Improving detection capabilities of doping agents by identification of new phase I and phase II metabolites by LC-MS/MS

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Abstract

Metabolic studies of doping agents have been traditionally performed by using gas chromatography coupled to mass spectrometry (GC-MS). In the last years, liquid chromatography coupled to mass spectrometry (LC-MS) has demonstrated to offer new possibilities to perform metabolic studies. The objective of this thesis was to study the metabolism (phase I and phase II) of different doping agents by LC-MS/MS in order to improve the detection capabilities of the studied substances.

For mesocarb, a thermolabile compound, the parent drug 19 metabolites were detected in urine including mono-, di- and tri-hydroxylated metabolites excreted free or conjugated with glucuronic acid and sulphate. For toremifene, an anti-estrogenic drug with poor electron ionization properties, the parent drug and 20 metabolites were detected in urine. The structure of most of the metabolites was proposed.

Anabolic androgenic steroids (AAS) metabolites conjugated with sulphate were investigated to improve the retrospectivity of the detection of these compounds. A study of hydrolysis and MS/MS behaviour of sulphate metabolites of AAS was performed, and sulphate conjugated metabolites of boldenone, methyltestosterone and metandienone were studied. Boldenone sulphate and epiboldenone sulphate were identified as boldenone metabolites in humans. They can be used as markers of exogenous boldenone administration. For

methyltestosterone, three new sulphate metabolites were detected and structures were proposed. One of them, 17β-methyl-5α-androstan-3α,17α-diol 3α-sulphate was detected in urine up to 21 days after methyltestosterone administration, improving three times the retrospectivity of the detection with respect to other previously reported long-term metabolites. Several new sulphate metabolites were detected after metandienone administration. One of them was characterized as 18-nor-17β-hydroxymethyl-17α-methylandrost-1,4,13-triene-3-one conjugated with sulphate, and it was detected up to 26 days after administration, improving the retrospectivity of the detection with respect to other long-term metabolites described.

The usefulness of LC-MS/MS for the detection and characterization of metabolites of doping agents has been demonstrated, especially for the study of new phase II metabolites and for metabolic studies of compounds where GC-MS shows relevant limitations.

Resum

Els estudis metabòlics de substàncies dopants han estat tradicionalment realitzats mitjançant l'ús de cromatografia de gasos acoblada a espectrometria de masses (GC-MS). En els últims anys, s'ha demostrat la utilitat de la cromatografia líquida acoblada a espectrometria de masses (LC-MS) per realitzar estudis de metabolisme. L'objectiu d'aquesta tesi va ser estudiar el metabolisme (fase I i fase II) de diferents substàncies dopants mitjançant LC-MS/MS per tal de millorar la capacitat de detecció dels compostos estudiats.

Per a mesocarb, compost termolàbil, es van detectar en orina el compost inalterat i 19 metabòlits incloent metabòlits mono-, di- i tri-hidroxilats excretats lliures o conjugats amb àcid glucurònic i sulfat. Per a toremifè, un fàrmac anti-estrogènic amb espectre de masses d'impacte electrònic amb pocs ions diganòstic, es van detectar el compost inalterat i 20 metabòlits en orina. Es va proposar l'estructura de la major part de metabòlits detectats.

Per tal de millorar la retrospectivitat de la detecció dels esteroides anabolitzants androgènics (AAS) es van estudiar els metabòlits conjugats amb sulfat. Es va realitzar un estudi de la hidròlisi i del comportament espectromètric dels metabòlits conjugats amb sulfat dels AAS. Es van estudiar els metabòlits conjugats amb sulfat de boldenona, metiltestosterona i metandienona. Es van identificar boldenona sulfat i epiboldenona sulfat com a metabòlits de boldenona en humans. Aquests metabòlits poden ser usats com a marcadors de

l'administració exògena de boldenona. Per a metiltestosterona, es van detectar i proposar les estructures de tres nous metabòlits conjugats amb sulfat. Un d'ells, el 17β-metil-5α-androstà-3α,17α-diol 3α-sulfat, va ser detectat en orina fins a 21 dies després de l'administració de metiltestosterona. Es van detectar diversos metabòlits de metandienona conjugats amb sulfat no descrits prèviament. Un d'ells, identificat com a 18-nor-17β-hidroximetil-17α-metilandrost-1,4,13-trien-3-ona conjugat amb sulfat, va ser detectat fins 26 dies després de l'administració. Tant per a metiltestosterone com per a metandienone, els metabòlits conjugats amb sulfat permeten millorar la retrospectivitat de la detecció respecte a altres marcadors descrits anteriorment.

S'ha demostrat la utilitat del LC-MS/MS per a la detecció i caracterització de metabòlits de substàncies dopants, especialment per a l'estudi de nous metabòlits de fase II i per a estudis de metabolisme de compostos que mostren limitacions en GC-MS.

Preface

Doping is the use of prohibited substances or methods to enhance the performance in sports. Doping is prohibited due to health risks for the athlete and to ensure that everyone competes with equal conditions. There is a list of prohibited substances and methods which is updated annually by the World Anti-Doping Agency. The task of doping control laboratories consist of the detection of the administration of these prohibited substances or the use of prohibited methods by the analysis of biological samples (normally, urine) from the athlete.

Doping agents are metabolized in the body in different extent, and the parent drug and/or the metabolites excreted in urine are the markers used to detect their administration. In doping control, the best marker of administration is not always the most abundant metabolite but the metabolite excreted in urine for longer period of time after administration. Therefore, metabolic studies are necessary in order to find the best markers of administration of each doping agent to improve the detection capabilities of each drug.

Metabolic studies of doping agents have been traditionally performed using gas chromatography-mass spectrometry (GC-MS), and the detection of most doping agents has been based for many years on the analysis of metabolites elucidated by GC-MS methods. GC-MS has some limitations for the study of the metabolism of drugs, such as the impossibility of analysis of thermolabile compounds, the difficulties on the analysis of polar compounds that need previous derivatization

and the impossibility to directly analyse phase II metabolites (conjugated metabolites). In addition, for some compounds the use of electron ionization, an universal hard ionization technique, results in few diagnostic ions limiting the usefulness of GC-MS for identification and characterization of new metabolites. In the last decade, liquid chromatography-mass spectrometry (LC-MS) has also been used for metabolic studies. LC-MS offers several advantages for metabolic studies with respect to GC-MS. Among them the possibility to directly analyse phase II metabolites and to the directly detect polar compounds, avoiding hydrolysis and derivatization steps, are the most important ones.

The aim of this thesis was to evaluate the metabolism of different doping agents using LC-MS technology in order to improve the detection capabilities of these compounds. Compounds where GC-MS show relevant limitations have been selected.

List of abbreviations

AAF: Adverse analytical finding

AAS: Anabolic androgenic steroids

AcetylCoA: Acetyl coenzyme A

API: Atmospheric pressure ionization

BM1: 5β-androst-1-en-17β-ol-3-one

CE: Collision energy

CID: Collision-induced dissociation

CV: Cone voltage

CYP: Cytochrome P450 isoform

ER: Estrogen receptor

ESI: Electrospray ionisation

EtAC: Ethyl Acetate

GC: Gas chromatography

GC/C/IRMS: Gas chromatography/combustion/isotope ratio mass spectrometry

GC-MS: Gas chromatography-mass spectrometry

GC-MS/MS: Gas chromatography-tandem mass spectrometry

GST: Glutathione-S-transferase

IOC: International Olympic Committee

IRMS: Isotope ratio mass spectrometry

ISL: International Standard for Laboratories

ISTD: Internal standard

LC: Liquid chromatography

LC-DAD: Liquid chromatography-diode array detection

LC-MS: Liquid chromatography-mass spectrometry

LC-MS/MS: Liquid chromatography coupled to tandem mass spectrometry

LOD: Limit of detection

LLE: Liquid-liquid extraction

MeOH: Methanol

MM: Molecular mass

MRPL: Minimal required performance limits

MS: Mass spectrometry

MBTFA: N-methyl-N-trimethylbis(trifluoroacetamide)

MSTFA: N-methyl-N-trimethylsilyltrifluoroacetamide

MT: Methyltestosterone

MTD: Metandienone

NAT: N-acetyltrasnferase

NL: Neutral loss

PAPS: 3'-phosphoadenosine-5'-phosphosulphate

PI: Precursor ion

QTOF: Quadrupole time-of-flight

RT: Retention time

SAM: *S*-adenosylmethionine

SERM: Selective estrogen receptor modulator

SIM: Single ion monitoring

SPE: Solid-phase extraction

SRM: Selected reaction monitoring

SULT: Sulfotransferase

TBME: *Tert*-butil methyl ether

TIC: Total ion chromatogram

TMS: Trimethylsilyl

TMSIm: Trimethylsilylimidazole

TOF: Time-of-flight

UDP: Uridine diphosphate

UGT: Uridine diphosphate-glucuronosyltransferase

WADA: World Anti-Doping Agency

XIC: Extracted ion chromatogram

Thesis structure

This thesis is structured in nine chapters. The first chapter includes the introduction, covering the background information on doping control analysis, metabolic studies and main aspects of analytical strategies to perform metabolic studies of doping agents. In the second chapter, the justification and objectives of the thesis are presented.

Chapters three to eight describe the experimental part of the thesis. Chapters three and four describe the works performed on mesocarb and toremifene metabolism, respectively. Chapters five to eight are devoted to the study of new sulphated metabolites of anabolic androgenic steroids (AAS). In chapter five, a comprehensive study of hydrolysis and MS/MS behaviour of sulphated AAS metabolites is presented. In chapters six, seven and eight, the studies of sulphated metabolites of boldenone, methyltestosterone and metandienone, respectively, are described. All experimental chapters, but chapter five, are published papers and the information included in the papers is presented.

