Control of anabolic steroids misuse in sport: potential of direct detection of phase II metabolites

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A mi familia, especialmente a ti aita

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"La satisfacción radica en el esfuerzo, no en el logro.

El esfuerzo total es una victoria completa"

Mahatma Gandhi

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Abstract

Anabolic androgenic steroids (AAS) are widely used by athletes to enhance sports performance. In this thesis, different studies were performed to evaluate the potential of the direct detection of phase II metabolites by LC-MS/MS to improve the detection of AAS misuse in sport.

First, the direct detection of glucuronide metabolites was investigated using metandienone, allowing the detection of thirteen glucuronides, one of them resistant to enzymatic hydrolysis. The proposed analytical strategy provides a comprehensive approach and it is recommended to be used for metabolic studies of other AAS.

Second, the detection of bisglucuronide conjugates of AAS was investigated. The mass spectrometric behavior was studied using synthesized AAS bisglucuronides to develop strategies for their detection. The formation of bisglucuronides was demonstrated in samples collected after norandrostenediol administration.

Finally, the role of sulfate metabolites to detect endogenous AAS administration was evaluated. A LC-MS/MS method was developed for the direct quantitation of fourteen endogenous steroid sulfates. The use of some ratios between steroid sulfates improved the retrospectivity after oral and intramuscular testosterone administration. Steroid sulfates should be included in the steroid profile to complement the current markers.

The results of the thesis demonstrate the increased role of the direct analysis of phase II metabolites of AAS in doping control.

Resumen

Los esteroides anabolizantes androgénicos (EAA) son ampliamente usados por los atletas para mejorar el rendimiento deportivo. En esta tesis se han desarrollado diferentes estudios en los que se evalúa el potencial de la detección directa de metabolitos de fase II por CL-EM/EM para mejorar la detección del abuso de los EAA en el deporte.

Primeramente, se ha evaluado la detección directa de metabolitos glucurónido para la metandienone, permitiendo la detección de trece glucurónidos, uno de ellos resistente a la hidrolisis enzimática. La estrategia analítica propuesta ofrece un estudio exhaustivo y se recomienda su uso para estudios metabólicos de otros EAA.

En segundo lugar, se ha evaluado la detección de conjugados bisglucurónidos de EAA. Se ha estudiado la ionización y fragmentación usando bisglucurónidos de EAA sintetizados con el fin de desarrollar estrategias para detectarlos. Estos conjugados se han detectado en muestras de orina recogidas tras la administración de norandrostenediol.

Finalmente, se ha evaluado la utilidad de los metabolitos conjugados con sulfato para detectar la administración de EAA endógenos. Se ha desarrollado un método CL-EM/EM para la cuantificación directa de catorce esteroides endógenos sulfato. El uso de algunos ratios entre sulfatos mejora la capacidad de detección tras la administración oral e intramuscular de

testosterona. Los esteroides sulfato deberían incluirse en el perfil esteroidal como complemento a los marcadores convencionales.

Los resultados de esta tesis demuestran el aumento del protagonismo del análisis directo de metabolitos de fase II de los EAA en el control de dopaje.

Preface

AAS are included in the list of prohibited substances by the World Anti-Doping Agency (WADA) due to their performing-enhancing properties and adverse health effects. They are widely misuse among athletes, being the most reported group of prohibited substances in doping controls. AAS are extensively metabolized and excreted in urine mainly as phase II metabolites.

The conventional methods for the detection of AAS are based on the analysis by gas and liquid chromatography-mass spectrometry after the hydrolysis with β-glucuronidase enzyme to release the corresponding phase I metabolites. Thus, only unconjugated and glucuronide metabolites hydrolysable under these conditions are detected. However, other phase II metabolites, such as glucuronides resistant to enzymatic hydrolysis, sulfates or other conjugates, are not detectable using these conventional methods. LC-MS/MS supposed an improvement in the AAS analysis thanks to the possibility of the direct detection of phase II metabolites. Some of these phase II metabolites are detected for long period of time (the so called long-term metabolites), expanding the detection capabilities of AAS misuse.

The aim of this thesis was to evaluate the potential of the direct detection of AAS phase II metabolites by LC-MS/MS technology in order to improve the detection of their misuse in sport.

List of abbreviations

AAF	Adverse analytical finding
AAS	Anabolic androgenic steroids
ABP	Athlete biological passport
ACN	Acetronitrile
ADAMS	Anti-doping administrations and management system
BG	Bisglucuronide
CE	Collision energy
CI	Chemical ionization
CID	Collision induced dissociation
CIL	Common ion loss
CL-EM/EM	Cromatografía líquida acoplada a espectrometría de masas en tandem
CV	Cone voltage
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
E. coli	Escherichia coli
EAAS	Endogenous anabolic androgenic steroids
EI	Electron ionization
ESI	Electrospray ionization
GC	Gas chromatography
GC/C/IRMS	Gas chromatography combustion isotope ratio mass spectrometry
GC-MS(/MS)	Gas chromatography (tandem) mass spectrometry
gluc	Dehydrated glucuronide moiety
HILIC	Hydrophilic interaction liquid chromatography
HRMS	High-resolution mass spectrometry
ISL	International Standard for Laboratories
ISTD	Deuterated internal standard
IT	Individual threshold
LC	Liquid chromatography
LC-MS(/MS)	Liquid chromatography (tandem) mass spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantitation
m/z	Mass to charge ratio
MeOH	Methanol

MM	Molecular mass
MRPL	Minimum required performance level
MS	Mass spectrometry
MSTFA	N-methyl-N-trimethylsilyl-trifluoroacetamide
MTD	Metandienone
NDIOL	19-nor-4-androstenediol
NL	Neutral loss scan
OH	Hydroxylation
ox	Oxidation
PI	Precursor ion scan
Q1/Q3	Quadrupole
q2	Collision cell
QC	Quality control
QqQ	Triple quadrupole analyzer
red	Reduction
RL	Population reference limit
RSD	Relative standard deviation
RT	Retention time
PAPS	3´-phosphoadenosine-5´-phosphosulfate
S/N	Signal to noise ratio
SIM	Selected ion monitoring
SPE	Solid phase extraction
SPME	Solid phase microextraction
SRM	Selected reaction monitoring
SULT	Sulfotransferases
T	Testosterone
T/E	Testosterone to epitestosterone ratio
TBME	*Tert*-butyl-methyl ether
TIC	Total ion chromatogram
TOF	Time of flight
UDPGA	Uridine-5´-diphosphoglucuronic acid
UGT	Uridine diphosphoglucuronosyl-transferases
UHPLC	Ultra-high performance liquid chromatography
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 and analytical strategies.

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

Five scientific papers representing the experimental and results of this thesis have been presented in chapters 3 to 7. Chapter 3 (Part I) is addressed to evaluate the direct detection of glucuronide metabolites of metandienone by LC-MS/MS. Chapter 4 (Part II) includes the study of MS behavior of bisglucuronide metabolites of AAS and the development of strategies for their detection in excretion study urines. Chapter 5, 6 and 7 (Part III) contain the development and validation of a quantitative LC-MS/MS method for endogenous steroid sulfate metabolites, population studies and, the evaluation of their potential as markers of T misuse after oral and intramuscular administration.

Chapter 8 comprises a general discussion of the results and suggestions for future work, and Chapter 9 the main conclusions extracted from the thesis results.

Finally, two annexes have been added containing the list of publications included in this thesis (Annex I) and, other publications by the author (Annex II).

INTRODUCTION

Chapter 1

1.1 Doping in sports

1.1.1 WADA and doping control

World Anti-Doping Agency (WADA) was established in 1999 as a single independent international agency composed and funded equally by the sport movement and governments of the world. WADA implemented a set of anti-doping rules, the World Anti-Doping Code, which is followed and accepted worldwide by most sporting and anti-doping organizations [1].

According to WADA, doping is defined as the occurrence of one or more of the anti-doping rule violations included in Article 2 of World Anti-Doping Code. The most frequent are "2.1 the presence of a prohibited substance or its metabolites or markers in an athlete's sample" and "2.2 the use or attempted use by an athlete of a prohibited substance or a prohibited method". Moreover, other additional situations are considered as doping such as "2.3 evading, refusing or failing to submit to sample collection, 2.4 whereabouts failures, 2.5 tampering or attempted tampering with any part of doping control, 2.6 possession of a prohibited substance or a prohibited method, 2.7 trafficking or attempted trafficking in any prohibited substance or prohibited method, 2.8 administration or attempted administration to any athlete in-competition of any prohibited substance or prohibited method, or administration or attempted administration to any athlete out-of-competition of any prohibited substance or any prohibited method that is prohibited out-of-competition, 2.9 complicity and 2.10 prohibited association".

The detection of the use of prohibited substances or methods is the task of doping control laboratories. These laboratories must achieve and maintain accreditation by WADA which is based upon compliance with international standards ISO/IEC 17025 and International Standard for Laboratories (ISL) [2, 3]. The objective of the ISL is to ensure valid test results and evidentiary data, and to achieve uniform and harmonized results and reporting among all accredited laboratories. Additionally, WADA publishes Technical Documents with specific technical requirements, addressing particular operational areas of the accredited laboratories [4].

1.1.2 The prohibited list

WADA publishes and revises annually the list of prohibited substances and methods [5]. The version of the prohibited list in 2018 is shown in Table 1.1 and is divided in different groups: substances and methods prohibited at all times (from S0 to S5 and M1 to M3); substances prohibited in-competition (from S6 to S9); and, substances prohibited in particular sports (P1). More than two hundred substances are forbidden.

A substance or method is included in the prohibited list when satisfy any two of the following three criteria: (I) it has the potential to enhance or enhances sport performance; (II) it represents an actual or potential health risk to the Athlete; and/or (III) it violates the spirit of sport (as defined in the Code [1]). Moreover, substances or methods which mask the effect or detection of prohibited

substances and, substances which has not been approved for human use are also prohibited.

Table 1.1. Substances and methods prohibited by WADA (The 2018 Prohibited List).

SUBSTANCES AND METHODS PROHIBITED AT ALL TIMES
SUBSTANCES
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
S5 Diuretics and masking agents
METHODS
M1 Manipulation of blood and blood components
M2 Chemical and physical manipulation
M3 Gene doping
SUBSTANCES AND METHODS PROHIBITED IN-COMPETITION
S6 Stimulants
S7 Narcotics
S8 Cannabinoids
S9 Glucocorticoids
SUBSTANCES PROHIBITED IN PARTICULAR SPORTS
P1 Beta-blockers

This thesis is focused on the detection of Anabolic Androgenic Steroids (AAS), included in S1 group (Anabolic Agents).

1.1.3 General analytical strategies in doping control

After the sample collection procedure (Figure 1.1), the samples are sent to WADA accredited laboratories in order to analyze them. Anti-doping laboratories have to apply analytical methodologies for the detection of all prohibited substances and methods at the required sensibilities. The analysis in doping control is generally performed using urine specimen as matrix due to the non-invasive

sample collection and the sufficient volume for analysis is generally available. In addition, drugs are present in relatively higher concentrations than in blood and, the detection time window can be enlarged since hydrophilic metabolites are also excreted in urine [6]. Blood samples are also collected and used for the analysis of blood parameters and for the detection of specific doping agents, such as human growth hormone or erythropoiesis stimulating agents. The different analytical strategies have to take into consideration the high sensitivity and selectivity required (due to the complex matrices used) for the analysis of compounds with wide range of physico-chemical properties and molecular weights, limited samples volumes and fast analysis time requirements.

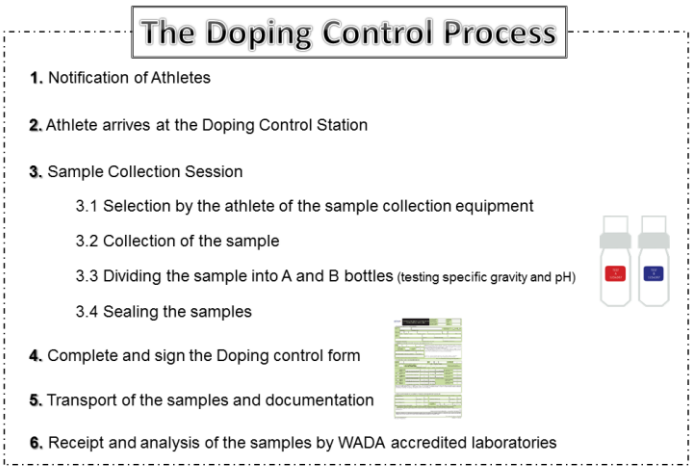

Figure 1.1. The doping control process.

In order to ensure that all accredited laboratories can report the detection of prohibited substances, their metabolites or their markers in a homogenous way, a minimum routine detection and

identification capability for testing methods has been established by WADA. This level is known as Minimum Required Performance Level (MRPL) [7]. MRPL values are established for each prohibited substance itself and/or its metabolites, markers or degradation products taking into account the metabolism, stability, pharmacokinetics and pharmacodynamics. WADA requires a limit of detection of 50% of the MRPL for all substances. The MRPL is not a threshold nor a reporting level. Thus, 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. The MRPLs range from 0.2-1000 ng/ml depending on the substance. In case of AAS, the MRPLs are set at 5 ng/ml and, 2 ng/ml for some specific steroids.

