with the newly identified metabolite, the ultimate aim of these studies is to “big-scale” synthesize the metabolite for use as reference materials. The availability of reference material can be of great importance for many issues of the doping analysis. Among them, the use of well characterized reference material is essential for the confirmatory analysis for the presence of an already known metabolite, the accurate quantification of the desired analytes and others such as the research for novel metabolites.

In addition, the use of standards allows for the study of their fragmentation behavior under different spectrometric conditions. Thus, the analysis of a standard with the product ion scan mode at different collision energies can reveal the presence of ion transitions that are specific for this compound. The use of these specific ion transitions can have an important impact on the development of both quantitative and qualitative methods based on SRM, since the analyte can be distinguished from other isobaric compounds included in the matrix. The study of the fragmentation behavior of similar compounds can also be the basis for the development of PrecIS or NL strategies used for the identification of novel metabolites by untargeted approaches.

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2. JUSTIFICATION AND OBJECTIVES

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2.1 Justification

Due to the wide misuse of anabolic androgenic steroids (AAS) in sports, there is a continuous need to improve the detection capabilities of their administration. Most AAS are extensively metabolized and they are mainly excreted in urine as phase II metabolites. For many AAS most of the metabolic profile remains unknown.

Studies on phase II steroid metabolism have been traditionally performed using gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-tandem mass spectrometry (LC-MS/MS), after hydrolysis of samples to release the phase I metabolites. Since most studies used β-glucuronidase enzymes, mainly glucuronoconjugated metabolites hydrolyzable under these conditions and non-conjugated excreted metabolites have been systematically studied.

In recent years, new phase II metabolites have been identified for some AAS using direct analysis by LC-MS/MS and some of them have shown to be useful as long-term markers. Some of the identified metabolites are sulfoconjugates and glucuronoconjugated metabolites poorly or not hydrolyzable using β-glucuronidases. These results demonstrate the need to re-evaluate AAS metabolism to study phase II metabolites not systematically studied up to now,

to look for new long-term metabolites, and to include them into the routine screening procedures.

Current screening methods for AAS are based on the hydrolysis with β-glucuronidase, liquid-liquid extraction and analysis by GC-MS, with previous derivatization, and in the last years, also by LC-MS/MS for those metabolites with poor GC-MS behavior. With this procedure only unconjugated metabolites and hydrolyzable glucuronic acid conjugates are detectable. Moreover, it is known that the time required to complete the hydrolysis varies between the different steroid glucuronides and the hydrolysis may also be incomplete in particular urine matrices due to enzyme inhibition. As a consequence, some metabolites may be underestimated due to incomplete hydrolysis. Furthermore, the current procedure is time consuming and requires the combination of two technologies to ensure detection of all compounds: the need for derivatization before GC-MS analysis limits the analysis of some phase I metabolites that do not form suitable derivatives; and, other phase I metabolites cannot be detected using LC-MS/MS due to the lack of ionizable groups. In addition, other phase II metabolites useful as long term metabolites (e.g. sulfates) cannot be analyzed using the current approach. Therefore, significant information is missed in the current AAS screening methods.

A method allowing the direct detection of all types of metabolites would improve the detection capabilities of AAS by incorporating the new long-term metabolites not monitored in the current screening conditions. LC-MS/MS is the most suitable technology to

develop a screening method based on the direct analysis of all steroid conjugates. Moreover, in response to the need of anti-doping community to improve the detection capabilities of the administration of AAS, it would be of interest to continue with the systematic study of the fraction of metabolites excreted as sulfate conjugates of other AAS.

During this thesis we will try to evaluate the potential of a different strategy that could overcome the limitations of the conventional methods and provide additional data on AAS metabolism useful for doping control analysis.

2.2 Objectives

The **main objective** of this doctoral thesis was to improve the detection capabilities of exogenous anabolic androgenic steroids in sports drug testing.

In order to reach this general and broad goal, **two specific objectives** were established:

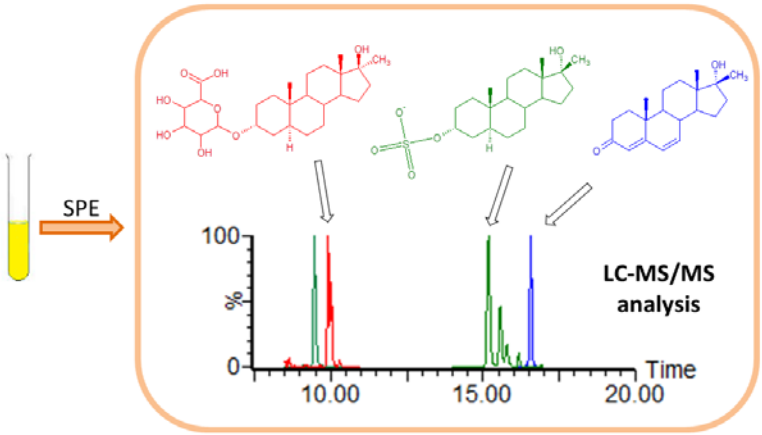
1. Development of an analytical methodology for the simultaneous detection of AAS phase I and phase II intact urinary metabolites using LC-MS/MS (PART I).