Finally, in chapter nine the main conclusions of each chapter and general conclusions are listed.

Three annexes are included containing three related published manuscripts. The first one is the study of analytical strategies for the study of steroid metabolism that includes the information described in chapter five of the thesis. The other two papers describe the study performed on boldione metabolism.

Chapter 1

Introduction

1.1. Doping control

Doping is the use of substances or methods to enhance the performance in sport. According to World Anti-Doping Agency (WADA) doping is defined as the occurrence of one or more of the anti-doping rule violations [1]. The most frequent are "*the presence of a prohibited substance or its metabolites or markers in an athlete's sample*" and "*the use or attempted use by an athlete of a prohibited substance or a prohibited method*". Additionally, other situations are also considered as doping such as "(i) *refusing or evading sample collection*, (ii) *violation of applicable requirements regarding athlete availability for out-of-competition testing*, (iii) *tampering or attempted tampering with any part of doping control*, (iv) *possession of prohibited substances and prohibited methods*, (v) *trafficking or attempted trafficking in any prohibited substance or prohibited method* and (vi) *administration, assisting, encouraging, aiding, abetting, covering up or any other type of complicity involving an anti-doping rule violation*".

WADA annually revise the list of banned substances and methods, divided in general categories or classes of prohibited substances and methods (Table 1.1) [2]. A substance or method is included on the Prohibited List of WADA when it exists medical or other scientific evidence, pharmacological effect or experience that the substance or method, alone or in combination with other substances or methods, has the potential to enhance or enhances sport performance; represents an actual or potential health risk to the athlete; violates the spirit of sport; or when has the potential to mask the use of other prohibited substances or prohibited methods [1].

The current prohibited list in sports includes ten groups of prohibited substances (non-approved substances, anabolic agents, peptide hormones, β-2-agonists, hormone and metabolic modulators, diuretics and masking agents, stimulants, narcotics, cannabinoids, glucocorticosteroids), three prohibited methods (manipulation of blood, chemical and physical manipulation, gene doping), and two groups of substances prohibited in particular sports (alcohol, β-blockers).

As can be observed in Table 1.1, doping classes from S0 to S5 and methods M1 to M3, are prohibited at all times (in- and out-of-competition); (e.g. anabolic agents including exogenous anabolic androgenic steroids (AAS); agents with anti-estrogenic activity); substances from S6 to S9 are prohibited only in-competition (e.g. stimulants); substances P1 and P2 are prohibited only in particular sports. In some particular cases, drugs are only prohibited when administered by some routes e.g. glucocorticosteroids which are prohibited when administered by oral, intravenous, intramuscular or rectal routes [2].

Anti-doping laboratories analyse samples collected from athletes to detect the presence of prohibited substances or the use of prohibited methods. Anti-doping laboratories must obtain and maintain accreditation from WADA to ensure production of valid test results and evidentiary data and to achieve uniform and harmonized results and reporting from all WADA accredited laboratories. WADA accreditation need a first accreditation according to the international standard ISO17025 (General requirements for the competence of

testing and calibration laboratories) and, additionally, to comply with specific operating standard for laboratory performance included in the International Standard for Laboratories (ISL) [3] WADA also publishes specific technical recommendations, addressing particular operational areas of the accredited laboratories, in technical documents (e.g. MRPL, identification criteria for qualitative assays, laboratory internal chain of custody, etc.) [4].

Table 1.1. Substances and methods prohibited by WADA in 2013.

WADA class		Example
Substances and methods prohibited at all times (in- and out-of-competition)		
S0	Non-approved substances	
S1	Anabolic agents *1. Anabolic androgenic steroids (AAS)* a) Exogenous AAS b) Endogenous AAS *2. Other anabolic agents*	Testosterone Boldenone Methyltestosterone Clenbuterol
S2	Peptide hormones, growth factors and related substances	Erythropoietin (EPO) Growth hormone (GH)
S3	β-2 agonists	Salbutamol
S4	Hormone and metabolic modulators	Exemestane Toremifene
S5	Diuretics and other masking agents	Probenenecid Furosemide
M1	Manipulation of blood and blood components	
M2	Chemical and physical manipulation	
M3	Gene doping	
Substances and methods prohibited in-competition		
S6	Stimulants	Mesocarb Methylhexaneamine
S7	Narcotics	Morphine
S8	Cannabinoids	THC
S9	Glucocorticosteroids	Methylprednisolone
Substances prohibited in particular sports		
P1	Alcohol	
P2	β-blockers	Alprenolol

1.1.2. Analytical strategy in doping control

Doping control laboratories have to develop analytical methodologies for the detection of all prohibited substances and methods at the required sensibilities.

The analytical strategy used in anti-doping control has to take into consideration the high sensitivity and selectivity required in complex matrices, involving mostly urine and blood samples, compounds with wide range of physicochemical properties and molecular weight, limited sample volumes and fast analysis time requirements (e.g. results within 24-48 h for important sport events).

Anti-doping control consists in the sequential application of screening and confirmation methods. Several screening methods, addressed to the detection of a wide number of compounds and/or metabolites with similar physicochemical properties, are applied to the sample [5-8]. This step must be fast, selective and sensitive, while avoiding false-negative results with a limited false-positive proportion (less than 10 %). The screening step provides an indication of the presence or absence of a doping agent.

When the presence of a doping agent is suspected, a confirmatory test, specific for the compound detected, must be performed. The confirmatory test should be at least as sensitive and preferably more specific than the screening test. Mass spectrometry (MS) is mandatory for confirmation purposes, whenever possible. Criteria to demonstrate the presence of prohibited substances using chromatography coupled

to MS are described in a WADA technical document. Comparison of relative retention times and relative abundances of three diagnostic ions for MS analysis or two diagnostic transitions for MS/MS analysis is needed [9].

In terms of sensitivity, WADA has defined the concept of minimal required performance limits (MRPL) which all accredited anti-doping laboratories should reach to ensure their results are reported homogeneously [10]. The MRPL is the concentration of a prohibited substance, marker or metabolite that anti-doping laboratories have to be able to detect and identify.

With urine samples, various sample preparation procedures can be considered, based on the analyte physicochemical properties and selected analytical method; examples of methods used in Barcelona's anti-doping laboratory are briefly described in Table 1.2. Liquid-liquid extraction (LLE) or solid-phase extraction (SPE) methods are commonly used in doping control analyses, allowing sample clean-up together with analyte pre-concentration. Sample clean-up extracts are, in general, directly compatible with liquid chromatography (LC), whereas derivatization steps are mandatory to make samples compatible with gas chromatography (GC) [11,12]. The derivatization step is performed with various strategies based on the targeted analytes [13]. For the detection of glucuronide conjugate metabolites, hydrolysis is performed by using β-glucuronidase enzymes, used to deconjugate the phase II metabolites.

The presence in a sample of a prohibited substance or its metabolites or markers or evidence of the use of prohibited method is defined as adverse analytical finding (AAF).

Some substances of the Prohibited List (e.g., salbutamol, formoterol or ephedrine) need quantitative measurements because they are considered doping agents only above a threshold concentration [14]. In addition, high levels of endogenous substances, such as some androgenic steroids, are quantified to estimate an adverse analytical finding. For other compounds, their presence in the matrix constitutes violation of the anti-doping rules. For some compounds, neither GC- nor LC-MS(/MS) techniques are able to differentiate endogenous substances from exogenous intake [15]. Therefore, various strategies have been explored. Statistical threshold levels or ratios have been established in doping control for e.g., testosterone. Isotope ratio mass spectrometry (IRMS), based on enrichment of ^{13}C, was introduced to distinguish between endogenous and synthetic steroid analogs. Indirect approaches using biomarkers of the exogenous administration of the drugs of abuse are a promising way to discriminate between endogenous and exogenous substances. The athlete's biological passport allows taking into account intra-individual variation of indirect markers and reveals modifications in the biological parameters induced by the administration of a banned substance [16].

Table 1.2. Screening methods used in Barcelona's anti-doping control laboratory.

	Substances detected	**Sample preparation**	**Instrumental analysis**
1A	Stimulants, narcotics	LLE TBME (alkaline pH) Salting-out effect	GC-MS
4B	AAS, β-agonists, corticosteroids, narcotics, stimulants, masking agents	Sample clean-up (Detectabuse) Enzymatic hydrolysis (*E. coli*) LLE TBME (alkaline pH)	GC-MS GC-MS/MS LC-MS/MS
5A	Diuretics, probenecid, stimulants	LLE EtAc (alkaline pH) Salting-out effect	LC-MS/MS
6B	hCG, LH		MEIA
EPO	EPO and analogues		Chemioluminiscence
MS044	β-blockers	Enzymatic hydrolysis (*E. coli*) LLE EtAc (alkaline pH)	LC-MS/MS
MS038	DEHP metabolites	Enzymatic hydrolysis (*E. coli*) LLE EtAc (acid pH)	LC-MS/MS

1.2. Metabolism of doping agents

Metabolism studies play an important role in drug discovery, pre-clinical and clinical development and it is necessary to identify the structures of metabolites [17]. This knowledge allows prediction of whether the compound may cause drug-drug interactions or be susceptible to marked interindividual variation in metabolism due to genetic polymorphism [18,19]. Metabolite identification is critical to many of these activities, to our understanding of the time course of drugs in the body, the structuring of dosage regimens, the pharmacology and toxicology of drug metabolites, and the interactions of multivalent drug combinations. The knowledge of the biotransformation pathways of the lead candidate to its metabolites is used to indicate, for example, the magnitude and duration of activity.