Drug testing procedures in anti-doping laboratories include initial testing and confirmatory analyses. First, initial testing procedures are applied to all samples in order to indicate the presence or absence of a doping agent. They should be able to detect a large variety of compounds and/or metabolites with similar physicochemical properties [8-10] at the concentrations required by WADA [7]. They must be fast, selective and sensitive, avoiding false negative results and minimizing false-positive. If a sample is considered suspicious in the initial testing procedure, a confirmatory analysis must be performed to assess the presence of the suspicious prohibited substance. Confirmatory methods should to provide sufficient selectivity for the detection of the suspect doping agent.

For several substances of the prohibited list (*non-threshold substances*), the detection of their presence in the matrix is considered evidence for their abuse and constitutes violation of the anti-doping rules. In this case, the sample is submitted to further confirmatory tests by (tandem) mass spectrometry (MS(/MS)) coupled to gas chromatography (GC) or liquid chromatography (LC). 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 previously established identification criteria are fulfilled [11].

However, other substances of the prohibited list need quantitative measurements either because they are considered as doping agents only above a certain concentration (*threshold substances* [12]: e.g., salbutamol, formoterol or ephedrine) or because they have endogenous nature [13] (e.g several androgenic steroids). Thus, for these substances, when the concentration exceeds the pre-established threshold the sample is considered suspicious. For the confirmation of *threshold substances*, in addition to the unequivocal identification of the analyte by the criteria mentioned in the previous paragraph, the concentration of the substance in the sample needs to be calculated from the average concentration of three aliquots of the sample and to report the result as AAF. The concentration has to be greater than the decision limit established for the substance taking into account its threshold and the maximum combined standard uncertainty [12].

For the endogenous compounds, the concentrations and ratios of testosterone (T) and some of its metabolites are measured in the screening method by gas chromatography (tandem) mass spectrometry (GC-MS(/MS)). If values fall out of the individual threshold established for each athlete using a Bayesian model, this may be an indication of misuse of endogenous steroids. The confirmatory analysis is performed by gas chromatography coupled to combustion isotope ratio mass spectrometry (GC/C/IRMS) which allows to distinguish between the endogenous origin and exogenous administration of synthetic steroid analogues [13].

1.1.4 Adverse analytical finding reports

Anabolic agents were included for the first time in the prohibited list in 1976 before the Montreal Olympic Games. Since then up to the last years, they are the most detected compounds in doping analysis, as shown in Figure 1.2. Moreover, an increment of the percentage of AAFs for hormone and metabolic modulators (up to 17% in 2016) has been also observed.

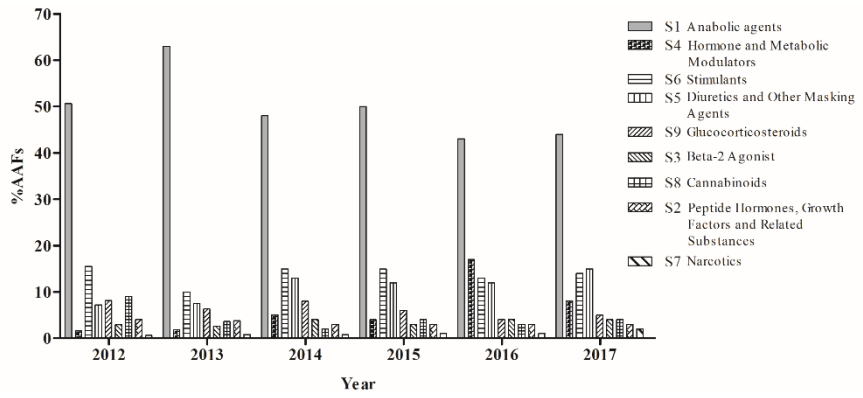

Figure 1.2. Percentage of AAFs of prohibited substance groups reported by WADA accredited laboratories in the last 6 years.

Throughout the years, T and stanozolol and, clenbuterol (other anabolic agents) have been the most frequently detected compounds among the S1 group. In the Testing Figures of 2017 [14] (Table 1.2), the cases of AAFs linked to stanozolol and nandrolone abuse were the most frequent with 284 and 205 occurrences, respectively. Moreover, AAFs associated with an exogenous origin of the steroid profile target compounds (such as T) was found in 158 occurrences. In previous annual reports, AAFs related to exogenous origin of T misuse (described as T/E>4) had also very high percentages of occurrence within their class (e.g. 55.5% in 2012 [15] and 59.6% in 2013 [16]).

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 like T, the findings may occur due to the detection of concentrations outside normal reference ranges and not necessary for a doping offense.

Due to the wide misuse of anabolic steroids, there is a strong push to enhance their detection. Research is focused on: the improvement of screening and confirmation methods, the identification of new metabolites to improve the detection capabilities and, the evaluation of new technologies to reduce the sample treatment and to enhance the sensitivity and selectivity.

Table 1.2. Occurrences of AAFs related to a specific anabolic agent published in Anti-doping Testing Figures Report in 2017 [14]

S1.1 Anabolic agents	**Occurrences**	**% within drug class**
Stanozolol	284	20%
Nandrolone (26 cases-GC/C/IRMS result for 19-norandrosterone is consistent with an exogenous origin)	205	14%
The GC/C/IRMS results is consistent with an exogenous origin	158	11%
Metandienone	133	9%
Drostanolone	119	8%
Oxandrolone	88	6%
Boldenone (25 cases-GC/C/IRMS result for boldenone and/or boldenone metabolites is consistent with an exogenous origin)	84	6%
Dehydrochloromethyl-testosterone	84	6%
Trenbolone	65	5%
Metenolone	64	4%
Mesterolone	37	3%
Clostebol	27	2%
Methasterone	13	1%
Fluoxymesterone	12	1%
1-androstenedione	11	1%
Deoxymethyltestosterone	11	1%
Methyltestosterone	11	1%
S1.2 Other Anabolic agents	**Occurrences**	**% within drug class**
Clenbuterol	294	78%
Ostarine	47	13%
LGD-4033	9	2%
Tibolone	8	2%
RAD140	6	2%
Ractopamine	4	1%
Andarine	3	1%
Zilpaterol	3	1%

1.2 Anabolic androgenic steroids

1.2.1 General aspects

Anabolic steroids are synthetic derivatives of the male hormone T. The general structure of anabolic steroids is based on the cyclopentanoperhydrophenanthrene core. This core is a carbon structure composed of three cyclohexane rings (A, B and C) fused with one cyclopentane ring (D) (Figure 1.3a). Most of the anabolic steroids contain a methyl group in C_{18} and C_{19} positions and a hydroxyl group or keto function in C_3 and C_{17} positions (Figure 1.3b-c).

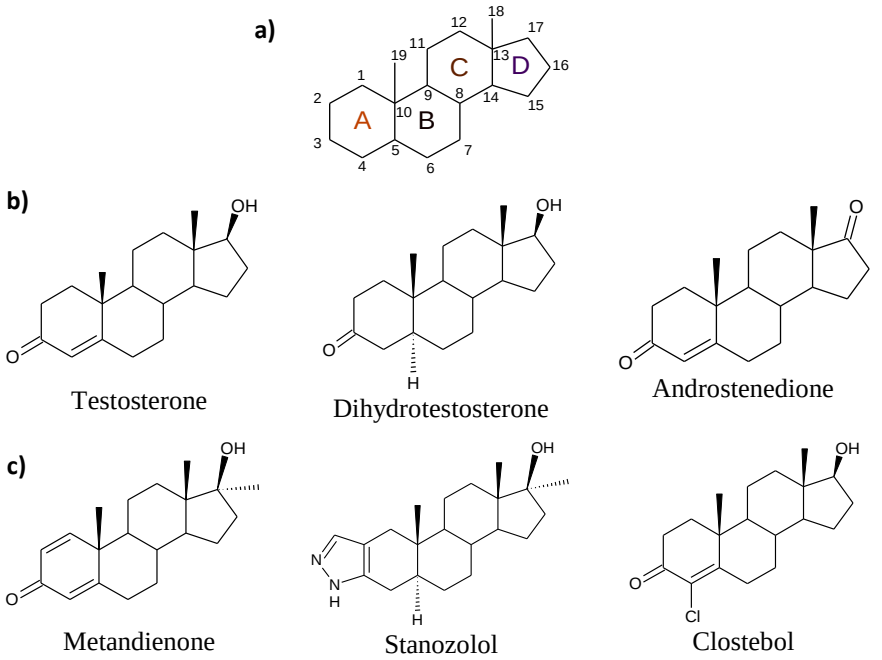

Figure 1.3. General structure for anabolic steroids (a). Endogenous AAS (b) and exogenous AAS (c).

AAS are divided in two groups: endogenous and exogenous. Endogenous AAS are substances naturally produced by the body. Among them, T is regarded one of the most important steroids as it is the primary male sex hormone and is responsible for the androgenic and anabolic effects observed during male adolescence and adulthood [17]. Prohormones or phase I metabolites of T (dehydroepiandrosterone (DHEA), androstenedione, androstenediol, dihydrotestosterone (DHT)) (Figure 1.3b) have been also reported as doping agents and included in the prohibited list. They are present in every sample, and the differentiation between the normal body levels and the ones resulted after exogenous administration is needed in doping control.

Contrary to the endogenous, the exogenous AAS refer to synthethic compounds that are not naturally produced by the human body (e.g. metandienone, stanozolol, clostebol, depicted in Figure 1.3c). They are the result of modifications in the T chemical structure such as: introduction of a methyl or ethyl group in the 17α-position; addition/removal of double bonds; reduction of the keto group; substitution using heteroatoms or halogens; addition of functional groups like heterocyclic rings; or removal of the C_{18} or C_{19} methyl group (Figure 1.4). In this category it should not be included the esterified or other preparations of endogenous AAS that, once inside the body, will liberate the free form of the naturally produced AAS.

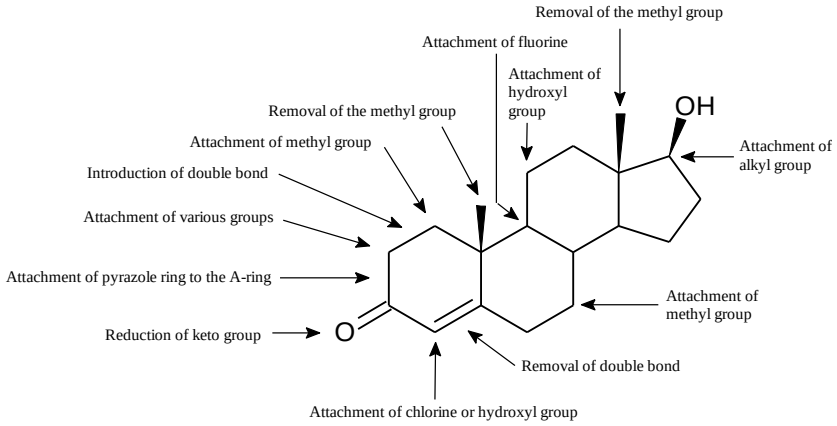

Figure 1.4. Common structural modifications of T.

1.2.2 Effects and adverse effects on health

The effects of T in the body can be divided into anabolic and androgenic effects. Besides the desired anabolic effects for those athletes taken steroids, they have also undesired androgenic effects. Although synthesis of AAS with structural modifications has been made to enhance anabolic and to decrease androgenic effects, they cannot be entirely separated [18]. Therefore, the accurate term for anabolic steroids should be "anabolic androgenic steroids", but for simplicity, the shorter term is normally used.

Anabolic functions are associated with protein building in skeletal muscle and bone, being the most important the increase in body dimensions (mainly in the upper region of the body: neck, thorax, shoulders and upper arm), lean body mass and muscle mass and strength [19].

Androgenic functions are responsible for the changes in the primary and secondary male sexual characteristics of humans. They are promoting male sexual differentiation during the first trimester of pregnancy, masculinization of the male at the puberty stimulating the growth of the testes and the male accessory reproductive glands and, maintenance of adult male sexual function. They are also responsible of secondary sexual characteristics such as the enlargement of the larynx (cause of a deepening of the voice), the growth of terminal hair (in the pubic, axillary and facial regions), an increase in sebaceous glands activity (leading to acne) and increment of aggressive behavior [20].

The use of AAS is not limited to elite athletes and seems that is increasing among recreational and amateur athletes, becoming nowadays a problem of public health [21-25]. Performance improvement, increment muscular development and strength, and also appearance are the mainly reasons for their misuse, even in general population.