2. Study of the sulfate fraction of AAS in order to improve the retrospectivity of the detection of these compounds. Target analytes were Clostebol, 4-chlorometandienone and Stanozolol (PART II).

PART I

3. Screening for anabolic steroids in sports: Analytical strategy based on the detection of phase I and phase II intact urinary metabolites by liquid chromatography tandem mass spectrometry

3. SCREENING FOR ANABOLIC STEROIDS IN SPORTS: ANALYTICAL STRATEGY BASED ON THE DETECTION OF PHASE I AND PHASE II INTACT URINARY METABOLITES BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY



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G. Balcells, O.J. Pozo, A. Esquivel, A. Kotronoulas, J. Joglar, J. Segura, R. Ventura. Screening for anabolic steroids in sports: analytical strategy based on the detection of phase I and phase II intact urinary metabolites by liquid chromatography tandem mass spectrometry. *J Chromatogr A.* **2015**, 1389, 65.

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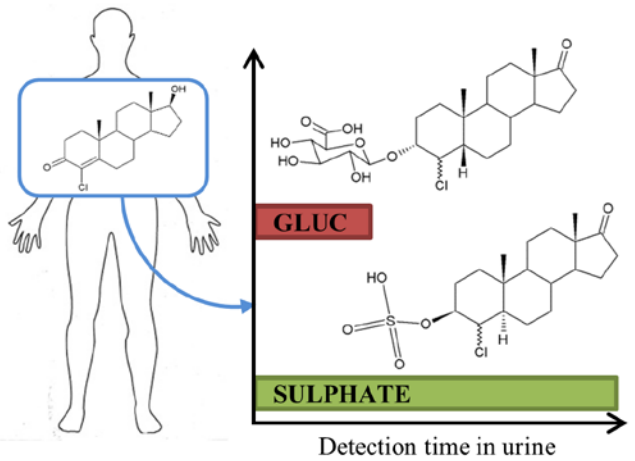
PART II

4. Detection and characterization of Clostebol sulfate metabolites in Caucasian population

5. Sulfate metabolites as alternative markers for the detection of 4-chlorometandienone misuse in doping control

6. Detection of Stanozolol *O*- and *N*-sulfate metabolites and their evaluation as additional markers in doping control

4. DETECTION AND CHARACTERIZATION OF CLOSTEBOL SULFATE METABOLITES IN CAUCASIAN POPULATION



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G. Balcells, O.J. Pozo, L. Garrostas, A. Esquivel, X. Matabosch, A. Kotronoulas, J. Joglar, R. Ventura. Detection and characterization of clostebol sulfate metabolites in Caucasian population. *J Chromatogr B.* **2016**, 1022, 54.

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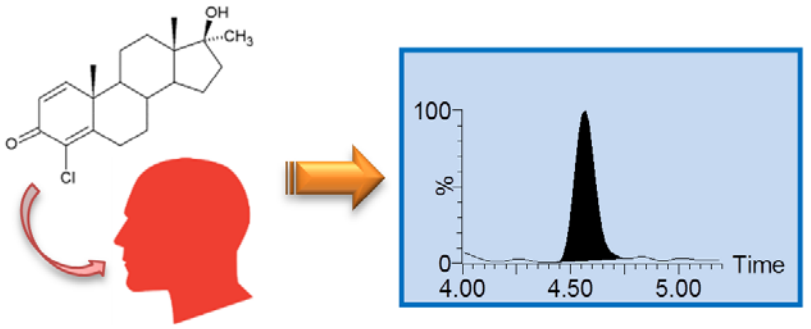
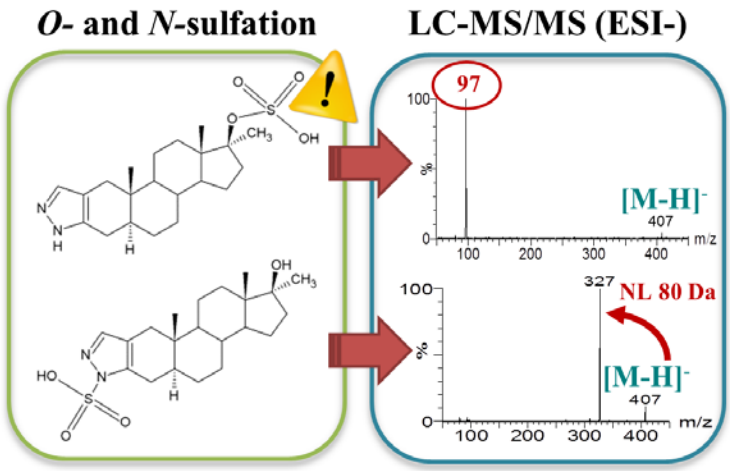
5. SULFATE METABOLITES AS ALTERNATIVE MARKERS FOR THE DETECTION OF 4-CHLOROMETANDIENONE MISUSE IN DOPING CONTROL