On the other hand, in the antidoping control field, and other areas of drug testing (drug of abuse testing, analysis of residues in food producing animals,…) metabolic studies are helpful in order to know the best marker of the administration of a drug. For exogenous doping agents, identification of the exogenous compound and/or its metabolites is sufficient to demonstrate the administration of the drug [3]. In this case, the most useful metabolite for detection of the drug misuse is not always the most abundant metabolite but the metabolite detectable for longer period of time after administration. For some doping agents, more than one metabolite are used as a marker to cover different excretion times. Therefore metabolic studies are needed to select the adequate markers to improve the retrospectivity of the detection of the compounds. Furthermore, some exogenous substances like glucocorticosteroids and β2-agonists are prohibited when administered by systemic routes but its use is allowed by other routes, (e.g. inhalation). In these cases, metabolic studies are useful in order to find specific metabolites that allow distinguishing the administration route [20-22]. For these reasons, metabolic studies are necessary in order to find the best markers of the administration.

For doping substances that are also produced endogenously studies are also needed to look for markers of the exogenous administration. The detection of the administration of an androgen that could be present normally in human body fluids is based upon the methodical evaluation of key parameters of the urinary profile of steroids. The direct confirmation comes from the measurement of $\delta^{13}C$ values reflecting the synthetic origin, ruling out a potential physiological anomaly [16].

1.2.1. General metabolic pathways

Drugs are xenobiotic compounds that are biotransformated in living systems into less toxic and more hydrophilic analogues to facilitate its excretion. The metabolism is a very important factor in the elimination of drugs from the body. Metabolism reactions are classified as either phase I or functionalization reactions and phase II or conjugation reactions. Both phase I and phase II reactions generally results in the loss of pharmacological activity, although in some cases results in the enhancement of activity [18,19,23,24]. Most common biotransformations are resumed in Table 1.3.

The enzyme systems involved in biotransformation of drug are located mainly in the liver. The liver is the principal organ responsible for xenobiotic metabolic reactions, though some of the enzymes and biotransformations occur in other tissues including the intestine, kidneys, placenta, lungs, and even the brain [18,19].

In phase I reactions, a drug suffers different types of irreversible chemical modifications including hydrolysis, reduction, and oxidation. As can be observed in Table 1.3, xenobiotics with esters and amide functionalities are hydrolysed by esterase and amidase enzymes, respectively, yielding free acids as products. They are also capable of process thioesters and carbamates as well [19]. Few drugs are subject to reductive reactions, which typically involve nitro-reduction and hydration. Nitro-reduction reactions are catalysed by cytochrome P450-reductase enzymes; Epoxide hydrolases are the enzymes which hydrate simple epoxides to two diols.

Drug oxidations are among the most prominent type of biotransformation. Mixed function oxidases catalyze the oxidation of diverse lipophilic drug substrates such as steroids, fatty acids, or hormones [18]. The most widely studied gene family of mixed-function oxidases and the one that participates more than any other in the oxidation of drugs is the cytochrome P450 family. The gene products of cytochrome P450 (CYP) enzymes are important in terms of the number of existing drugs that they process, their substrate specificity, polymorphism, and tendency to be determinants in drug-drug interactions [18,19]. P450 enzymes catalyze N-, O- and S-dealkylation, aliphatic hydroxylation, aromatic hydroxylation, N-hydroxylation, N-oxidation, deamination and dehalogenation. The three key cytochrome P450 or CYP families responsible for drug metabolism in humans are CYP1, CYP2, and CYP3. The knowledge of CYP genotypes should be useful in predicting individual phenotypes that will help in the selection of the most appropriate drug dosages for each person.

Phase II reactions are considered to be synthetic in nature since they result in the formation of a metabolite with an increased molecular weight in addition to being more polar and hydrophilic. Phase II conjugation reactions lead to the formation of a covalent linkage between a functional group on the parent compound or phase I metabolite with a polar compound. As resumed in Table 1.3, phase II reactions include glucuronidation, sulfonation, acetylation, gluthatione conjugation, and methylation [18,19,23].

Glucuronidation is catalyzed by uridine diphosphate (UDP)-glucuronosyltransferases or UGTs and uses uridine-5'-diphosphoglucuronic acid as the co-substrate. These enzymes catalyze the transfer of glucuronic acid from the cofactor UDP-glucuronic acid to a substrate to form β-D-glucopyranosiduronic acids (glucuronides). The distribution pattern of UGTs is mostly similar to that of the CYPs. Like the CYPs, the UGTs are a superfamily of enzymes. In humans there are three subfamilies known as UGT1A, UGT2B, and UGT2A. The liver is the major site at which glucuronisation occurs although UGTs are also located in brain, kidney, breast tissue, and prostate as well. The generation of glucuronides can be formed through alcoholic and phenolic hydroxyl groups, carboxyl, sulfuryl, and carbonyl moieties, as well as through primary, secondary, and tertiary amine linkage and nucleophilic carbons (Table 1.3) [18,19,25]. UGTs play a significant role in phase II detoxification of drugs in humans, and are responsible for more than one-third of all the phase II metabolism of drugs.

Sulfoconjugation (or sulfonation) constitutes an important pathway in the metabolism of numerous both exogenous and endogenous compounds. Enzymes mediating xenobiotic sulfonation are the sulfotransferase (SULT) enzymes. SULTs comprise a superfamily of enzymes; four human SULT families, SULT1, SULT2, SULT4 and SULT6, have been identified. The source of the sulfonyl group (SO_3^-) is adenosine 3'-phosphoadenosine-5'-phosphosulphate (PAPS), generally to a hydroxyl on an acceptor group. (Table 1.3). SULTs are localised in different tissues such liver, brain, breast, intestine, lung, adrenal gland, or kidney among others [26]. Sulfonation can be formed

through N-hydroxyarylamines, N-hydroxy heterocyclic amines, and hydroxyl methyl polycyclic aromatic hydrocarbons, phenols and primary and secondary alcohols.

Compared to sulfonations and glucuronidations, acetylations are modest in terms of the number and variety of substrates, but remain significant in a toxicological perspective. In humans, acetylation reactions are catalyzed by two *N*–acetyltransferase isoenzymes (NATs), *N*–acetyltransferase 1 (NAT1) and 2 (NAT2). NATs are cytosolic enzymes found in many tissues such as liver, intestine, blood cells, placenta or skin [27]. These enzymes transfer acetate from acetyl coenzyme A (acetylCoA) to primary amines, hydrazines, sulfonamides, and aromatic amines (Table 1.3).

Methylation is a common but generally minor pathway of xenobiotic biotransformation. Unlike most other conjugative reactions, methylation often does not dramatically alter the solubility of substrates and results either in inactive or active compounds. Methyltransferases or methylase, transfers a methyl group from a donor to an acceptor (Table 1.3). The co-factor required to form methyl conjugates is *S*-adenosylmethionine (SAM), which is primarily formed by the condensation of ATP and L-methionine. A large number of both endogenous and exogenous compounds can undergo *N*-, *O*-, *S*- and arsenic-methylation during their metabolism [23].

The glutathione-S-transferases (GSTs) catalyze the transfer of glutathione to reactive electrophiles, a function that serves to protect cellular macromolecules from interacting with electrophiles that

contain electrophilic compounds such as ketones, quinones, sulfoxides, esters, peroxides, and ozonides and protects the cellular environment from damage [28]. A common product resulting from glutathione conjugation is a mercapturic acid. The superfamily of human soluble GSTs is further divided into eight separate classes: Alpha (A1-A4), Kappa (K1), Mu (M1-M5), Pi (P1), Sigma (S1), Theta (T1-T2), Zeta (Z1) and Omega (O1-O2) [29]. Most members of both glutathione transferase superfamilies have been found to be genetically polymorphic.

Table 1.3. Examples of most common phase I and phase II reactions.

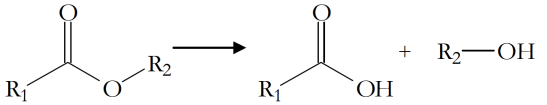
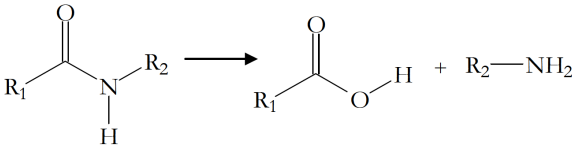
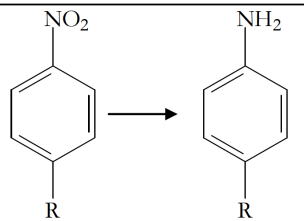
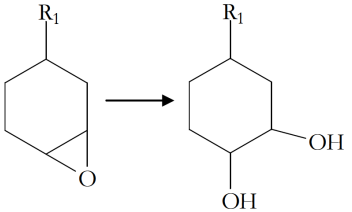
Phase I biotransformations				
	Type of reactions	Biotransformation	Enzyme	Example
Hydrolysis				
1.	Ester		Esterase	Cocaine
2.	Amide		Amidase	Indapamide
Reduction				
1.	Nitro reduction		Cytochrome P450-reductase	
2.	Hydration		Epoxide hydrolase	

Table 1.3. (Continued).