AAS misuse has been related to a variety of different side effects, dependent on the structure of the steroid, the dose, the frequency of use, the sex and age at the initiation use. Some adverse effects of AAS abuse are reversible, normally related with cosmetic problems. However, others are irreversible and may induce serious damage. Among other, the main adverse effects include: endocrine effects (testicular atrophy, sterility, gynecomastia in males, virilisation in females), cardiovascular effects (myocardial infarction,

hypertension, thrombosis, sudden cardiac death), neuropsychiatric effects (anxiety, depression, hostility, aggressiveness, psychosis), liver effects (liver tumours, impaired function), bone effects (stunting of linear growth in children), skin effects (acne) and vocal chords effects (deepening of the voice in women) [19, 20, 26, 27]. Most of these adverse effects have appeared with therapeutic recommended doses; thus, more serious consequences might happen in people administering large doses during long periods of time such as those recommended to improve sport performance.

The therapeutic use is quite limited and may vary between steroids: hypogonadism, some anemia cases, hereditary angioneurotic oedema, same breast cancers, osteoporosis, some cases of posttraumatic and postchirurgical weakness states and more recently as androgen replacement theraphy in aging [28-30]. In some situations, the efficacy has not been completely established or other better therapies exist.

1.3 AAS metabolism

After administration, AAS are extensively modified by several metabolic reactions (phase I and Phase II) in order to inactivate the drug (less toxic, less active) and to facilitate its elimination from the body (more polar). Some intermediate metabolites can remain biologically active. Most of the steroid metabolism takes place in the liver although some metabolic reactions also occur in the kidney, skin or other tissues (brain, adipose and reproductive

tissues) [20]. In man, the bulk of steroid metabolites appear in urine. These metabolic reactions are catalyzed by a large number of enzymes [31]. The most important metabolic reactions were reviewed in depth by Schänzer [32].

1.3.1 Phase I metabolism

Phase I reactions involve oxidation, reduction or hydroxylation to introduce reactive and polar groups into the steroid. Several of these reactions are catalyzed by the cytochrome P450 (CYP). The most common phase I metabolic reactions are depicted in Figure 1.5 [32].

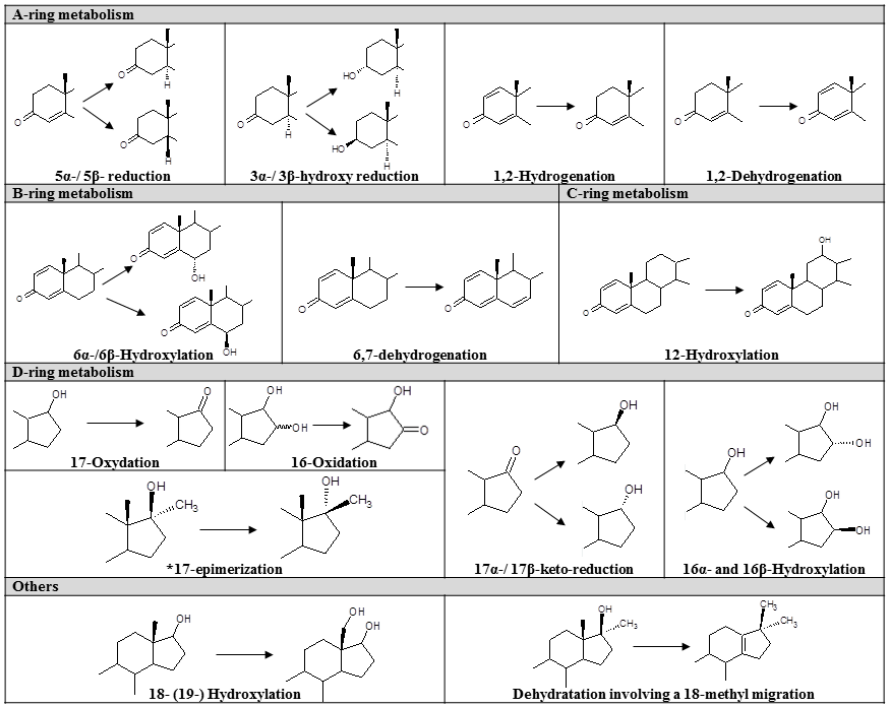

Figure 1.5.Common phase I metabolic reactions of AAS.

1.3.2 Phase II metabolism

Phase II reactions, also named as conjugation reactions, consist on the conjugation of the anabolic steroid or its phase I metabolite with very polar molecules. In human, glucuronidation and sulfation (Figure 1.6) are the major phase II reactions [33]. Other minor phase II metabolic pathways have been described such as acetylation, conjugation with glutathione, N-acetylglucosamine or amino acids or bisconjugation [34-40].

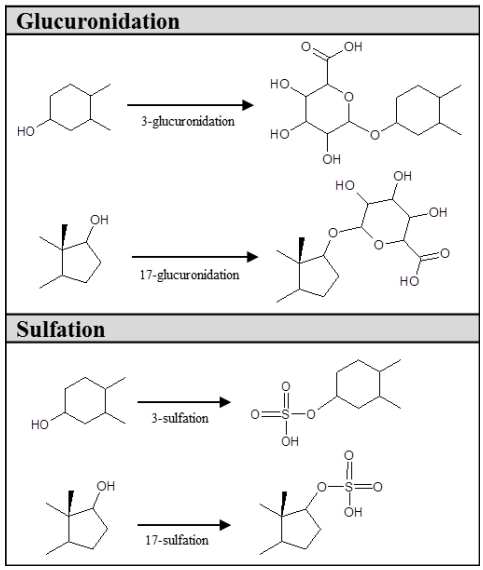

Figure 1.6. Common phase II metabolic reactions of AAS.

1.3.2.1 Glucuronidation

Glucuronidation is consider the most predominant pathway in human metabolism and mostly irreversible. It is catalyzed by uridine diphosphoglucuronosyl-transferases (UGT) and uses uridine-5´-diphosphoglucuronid acid (UDPGA) as the co-substrate. The reaction leads to the attachment of the highly polar glucuronide

acid moiety to the steroid structure to form a β-glycosidic bond (Figure 1.7). The liver is the major site at which glucuronidation occurs although UGTs are also located in kidney, brain, lung, skin, breast tissue, and prostate as well [41]. The most important UGT enzymes involved in glucuronidation of steroids are UGT1A and UGT2B. The glucuronide moiety can be bound to different nucleophilic functional groups (hydroxyl, carboxyl, thiol, phenol or amino groups) on the parent aglycone, giving rise to different glucuronide isomers [42, 43]. Evidence of the regio- and stereoselectivity of UGTs have been shown, especially for UGT2B enzymes [42, 44, 45]. For example, UGT2B7 enzyme conjugates only at 3-hydroxy position, UGT2B15 only at 17-hydroxy position whereas UGT2B17 conjugates both the 3-hydroxy and 17-hydroxy position. Differentiation among isomers might be important as they can have different pharmacological activity (as same as for sulfates).

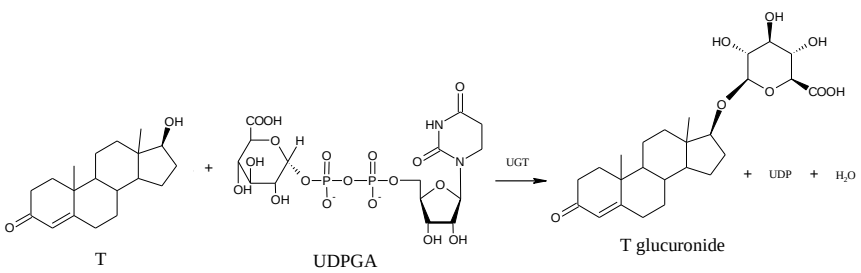

Figure 1.7. A UGT-catalysed glucuronide conjugation between T and UDPGA.

1.3.2.2 Sulfation

Sulfation also plays an important role in the human metabolism of both endogenous and exogenous compounds. Sulfation is catalyzed by sulphotransferase enzymes (SULT), which transfer the sulfo moiety (SO_3) from a co-substrate, 3´-phosphoadenosine-5´-phosphosulfate (PAPS), to the steroid (Figure 1.8). There are two classes of SULTs in mammals: membrane-bound and cytosolic. Five of cytosolic SULT (SULT1A1, SULT1E1, SULT2A1, SULT2B1a and SULT2B1b) are involved in the sulfation of steroids. SULTs are localised in different tissues such liver, brain, breast, intestine, lung, adrenal gland, or kidney among others [46]. Sulfation generally takes place through hydroxyl group of the steroids. In contrast to glucuronidation, several steroids can be metabolized by the same SULT enzyme. Moreover, the reaction can be reverted due to the presence of sulfatases [47].

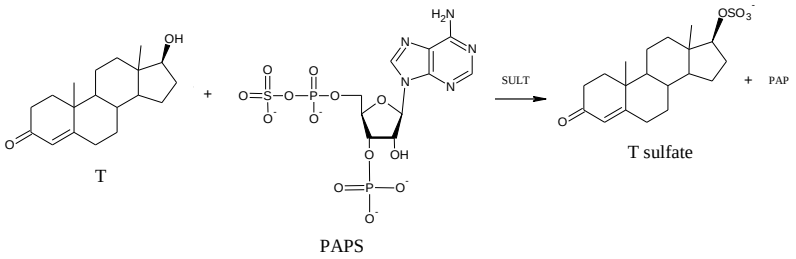

Figure 1.8. A SULT-catalysed sulfation between T and PAPS.

Regarding the configuration of phase II metabolites, 3α-hydroxy steroids are usually conjugated with glucuronic acid whereas 3β-hydroxy steroids are mainly excreted as sulfates (such as DHEA). 17β-hydroxy steroids conjugate with glucuronic acid as well as with sulfo-moiety [32]. Moreover, the conjugation is not restricted to 3- and 17-position, for instance 16βhydroxy-stanozolol 16β-sulfate was found in urine after stanozolol administration [48]. Although O-conjugates are the most abundant for steroids, N-conjugates also exist (such as 4β-hydroxy-16-oxo-stanozolol-N-sulfate) [48].

It is worth to notice that 17-epimerization (shown in Figure 1.5) is not a metabolic reaction but it is a product of urinary degradation of a 17β-sulfate metabolite of 17α-methyl steroids. 17-epimers for several 17β-hydroxy-17α-methyl steroids (e.g. fluoxymesterone, bolasterone, metandienone, methyltestosterone, oxandrolone, stanozolol or 4-chlorometandienone) have been observed [32, 49, 50]. In a first step the 17β-sulfate is removed generating a stabilized cation and from this cation several eliminations or substitutions can be performed generating different compounds (such as 18-nor-17,17-dimethyl analogues) and the corresponding 17-epimer (17α-hydroxy-l7β-methyl steroid). They are also useful for doping control analysis because they are urinary markers for steroid misuse, and furthermore, some of them are long term metabolites, such as the case of 17β-methyl-5α-androstan-3α,17α-diol 3α-sulfate (methyltestosterone metabolite) [51].

1.3.3 Genetic polymorphism

Genetic polymorphism may alter the expression or the activity of a specific metabolic enzyme, thus, it can affect the metabolism of AAS [33]. For example, the deletion polymorphism of UGT2B17 gene is associated with significantly lower glucuronidation rates of T, which decreases the excretion of T glucuronide but does not affect epitestosterone glucuronide. For the UGT2B17 gene, it presents an unusually high degree of geographic variation, with a high deletion frequency in East Asian populations, intermediate in European/West Asian, and low frequency in the African [52]. This inter-ethnic variation in metabolism has implications for the detection of T administration [53], using the testosterone/epitestosterone (T/E) ratio as a marker. For example, it was demonstrated that approximately 40% of the individuals having a deletion of the UGT2B17 gene would never reach the cut-off ratio of 4 (established by WADA [13] in the past) even after an elevated dose of T [54]. Polymorphism in SULT enzymes has been also reported, especially for SULT1A1 which differ between ethnics [55].

1.4 Detection of AAS in doping control

The detection of AAS in doping control is commonly performed in urine and the analytical strategies to detect them have continuously improved.

Exogenous and endogenous AAS have quite different issues regarding to the detection. For exogenous AAS, the presence of the

substance or of one of its metabolites in the biological sample is considered sufficient to report an AAF, hence qualitative determination is needed. On the other hand, for endogenous AAS, in addition to the detection of the compound in urine, the exogenous origin needs to be demonstrated to declare an AAF. The exogenous origin is demonstrated using GC/C/IRMS analysis which is based in the different content of ^{13}C between the compounds produced in the body and the synthetic compounds of pharmaceutical preparations. Suspicious samples to be analyzed by GC/C/IRMS are selected based on the concentrations of endogenous AAS and their metabolites in urine, thus, a quantitative method is required.

1.4.1 Exogenous AAS

1.4.1.1 Detection of anabolic steroids in doping analysis

Detection of anabolic agents was implemented during the Montreal Olympic Games (1976) and mainly performed by radio-immunoassay (RIA). These methods had several serious limitations such as the low selectivity, sensitivity and specificity [56].

The introduction of GC-MS for the detection of AAS and their metabolites was an important progress in doping control. It combined the required analytical sensitivity and specificity to distinguish between different steroid metabolites. Hydrolysis, purification and preconcentration, and derivatization of the samples are needed prior to the analysis by GC-MS [57, 58]. Most of the methods routinely used in doping control laboratories are based on this concept [56, 59, 60]. Published GC-MS(/MS) methods are usually

operating in selected ion monitoring (SIM) or selected reaction monitoring (SRM) mode, and allow for the simultaneous detection of different metabolites for more than 50 anabolic steroids, including endogenous and exogenous at the required concentration levels.