This capther has been adapted from:
G. Balcells, C. Gómez, L. Garrostas, O.J. Pozo, R. Ventura. Sulfate metabolites as alternative markers for the detection of 4-chlorometandienone misuse in doping control. *Drug Test Anal*. **2016**, doi: 10.1002/dta.2101.

Balcells G, Gómez C, Garrostas L, Pozo ÓJ, Ventura R. Sulfate metabolites as alternative markers for the detection of 4-chlorometandienone misuse in doping control. Drug Test Anal. 2016 Sep 30. doi: 10.1002/dta.2101.

6. DETECTION OF STANOZOLOL *O*- AND *N*-SULFATE METABOLITES AND THEIR EVALUATION AS ADDITIONAL MARKERS IN DOPING CONTROL



This capther has been adapted from:
G. Balcells, X. Matabosch, R. Ventura. Detection of stanozolol *O*- and *N*-sulfate metabolites and their evaluation as additional markers in doping control. **2016**, doi: 10.1002/dta.2107.

Balcells G, Matabosch X, Ventura R. Detection of stanozolol O- and N-sulfate metabolites and their evaluation as additional markers in doping control. Drug Test Anal. 2016 Oct 7. doi: 10.1002/dta.2107

7. DISCUSSION

7. DISCUSSION

7.1 General discussion

This thesis deals with two different aspects related to the improvement of the detection capabilities of exogenous AAS in sports drug testing. In the first place, an alternative methodology to screen for these substances, with a different perspective compared to the conventional screening procedures, was developed. In the second place, metabolic studies of three exogenous AAS, clostebol (CLO), 4-chlorometandienone (4Cl-MTD) and stanozolol (STAN) were performed in order to look for new long-term metabolites to include into the above mentioned methodology.

The alternative method, described in Chapter 3, is based on LC-MS/MS analysis. The method offers a number of advantages and it also presents some limitations. In comparison with the conventional screening methods, which are still used in all anti-doping laboratories [1,2], it establishes a new approach to achieve a less time consuming, more robust and more comprehensive detection of exogenous AAS.

The LC-MS/MS method allows for the intact detection of all phase II metabolites. Even if their corresponding phase I metabolites do not ionize, the glucuronide or sulfate moieties help in the ionization of the phase II metabolites. Therefore, many of the problems observed when monitoring the phase I metabolites (derivatization issues (e.g. trenbolone), analytes with poor GC-MS properties (e.g. STAN) or ionization difficulties by LC-MS (e.g. androstanediols))

are overcome when monitoring the corresponding phase II metabolites. Moreover, the hydrolysis step is not required and this is especially important for those phase II metabolites that do not hydrolyze with β-glucuronidase (e.g. sulfate metabolites and some glucuronides resistant to enzymatic hydrolysis) [3-6]. These metabolites have been underestimated for many years because they cannot be detected using conventional methods.

After validation, the method showed to be fit for purpose. Furthermore, the analysis of excretion study urines worked as a proof-of-concept to demonstrate that any type of metabolite irrespective of their nature (unconjugated, glucuronides and sulfates) could be incorporated into the method.

Even though the enzymatic hydrolysis step is not carried out, a simple sample preparation consisting of an off-line SPE was required with the aim of pre-concentrating the sample. This strategy showed good extraction recoveries for all types of phase II metabolites and, even more importantly, it could be easily automatable in the future with an on-line SPE system. Although this is a much simple and less time-consuming sample preparation procedure (without hydrolysis and derivatization steps), the continuous improvement in instrumentation sensitivity could allow for the implementation of a dilute-and-shoot (DS) or a direct injection approach for which no sample preparation is needed in the forthcoming years.

Depending on the instrumentation available and the levels required by WADA (continuously being updated), a compromise between doing a pre-concentration step or using direct injection to reach the required detection levels has to be met. The latter would be a more appropriate approach for a screening method and also, it would enable the combination of the method with other direct injection or DS methods already used in doping control analysis to move forward to the development of a multi-analyte screening method [7]. Nevertheless, the direct injection approach can also have some limitations. Besides an increased suppression of the ionization due to the complexity of the urine matrix, the injection of a large number of samples can also be detrimental for the instruments [1].