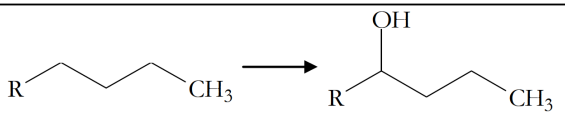
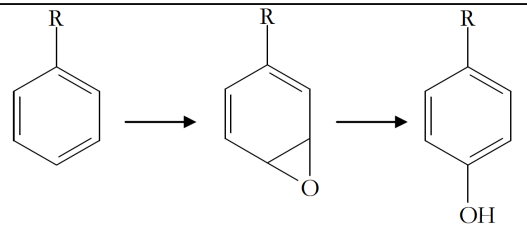
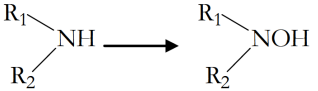
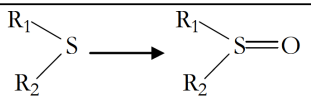
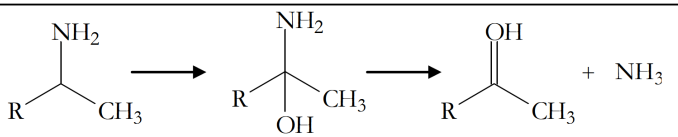
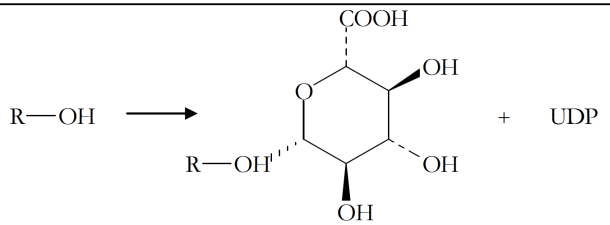
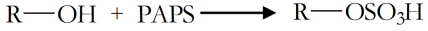
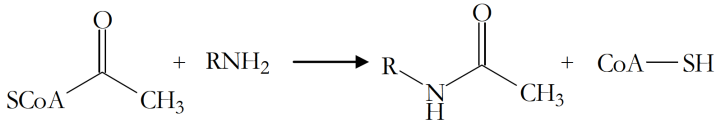
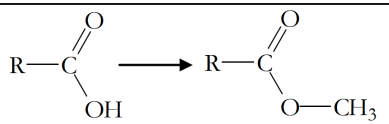
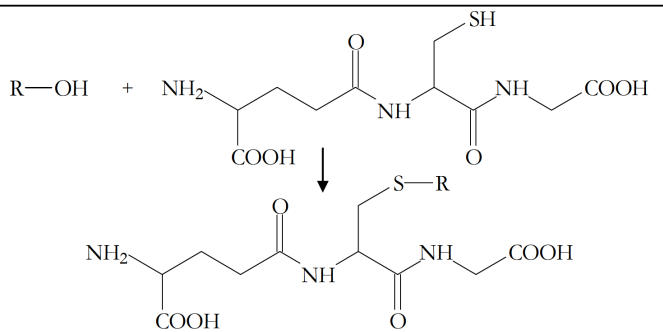
Oxidation				
1.	N-dealkylation	$RNHCH_3 \longrightarrow RNH_2 \longrightarrow CH_2O$	Mixed function oxidase (cytochrome P450)	Tamoxifen
2.	O-dealkylation	$R{-}OCH_3 \longrightarrow R{-}OH \longrightarrow CH_2O$	Mixed function oxidase (cytochrome P450)	Codeine
3.	Aliphatic hydroxylation		Mixed function oxidase (cytochrome P450)	Phenobarbital
4.	Aromatic hydroxylation		Mixed function oxidase (cytochrome P450)	Amphetamine
5.	N-Oxidation		Mixed function oxidase (cytochrome P450)	Meperidine
6.	S-Oxidation		Mixed function oxidase (cytochrome P450)	Omeprazol
7.	Deamination		Mixed function oxidase (monoamine oxidase)	Diazepam

Table 1.3. (Continued).

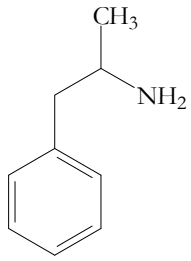
Phase II biotransformations				
Conjugation reactions				
1.	Glucuronidation		UDP-Glucuronosyl transferases	Morphine
2.	Sulfation		Sulfotransferases	AAS
3.	Acetylation		N-acetyltransferases	Sulfonamides
4.	Methylation		Methyltransferase	L-Dopa
5.	Glutathione conjugation		Glutathione-S-transferase	Ethacrynic acid

1.2.2. Metabolism of specific doping agents

1.2.2.1. Stimulants

Stimulants, such as amphetamines (Figure 1.1) and other sympatomimetic drugs, was one of the firsts groups of drugs prohibited in the doping control field. These substances may be used to reduce tiredness and increase performance, endurance, alertness, competitiveness and aggression [30]. Traditionally they have been used shortly before competition to directly improve performance. Therefore, they are currently only prohibited in-competition.

Figure 1.1. Amphetamine structure



The structure of these drugs is closely related to that of endogenous cathecolamines. They are phenylalkylamine derivatives with substituents in different positions: in the amine group (e.g. methamphetamine), in the phenyl ring (e.g. methoxyphenamine) and in α and β carbon atoms of the side chain (e.g. methylephedrine). Most of stimulants have a basic character, are relatively short-acting and although they can undergo phase I and phase II metabolism, they are mainly excreted as the parent drug in urine [31]. They are metabolised

in the liver by reactions of hydroxylation affecting the aromatic ring and the alkyl chain, deamination and N-dealkylation reactions, and further conjugation with glucuronic acid and, to a lesser extent, with sulphate [12].

Mesocarb is one of the examples of a drug with stimulant effect on the central nervous system included in the list of prohibited substances of the WADA [2]. Few studies of mesocarb metabolism have been reported in literature [32-35]. The limitation of detection of mesocarb and its hydroxylated metabolites by GC-MS, is their thermal unstablility and they decompose at the injection port of GC-MS.

1.2.2.2 Hormone antagonist and modulators

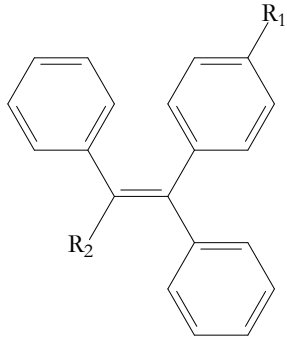
Selective estrogen receptor modulators (SERMs) are a class of compounds that can act as estrogen receptor (ER) agonists in some tissues while acting as ER antagonist in others. Currently, there are two main chemical classes of SERMs: triphenylethylene derivates (e.g. clomiphene, toremifene and tamoxifen) (Figure 1.2) and benzothiophene derivates (e.g. raloxyfen).

Anti-estrogen drugs are effective for prevention and as adjuvant treatment of estrogen-dependent breast cancer, for the treatment and prevention of post-menopausal osteoporosis and cardiovascular disease [36]. In males, the anti-estrogenic substances may cause an increase of the endogenous production of androgens by stimulating the gonadotrophines release. In athletes the use of antiestrogenic

compounds may compensate an extensive abuse of anabolic androgenic steroids

Very few metabolic studies have been reported for these substances [37-42]. The main reactions described were hydroxylations, N-dealkylations and N-oxidations. One of the main limitations of detection of toremifene and metabolites is its poor electron ionization and GC-MS properties.

Figure 1.2. General structure of triphenylethylene derivates.



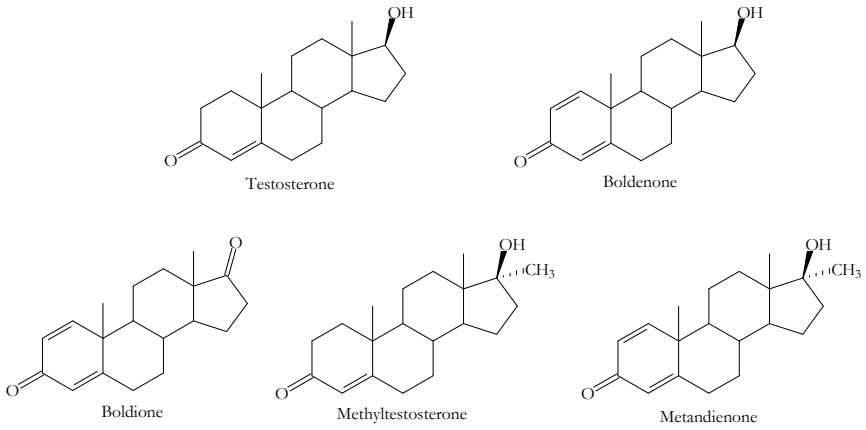
	R_1	R_2
Clompiphene	$OCH_2CH_2N(CH_2CH_3)_2$	Cl
Toremifene	$OCH_2CH_2N(CH_3)_2$	CH_2CH_2Cl
Tamoixifen	$OCH_2CH_2N(CH_3)_2$	CH_2CH_3

1.2.2.3. Anabolic steroids

Testosterone (Figure 1.3) is the principal anabolic androgenic steroid (AAS) in humans. It is produced mainly in the testis and it is involved in the development of several tissues and processes [12]. AAS structures are based testosterone structure. Androgens are the responsible for a wide variety of functions, of which the most

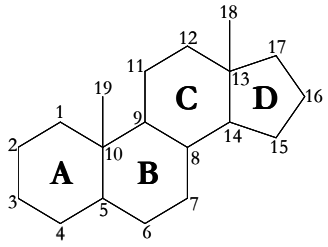
important are promoting male sexual differentiation during the first trimester of pregnancy, masculinization of the male at the puberty, and maintenance of adult male sexual function. Androgens also promote the assimilation of protein, thus androgens are anabolic agents.

Figure 1.3. Structures of testosterone and some exogenous AAS.



AAS are included in the list of forbidden substances in sport due to their performance enhancing because of increase muscle mass, which in turn provides greater strength and speed. They are the group of forbidden substances most frequently detected in antidoping control analyses reflecting the wide misuse of these compounds as performance enhancing drugs in sports by athletes [43].