The hydrolysis is performed with β-glucuronidase enzyme obtained from *Escherichia coli (E.coli)* and the extraction is based on a liquid-liquid extraction (LLE) using an organic solvent such as diethylether or *tert*-butyl methyl ether. The derivatization step, developed by the group of Prof. Donike, is based on the formation of trimethylsilylated steroid derivatives of both the hydroxyl and the keto groups, through the formation of the enol. Trimethylsylilation is normally performed with a mixture containing N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), ammonium iodide (NH_4I) and ethanethiol [57, 58]. This derivatization step requires anhydrous conditions to assure complete efficiency (reagents and the formed derivatives are sensitive to water) and high temperatures (≈60ºC) in order to enolyse the more stable keto functions of the molecules.

However, these methods based on GC-MS analysis have also several limitations. First, only volatile and termally stable compounds can be analyzed by GC-MS. Moreover, only free and glucuronide metabolites hydrolysable with β-glucuronidase enzyme are detected. Therefore, glucuronides resistant to enzymatic hydrolysis, sulfates or other conjugates are not detected by these

methods. Due to this limitation, historically these compounds have not been systematically determined. Furthermore, the percentage of hydrolysis of each glucuronide may depend on various factors such as the affinity of the β-glucuronidase for the glucuronide as well as the incubation time, temperature and the pH conditions [61]. Also the differences between β-glucuronidase enzyme preparations [62-64] or the presence of enzyme inhibitors in the urine matrix [65] may plan an important role on the efficiency of the enzymatic hydrolysis.

Several limitations are also associated with the derivatization. The most important one is, possibly, that several AAS and metabolites are not properly derivatized, mainly those with a high number of hydroxyl groups or with high conjugation in the keto function. Adittionally, the derivatization process may result in more than one reaction product for some steroids [66] or in chemical rearrangement of other [67]. All these variables can have a great impact on the analysis and might lead to the underestimation of the real concentration of different glucuronides.

Apart from GC-MS(/MS), also chemical ionization (GC-CI-MS/MS), atmospheric pressure chemical ionization (GC-APCI-MS/MS) or atmospheric pressure photoionization (GC-APPI-MS/MS) coupled to HRMS analyzers have been also used for steroid analysis [68-70]. Although, the use of GC-MS(/MS) for the detection of steroid misuse is still the gold standard in doping control laboratories, the irruption of liquid chromatography tandem mass spectrometry (LC-MS/MS) supposed a great advantage over

GC-MS, reducing the sample preparation procedures and the analytical times, and the improvement of sensitivity [71]. The firsts commercial LC-MS instruments in doping control laboratories were employed for the detection of substances for which GC-MS detection was difficult or impossible, such as thermolabile and very polar compounds (e.g. stanozolol, fluoxymesterone, oxandrolone) [72-75], and also for compounds with high level of conjugated double bonds (e.g. trenbolone, methyldienolone, methyltrienolone).

Nowadays, both GC-MS and LC-MS technologies are used in the routine control analysis for the detection of AAS misuse (Figure 1.9).

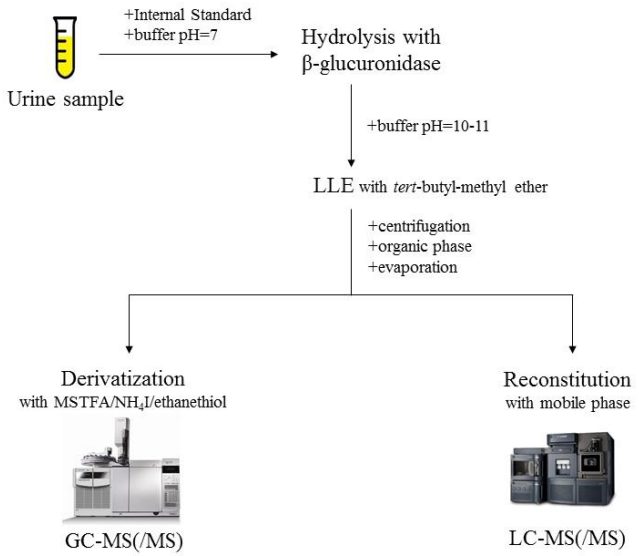

Figure 1.9. Common procedures used for the screening analysis of AAS in doping control laboratories.

1.4.1.2 Direct detection of AAS by LC-MS/MS

The use of LC-MS/MS analytical methods in the analysis of steroids is increasing due to the benefits associated to this technique. Among them, the most important one is probably that LC-MS/MS allows for the direct detection of phase II metabolites avoiding the enzymatic hydrolysis and derivatization steps [72, 76]. Hence, glucuronides resistant to enzymatic hydrolysis, sulfates or other conjugates (cysteine conjugates, bisconjugates) can be detected. A high number of these conjugates, which cannot be detected using the traditional procedures, have been recently identified by direct analysis [48, 51, 77-83]. Moreover, some of them have shown to be useful as long-term metabolites (metabolites detectable for the longest period of time after administration) such as for metandienone, methyltestosterone or stanozolol [51, 79, 80]. As an example, methyltestosterone misuse is commonly detected by GC-MS of its two main metabolites excreted as glucuronide conjugates (17α-methyl-5α-androstan-3α,17β-diol and 17α-methyl-5β-androstan-3α,17β-diol). The evaluation of sulfate fraction of methyltestosterone allowed the identification of 17β-methyl-5α-androstan-3α,17α-diol sulfate, which improved the detection times up to 21 days, whereas the conventional metabolites only were detected up to 6 days [51].

Screening methods based on direct detection of phase I and phase II metabolites of some AAS have been recently described [84, 85]. In comparison with the conventional indirect LC-MS and GC-MS methods, they establish a new approach to achieve a less time

consuming, more robust and more comprehensive detection of exogenous AAS. Moreover, as previously commented, some of phase II metabolites are long-term metabolites. Thus, the inclusion of these metabolites in the screening methods supposed an improvement in the detection capabilities of AAS misuse. Particularly, a screening method developed by our group allows the direct detection of 36 metabolites (7 unconjugated, 19 glucuronides and 10 sulfates) corresponding to 15 AAS using LC-MS/MS [84]. Moreover, due to the ongoing development of increasingly powerful analytical instruments as provided by TOF or orbitrap, direct injection or dilute-and-shoot methods have been already used for the detection of AAS [85, 86]. Tudela et al. proposed a method for the detection of 32 metabolites, excreted free or conjugated, based on dilute-and-shoot strategy and analysis by liquid chromatography-high resolution mass spectrometry [85]. Besides the reduced sample preparation required using dilute-and-shoot or direct injection, HRMS instruments allows the development of multi-target screening methods, such as presented by Gorgens et al. to screen for more than 200 analytes of various classes of doping agents including two conjugated metabolites from stanozolol and metandienone [86]. HRMS analyzers have been also applied for the detection of novel markers of exogenous and also for endogenous AAS [80, 87-89].

1.4.2 Endogenous AAS

1.4.2.1 Progress in endogenous AAS detection

In 1983 Donike *et al.* reported that the ratio of urinary concentration of T to its epimer epitestosterone (T/E) excreted as glucuronides was severely altered after the administration of these substances [90]. Their urinary concentrations suffered changes that results in an increased T/E value that could be detected by the adequate methods (GC-MS analysis). Based on the analysis of 2700 urine samples, an average basal value for the T/E was found to be around 1 and their values showed a logarithmic normal distribution with an upper limit value lower than 6 [90, 91]. The IOC established a T/E threshold value of 6, considering higher value than this limit as sufficient proof for endogenous AAS (EAAS) abuse. However, natural T/E values that exceeded the established threshold of 6 were reported [91-93], highlighting the limitations of the use of universal threshold values for T/E. Based on these results it was decided to investigate those T/E values between 6 and 10, meanwhile any sample with T/E higher than 10 was automatically declared as positive. In 2004, the introduction of GC/C/IRMS technique allowed for the differentiation between the exogenously administered steroids to those produced naturally in the body by their different relative content of ^{13}C. Due to the difficulties to apply GC/C/IRMS to every urine sample (high volume of urine required and time-consuming methods needed), WADA decided to use the threshold value of 4 for T/E as initial testing method to select suspicious samples, and the use of GC/C/IRMS only as confirmatory method. Additionally, the determination of urinary androsterone (A), etiocholanolone

(Etio) and DHEA excreted as glucuronides was implemented for the evaluation of a sample [94]. The reference ranges [95] and intra-individual stability of these parameters were also studied [56, 59].

However, various population studies demonstrated a distribution with two different lognormal populations due to genetic polymorphism between ethnicities [52, 96-98]. Some individuals would not exceed the threshold value of 4 even after an elevated dose of T [54]. Therefore, the universal cut-off limits based on population reference ranges were questioned whereas the idea of individual limits gained support [99, 100]. Moreover, these studies showed the high intra-individual stability of the measured T metabolites.

Thus, in 2007, an adaptive Bayesian model to evaluate longitudinally the individual values of the steroid profile markers was proposed by Sottas et al. [101]. The model progressively adapts the calculated upper and lower reference levels evolving from a population-based to a subjected-based approach as the number of recorded measurements per athlete grows. So, individual limits of the marker concentration or value can be established for every athlete and unexpected changes in his/her steroid profile can be detected. In many cases, this model showed a decrease of the number of false negative or atypical findings [102] and improvement of the detection time for the EAAS misuse [103, 104]. Hence, in 2014, the Bayesian adaptive model was applied by WADA to the Athlete Steroidal Passport, a module of the Athlete Biological Passport for recording the individual steroid profile of an athlete [105]. This

process is possible due to the informatics system Anti-Doping Administration & Management System (ADAMS) that collects and reports all the necessary information.

Currently, WADA has established a steroid profile composed by the urinary concentrations of T, E, A, Etio, 5α-androstane-3α,17β-diol (5αAdiol) and 5β-androstane-3α,17β-diol (5βAdiol) in the free and glucuronide fractions, and the ratios T/E, A/T, A/Etio, 5αAdiol/5βAdiol and 5αAdiol/E [105]. Today, the T/E is the most extensively used marker and its values are usually used as reference for the detection of T or other EAAS misuse.

1.4.2.2 EAAS routine analytical testing

An initial testing procedure is conducted to estimate the steroid profile of the athlete. In the case of an alteration in the steroid profile determined by the Adaptive model not supported in principle by any physiological reason, a confirmation procedure is mandatory.

The initial testing procedure for EAAS detection is conducted by GC-MS or GC-MS/MS instrumentation. The method consists on the quantitation of free and glucuronide metabolites including in the steroid profile after enzymatic hydrolysis and derivatization steps (see section 1.4.1.1 and Figure 1.9).

The confirmation procedure for the exogenous administration of EAAS is performed by GC/C/IRMS [106]. Exogenous steroids have a

low ^{13}C content which is significantly different from the endogenous steroids. GC/C/IRMS allows the differentiation between them by their relative content of ^{13}C.

1.4.2.3 Alternative tools/markers for the screening of EAAS misuse

The effective detection of the use of EAAS is one of the current major challenges in doping control. Research is focused in the identification of additional markers [81, 104, 107-113], optimization of sample preparation strategies for screening and confirmatory analysis [114-116], and the identification of factors potentially influencing the steroid profile [61, 117].

Regarding the identification of additional markers of EAAS administration, several studies have been conducted during the last years in order to investigate potential alternatives to the T/E that would increase the detection time of the EAAS misuse, i.e. would increase the period of time in which the misuse can be detected, and/or would be specific to a certain administration route

In a study conducted by Van Renterghem et al., the ratios 6α-OH-androstenedione/16α-OH-DHEA, 7α-OH-T/7β-OH-DHEA, 4-OH-androstenedione/16α-OH-androstenedione and DHT/5β-androstane-3α,17β-diol were proposed as additional markers for the detection of oral T misuse. However, these markers showed a large heterogeneity in response to T administration, with the T/E ratio providing the longest detection time in some volunteers, while other

markers extended the detection window in other volunteers [104]. These hydroxylated metabolites were also evaluated after DHT gel and DHEA oral administration. For DHT administration, DHT/5βαβ-Adiol is the marker that provided the longest detection time in 3 out of 6 volunteers. In case of DHEA, 5βαβ-Adiol/5ααβ-Adiol resulted in the longest detection in 4 out if 6 volunteers [118].

During the last years, the direct detection of phase II metabolites of EAAS is gaining importance as happens with exogenous AAS. In a work performed by our group four T metabolites conjugated with cysteine were identified and characterized in urine [107, 108]. The usefulness of these metabolites after the administration of different EAAS (T, DHT, DHEA) and administration routes (oral, intramuscular, transdermal) was evaluated [109, 110, 112, 113]. The evaluation of markers between cysteinyl metabolites based on population reference limits allowed improving the detection times of a single oral dose (120mg) of T between 3 to 6 times when comparing with traditional T/E. Furthermore, the use of ratios allowed the detection in individuals where no increase of T/E was observed after the oral administration. The use of individual thresholds and the application of some ratios allowed the detection of T transdermal administration whereas using the common marker T/E the detection remained impossible. In contrast, the evaluation of these conjugates after administration of other EAAS, such as DHT gel and DHEA oral, did not improve the detection times compared with the common markers of the steroid profile.