During method development, the direct injection approach was tested but not enough sensitivity was achieved for some of the metabolites (mainly glucuronides) in order to be compliant with WADA requirements [8]. Related to that and taking into account that these MRPLs have been established by WADA considering the phase I metabolites, re-evaluation of the MRPLs considering phase II metabolites should be performed. At least for sulfate metabolites, it seems MRPLs should be lower. And this is important because they are already being monitored in some anti-doping laboratories.

Regarding instrumentation, the mass analyzer used to develop this method was a triple quadrupole (QqQ) instrument working in SRM acquisition mode. This operational mode exhibited high sensitivity and permitted the selection of the most selective and sensitive ion transitions for each of the analytes included in the method. Since

phase II metabolites have a common fragmentation, some endogenous compounds can share some of the ion transitions compromising the detection of some of the metabolites, as it was observed during the development of the method. Therefore, the in-depth study of the MS behavior of the desired analytes under different MS conditions was essential to find out the most specific and sensitive ion transitions. In this work, the detection of all compounds was accomplished by using different strategies that consisted of both, the selection of more specific precursor ions (such as in-source fragments) for some of the compounds and, the use of more characteristic product ions for other analytes. For example, the discovery of the NL of hydrochloric acid (HCl) (36 Da) for those sulfate metabolites with a reduced double bond in C4-C5 during the study of CLO sulfate metabolites (Chapter 4) was useful to incorporate the most sensitive and specific ion transitions $[M-H]^- \rightarrow [M-H-36]^-$ into the method. This is particularly important when using SRM methods for which the number of metabolites/ion transitions is an intrinsic limitation.

This could be overcome using HRMS analyzers (TOF or Orbitrap). These instruments are commonly used in full-scan acquisition mode permitting the development of comprehensive screening methods due to the theoretically unlimited number of analytes that can be tested. Moreover, retrospective evaluation of the data is possible without the need of repeating the analysis. In addition, the effort required to include new analytes is minimized, since only the accurate mass of the expected ion species of the analyte needs to be known for the evaluation of the data. The two main considerations

in the development of highly comprehensive screening procedures by LC-HRMS come from the sample preparation procedure and the ionization efficiency of the analytes. A previous published method using DS and LC-HRMS analysis [9] showed higher LODs for most of the unconjugated, sulfate and glucuronide metabolites. Therefore, our results demonstrate the importance of using a sample preparation procedure and more selective ion transitions, to achieve the required sensitivity. Moreover, and specifically for one of the compounds (BOLD-S), the use of a QqQ permitted a better detectability (lower LOD) in comparison to HRMS method due to the presence of endogenous interferences when using either $[M+H]^+$ or $[M-H]^-$ as diagnostic ions. Nonetheless, new versions of high resolution instruments with the presence of a quadrupole are already in the market and in use in some anti-doping laboratories [10]. They offer the possibility to perform precursor ion selected MS/MS experiments and thus, they could also be suitable instruments to develop a similar method without these issues.

Regardless of the type of instrument used, screening methods need to ensure their major effectiveness. Consequently, studies elucidating the still largely incomplete picture of the (urinary) metabolic profile of AAS have to be conducted, aiming at complementing the list of optimal target analytes (long-term metabolites) for doping controls.

Looking for new markers

Metabolic studies are of utmost importance to identify and characterize new or alternative target analytes which could expand the detection window of AAS misuse. Anti-doping laboratories have relied for many years on the detection of metabolites identified in the 1980s by GC-MS methods, and more recently, by LC-MS methods. However, some new phase II metabolites have been recently described by LC-MS/MS. Some of these metabolites are better targets in comparison to the conventional metabolites and so, there is the need to continuously re-evaluate the metabolism of these substances. Furthermore, metabolic studies are also important to contribute in the growing knowledge of AAS metabolism.

When performing metabolic studies, several strategies (targeted and untargeted) can be set up depending on whether there is or not an *ex ante* hypothesis of the metabolite structure. The aim of the metabolic studies comprised in this thesis (chapter 4, 5 and 6) was to identify new sulfate metabolites and evaluate them as markers of AAS misuse. Thanks to the accumulated knowledge about AAS phase II metabolites and, in particular, about AAS sulfoconjugates, targeted strategies were employed but different protocols were used in each chapter.