Figure 1.4. General structure and carbon numering convention in steroids.



Metabolism of AAS has been extensively reviewed [44-48]. In Table 1.4, most important phase I metabolic reactions of AAS are described. In Figure 1.4, the carbon numbering convention of steroids is described. Phase I metabolism involves modifications (reductions, oxidations and hydroxylations) in the four rings of the steroidal structure as can be seen in Table 1.4. Among others, phase I reactions includes the C-4,5 double bond reduction to 5α and 5β-androstane isomers and the rapid reduction of the 3-keto group by 3-hydroxy-dehydrogenase enzymes, or the introduction of hydroxyl groups in different positions of the molecule. Some phase I metabolites are excreted in free form in urine, however in most of the cases phase II metabolism takes place [12,45,48].

Table 1.4. Phase I metabolic reactions of AAS.

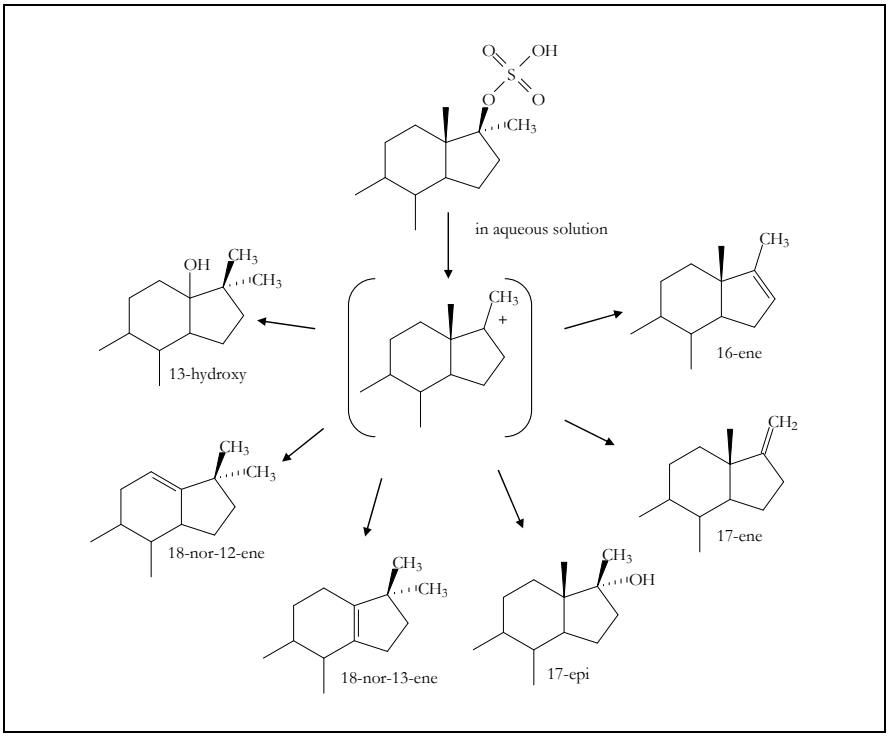
	Phase I metabolism	**Examples**
A ring	5α- and 5β- reduction	Testosterone
	3α- and 3β-hydroxy reduction	Nandrolone
	1,2-hydrogenation of 3-keto-abdrost-1,4-diene steroids	Metandienone
	1,2-dehydrogenation of 3-keto- 4-ene steroids	Boldenone
	Others (special A-ring modified)	Stanozolol
B ring	6β-hydroxylation	Metandienone Fluoxymesterone
	6,7-dehydrogenation	Metandienone
C ring	12-hydroxylation	4-chlor-1,2-dehydro-17α-methyltestosterone
D ring	17-oxidantion of the 17β-hydroxy group	Boldenone
	17β-hydroxylation of the 17-keto steroids	
	17α-hydroxylation of the 17-keto steroids	Trenbolone
	16α- and 16β-hydroxylation	Stanozolol
	16-oxidation	4-chlor-1,2-dehydro-17α-methyltestosterone
	18- (19-) hydroxyltation	Mesterolone

In phase II metabolism, phase I metabolites are further conjugated by glucuronidation and sulfatation. The main described phase II metabolites of AAS are conjugates with glucuronic acid. In the metabolism of AAS, reduction of the 3-keto group yields mainly the 3α-hydroxy configuration. The 3α-hydroxy steroids are conjugated with glucuronic acid regardless of whether the steroid has a 5α- or 5β-configuration. On the other hand, 3β-hydroxy steroids are mainly excreted as sulphates. 3α-O-β-Glucuronides are the major metabolites of AAS; however, some androgens are excreted also as sulphates, e.g., androsterone, etiocholanolone, epiandrosterone (major metabolite excreted), testosterone, and epitestosterone. Some AAS with a secondary 17β-hydroxy group or with tertiary 17β-hydroxy group in 17β-hydroxy-17α-methyl are conjugated with glucuronic acid (e.g. testosterone, methenolone, drostanolone and clostebol). For most of them, sulphated fraction has not been comprehensively studied due to the difficulties in the hydrolysis of sulphates to phase I metabolites.

AAS with a secondary 17β-hydroxy group or with tertiary 17β-hydroxy group in 17β-hydroxy-17α-methyl are frequently conjugated with sulphate [47,49-51]. In case of tertiary 17β-hydroxy group in 17β-hydroxy-17α-methyl steroids the 17β-sulphate is sterically influenced and descomposes in urine to yield several dehydration products (e.g. 18-nor-17,17-dimethyl analogues) and the corresponding 17-epimeric isomer (17α-hydroxy-l7β-methyl) (Figure 1.5). 17-Epimerization and metabolites resulting from decomposition have been demonstrated for several 17β-hydroxy-17α-methyl steroids: fluoxymesterone, bolasterone, metandienone, methyltestosterone, oxandrolone, stanozolol or 4-clhorometandienone [47,49-51].

Labile conjugates where described for some AAS (e.g. metandienone and boldenone) in 6β-hydroxy metabolites [47,52]. Labile conjugates have been recently identified for testosterone as Cysteine conjugates [53].

Figure 1.5. Degradation of the 17β-sulphate of tertiary 17β-hydroxy groups, with rearrangement of 18-nor-17,17-dimethyl-13-ene, 18-nor-17,17-dimethyl-12-ene, 16-ene, 17-ene, 13-hydroxy-17,17-dimethyl and 17-epimeric steroids.



As examples of AAS metabolim, the up to now available information of the AAS studied in this thesis (boldenone, methyltestosterone and metandienone) is described below.

- Boldenone

Different metabolic studies of boldenone in man have been reported. After oral administration of boldenone, the parent compound, 5β-androst-1-en-17β-ol-3-one (BM1), 5β-androst-1-en-3α,17β-diol, 5β-androst-1-en-3α-ol-17-one and 5β-androst-1-en-6β-ol-3,17-dione have been detected in human urine excreted as conjugates with glucuronic acid [47]. Other metabolites have been reported at low concentrations after treatment of the urine with potassium carbonate: 5β-androst-1-en-3,17-dione, 5α-androst-1-ene-3,17-dione, androsta-1,4-diene-3,17-dione, androsta-1,4-diene-6β,17β-diol-3-one, and androsta-1,4-diene-6β-ol-3,17-dione [52]. Among these compounds unchanged boldenone and its main metabolite, BM1, are normally used to detect boldenone misuse in doping control analyses, because they are the compounds detectable for the longest period of time after administration according to the data available up to now [52].

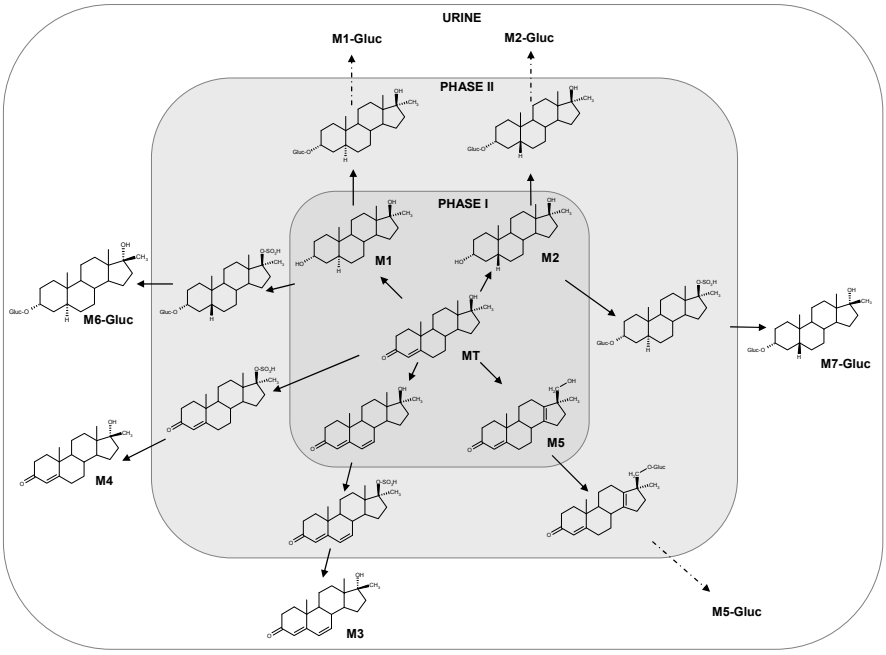
- Methyltestosterone

The use of methyltestosterone is normally screened by the detection of the two main metabolites, 17α-methyl-5α-androstan-3α,17β-diol (M1) and 17α-methyl-5β-androstan-3α,17β-diol (M2) (Figure1.6) in the glucuronide fraction.