Later, the presence of two glucuronides (6β-hydroxyandrosterone 3-glucuronide and 6β-hydroxyetiocholanolone 3-glucuronide) that increased after oral T administration and greatly resisted to enzymatic hydrolysis with β-glucuronidase was also described by our group [81, 119]. In recent studies, these metabolites together with the main glucuronides included in the steroid profile and their ratios have been evaluated in different excretion studies. In case of oral T misuse, ratios between T and epitestosterone glucuronides in denominator with 6β-hydroxyandrosterone 3-glucuronide and 6β-hydroxyetiocholanolone 3-glucuronide presented much larger detection times than those obtained by the established T/E marker for all volunteers, extending the detection capability up to 96h [111]. Quantitative method for these metabolites has been recently applied in urine samples after T intramuscular and transdermal administration in Caucasian and Asian population. However, detection times reached were close to the ones obtained with T/E [112, 113].

In addition, steroid sulfate fraction has been also investigated [87, 120-122]. The main limitation of preceding studies is that they are only focused on some of the possible sulfate metabolites and do not cover others that could be of interest. After administration of DHEA to seven healthy volunteers, an increase in the concentrations of epiandrosterone sulfate, and some androstanediol and androstenediol sulfates was observed [123]. In another study, Etio sulfate was revealed to be promising to improve detection after oral T administration in individuals of different ethnicities [121].

Moreover, in a recent study with four volunteers using IRMS measurements, epiandrosterone sulfate was identified as the biomarker allowing for a more prolonged detection of oral T misuse in subjects of different genotypes of the UGT2B17 enzyme [122] up to 5 days compared to 1 day if currently drug testing procedure were used. Excretion studies after application of T (n=2) and after oral administration of 4-androstenedione (n=1), DHT (n=1), epiandrosterone (n=1) and DHEA (n=1) was also evaluated using IRMS. The results also demonstrated that the response of epiandrosterone excreted as sulfate was found to be significantly influenced for longer periods of time [120, 124].

Nowadays, the sample treatment and strategies used for sports drug testing are been optimized in order to simplify the initial testing procedures. For instance, a simplified procedure for IRMS screening analysis was developed, allowing the analysis of a larger volume of samples in normal routine conditions [115]. Also, HRMS analyzers allow the accurate quantitation of EAAS using dilute-and-shoot or direct injection approaches, such as proposed by Abushareeda et al. [116]. Other strategy, conducted by Jardines et al. [114], consists on the inclusion of the IRMS data into ADAMS and to apply the same Bayesian statistical treatment used for the longitudinal evaluation of T/E ratio and the other main markers of the steroid profile.

1.5 Metabolic studies of AAS

Metabolic studies are essential to identify as many metabolites as possible and to select the best marker of the administration. For the detection of AAS misuse in doping control analyses, the best marker is not always the most abundant but the metabolite excreted for the longest period after the administration of the drug. These metabolic studies are based on the following steps: detection of potential metabolites (section 1.5.1 and 1.5.2); study of the excretion profile of each metabolite to evaluate their detection times (section 1.5.3); and confirmation of the structure by synthesis of the authentic material (section 1.5.4).

Metabolism of AASs is normally studied with human urine samples collected after the administration of the drug (*in vivo*). However, this methodology is limited by the availability of human excretion urines due to ethical considerations. An approach based on the use of chimeric mouse with humanized liver for metabolic studies have been successfully validated and applied for the detection of several AAS metabolites [125-128]. On the other hand, *in-vitro* studies using microsomes or fungus (*Cunninghamella elegans*) provide a feasible alternative and metabolism of several steroids has been studied by this system [129-131]. Higher concentrations can be used on the metabolic studies using either animal models or *in vitro* systems helping to the detection of AAS. However, the main disadvantages are the small volume of sample obtained and the impossibility to directly extrapolate the results to the function of the human organism. For both cases, comparison between a blank (generally a

pre-administration sample) and a post-administration sample was used to detect the metabolites of the drug. Only peaks detected in post-administration samples with consistent MS behavior of an AAS-metabolite structure are considered AAS metabolites.

A general overview of evaluation of phase I and phase II metabolism will be explained, greater emphasizing in phase II metabolism, which is the focus of this thesis.

1.5.1 Evaluation of phase I metabolism

Most of the metabolic studies published for AASs focus on the detection of phase I metabolites. Firstly, GC-MS was used for the analysis of phase I metabolites because it was the unique available technique. Since the introduction of LC-MS, it was also used for metabolic studies. The sample treatment, the mass spectrometric behavior and, the different strategies applied for the detection of phase I metabolites using GC-MS and LC-MS are summarized in Table 1.3.

Table 1.3. Overview of metabolic studies using GC-MS and LC-MS of phase I metabolites of AAS.

	GC-MS analysis	LC-MS analysis
Sample treatment	**Hydrolysis** (see section 1.5.2)	
	LLE with TBME in alkaline conditions [132] / **SPE** with C18, mixed-mode cartridges [133]. Others: SPME and SPE+LLE[134, 135]	
	Derivatization common procedure with MSTFA Others: methyloxime-TMS derivatives [136]	Only derivatization to convert poorly-ionizable analytes into easily-detectable analytes (increase the sensitivity)
MS behaviour	**EI**. Fragmentation extensively investigated [73, 132, 138]. Softer ionization methods have been successfully tested for the target detection of some AAS [137, 138].	**ESI** (most frequent) and APCI. - Only conjugated keto-function and/or nitrogen atom ionized as $[M+H]^+$ - Formation of adducts (such as $[M+NH_4]^+$, $[M+Na]^+$, $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$) is necessary for AAS without these functions [74]. Several studies showed the fragmentation of steroids [139, 140].
Strategies	Evaluation **full scan** chromatogram of post-administration samples[141, 142] ✓ Detect unforeseen metabolic biotransformation χ Poor sensitivity Evaluation of **extracted ion chromatograms** (EIC) corresponding to common ions [143] **Targeted analysis** using SIM (GC-MS) or SRM mode (GC-MS/MS) [143] **Synthesis** of the potential metabolites and checking by a target analysis [144] ✓ High sensitivity Prediction of metabolism with **principal-component analysis** [145] ✓ Metabolism of a certain AAS can be broadly predicted.	Evaluation of **full scan** chromatograms with nominal single mass analyzers χ Low sensitivity and selectivity χ Detection only the abundant metabolites **Targeted analysis** using SRM ✓ High sensitivity and selectivity χ Only predicted metabolites **Open detection methods** [78] χ Lower sensitivity and selectivity ✓ Unknown metabolites (but only which produce these common ions or losses)

1.5.2 Evaluation of phase II metabolism

Phase II metabolism have been normally studied using indirect methods consisting of a specific hydrolysis of the conjugates to release the phase I metabolite, which have been then analyzed by GC-MS or LC-MS (as explained in section 1.5.1). However, the introduction of LC-MS(/MS) allows the direct analysis of phase II metabolites skipping the hydrolysis step.

A revision of indirect and direct methods applied for the detection of glucuronides, sulfates and other conjugates will be described.

1.5.2.1 Glucuronides

Indirect methods

Generally glucuronides have been detected after enzymatic hydrolysis with β-glucuronidase preparations from various sources, being the gold standard from *E.coli* [65, 75]. Several glucuronide metabolites were described using indirect methods [32]. For example, 17-epimetendiol and 18-nor-17β-hydroxymethyl-17α-methyl-5β-androsta-1,4,13-triene-3-one glucuronide metabolites of metandienone [146, 147] or, 17α-methyl-5α-androstan-3α,17β-diol and 17α-methyl-5β-androstan-3α,17β-diol glucuronide metabolites of methyltestosterone [148]. As previously commented, efficiency of the hydrolysis may be influenced by several factors (incubation time, temperature, pH, salts concentration) but it is substantially affected by the enzyme preparations due to the possible interconversion of some steroids [75, 149]. Chemical hydrolysis (e.g., use of sodium periodate and acidic hydrolysis) have been also reported [150, 151].

However, enzymatic hydrolysis is preferred because it avoids undesired effects shown by chemical hydrolysis (e.g., degradation of some analytes and increase of matrix interferences arising from the degradation of macromolecules). After the hydrolysis step, the extraction and the detection of phase I metabolites is performed as explained in section 1.5.1.

Apart from the loss of information about the distribution of the different sites of conjugation, glucuronide metabolites partially or totally resistant to enzymatic hydrolysis cannot be detected using indirect methods. However, these conjugates can be detected by direct analysis such as reported for T or stanozolol [80, 81].

Direct analysis

The clean-up of the samples and the pre-concentration of the analytes to reach the required sensitivity are critical steps for the direct detection of phase II metabolites. LLE, SPE or liquid-phase microextraction have been described for the targeted detection of AAS glucuronide metabolites [152, 153].

Glucuronides are usually ionized using electrospray both in negative and positive modes (Table 1.4) [74]. Conjugates with a 3-keto function exhibited the $[M+H]^+$ ion, whereas steroid glucuronides with 17-keto function or unconjugated 3-keto moiety ionized preferentially by adduct formation ($[M+NH_4]^+$). In negative mode, all glucuronides ionized as $[M-H]^-$ due to the deprotonation of the acidic carboxylic group on glucuronide moiety [81]. Most of the

common ions and losses observed in the CID analysis in both modes coming from the glucuronide moiety, but at high collision energies ions of the free AAS structure are also formed (Table 1.4). Among others, T and stanozolol resistant glucuronide metabolites have been described using direct analysis [80, 81].

1.5.2.2 Sulfates

Indirect methods

Unlike glucuronides, there is no universal method for the cleavage of all steroid sulfates. The hydrolysis of sulfate conjugates is also performed using enzymatic or chemical hydrolysis. Different enzyme with sulfatase activity have been reported [75, 154], the preparations from *Patela vulgate* and *Helix Pomatia* with both β-glucuronidase and arylsulfatase activities are the most commonly used [155]. Depending on the position of the sulfate moiety and the α/β configuration, some sulfates show partial or total resistance to enzymatic hydrolysis. The enzymatic hydrolysis with *Helix Pomatia* can also lead to steroid conversion, degradation, and artifact formation. Chemical hydrolysis is achieved using acid conditions (hydrochloric or sulfuric). The hydrolysis is also influenced by temperature, time and acid selected [75]. Other chemical hydrolysis were tested such as the use of trimethylchlorosilane for the hydrolysis or methanolysis for the cleavage of both glucuronide and sulfate in a single step [75, 156]. This approach has had little impact in doping control methodologies, mainly because the development of a robust analytical method for GC-MS analysis of steroids originating from their sulfates has not

proven to be simple. In spite of all these limitations, sulfate metabolites have been described for some endogenous [123] and exogenous AAS [32] using GC-MS methods.

Direct analysis

Sulfate metabolites are successfully extracted from matrix with polar solvents like ethyl acetate [51, 77]. SPE (e.g., with quaternary ammonium or C18 cartridge) are also used [156, 157]. Moreover, as well as for glucuronides, the aqueous nature of the urine allows for applying dilute-and-shoot strategies for metabolic studies [85].

Steroid sulfates are almost exclusively ionized in negative mode as $[M-H]^-$ [77] (Table 1.4). In positive mode, only steroid sulfates with a conjugated 3-keto group ionized as $[M+H]^+$. Formation of characteristic adducts in each ionization mode has been also observed [158]. Common ions and common losses of sulfates are depicted in Table 1.4. For some steroid sulfates, specific neutral losses were observed (e.g., loss of a methyl group for boldenone sulfate). Several studies have identify new sulfate metabolites using direct detection for AAS, such as boldenone, methenolone, methyltestosterone, metandienone, clostebol, stanozolol or 4-chlorometandienone [48, 51, 77, 79, 82, 83, 88].

Table 1.4.Mass spectrometric behaviour of glucuronide and sulfate metabolites of AAS.

Conjugate	ESI mode	Precursor ion	Common losses (Da)		Common ions (*m/z*)	
Glucuronides	ESI+	$[M+H]^+$	$[M+H\text{-gluc}]^+$	176	$[gluc+H]^+$	141
			$[M+H\text{-gluc-}H_2O]^+$	194	$[gluc+H\text{-}H_2O]^+$	159
			$[M+H\text{-gluc-}2H_2O]^+$	212	$[gluc+H\text{-}2H_2O]^+$	177
		$[M+NH_4]^+$	$[M+NH_4\text{-}NH_3\text{-gluc}]^+$	193		
			$[M+NH_4\text{-}NH_3\text{-gluc-}H_2O]^+$	211		
			$[M+NH_4\text{-}NH_3\text{-gluc-}2H_2O]^+$	229	Those of the free AAS	
	ESI-	$[M-H]^-$	$[M-H\text{-gluc}]^-$	176	$HOCH_2CO_2^-$	75
					$[gluc\text{-}H\text{-}H_2O\text{-}CO_2]^-$	85
					$[gluc\text{-}H\text{-}H_2O\text{-}CO_2\text{-}CO]^-$	113
					$[gluc–H–H_2O]^-$	157
					$[gluc–H]^-$	175
Sulfates	ESI+	$[M+H]^+$	$[M+H\text{-}SO_3]^+$	80	Those of the free AAS	
			$[M+H\text{-}SO_3\text{-}H_2O]^+$	98		
	ESI-	$[M-H]^-$	-	-	HSO_4^-	97
					SO_3^-	80

*gluc: dehydrated glucuronide moiety

1.5.2.3 Other conjugates

Additional metabolic pathways such as conjugation with glutathione, N-acetylglucosamine or bisconjugation (bisglucuronides, diglucuronides, bissulfates and bisconjugates) have been described [34-40].