The different targeted methodologies are discussed in detail below. In chapter 4, several acquisition modes: PrecIS, NL and SRM were used to ensure the detection of the maximum number of CLO sulfate metabolites. Each of the approaches has pros and cons and

one need to pay close attention to ensure data evaluation is properly done depending on the acquisition mode used. For instance, the application of a PrecIS of *m/z* 97, common to all sulfate conjugates, can let to the detection of many peaks in a post-administration sample and careful assessment is needed to confirm they do not come from endogenous sulfates or unrelated substances having the same product ion. Furthermore, the results of this thesis demonstrated that NL and PrecIS were less sensitive strategies compared to SRM methods and could only enable the detection of the most abundant metabolites. Regarding the SRM methods, although they were much more sensitive in comparison to open scan methods (NL and PrecIS) and they provided the best results in our studies, their main limitation is that they are blind to the non-targeted metabolites (only the calculated masses are included). Thus, combination of the different acquisitions modes is advisable.

In Chapter 5 the aim was to investigate if any of the already known 4Cl-MTD phase I metabolites described after hydrolysis with β-glucuronidase enzymes could be also excreted as a sulfate metabolite. This hypothesis was based on a previous work about metandienone metabolism where the described sulfoconjugate corresponded to the earlier reported long-term metabolite obtained from the glucuronide fraction [5]. In this study, only a theoretical SRM method was used and it included both, the common ion transition for sulfate metabolites ($[M-H]^- \rightarrow 97$) and specific ion transitions based on the A-ring structure of the expected metabolites. For those metabolites with a 3-keto-4-Cl-1,4-diene structure, ion transitions to the NL of a methyl group (similar to

boldenone [3]) were applied whereas for those metabolites with a double bond in C4-C5 (similar to the CLO metabolites in chapter 4) ion transitions of the NL of HCl were incorporated. Interestingly, the ion transitions to the common fragmentation were highly interfered by endogenous compounds and only the ion transitions to the specific losses were useful. These results demonstrated the importance of making use of previous data and studying in-depth the MS behavior of new metabolites to generate knowledge useful to identify similar metabolites in future studies.

Another possibility to start a metabolic study is to synthetize some potential metabolites, study their MS behavior and develop methods to detect new metabolites. This approach was used in Chapter 6 to identify new STAN sulfate metabolites. Sulfation of the phase I metabolites commercially available as standards was performed in order to obtain MS data useful to develop analytical strategies to detect potential STAN sulfate metabolites. Only sulfate metabolites conjugated in the hydroxyl groups (*O*-sulfates) were synthetized. Other sulfates such as *N*-sulfate metabolites were not obtained. This was probably due to the synthesis conditions employed. Moreover, from the obtained products, only one product was stable (16STAN-S), the rest of metabolites which had 17α-methyl-17β-sulfate structure were unstable in aqueous solutions as previously described [11]. All in all, *N*-sulfates were expected in the metabolism of STAN. For this reason, a bibliographic search was performed to develop analytical strategies that would cover both, *O*- and *N*-sulfates. With the obtained information, open scan methods (PrecIS and NL) and a theoretical SRM method were employed.

Again, as in the other two studies, the SRM gave better results and resulted in the identification of eleven new sulfate metabolites.

After the detection of the novel metabolites, their detection window needs to be assessed in urine samples from healthy human volunteers who have been administered the AAS. The aim is to identify the metabolite detected for the longest period of time for each AAS ensuring it is representative for all individuals and conditions. For this reason, the generation of samples is another important aspect of the metabolic studies. These studies need to be performed according to clinical protocols approved by ethical committees and the request, evaluation and approval can take very long time.

Several factors can have an impact in the metabolism of an administered drug and they need to be considered. For instance: the inter-individual and inter-ethnic variations; the administration route (e.g. oral, intramuscular, etc); the dose administered (e.g. single dose, multi-dose, etc) among other ones (gender, age, etc).

To the extent possible, these factors have been considered in the metabolic studies performed in chapter 4, 5 and 6. Inter-individual variations have been covered by the analysis of several volunteers in each case, n=4 (chapter 4), n=2 (chapter 5) and n=6 (chapter 6). The fact that these studies have involved several volunteers must be highlighted because most of the published metabolic studies up to now have only included one or sometimes two volunteers.