Other minor metabolites detectable by LC-MS/MS like 17α-hydroxy-17β-methylandrost-4,6-dien-3-one (M3) or 17α-hydroxy-17β-methylandrost-4-en-3-one (M4) [54] have been reported, (Figure 1.6). In addition to that, other metabolites like 17β-hydroxymethyl-17α-

methyl-18-norandrosta-4,13-dien-3-one (M5) [55] or 17β-methyl-5α-androstan-3α,17α-diol (M6) and 17β-methyl-5β-androstan-3α,17α-diol (M7) [50] have been reported by using GC-MS/MS or GC-MS after β-glucuronidase hydrolysis (Figure 1.6). While M5 is excreted as glucuronide, the occurrence of M3, M4, M6 and M7 in urine can be explained by epimerization at the C17 after conjugation with a sulphate moiety. As mentioned above, 17β-sulphates are spontaneously hydrolysed in urine to several dehydratation products, and to the 17α-hydroxy-17β-methyl epimers [49,50] (Figure 1.6).

Figure 1.6. Metabolism of methyltestosterone.



Among all these metabolites described for methyltestosterone, it was demonstrated that the LC-MS/MS detection of M3 provides the highest retrospectivity [54]. This fact shows the potential usefulness of

metabolites conjugated with sulphates for the long-term detection of methyltestosterone misuse. A comprehensive study of sulphate metabolites for methyltestosterone has not been performed up to now.

- Metandienone

The metabolism of metandienone has been extensively studied [45,47,55,56,57]. The main metabolic pathways described are depicted in Figure 1.7. The metabolism of metandienone was first published by Rongone and Segaloff [57] who described 6β-hydroxymetandienone (I) and 17-epimetandienone (II) as metabolites of metandienone in humans that are detected in urine in free form [56]. 17-epimerization as well as formation of 18-nor-17,17-dimethyl analogs were demonstrated to result from degradation and rearrangement of 17β-sulphate conjugates in urine [49,50]. 6β-Hydroxy-17-epimetandienone (III) resulting also from the formation of a 17β-sulphate, is detected in post-administration samples in free form [56].

Different phase I metabolites resulting from reduction of the A ring (metabolites IV, VI and IX) have been described [56]. Metabolite IV (17β-hydroxy-17α-methyl-5β-androst-1-en-3-one) is excreted in urine as a 17β-glucuronide and as a 17β-sulphate, that is decomposed in urine to the C17 epimer, 17α-hydroxy-17β-methyl-5β-androst-1-en-3-one (V), and 18-nor-17,17-dimethyl-androsta-1,4,13-triene-3-one. Further reduction of the A ring results in the formation of 17α-methyl-5β-androst-1-en-3α,17β-diol (VI) that is conjugated with glucuronic acid at the 3α position and with sulphate at the 17β

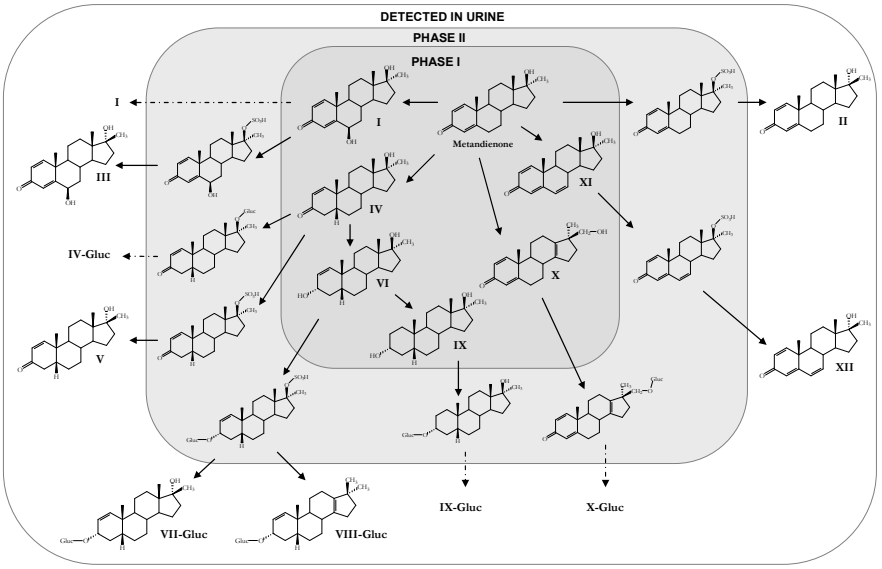
position. This metabolite is decomposed in urine to form 17-epimetendiol glucuronide (VII-Gluc) and 18-nor-epimetendiol glucuronide (VIII-Gluc). Additional reduction of the A ring results in the formation of 17α-methyl-5β-androstan-3α,17β-diol (IX) which is also a metabolite of methyltestosterone and it is excreted in urine as a glucuronoconjugate. The 17-epimer of metabolite IX, 17β-methyl-5β-androstan-3α,17α-diol and other compounds resulting from decomposition of a 17β-sulphate (18-nor-17,17-dimethyl-androsta-13-ene-3α-ol) were also detected as glucuronide conjugates in urine and, therefore, were also result of 3α-glucuronoconjugation and 17β-sulphatation [56]. Moreover, dihydroxylated metabolites have been also described as 6β,16β-dihydroxy-metandienone, 6β,16α-dihydroxy-metandienone, 6β,16β-dihydroxy-epimetandienone and 6β,12α-dihydroxy-metandienone [56].

More recently, a long-term metabolite excreted in the glucuronide fraction, 18-nor-17β-hydroxymethyl-17α-methyl-5β-androsta-1,4,13-triene-3-one (X), was described [55], which is observed up to 19 days after metandienone administration. Finally, 6-ene-epimetandienone (XII) resulting from 6,7-dehydrogenation and the formation of a 17β-sulphate has been reported [58].

Several of the metabolites reported (Figure 1.7) have 17β-methyl-17α-hydroxy or 18-nor-17,17-dimethyl structures. As indicated above, they result from the spontaneous hydrolysis of 17β-sulphates in urine. In the case of metandienone, some metabolites having longer retrospectivity (VII and XI) arise from the formation of sulphate conjugates. This fact shows the potential usefulness of sulphate

conjugate metabolites for the long-term detection of metandienone misuse. A comprehensive study of sulphate metabolites of metandienone has not been performed up to now.

Figure 1.7. Schema of main metabolic pathways of metandienone metabolism.



1.3. Analytical strategies for metabolic studies of doping agents

Metabolic studies of doping agents are needed in order to look for markers of the administration of the drug. In addition, for some specific groups of prohibited substances (e.g. glucocorticosteroids or β2-agonists), metabolic studies are needed to look for specific markers of the administration routes in order to distinguish between forbidden administration ways from allowed use.

As mentioned in previous paragraphs, the best marker of the administration of a doping agent is not always the most important metabolite but the metabolite excreted for longer times after administration. For this reason, metabolic studies of doping agents are addressed to the detection and identification of as much as metabolites as possible, even those representing a low percentage of the dose administered (less than 1 % of the dose). Then, the excretion profiles of the metabolites are measured using excretion study samples, and the usefulness of a given metabolite for doping control purposes is measured according to its excretion time. For this reason, even metabolites representing less than 1 % of the administered dose may be of interest if their excretion times are long.

Most information on metabolism of doping agents comes from the pharmaceutical industry and it was obtained during the development of the drug. In these cases, the methodology used depend on the years were the drugs were developed from thin-layer chromatography, liquid chromatography with ultraviolet or radioactivity detectors, and gas-chromatography in the sixties and seventies, to gas chromatography coupled to mass spectrometry (GC-MS) in the eighties and to liquid chromatography coupled to different mass spectrometry techniques (LC-MS) in the lasts years. In spite of the low analytical capabilities of the technologies used in the former years, in general the quality of results was very good allowing for the identification of the most important metabolites of the drugs under study at that moment.

First studies on metabolism of doping agents performed by antidoping control laboratories were published at the late seventies-early eighties. The most powerful methodology available at that moment was GC-MS. GC-MS offers high resolution, high sensitivity and high specificity, however it has some limitations. First, it requires derivatization of polar compounds to make them amenable for analysis. In addition, thermolabile compounds cannot be analysed. This is the reason why the most important phase II metabolites (glucuronides and sulphates) can not be directly analysed using GC-MS. As a consequence, studies of phase II metabolism have been traditionally performed using specific hydrolyses of the conjugates to release the phase I metabolite that have been then studied by GC-MS or LC-MS, in the lasts years. The introduction of LC-MS technology in the mid nineties introduced new possibilities to perform metabolic studies such as the direct analysis of very polar compounds without the need of derivatization or the direct analysis of conjugated metabolites without a previous hydrolysis.

Metabolic studies of doping agents are performed using classical models, *in vitro* and, mainly, *in vivo* studies in healthy volunteers. In recent years, *in vivo* animal models based on mice with functional human hepatocytes have been successfully employed to the study of human metabolism of some AAS [59].

One of the main limitations for the studies using human volunteers is the obtaining of consent of the Ethical Committees because some of the drugs are not commercialized for human use. For this reason,

metabolic studies of doping agents are generally described using a low number of volunteers.

1.3.1. Gas chromatography-mass spectrometry

The introduction of gas chromatographic (GC) methods in the early sixties allowed a qualitative advance in the potentiality for detection of either the drugs or their marker metabolites. The identifications were initially carried out only on the basis of retention times and the use of non-selective detection. In the 1980s, gas chromatography coupled to mass spectrometry (GC-MS) has become the most powerful method for the identification and determination of relatively apolar organic molecules, especially in complex matrices such as biosamples [60]. GC-MS was the gold standard in all fields of analytical toxicology owing to its high identification and separation power combined with high sensitivity.