Conjugates with cysteine and N-acetylcysteine can be indirectly detected by release of the cysteine moiety by alkaline treatment of the sample [39]. Once the conjugate is released, strategies for the detection of phase I metabolites (see section 1.5.1) can be applied [78, 107]. The direct detection of AASs conjugated with cysteine and N-acetylcysteine has also been reported, such as for boldione or T [39, 159]. For these metabolites, the sensitivity for direct detection is clearly less than the obtained by indirect methods [39].

Other phase II metabolites not systematically studied for AAS are bisglucuronides, diglucuronides, bissulfates and bisconjugates.

Glucuronidation reaction could proceed on another functional group of monoglucuronide leading to bisglucuronides or diglucuronides. In the first type, two separated functional groups on the same molecule are conjugated with glucuronic acid, and they have been described for compounds containing several hydroxyl groups such as bilirubin, morphine or estradiol [36, 160, 161]. Most of AAS phase I metabolites also contain several hydroxyl groups and, for this reason, the formation of bisglucuronides may also be expected. In the second type, glucuronidation occurred two times at a single

functional group of the aglycone [37, 38]. Diglucuronide metabolites have been described in some *in vitro* studies for endogenous steroids, such as estrone, T and DHT [36-38].

The formation of bisconjugated metabolites (conjugation of a functional group with glucuronic acid and a second functional group with sulfate) has been described for some AAS. AAS having a 17β-hydroxy-17α-methyl feature in their structure form the 17β-sulfate that decomposes in urine to yield several dehydration products (e.g. 18-nor-17,17-dimethyl-13-ene) and the corresponding 17-epimer (17α-hydroxy-17β-methyl). For metandienone, both the 17-epimer (17β-methyl-5β-androstan-3α,17α-diol) and dehydration product (18-nor-17,17-dimethyl-androsta-13-ene-3α-ol) were also detected in urine as glucuronide metabolites [146, 162], demonstrating the simultaneous 3α-glucuronidation and 17β-sulfation. In case of methyltestosterone, 17β-methyl-5α-androstan-3α,17α-diol and 17β-methyl-5β-androstan-3α,17α-diol have also been reported as 3α-glucuronide metabolites, therefore the simultaneous 3α-glucurononidation and 17β-sulfation were also provided [50]. These bisconjugated metabolites only can be detected using the conventional methods if the hydrolysis of glucuronide moiety is accomplished. However, up to now, bisconjugates have not been directly detected.

Finally, bissulfates (two separated functional groups conjugated with sulfate) metabolites have been also proposed in the literature. As example, for methyltestosterone 17β-methyl-5α-androstan-

3α,17α-diol and 17β-methyl-5β-androstan-3α,17α-diol were excreted in urine as 3α-sulfates, resulting from sulfation in 3α- and 17β-position. It is important to note that 17β-methyl-5α-androstan-3α,17α-diol 3α-sulfate is the long-term metabolite for the detection of methyltestosterone misuse [51].

These metabolites cannot be detected using the conventional approaches due to the challenge with the hydrolysis of sulfates. Recently a study conducted by McLeod et al. in collaboration with our group, proposed a method for the direct detection of bissulfate metabolites, describing bissulfates of estriol, pregnanediol and tibolone [40].

1.5.2.4 LC-MS/MS strategies for the detection of steroid metabolites

In general, most of the MS strategies for the reevaluation of steroid metabolism require the comparison of samples collected before and after drug administration. Targeted or untargeted methods can be applied for this purpose (Figure 1.10).

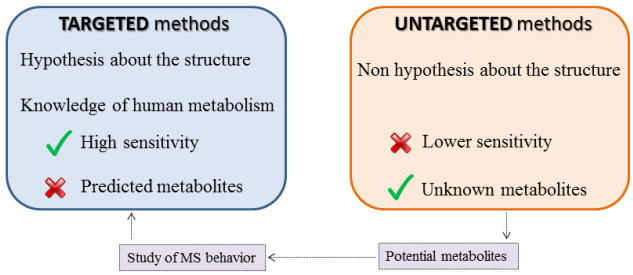

Figure 1.10. Targeted and untargeted methods for the detection of unknown metabolites.

The triple quadrupole mass spectrometer (QqQ) is usually the instrument of choice for targeted methods due to its high sensitivity and specificity when working in the selected reaction monitoring (SRM) mode (Figure 1.11). Moreover, different acquisition modes, precursor ion (PI) and neutral loss (NL) scan, have proven useful in several untargeted scenarios, among them the detection of unknown metabolites which their nature can be, to some extent, predicted (e.g. detection of phase II metabolites of steroids) (Figure 1.11).

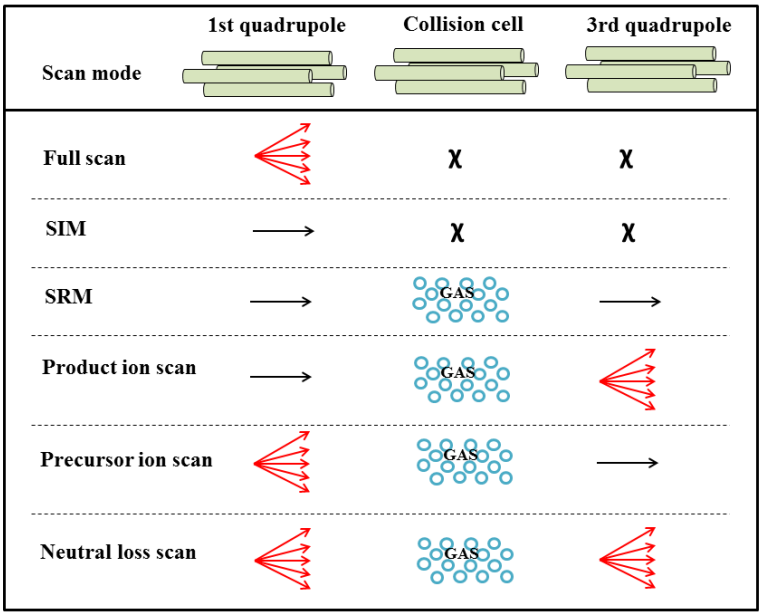

Figure 1.11.Acquisition modes available for QqQ analyzer.

Additionally, untargeted approaches frequently take advantage of the analytical properties of high resolution analyzers such as TOF or Orbitrap system. Normally these strategies compare full-acquisition chromatograms obtained for a negative and positive sample, allowing the detection of potentially any compound present in the

sample with the main limitation being the ionization of the molecule. However, they also have additional limitations. Thus, analytes which require a specific chromatographic method can be difficult to detect. Moreover, the elucidation of the structure of the metabolite remain the main bottlenecks for untargeted approaches.

This section will focus on a brief review of strategies performed typically in a QqQ instrument for the detection of phase II metabolites of steroids.

Precursor ion (PI) scan strategies

In the PI scan methods, the first quadrupole (Q1) scans all ions across a mass range generated in the ion source, the collision cell (q2) fragments all these ions, and then the third quadrupole (Q3) selects one specific product ion.

The common fragmentation behavior of phase II metabolites (Table 1.4) promotes the development of PI strategies for the open detection of metabolites without any restriction in the metabolite structure rather than the conjugation moiety. Thus, PI scan of *m/z* 113, 85 and 75 in negative mode and, *m/z* of 177, 159 and 141 in positive mode can be used for the open detection of glucuronides. In the case of sulfates, PI scan method of *m/z* 97 in negative mode is the most widely used.

The main advantage of PI strategies is the fact that, although guided, they can be considered as untargeted approaches, so

compounds with unknown *m/z* but with a known fragmentation (as phase II metabolites, Table 1.4) are detected as potential metabolites. On the other hand, the main limitation is the limited structural information obtained because most of the product ions are related with the conjugated moiety. For this reason, in order to establish the metabolite structure, the hydrolysis of the metabolite and analysis of the free steroid by either GC-MS or LC-MS is normally performed.

Neutral loss (NL) scan strategies

In this operation mode, the instrument scan a mass range in Q1, fragment all ions in the q2, and then Q3 scan with a constant difference of *m/z*, corresponding to the selected loss. This strategy allows for the identification of all precursor ions that have lost a selected mass, which might be correlated to a common chemical structure. Similar to precursor ion, this scan mode is useful for the detection of compounds sharing a common structure.

Common losses are appearing in the CID spectra of phase II steroid metabolites. NL of 176, 194, 211 and 229 Da in positive mode can be used for the open detection of glucuronide metabolites. Regarding sulfates, NL of 80 Da is common in positive mode, but is limited to steroid sulfates that are easily ionizable (i.e. with a conjugated keto moiety) (Table 1.4). Glucuronides and sulfates for both exogenous [48, 79, 82] and endogenous AAS [81, 163] have been described using open scan methods (PI and NL).

Moreover, PI and NL from the free AAS structure have also been described for some AAS metabolites, so these strategies can be also applied for their detection. As an example, one study conducted by our group [82] revealed a NL of 36/38 Da (loss of HCl) in negative mode for the detection of sulfates metabolites containing chlorine (Cl). Using this approach three metabolites were detected in excretion study urines after clostebol (4-chlorotestosterone) oral administration. Also the CID behavior revealed the presence of the ions *m/z* 131 and 143 for clostebol metabolites with unmodified A-ring and having a 3-keto function, so PI scan methods were also developed. Other example is the NL scan of 20 Da (loss of HF) which was applied for the specific detection of fluoxymesterone metabolites [164].

Theoretical SRM strategies

The knowledge about the metabolic pathways of steroids and their ionization and CID behavior is the key of this type of approach. Based on this knowledge, metabolites are predicted and then, theoretical SRM methods for the detection of these metabolites are developed. The main limitation is that only predicted metabolites following an expected MS behavior will be detected. Against this limitation, the main advantage of this strategy is the sensitivity, being the most sensitive acquisition of MS modes. Thus, SRM is commonly used for both qualitative and quantitative methods for determination of exogenous [84, 165] and endogenous AAS [153, 166]. Moreover, this approach has been successfully employed for the study of the metabolism of several steroids such as boldenone,

methytestosterone, metandienone, stanozolol, clostebol or 4-chlorometandienone [48, 51, 77, 79, 82, 83].

Product ion scan

Product ion scan is useful to obtain the largest amount of structural information in order to characterize the structure of the metabolites. In the product ion scan, a precursor ion is selected in Q1, fragment at adequate collision energy in q2 and then Q3 scan all ions across a mass range.

A product ion scan can also be performed in QTOF instruments obtaining all the product ions with accurate mass and, therefore, it improves the structural information of the ions.

1.5.3 Evaluation of detection times

After the detection of the new metabolites, the excretion profiles are evaluated in order to stablish the detection times. Detection time is the time that each metabolite is detected in the urine samples after administration of the AAS. Excretion study samples from different individuals, ethnics, doses and routes of administrations are used. Further, the detection times of the new metabolites are compared with those of the conventional metabolites monitored using the conventional methods in anti-doping controls, in order to stablish the best marker for long-term detection of AAS misuse, meaning the most retrospective metabolite.

1.5.4 Confirmation of structure by synthesis

The structure of the most interesting metabolite should be confirmed. 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. Thus, the hydrolysis of the conjugate and the subsequent analysis of the free steroid by either GC-MS(/MS) or LC-MS(/MS) is usually performed to stablish the structure of the metabolite. However, the chemical synthesis of the metabolite is the ultimate confirmation of its structure. The comparison of the MS spectrum of the reference material and the investigated metabolite together with a correct characterization is required. Currently, the nuclear magnetic resonance spectroscopy (NMR) is one of the most versatile and commonly used techniques for the characterization of the structure.

The synthesis of standards allows for the study of their ionization and fragmentation behavior under different spectrometric conditions and this knowledge can also be the basis for the development of new open detection strategies for the identification of new metabolites. Moreover, the analysis of the product ion mass spectra of a standard at different collision energies can reveal the presence of specific ion transitions for this compound, an important tool on the development of qualitative and quantitative methods.

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JUSTIFICATION

AND OBJECTIVES

Chapter 2

2.1. Justification

A continuous improvement in the detection capabilities of AAS misuse is needed, because they are the most commonly detected substances in doping control analyses in sports. Most AAS are widely metabolized and excreted in urine mainly as phase II metabolites.