Regarding the inter-ethnic variations, during the last two decades, it has been widely demonstrated that glucuronidation of certain AAS (e.g. testosterone [12]) can be severely affected by genetic variations among populations. Although it seems sulfation is not that significantly affected, some differences have also been observed [13]. Therefore, it is crucial to evaluate volunteers from different ethnicities. In this sense, while performing the study of the CLO sulfate metabolites (Chapter 4), another work on CLO sulfoconjugates was published by the Anti-doping Laboratories of Beijing and Madrid [14]. That study involved only one Chinese volunteer and described a long-term metabolite detected up to 25 days that shared the same molecular mass as the one described in our work. Since its characterization was only based on the MS data obtained after LC-QTOF analysis, after publication of our results, we started collaboration with the Anti-doping Laboratory in Beijing from which we received some CLO excretion study urines to investigate. The results of these experiments demonstrated both populations shared the same long-term metabolite. Thus, with the current data, the described metabolite is considered the long-term marker for the detection of CLO misuse irrespective of the ethnic population. However, to finally confirm the suitability of this CLO long-term sulfate metabolite, more excretion studies involving volunteers from other ethnic populations (different from Caucasian and Chinese) should be performed. Nonetheless, the inclusion of this metabolite into screening methods such as the one developed in this thesis is advisable and the analytical data presented in Chapter 4 is sufficient to allow the detection of the long-term metabolite in

urine samples collected after CLO administration by other anti-doping laboratories. Although in chapter 5 and 6, inter-ethnic variations could not be studied, these studies are required in order to investigate if the same metabolic profiles are shared or they differ among populations. In the latter case, two different long-term metabolites for the same substance should be included into screening methods.

Another drawback observed in Chapter 5 was the impossibility of determining the detection time of the described metabolites because most metabolites were still detectable in the last collected sample corresponding to day 8. In this case, the question of retrospectivity remains open and, generation of excretion study samples with longer collection time periods to investigate the excretion profiles is required.

The administration routes as well as the dose administered are other factors of variability that should always be considered when studying AAS metabolism. In chapter 6, the excretion studies involved oral or intramuscular administration of the drug in several volunteers whereas different administrations, using single or multiple doses, were evaluated in Chapter 4.

As mentioned before, apart from the discovery of new long-term metabolites to be included into screening methods, metabolic studies are also very important to bring in new insights in the metabolism of AAS, such as the discovery of new bio-transformation pathways (e.g. conjugation with cysteine [15]) or

new types of metabolites. As an example, the results obtained in Chapter 5 showed that one of the metabolites that has only been reported to be excreted as a free metabolite was also excreted as a sulfate. Moreover, for another metabolite reported to be excreted conjugated with glucuronic acid, this study proved its excretion also takes place as a sulfate. In the case of the STAN (Chapter 6) new *N*-sulfates were identified. These metabolites could be additional targets in the detection of human doping or be useful markers in other fields such as horse doping or cattle feeding for which STAN sulfation is more relevant [16]. These results demonstrate the high complexity of the AAS metabolism and the need to constantly revising it.

From the three studies performed, only the CLO sulfate metabolite had longer retrospectivity (31 days) compared to the other CLO metabolites. Regarding 4Cl-MTD further work is needed to investigate which would be the most interesting metabolite to include into the screening methods. Finally, for STAN, although new sulfate metabolites were identified, the previously described long-term epistanozolol-*N*-glucuronide provides better retrospectivity.

The ultimate aim of the metabolic studies is to synthetize the metabolites for the final confirmation of the structure. In collaboration with the "Department of Biological Chemistry and Molecular Modeling-CSIC" in Barcelona, the chemical synthesis of the CLO sulfate metabolite (Chapter 4) was attempted. Although different approaches were tested, none of them resulted in the

synthesis of the described metabolite and this will be the aim of a new research project conducted in our laboratory. Although simplified routes of reference material preparation have been recently described [17], our experience gave evidence of the complexity and difficulty of AAS synthesis. Apart from the confirmation of the structure, synthesis is also required to end up including these metabolites into doping control testing methods and performing proper validations. In fact, the validation of the developed method was limited to those compounds with reference material commercially available that represented 23 of the 36 metabolites included into the method.

In the recent years, the anti-doping community has had a very active role in the detection and characterization of new long-term phase II metabolites. The implementation of long-term metabolites into sport drug testing together with the use of highly sensitive detection methods in screening procedures has helped in the prolongation of the detection windows. This has resulted in an enormous increase of AAF. Thus, implementation of these new analytical technologies and alternative target compounds is advisable to all doping control laboratories.

Implementation of the method in routine analysis

The method developed in this thesis can readily be implemented to WADA accredited laboratories as it is a direct sensitive approach for the analysis of exogenous AAS. In fact, our Anti-doping Laboratory in Barcelona got it into ISO 17025 accreditation in early

2016. Although the ultimate aim of the method is to be used as a screening procedure in doping control analysis, at the moment, it is in used with two different purposes.