Several limitations are associated with GC-MS analysis. The need of derivatization of polar compounds and the impossibility of the direct analysis of conjugated metabolites are the most important limitations for the performance of metabolic studies by GC-MS.

Volatile and thermostable molecules on the WADA Prohibited List are directly compatible with GC-MS, while non-volatile compounds can be effectively analysed by GC if derivatized first [6,61]. Furthermore, in case of AAS, there are some difficulties to detect some of them by common GC-MS methods due to problems of derivatization of steroids with large conjugated systems, with a high

number of hydroxyl groups or with high conjugation in the keto function.

The need to improve GC properties of target analytes and to obtain supporting information that would provide additional confidence in analytical results led to the development of various derivatization strategies, which improved chromatographic peak shapes and yielded additional data characterizing a substance.

Silylation is the most widely used derivatization procedure for GC-MS analyses. Silyl derivates are formed mainly in -OH, -SH or -NH groups. The most common silylation procedure is the formation of trimethylsilylted derivates. For AAS, trimethylsylilation is normally performed with a mixture containing N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), ammonium iodide and ethanethiol. Under these conditions, both the hydroxyl and the keto groups are derivatized [13,62]. Through this procedure, hydroxyl and carbonyl groups are converted, respectively, in the corresponding trimethylsilyl (TMS) and enol-TMS ethers. Nevertheless, the failure of some compounds to provide a single reaction product, the chemical rearrangement of others, and the fact that both hydroxyl and carbonyl functions are converted to the same derivative, curtail the structural information resulting from this approach [63]. Other reagents like trimethylsilylimidazole (TMSIm) has strong silylation power for hydroxyl and carboxyl groups but does not react with amino groups nor promote enol-TMS ether formation.

Selective N-trifluoroactil(TFA)-O-TMS derivative formation has been described for phenolalkylamines, hydroxyamines and amino acids [13]. The trimethylsilylating reagent used was MSTFA, followed by *N*-methylbis(trifluoroacetamide) (MBTFA) as trifluoroacylating agent. The derivatives are very stable in solution and show excellent gas chromatographic properties.

Since elucidation is the goal in metabolic studies, other derivatization protocols could be more useful. For example, formation of methoxime derivates of ketone groups prior to trimethylsilylation of hydroxyl groups has been widely used to analyze corticosteroids [64]. For instance, the formation of methyloxime-TMS derivates commonly employed in clinical steroid profiling permits the determination of the number of hydroxyls and carbonyl groups [65]. The formation of specific derivatives for each functional group provides valuable structural information.

Acylation is another commonly used derivatization procedure used in GC-MS. Acylated derivated can be obtained from alcohols, amines, amides, thiols, phenols, sulfonamides or aromatic rings. Acetylation has been used to derivatize, for example, β-blockers and their metabolites [66].

Another limitation of GC-MS analysis for metabolic studies is the poor electron ionization mass spectra obtained for some compounds. Electron ionization is an universal hard ionization technique and in some cases it results in few diagnostic ions. The mass spectrum of many stimulants, for example, shows one major low ion and other

poor diagnostic ions because of its very low intensity. Characteristic fragment ions at m/z 44, 58, 72, 86 or 100 can be obtained for different phenylethylamine compounds depending on the substituents at the nitrogen atom and the carbon atom in the α position [12]. Some anti-estrogenic drugs, for example, have a dialkylaminoalkyl side chain and the electron ionization mass spectra results also in one major low mass ion coming from this side chain. The use of different derivatization methods before GC-MS analysis has been described to produce sufficient diagnostic ions for identification purposes of some compounds [67,68]

Suitable sample preparation is the most important prerequisite in the GC-MS analysis of biosamples. It involves isolation and, if necessary, cleavage of conjugates and derivatization of the drugs and their metabolites [47]. Hydrolysis procedure is applied in order to deconjugate the phase II metabolites. After being released, phase I metabolites have to be isolated from the complex urine matrix. The hydrolysis of conjugates is a fundamental step in many of the analytical methods used and is performed prior to the analysis and structure elucidation of the phase I metabolites [69]. Ideally, the whole amount of the metabolites should be extracted while minimizing the co-extraction of interferents present in the urine.

Phase II metabolites have been normally studied using indirect methods consisting in a hydrolysis procedure to cleave the conjugates and to release the corresponding phase I metabolites. Hydrolysis can be carried out by enzymatic or chemical means depending on the compounds studied.

Both acidic and enzymatic hydrolysis using β-glucuronidase from different sources (e.g. *Patella vulgate*, *Helix pomatia, Escherichia coli*) have been reported for studies of opioids (e.g. codeine and morphine) [70,71]. Acid hydrolysis presented higher recoveries in opioid hydrolysis. Enzymatic hydrolysis is the most common hydrolysis procedure used in metabolic studies of compound such as AAS. β-Glucuronidase from *Escherichia coli*, β-glucuronidase-arylsulphatase from *Helix pomatia* have been the most used enzymes. Most metabolites of AAS conjugated with sulphate are resistant to hydrolysis by *Helix pomatia* juice, so they will not be detected in studies where this type of hydrolysis is used. For hydrolysis of sulphates, solvolysis procedures have been described [72].

Most metabolic studies used hydrolysis with enzymes with β-glucuronidase activity, thus mainly conjugates with glucuronic acid have been studied, even when using LC-MS analysis. Except for specific compounds, other phase II metabolites, such as sulphate conjugate metabolites, have not been comprehensively studied mainly due to the difficulties in the hydrolysis of sulphates to phase I metabolites [47].

The use of selected ion monitoring (SIM) of the most abundant, characteristic and/or specific fragment ions of target compounds has enabled the sensitive qualitative and quantitative detection [73]. Acquisiton in SIM mode is used in single quadrupole instruments (GC-MS) or selected reaction monitoring (SRM) mode in triple quadrupole instruments (GC-MS/MS).

The acquisition of scan mode with nominal single mass analyzers (single quadrupole or ion trap) is also used for the detection of metabolites. However, the usefulness of this approach is limited to the detection of the main metabolites. Thus, as an example, the detection and characterization of three metabolites of boldione was possible using this approach [74]. Therefore, even minor metabolites might be detected by this strategy. Obviously, the main drawbacks are the impossibility of detecting unexpected metabolites and the need of previous knowledge about the drug metabolism [45,50,55]. If the drug is unknown, the full-scan mode is the method of choice, as comparison of the full mass spectra with reference spectra is necessary [75].

1.3.2. Liquid chromatography-mass spectrometry

Over the last decade the technique of liquid chromatography-tandem mass spectrometry (LC-MS/MS) has slowly grown in importance in doping analysis. It was initially utilised on substances that were difficult to detect by other techniques, for the more polar banned substances and thermally labile stimulants or substances, such as the diuretics and corticosteroids, for which GC-MS detection was difficult [5,15,76,77]. The use of LC-MS/MS provides several advantages in comparison with GC-MS. LC-MS/MS has the possibility to directly analyse the phase II metabolites, avoiding hydrolysis, and very polar compounds avoiding derivatization steps [78-80]. Avoiding the derivatization step, detection of metabolites which present difficulties in the derivatization, like polyhydroxylated metabolites, is possible

[81]. LC-MS provides more sensitivity, the analytical time is generally shorter in comparison with GC-MS procedures and necessitates less tedious sample preparation procedures due to the high analyte selectivity of LC-MS/MS [78,79,82]. Working in gradient mode, LC-MS/MS allows a rapidly and efficient separation of drugs and related substances [15]. Due to the reduced analysis time, the peaks become narrow and high acquisition mass analysers with fast acquisition and detector responses, such as triple quadrupole [82].

Other benefits of LC-MS/MS over GC-MS are that increase the possibility to detect a wider number of drugs relevant for doping control, eliminates predictable sources of error, and eliminates the quantitative recovery problems associated with the hydrolysis and derivatization steps of GC-MS. Furthermore, LC-MS allows the determination of thermally labile compounds, chemically unstable and/or volatile drugs [62,78-80].

In recent years, LC-MS/MS has emerged as the most accurate method for measuring small molecules. Research on the detection of all groups of doping agents has been investigated by LC-MS and routine LC-MS screening applications are now available for almost all classes of doping agents. Improvements in MS technology with respect to scan speed, sensitivity, and polarity switching allows for the combined detection of different classes of doping agents [69,73].

Electrospray ionisation (ESI) LC-MS/MS is the most widely used source for doping control because it is dedicated to polar compounds, many drugs are quite polar and their metabolites are even more polar,

and offers high sensitivities respect other ionization sources. As the biotransformation may lead to significant changes in the ionization characteristics of the analytes, data acquisition in both positive and negative mode is normally performed in metabolic studies [69]. With the constantly increasing number of analytes and applications of LC-MS/MS, detailed knowledge of mass spectrometric behaviour and dissociation pathways of target compounds was required. Numerous studies were dedicated to the elucidation of fragmentation or deprotonation followed by collision-induced dissociation (CID) [15,81,83-85]. LC-MS/MS offers the possibility to apply open methods (neutral loss and precursor ion scan methods) for the detection of metabolites having a common chemical structure.

Studies on fragmentation pathways of relevant drugs have been of particular interest for doping control laboratories as they have provided the necessary data to unambiguously determine prohibited compounds in collected samples [83]. The key to structure identification approaches is based on the fact that metabolites generally retain a significant part of the core structure of the parent drug. Therefore, the parent drug and its corresponding metabolites would be expected to undergo similar fragmentations and to produce mass spectra that indicate major substructures, and structure-related MS-MS analysis modes can be applied to screen biological samples for metabolites with certain features in common with the parent drug [17].