Studies on AAS metabolism has been evaluated for many years by GC-MS or LC-MS(/MS) using indirect methods, i.e. after hydrolysis of samples, mainly using β-glucuronidase enzymes to release the phase I metabolites, allowing the detection of free and glucuronide metabolites hydrolysable under these conditions. However, glucuronide metabolites resistant to enzymatic hydrolysis, sulfates and other conjugates have not been comprehensively studied.

The irruption of the LC-MS has shown several possibilities such as the direct detection of phase II metabolites, avoiding hydrolysis and derivatization steps. Some of these phase II metabolites (glucuronides, sulfates), despite being minor metabolites and undetectable when using traditional methods, provide longer retrospectivity compared to the conventional metabolites.

In addition, LC-MS technology allows the simplification of initial testing and confirmation procedures used in anti-doping controls through the direct analysis of phase II metabolites.

These results suggest the need to re-evaluate of phase II metabolism of exogenous and endogenous AAS using direct analysis by LC-MS technology, in order to identify new markers and to evaluate their usefulness to detect AAS administration and also to allow their incorporation in the initial testing analysis based in LC-MS methods used in doping controls.

2.2. Objectives

The main objective of this thesis was to evaluate the usefulness of the direct analysis of phase II metabolites of AAS to improve the detection of their misuse in sport.

The thesis has been divided in three different parts and specific objectives for each part are proposed to reach this goal:

❖ **Part I.** Evaluation of the direct detection of AAS glucuronide metabolites, including those resistant to enzymatic hydrolysis, using LC-MS/MS methods. The study was performed with metandienone.

❖ **Part II.** Development of LC-MS/MS strategies for the detection of bisglucuronide metabolites of AAS:

- Synthesis and study of ionization and collision-induced dissociation behavior of AAS bisglucuronide metabolites.
- Application of developed strategies for the detection of bisglucuronide metabolites after norandrostenediol oral administration.

❖ **Part III.** Evaluation of endogenous steroid sulfate metabolites as markers of the administration of endogenous AAS.

- Development and validation of a method for the quantitation of endogenous steroid sulfate metabolites.
- Evaluation of sulfates as potential markers of T misuse after different administration routes and in different ethnicities.

LC-MS/MS DETECTION OF UNALTERED GLUCURONOCONJUGATED METABOLITES OF METANDIENONE

Chapter 3

This chapter has been published as:

Esquivel A, Pozo O.J, 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.* 2017;9:534-544.

IONIZATION AND COLLISION INDUCED DISSOCIATION OF STEROID BISGLUCURONIDES

Chapter 4

This chapter has been published as:

Esquivel A, Matabosch X, Kotronoulas A, Balcells G, Joglar J, Ventura R. Ionization and collision induced dissociation of steroid bisglucuronides. *J Mass Spectrom*. 2017;52:759-769.

DIRECT QUANTITATION OF ENDOGENOUS STEROID SULFATES IN HUMAN URINE BY LIQUID CHROMATOGRAPHY-ELECTROSPRAY TANDEM MASS SPECTROMETRY

Chapter 5

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Esquivel A, Alechaga E, Monfort N, Ventura R. Direct quantitation of endogenous steroid sulfates in human urine by liquid chromatography-electrospray tandem mass spectrometry. *Drug Test Anal*. 2018;1-10.

SULFATE METABOLITES IMPROVE RETROSPECTIVITY AFTER ORAL TESTOSTERONE ADMINISTRATION

Chapter 6

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Esquivel A, Alechaga É, Monfort N, Ventura R. Sulfate metabolites improve retrospectivity after oral testosterone administration. Drug Test Anal. 2019 Mar 20;11(3):392–402. DOI: 10.1002/dta.2529

EVALUATION OF SULFATE METABOLITES AS MARKERS OF INTRAMUSCULAR TESTOSTERONE ADMINISTRATION IN CAUCASIAN AND ASIAN POPULATIONS

Chapter 7

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Esquivel A, Alechaga É, Monfort N, Yang S, Xing Y, Moutian W, et al. Evaluation of sulfate metabolites as markers of intramuscular testosterone administration in Caucasian and Asian populations. Drug Test Anal. 2019 May 8;dta.2598. DOI: 10.1002/dta.2598

DISCUSSION

Chapter 8

8.1 Discussion

Doping control laboratories need a continuous improvement of the detection of both exogenous and endogenous AAS misuse because they are widely used by athletes. AAS are metabolized and excreted in urine mainly as phase II metabolites. Anti-doping laboratories have relied for many years on the detection of glucuronide metabolites identified by GC-MS methods and, more recently, by LC-MS methods using indirect methods, i.e after hydrolysis with β-glucuronidase enzyme to release the phase I metabolites. In recent years, a high number of new phase II metabolites has been directly detected using LC-MS technology, being some of them long-term metabolites [1-8]. For this reason, the objective of the thesis was the evaluation of the direct analysis of phase II metabolites of exogenous and endogenous AAS to improve the detection of their misuse in sport.

Glucuronides are the most abundant phase II metabolites of AAS. As mentioned before, indirect methods using β-glucuronidase enzymes were used in metabolic studies to detect new glucuronide metabolites and, therefore, only glucuronides hydrolysable under these conditions were detected. However, glucuronide metabolites of stanozolol and T which are poorly or not hydrolyzed using β-glucuronidase enzyme have been recently described using LC-MS/MS [4, 9]. Thus, there is a need to re-assess the AAS glucuronide metabolites, and the first objective of the thesis was to evaluate LC-MS/MS strategies to directly detect steroid glucuronides to overcome the limitations of the indirect detection (Chapter 3).

Metandienone is one of the most frequently detected AAS in doping controls and it was used as a model compound.

Based on the common ionization and CID behavior of steroid glucuronides, different MS strategies were developed for their detection using QqQ instruments: open scan methods based on NL and PI scan, and target SRM methods using characteristic ion transitions of potential metabolites. As expected, the application of SRM methods resulted in a more sensitive methodology to detect potential metabolites (13 metabolites) when compared with PI and NL methods (1 metabolite using total ion chromatogram (TIC), and 7 metabolites using extracted ion chromatogram (XIC)). Open scan methods are the best approach to detect unknown major metabolites, including those resulting from unforeseen metabolic pathways, when TIC data from pre- and post-administration samples can be compared. However, for minor metabolites, the low sensitivity is nowadays an important limitation and, strategies for the detection of predicted metabolites (e.g. evaluation of XIC of PI and NL methods, or SRM methods) are required. In order to obtain the maximum information about the metabolism of a specific AAS, the combination of both methodologies seems to be the most suitable approach.

Using LC-MS/MS a higher number of metandienone glucuronide metabolites than those previously reported were detected. However, considering the detection times after metandienone administration, the most interesting glucuronide metabolites were those already

described using the indirect analysis by GC-MS. Among the 13 metabolites detected, one of them was resistant to hydrolysis with β-glucuronidase however it showed a short detection time. Therefore, LC-MS/MS has shown to be useful to directly detect unknown metandienone glucuronide metabolites, including those resistant to enzymatic hydrolysis, and its use is recommended in future for a comprehensive evaluation of the glucuronide fraction in metabolic studies of other AAS.

The LC-MS technology enables a simplification of the initial testing and confirmation procedures of doping control, being the direct analysis of the urine sample after dilution ('dilute-and-shoot') the most common approach for most doping agents. In the case of AAS, the analysis by GC-MS/MS after hydrolysis and derivatization is still being used because some phase I metabolites (e.g., completely reduced metabolites) do not ionize using ESI. However, these phase I metabolites are excreted in urine conjugated with glucuronic acid or sulfate, and these conjugates can be ionized. Therefore, the detection of the phase II metabolites overcomes some of the problems observed when monitoring the phase I metabolites. Initial testing procedures based on the direct analysis of some AAS phase II metabolites by LC-MS technologies have already been described [10-12].

In this line, in Chapter 3 the retrospectivities obtained using the direct analysis of steroid glucuronides of metandienone by LC-MS/MS were also evaluated and compared with those obtained

using the analysis of the corresponding aglycones by GC-MS/MS. The results showed that the retrospectivity obtained by each technique was dependent on the metabolite: epimetendiol (MVII) was detected during the same time by both technologies; metabolite MI was detected for longer time by LC-MS/MS; and metabolite MII was detected for longer by GC-MS/MS. It is worth to notice that the preconcentration step of the GC-MS/MS method was higher than the one used with the LC-MS/MS method (50 vs 10) and this may be one of the reasons of the longest detection of some metabolites using the GC-MS/MS method. These results demonstrate the potential of the direct analysis of glucuronides in initial testing procedures in doping control. Although nowadays the sensitivity obtained is limited for some of the glucuronides and a preconcentration of the sample is needed, it is expected that instrumentation developments leading to more sensitive equipment will help in the inclusion of these compounds in initial testing procedures, even using dilute-and-shoot strategies.

One of the limitations to incorporate phase II metabolites in the testing procedures is the lack of reference standards and analytical data. In Chapter 3, analytical data of the intact glucuronides was presented for first time for some of the metabolites. These data will be useful for anti-doping laboratories to incorporate them in routine initial testing and confirmation procedures.

The formation of bisconjugated metabolites of AAS is known. Bissulfates and bisconjugates (glucuronide and sulfate) have been

described for some AAS. Two 3α-sulfate metabolites described for methyltestosterone have 17α-hydroxy structure and, hence, they result from the degradation in urine of a 17β-sulfate; therefore, the initial metabolite was a bissulfate (3α,17β-bissulfate) [2]. Epimethendiol 3α-glucuronide and some glucuronide metabolites of methyltestosterone and other AAS have also 17α-hydroxy structure, resulting from degradation of 17β-sulfates in urine, and, therefore, the initial metabolite was a 3α-glucuronide-17β-sulfate [2, 3]. In consequence, it might be expected that BG metabolites are also formed for AAS or their phase I metabolites, containing two hydroxyl groups in the structure. BG metabolites have been already described for some compounds [13-15], however, they have not been studied for AAS. For that reason, another objective of the thesis was to study the detection of BG metabolites of AAS (Chapter 4).

In order to develop LC-MS/MS strategies to detect BGs of AAS in urine, the ionization and CID behavior of steroid BGs was studied using synthetized metabolites. The chemical synthesis of steroid BGs was tested for 23 AAS phase I metabolites, and it was successfully for 19. Metabolites with 17α-methyl structure did not form BGs, probably due to steric effects that avoid the conjugation in the 17β-hydroxy group. This fact might indicate that BGs of these phase I metabolites are not formed in the body either for the same reason. A common ionization behavior was observed with single and double charged molecular ions in negative mode, as observed for steroid bissulfates [16]. The most important product ions observed after CID were result of either the neutral/ion losses of the

glucuronide moieties or ions coming from the glucuronide moiety. The low natural abundance of double-charged metabolites and the use of ion transitions with an increase of *m/z* made the monitoring of these common ion losses a very specific strategy for the detection of these conjugates, as reported also for bissulfate metabolites [16]. Ions from the fragmentation of steroid structure were not detected and, therefore, the identification of steroid structure using product ion scan data would be difficult.

Based on the MS behavior, targeted and untargeted MS strategies can be developed for the detection of BGs. In our study, as a proof of concept, only a theoretical SRM method for the targeted detection of NDIOL BG metabolites was developed, resulting in the detection of one minor BG metabolite in the first hours after administration. Taking into account the MM of the metabolite, the most feasible structure for the metabolite is hydroxy-NDIOL, hydroxy-androsterone or hydroxy-etiocholanolone. Estranediols are phase I metabolites of NDIOL and we expected the formation of BG metabolites for these aglycones. However, they were not detected probably because estranediols are minor metabolites and even if BGs are formed the sensitivity of the method was not sufficient to detect them. Additionally, the developed strategy was also applied to samples collected after oral administration of nandrolone (2 volunteers) and testosterone (3 volunteers) (results not presented in this thesis), and unfortunately, BG metabolites were not detected. These results suggest that bisglucuronidation is a minor metabolic pathway for steroids. The substrate regio- and

stereoselectivity of UGTs together with the stiffness of the UGT site of action (e.g. UGT2B7 enzyme conjugates only at 3-hydroxy position, whereas UGT2B15 only at 17-hydroxy position) can explain the low amounts of steroid BGs found in urine. Nevertheless, these low concentrations do not discard the potential use of BGs as markers of the administration of specific AAS.

In the last years, many of the studies to detect new phase II metabolites of AAS by LC-MS have been focused on the detection of sulfate metabolites of exogenous AAS [1-3, 6-8]. However, studies on the role of sulfate metabolites in the detection of endogenous AAS misuse are scarce. For that reason, the last objective of the thesis was to evaluate the potential of endogenous steroid sulfate metabolites as markers of the administration of endogenous AAS. The detection of endogenous AAS misuse is one of the main challenges in doping control and it is currently performed using the steroid profile. However, improvements are needed to prolong the detection windows and the steroid profile should not be seen as a static tool since the inclusion of additional metabolites may improve the detection capabilities of EAAS abuse.