First, as a method to monitor long-term metabolites that are not covered in any screening procedure in the laboratory but they are better targets than those monitored in the conventional GC-MS(/MS) and LC-MS(/MS) methods. Thus, the method works as a complement to other doping control assays for the determination of exogenous AAS. Second, it is also implemented as a confirmatory method. When considering this second possibility, the only metabolites that cannot undergo confirmatory procedures by using this approach are some of the sulfate metabolites having only one diagnostic ion transition available. For confirmation analysis of these sulfates, it would be necessary to develop procedures to be compliant with WADA requirements for confirmation of the identity [18]. The confirmation procedures may include either derivatization of the sulfate conjugates in order to obtain at least two diagnostic ion transitions or; solvolysis of the sulfate metabolites, derivatization and analysis by GC-MS/MS or LC-MS/MS. Confirmation procedures are currently under investigation.

Although this method included the maximum number of phase II metabolites (commercially available or recently described metabolites that were identified through excretion study urines), it is obvious that it is not a complete method that covers all the

exogenous AAS included in the WADA Prohibited List. However, this work forms the base for the final AAS screening method.

Finally, the steps required before the definitive implementation of the screening method in a routine basis would be the following ones:

i) continue the re-evaluation of AAS metabolism by LC-MS/MS
ii) determine the best markers of administration for each AAS (the metabolite detected for the longest period of time)
iii) synthetize the reference materials of the long-term metabolites

Meanwhile, newly described phase II long-term metabolites can be continuously incorporated into the method by using excretion study urines. The lack of reference material does not prevent their inclusion. We have included some analytes of which we did not have reference material available (metabolites of boldenone, metandienone, methyltestosterone, STAN and CLO). The detection of these metabolites requires a dedicated study of the excretion study samples. For those phase II metabolites described using indirect methods (e.g. specific hydrolysis of the urine), only data of the phase I metabolites is available in the literature. Thus, the detection of the corresponding phase II metabolite (glucuronide or sulfate) is required to include them into the method. Although not all the Anti-doping Laboratories have excretion study urines available, it is common practice to share these samples among laboratories with the aim to facilitate the identification and incorporation of these metabolites into screening methods.

Updating, expanding and improving tests methods for the detection of substances and methods of doping is an essential aspect for the prevision of adequate analytical platforms in today's sports drug testing programmes. In this line, implementing the best possible analytical approaches and most recent scientific information is deemed crucial.

7.2 Suggestions for future work

Based on the results reported in this thesis, several research lines can be suggested:

- Evaluation of other mass analyzers (e.g. QTOF, QOrbitrap...) to develop the screening method for the simultaneous detection of phase I and phase II intact urinary metabolites of AAS.
- Evaluation of the possibility of a multi-screening analyte method.
- Incorporation of endogenous phase II metabolites into the developed methodology.
- Extended study of the long-term detectability of the metabolites found in this thesis by the analysis of urines collected from excretion studies with longer collection time periods and involving volunteers from different origins.
- Re-evaluation of the metabolism of other AAS.
- Testing metabolomics based approaches as alternatives to metabolic studies to detect new phase II metabolites.

7.3 References

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8. CONCLUSIONS

8. CONCLUSIONS

The results obtained from this thesis contribute to improving the screening of exogenous AAS through both, an alternative analytical methodology and the description of new phase II metabolites.

Part 1: Alternative analytical methodology

1. A UHPLC-MS/MS screening method was developed, optimized and validated for the simultaneous detection of phase I and phase II intact urinary metabolites of exogenous AAS. The method improves the detection capabilities through the monitoring of important phase II long-term metabolites.

2. The analysis of methyltestosterone and stanozolol excretion study urines worked as a proof-of-concept to show that any metabolite irrespective of its nature (unconjugated metabolites, glucuronides and sulfates) can be readily incorporated into the method.

3. This screening method represents a much simple approach with a less-time consuming sample preparation.

Part 2: Looking for new markers

4. The in-depth study of the ionization and fragmentation mass spectrometric behavior of some of the detected sulfate metabolites was essential to discover specific neutral losses that increased the sensitivity and specificity of the detection of new metabolites.

To detect unknown metabolites neutral loss and precursor ion scan methods, or SRM methods including theoretical ion transitions can be used. SRM methods offer a more sensitive detection although unexpected metabolites cannot be detected. The combination of the different strategies is desirable.

Clostebol

5. Several sulfate metabolites were detected after the administration of clostebol to four Caucasian volunteers. One of them was characterized as 4ζ-chloro-5α-androst-3β-ol-17-one 3β-sulfate. This metabolite was the only one detected up to the last collected sample of all excretion studies, being 31 days for two of them. Thus, it is a long-term metabolite and its inclusion to screening methods is advisable.

4-chlorometandienone

6. For 4-chlorometandienone, six new metabolites conjugated with sulfate were detected in urine. Three of the identified metabolites were characterized by LC-MS/MS and GC-MS/MS. Five out of the six were detected up to the last collected sample (8 days) after administration demonstrating high potential to be long-term metabolites.