The first objective was to develop an analytical method able to quantify the urinary endogenous steroid sulfates related with T metabolism to obtain reliable data for the evaluation (Chapter 5). The analysis of sulfate metabolites of steroids is known to be difficult. The indirect approach has important limitations because there is no universal method to hydrolyze all steroid sulfates and, in

addition, the hydrolysis methods available provide different hydrolysis efficiency, depending on the structure of the analyte [17, 18]. Therefore, the application of indirect methods may lead to unreliable results for some of the analytes and, for that reason, the direct analysis of steroid sulfates using LC-MS was mandatory. The method included a sample preparation specific for steroid sulfates, where the most abundant glucuronide metabolites were eliminated to avoid interferences during analysis. Liquid chromatographic conditions needed also to be accurately optimized to obtain good peak shapes of steroid sulfates and adequate resolution between all isomeric compounds (A-S/Etio-S and epiA-S/DHT-S).

The developed method provided an accurate quantitation of eleven steroid sulfates commercially available in a wide range of concentrations (0.5-10000 ng/mL). It is the most comprehensive method described up to now since the reported methods were only focused on a few number of endogenous sulfate metabolites [17, 19-27]. In addition, taking advantage of the common ionization and CID behavior of steroid sulfates, three androstanediol sulfate metabolites were detected in urine samples using theoretical transitions. The structure of these metabolites needs to be elucidated. The estimation of the concentration of these analytes was performed using a calibration curve of 5α-androstane-3β,17β-diol 17β-sulfate (Diol-S), the only androstanediol sulfate available as reference standard and not detected in urine samples.

Basal concentrations of endogenous sulfate metabolites were quantified using 75 urine samples from healthy volunteers (males and females of Caucasian ethnic and Asian males) providing additional data to evaluate the usefulness of these metabolites as markers of EAAS misuse. Basal concentrations of some of the metabolites were reported for the first time (epiA-S, DHA-S, 6βOHA-S, and the three androstanediol sulfates). These basal samples were used to establish population reference limits of endogenous steroid sulfates to evaluate detection times in administration studies, however, due to the low number of basal samples studied and the high inter-individual variability observed, these limits have to be taken with caution. A larger number of urines need to be studied to establish adequate population reference limits.

The potential of sulfate metabolites to detect EAAS misuse was evaluated in samples collected after oral and intramuscular T administration to healthy volunteers (Chapters 6 and 7, respectively). Our results demonstrated that some of the sulfate metabolites improve the detection capabilities compared with the currently established steroid profile. In general, the use of sulfate ratios provided better results than those obtained with individual concentrations. A low intra-individual variability of the markers is desirable for the detection of EAAS misuse by the use of individual thresholds. In our studies, although high intra-individual variability was observed for sulfates, which could be a hindrance, they can

substantially increase the detection times with respect to conventional T/E ratio.

Different behavior was observed after both administration routes. Whereas after oral administration the concentrations of most of the sulfates drastically increased after 4-8h after administration, in case of intramuscular administration any significant increment was observed. The reason might be the activation of the metabolic pathways leading to sulfate metabolites only after the administration of a large amounts of T in a short period of time as in the case of oral administration.

Several factors may influence the metabolism and some of them have been considered in our studies. In order to cover inter-individual variability, several volunteers were evaluated (5 in the oral study and 12 in the intramuscular study). This must be highlighted because most of the published studies regarding the usefulness of sulfates have included a reduced number of volunteers [19, 20, 26, 28-30]. Moreover, possible inter-ethnic variations have been considered in the study of intramuscular administration, where 6 Caucasian and 6 Asian volunteers were studied. Our results demonstrated that the potential of steroid sulfate metabolites as markers of T intramuscular administration highly depends on the volunteer and the ethnicity of the volunteer. Most of the selected ratios improved the detection capabilities of T misuse for all Caucasian volunteers, whereas for Asian, only some of them provide longer detection times than those obtained with T/E ratio

and only for three of the volunteers. The low sulfate concentrations observed generally for Asian volunteers precluded the evaluation of the detection times involving these ratios. Inter-ethnic variations need to be also studied after oral T administration.

Different useful ratios between sulfate metabolites were obtained to detect oral and intramuscular administrations. After oral T administration, A-S/E-S, epiA-S/E-S, Diol-S1/E-S, epiA-S/DHA-S and Diol-S1/DHA-S provided significantly prolonged detection times compared with T/E, some of them up to 144h after administration. On other hand, for intramuscular T administration, the better results were obtained with A-S/E-S, A-S/T-S, Etio-S/T-S, DHA-S/T-S and epiA-S/T-S ratios, being A-S/T-S which provided the longest detection times for both populations. Conversely, the same ratio detected as free and glucuronoconjugated fraction, A/T, previously reported as a potential marker for T intramuscular misuse [31], was not found suitable in these studies [32]. These facts suggest the wide variability of metabolic pathways between volunteers and ethnicities, showing that the simultaneous evaluation of free, glucuronide and sulfate fraction of the steroid profile in the initial testing procedures could be an advisable strategy to improve the detection capabilities of EAAS misuse

Combining the results obtained for both administration routes, it can be concluded that the determination of T-S, E-S, A-S, Etio-S, epiA-S, DHA-S and Diol-S1 might complement the current steroid profile since several ratios involving these markers provide better

detection times than the current T/E in both administration routes. It is worth to notice that during the first hours (4-8h after oral, and 1-3 days after intramuscular) negative results were observed for the detection of T administration using sulfate ratios. However, after these times, they maintained detectability for longer than the established T/E ratio. That could mean that sulfation is a secondary metabolic pathway that appears later, but it also took longer to return to normal activity. Therefore, the current markers of the steroid profile can detect the T misuse in the early hours after administration and sulfate markers provide detectability in the long term.

For all these reasons, sulfate metabolites can adequately complement the currently used markers of the steroid profile. However, due to the inter-individual variability, studies with a high number of volunteers are recommended to be conducted. Extended evaluations of the intra-individual variability under different conditions (e.g. after exercise or during the circadian and menstrual cycle) and the inter-individual variability (a high number of population urine samples) of the markers should be studied. In addition, the influence on the excretion of sulfate metabolites of several factors such as alcohol consumption should also be considered. The use of combined ratios between glucuronide and sulfate metabolites needs also to be evaluated.

In order to include sulfate metabolites in the steroid profile, the quantitation of these metabolites needs to be performed during the

initial testing procedures. The method used in the present study to quantify steroid sulfates is too complex to be applied in routine anti-doping analysis and a simpler method needs to be developed. As mentioned, the conventional procedure for the quantitation of the steroids included in the steroid profile (GC-MS after hydrolysis with β-glucuronidase hydrolysis and derivatization) is not useful for the quantitation of steroid sulfates. However, it has been recently demonstrated that accurate quantitation of some endogenous steroid sulfates can be achieved in initial testing procedures based on urine dilution and LC-MS technology [33]. The use of more sensitive mass spectrometers with wide linear range will help in the incorporation of the sulfate metabolites in the initial testing procedures.

In summary, the results of the thesis demonstrate the increased role of the direct analysis of phase II metabolites of AAS in doping control. On the one hand, the direct analysis of these metabolites will provide a more comprehensive evaluation of the phase II metabolism of AAS to look for new markers of the administration. And on the other hand, the direct analysis of the phase II metabolites during the initial testing and confirmation procedures will enable a much simpler approach and will provide longer detection times for some exogenous and endogenous AAS.

8.2 Suggestions for future work

Based on the results reported in this thesis, several lines can be proposed for the future investigation:

- Evaluation of the presence of glucuronides resistant to enzymatic hydrolysis after the administration of other AAS using the developed methodology.
- Extended study of bisglucuronide metabolites of other AAS.
- Evaluation of other conjugates of AAS, such as diglucuronides or bisconjugates.
- Evaluation of endogenous steroid sulfate metabolites as potential markers of T misuse in other administration routes (such as transdermal) and after the administration of other EAAS in volunteers from different ethnicities.
- Identification of androstanediol sulfates of interest.
- Increase the number of basal samples to provide adequate population levels of endogenous steroid sulfates.
- Development of methods for the quantitation of endogenous steroid sulfates to incorporate them in the initial testing procedures.
- Evaluation of the usefulness of the combination of glucuronides and sulfates in the steroid profile.
- Re-evaluation of the phase II metabolism of other exogenous and endogenous AAS.

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CONCLUSIONS

Chapter 9

Conclusions

The most important conclusions of this thesis are summarized below.

Part I: Glucuronides

1. LC-MS/MS showed to be useful for the direct detection of unknown metandienone glucuronide metabolites, including those resistant to enzymatic hydrolysis, not detectable using the conventional indirect methods.

2. Thirteen glucuronide metabolites were detected in urine with detection times between one and twenty two days. One of them was resistant to enzymatic hydrolysis with β-glucuronidase, however it was only detected up to four days after administration.

3. Although the three glucuronide metabolites detected for the longest period of time were previously reported, for some of them, analytical data of the intact phase II metabolites were presented for the first time.

4. The proposed strategies provide a comprehensive approach for the detection of glucuronide metabolites, and they are recommended to be used for metabolic studies of other exogenous AAS.

Part II: Bisglucuronides

5. The electrospray ionization and CID behavior of steroid BGs was studied using synthesized AAS BG metabolites. A characteristic pattern was observed for all the compounds. In positive mode, the adduct $[M+NH_4]^+$ was observed, and in negative mode $[M-H]^-$ and $[M-2H]^{2-}$ ions were formed. The most important product ions observed were result of either the neutral/ion losses of the glucuronide moieties or ions coming from the glucuronide moiety.

6. The characteristic ionization and dissociation pattern may be the basis to develop targeted and untargeted MS strategies to detect steroid BGs. As a proof of concept, one BG metabolite was detected after oral administration of norandrostenediol. The results demonstrate the usefulness of the analytical strategy to detect BG metabolites and the formation of these metabolites after AAS administration.

7. Additional studies are needed to evaluate the potential of BG metabolites to detect the administration of AAS.

Part III: Sulfates

8. A LC-MS/MS method was optimized and validated for the direct quantitation of fourteen endogenous steroid sulfates in urine. The method was suitable for compounds present in a wide range of concentrations (0.5-10000 ng/mL).

9. Basal levels of steroid sulfates in male and female urines were quantified, providing additional data to evaluate the usefulness of these metabolites as markers of EAAS misuse.

10. The potential of endogenous steroid sulfates to improve the detection of oral T administration was evaluated in Caucasian volunteers. The concentration of epiA-S and the ratios A-S/E-S, epiA-S/E-S, Diol-S1/E-S, epiA-S/DHA-S and Diol-S1/DHA-S significantly improved the detection times (up to 144 h, in some cases) compared to the conventional T/E.

11. The usefulness of sulfate metabolites to detect T intramuscular administration was evaluated in Caucasian and Asian volunteers. The ratios A-S/E-S, Etio-S/E-S, A-S/T-S, Etio-S/T-S, DHA-S/T-S and epiA-S/T-S presented similar or better detection times than those obtained with T/E for most of the volunteers of both populations, being A-S/T-S the most promising marker of T intramuscular administration.

12. Steroid sulfate metabolites should be included in the steroid profile to complement the current markers, because they improve the retrospectivity of both oral and intramuscular T administration.

General conclusions

13. The results of the thesis demonstrate the increased role of the direct analysis of phase II metabolites of AAS in doping control.

14. The direct analysis of phase II metabolites will provide a more comprehensive evaluation of the phase II metabolism of AAS to look for new markers of the administration.

15. The direct analysis of the phase II metabolites during the initial testing and confirmation procedures will enable a much simple approach for testing and will provide longer detection times for some exogenous and endogenous AAS.

ANNEXES

ANNEX I: Scientific publications included in this thesis

- **Esquivel A**, Pozo O.J, 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*. 2017;9:534-544.

- **Esquivel A**, Matabosch X, Kotronoulas A, Balcells G, Joglar J, Ventura R. Ionization and collision induced dissociation of steroid bisglucuronides. *J Mass Spectrom*. 2017;52:759-769.

- **Esquivel A,** Alechaga E, Monfort N, Ventura R. Direct quantitation of endogenous steroid sulfates in human urine by liquid chromatography-electrospray tandem mass spectrometry. *Drug Test Anal*. 2018;1-10.

- **Esquivel A,** Alechaga E, Monfort N, Ventura R. Sulfate metabolites improve retrospectivity after oral testosterone administration. *Drug Test Anal*. 2018. Accepted.

- **Esquivel A**, Alechaga E, Monfort N, Yang S, Xing Y, Moutian W, Ventura R. Evaluation of sulfate metabolites as markers of intramuscular testosterone administration in Caucasian and Asian populations. Submitted to *Drug Test Anal*.

ANNEX II: Other publications by the author

- **Esquivel A**, Balcells G, Pozo O.J, Gómez C, Kotronoulas A, Joglar J, Segura J, Ventura R. New LC-MS/MS screening for methyltestosterone: direct detection of phase I and phase II metabolites in urine, in: Recent Advances in doping Analysis (23), Sportverlag Strauβ, Köln, 2015, 178-182.

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