Stanozolol

7. Eleven new sulfate metabolites were detected after the administration of stanozolol providing novel information about ionization and fragmentation behavior of *N*-sulfates.

Although these sulfate metabolites do not improve the retrospectivity of the detection, this study provided very valuable data on the excretion profiles of six different volunteers after oral or intramuscular administration. Moreover, it provided new insights into stanozolol metabolism.

General conclusions

8. The developed method has been implemented into routine analysis but cannot totally substitute the conventional screening methods until all prohibited steroids (exogenous and endogenous) would be incorporated and their reference material available.

9. The re-evaluation of AAS metabolism needs to continue to look for new metabolites that could improve the detection capabilities of the detection of AAS misuse.

9. ANNEXES

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9.1 ANNEX I: Supplementary material

Balcells G, Pozo OJ, Esquivel A, Kotronoulas A, Joglar J, Segura J, Ventura R. Screening for anabolic steroids in sports: analytical strategy based on the detection of phase I and phase II intact urinary metabolites by liquid chromatography tandem mass spectrometry. Appendix A. Supplementary data. J Chromatogr A. 2015 Apr 10;1389:65-75. doi:10.1016/j.chroma.2015.02.022

Balcells G, Pozo OJ, Garrostas L, Esquivel A, Matabosch X, Kotronoulas A, Joglar J, Ventura R. Detection and characterization of clostebol sulfate metabolites in Caucasian population. Appendix A. Supplementary data. J Chromatogr B Analyt Technol Biomed Life Sci. 2016 Jun 1;1022:54-63. doi: 10.1016/j.jchromb.2016.03.028

Balcells G, Matabosch X, Ventura R. Detection of stanozolol O- and N-sulfate metabolites and their evaluation as additional markers in doping control. Supporting information. Drug Test Anal. 2016 Oct 7. doi: 10.1002/dta.2107

9.2 ANNEX II: Scientific publications included in this thesis

1. **G. Balcells**, X. Matabosch, R. Ventura. Detection of stanozolol *O*- and *N*-sulfate metabolites and their evaluation as additional markers in doping control. Drug Test Anal, (2016) doi: 10.1002/dta.2107.

2. **G. Balcells,** C. Gomez, L. Garrostas, O.J. Pozo, R. Ventura. Sulfate metabolites as alternative markers for the detection of 4-chlorometandienone misuse in doping control. Drug Test Anal, (2016) doi: 10.1002/dta.2101.

3. **G. Balcells**, O.J. Pozo, L. Garrostas, A. Esquivel, X. Matabosch, A. Kotronoulas, J. Joglar, R. Ventura. Detection and characterization of clostebol sulfate metabolites in Caucasian population. J Chromatogr B, 1022 (2016) 54-63.

4. **G. Balcells**, O.J. Pozo, A. Esquivel, A. Kotronoulas, J. Joglar, J. Segura, R. Ventura. Screening for anabolic steroids in sports: analytical strategy based on the detection of phase I and phase II intact urinary metabolites by liquid chromatography tandem mass spectrometry. J Chromatogr A, 1389 (2015) 65-75.

9.3 ANNEX III: Other publications by the author

1. M.D. McLeod, C.C. Waller, A. Esquivel, **G. Balcells**, R. Ventura, J. Segura, O.J. Pozo. A constant ion loss scan method for the untargeted detection of bis-sulfate metabolites. Anal Chem, (2016) submitted.

2. Esquivel, O.J. Pozo, L. Garrostas, **G. Balcells**, C. Gomez, A. Kotronoulas, J. Joglar, R. Ventura. LC-MS/MS detection of unaltered glucuronoconjugated metabolites of metandienone. Drug Test Anal, (2016), doi: 10.1002/dta.1996.

3. **G. Balcells**, O.J. Pozo, R. Ventura. High-resolution mass spectrometry in doping control. In: Applications of Time-of-Flight and Orbitrap Mass Spectrometry in Environmental, Food, Doping, and Forensic Analysis, Elsevier, Amsterdam, 2016, pp. 91-117.

McLeod MD, Waller CC, Esquivel A, Balcells G, Ventura R, Segura J, Pozo OJ. A constant ion loss method for the untargeted detection of bissulfate metabolites. Anal Chem. 2016 Dec 16

Esquivel A, Pozo OJ, Garrostas L, Balcells G, Gómez C, Kotronoulas A, Joglar J, Ventura R. LC-MS/MS detection of unaltered glucuronoconjugated metabolites of metandienone. Drug Test Anal. 2016 May 30. doi: 10.1002/dta.1996

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