Product-ion scan methods provide the specific product ions and neutral losses which can be assigned to substructures of the molecule. Further information is obtained, by the observation of sequential

neutral losses. In studies of metabolism, searching for new metabolites, precursor-ion scan methods can be applied to search for metabolites that share common fragments, while the neutral-loss analysis mode can be used to screen for metabolites that share common losses [69,77]. For example, for the detection of conjugated metabolites, glucuronide and sulphate conjugates are readily fragmented in MS-MS by the formation of either conjugate-specific fragment ions, e.g., m/z 97 for sulphates and m/z 85 and 113 for glucuronides in the negative-ion mode, or conjugate-specific neutral losses, e.g., 80 and 176 Da for sulphates and glucuronides, respectively [53,69].

Another invaluable tool in metabolite identification is the Q-TOF, which provides accurate-mass determination in both MS and MS-MS mode [69]. Since TOF instruments acquire full scan data only can give good high resolution data, useful information relating to possible use is stored in this files. The use of the high resolution ability of these instruments reduces the background noise encountered in low resolution LC-MS scanning and so gives the ability to detect quite low levels of substances, for which the selectivity of LC-MS/MS would normally require. The stored data can be re-interrogated at a later date, once information about new designer steroids is obtained. This procedure is quite useful in detection of designer steroids once it is know what steroid to investigate and readily gives good information on metabolism pathways [86].

LC-MS/MS has a number of features that can be effectively exploited in sports drug testing or that offer good perspectives of application in

metabolic studies. LC-MS/MS appears to be the most suitable and powerful technique for unambiguous identification of such hydrophilic and polar compounds e.g. glucuronides and sulphate conjugates [87].

1.4. References

[1] WADA. (2009). World Anti-Doping Code. http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-The-Code/WADA_Anti-Doping_CODE_2009_EN.pdf

[2] WADA. (2012). The World Anti-Doping Code. The 2013 Prohibited List. International Standard. http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/2013/WADA-Prohibited-List-2013-EN.pdf

[3] WADA. (2012) International Standards of Laboratories. http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/ISL/WADA_Int_Standard_Laboratories_2012_EN.pdf

[4] WADA. (2013). Technical Document - TD2013INDEX. http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/Technical_Documents/WADA-TD2013-INDEX-EN.pdf

[5] Ventura, R., Roig, M., Monfort, N., Sáez, P., Bergés, R., Segura, J. (2008). High-throughput and sensitive screening by ultra-performance liquid chromatography tandem mass spectrometry of diuretics and other doping agents. *Eur. J. Mass Spectrom.*, 14, 191-200.

[6] Kolmonen, M., Leinonen, A., Pelander, A., Ojanperä, I. (2007). A general screening method for doping agents in human urine by solid phase extraction and liquid chromatography/time-of-flight mass spectrometry. *Anal. Chim. Acta*, 585, 94-102.

[7] Vonaparti, A., Lyris, E., Angelis, Y. S., Panderi, I., Koupparis, M., Tsantili-Kakoulidou, A., Peters, R. J. B., Nielen, M. W. F., Georgakopoulos, C. (2010). Preventive doping control screening analysis of prohibited substances in human urine using rapid-resolution liquid chromatography/high-resolution time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.*, 24, 1595-1609.

[8] Van Eenoo, P., Van Gansbeke, W., De Brabanter, N., Deventer, K., Delbeke, F. T. (2011). A fast, comprehensive screening method for doping agents in urine by gas chromatography-triple quadrupole mass spectrometry. *J. Chromatogr. A*, 1218, 3306-3316.

[9] WADA. (2010). Technical Document – TD2010IDR http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/Technical_Documents/WADA_TD2010IDCRv1.0_Identification%20Criteria%20for%20Qualitative%20Assays_May%2008%202010_EN.doc.pdf

[10] WADA. (2012). WADA Technical Document - TD2013MRPL. http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/Technical_Documents/WADA-TD2013MRPL-Minimum-Required-Performance-Levels-v1-2012-EN.pdf

[11] Schänzer, W. (1998). Sports: Detection of exogenous anabolic steroids. In: S.B. Karch (Ed.), *Drug abuse handbook*, CRC, Boca Raton, Chapter 9, 671-689.

[12] Segura, J., Ventura, R., Marcos, J., Gutiérrez-Gallego, R. (2008). Doping substances in human and animal sport. Chapter 21. Boguz, M.J. (Ed.) Forensic Science. *Handbook of analytical separations*, 6, 699-744.

[13] Segura, J., Ventura, R., Jurado, C. (1998). Derivatization procedures for gas chromatographic-mass spectrometric determination of xenobiotics in biological samples, with special attention to drugs of abuse and doping agents. *J. Chromatogr. B*, 713, 61-90.

[14] WADA (2013). Technical Document – TD2013DL. http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/Technical_Documents/WADA-TD2013DL-Decision-Limits-for-the-Confirmatory-Quantification-Threshold-Substances-2.0-EN.pdf

[15] Thevis, M., Schänzer, W. (2007). Current role of LC-MS(/MS) in doping control. *Anal. Bioanal. Chem.*, 388, 1351-1358.

[16] Thevis, M. (2010). Mass spectrometry in sports drug testing: Characterization of prohibited substances and doping control analytical assay. *Wiley*, 275-339.

[17] Lee, M. S., Kerns, E. H. (1999). LC/MS applications in drug development. *Mass Spectrom. Rev.*, 18, 187-279.

[18] Bachmann, K., (2008). Drug Metabolism. Hacker, M., Messer, W., Bachmann, K. (Eds.) *Pharmacology. Principles and practice.* 8, 131-173.

[19] Ghosal, A., Ramanathan, R., Kishnani, N. S., Chowdhury, S. K., Alton, K. B. (2005). Chowdhury, S.K. (Ed.) Cytochrome P450 (CYP) and UDP-Glucuronosyltransferase (UGT) enzymes: role in drug metabolism, polymorphism, and identification of their involvement in drug metabolism. *Progress in Pharmaceutical and Biomedical Analysis (6) Identification and Quantification of Drugs, Metabolites and Metabolizing Enzymes by LC-MS*, 12, 295-336.

[20] Bergés, R., Segura, J., Ventura, R., Fitchm K. D., Morton, A. R., Farré, M., Mas, M., de La Torre, X. (2000). Discrimination of prohibited oral use of salbutamol from authorized inhaled asthma treatment. *Clin. Chem.* 46, 1365-1375.

[21] Ventura, R., Damasceno, L., Farré, M., Cardoso, J., Segura, J. (2000). Analytical methodology for the detection of β2-agonists in urine by gas chromatography-mass spectrometry for application in doping control. *Anal. Chim. Acta*, 418, 79-92.

[22] Matabosch, X., Pozo, O. J., Pérez-Mañá, C., Farré, M., Marcos, J., Segura, J., Ventura, R. (2012). Discrimination of prohibited oral use from authorized inhaled treatment of budesonide in sports. *Ther. Drug Monit.*, 35, 118-128.

[23] Jancova, P., Siller, M. (2012). Phase II drug metabolism. Paxton, J. (Ed.) *Topics on drug metabolism.* 35-60.

[24] Gomes, R. L., Meredith, W., Snape, C. E., Sephton, M. A. (2009). Conjugated steroids: analytical approaches and applications. *Anal. Bioanal. Chem.*, 393, 453-458.

[25] Kuuranne, T., Kurkela, M., Thevis, M., Schänzer, W., Finel, M., Kostiainen, R. (2003). Glucuronidation of anabolic androgenic steroids by recombinant human UDP-glucuronosyltransferases. *Drug Metab. Dispos.*, 31, 1117-1124.

[26] Gamage, N., Barnett, A., Hempel, N., Duggleby, R. G., Windmill, K. F.; Martin, J. L., McManus, M. E. (2006). Human sulfotransferases and their role in chemical metabolism. *Toxicol. Sci.*, 90, 5-22.

[27] Dupret, J. M., Rodrigues-Lima, F. (2005). Structure and regulation of the drug–metabolizing enzymes arylamine N-acetyltransferases. *Curr. Med. Chem.*, 12, 311-318.

[28] Van Bladeren, P. J. (2000). Glutathione conjugation as a bioactivation reaction. *Chem. Biol. Int.*, 129, 61-76.

[29] Hayes, J. D., Flanagan, J. U., Jowsey, I. R. (2005). Glutathione transferases. *Annu. Rev. Pharmacol. Toxicol.*, 45, 51-88.

[30] Thevis, M., Sigmund, G., Geyer, H., Schänzer, W. (2010). Stimulants and doping in sport. *Endocrinol. Metab. Clin. N. Am.*, 39, 89-105.

[31] Van Eenoo, P., Delbeke, F. T. (2010). β-Adrenergic stimulation. D. Thieme and P. Hemmersbach (eds.), *Doping in Sports, Handbook of Experimental Pharmacology*, 195, 227-249.

[32] Polgár, M., Vereczkey, L., Szporny, L., Czira, G., Tamás, J., Gács-Baitz, E., Holly, S. (1979). Metabolism of mesocarb in the rat. *Xenobiotica*, 9, 511-520.

[33] Ventura, R., Nadal, T., Alcalde, P., Segura, J. (1993). Determination of mesocarb metabolites by high-performance liquid chromatography with UV detection and with mass spectrometry using a particle-beam interface. *J. Chromatogr.*, 647, 203-210.

[34] Appolonova, S. A., Shpak, A. V., Semenov, V. A. (2004). Liquid chromatography-electrospray ionization ion trap mass spectrometry for analysis of mesocarb and its metabolites in human urine. *J. Chromatogr. B*, 800, 281-289.

[35] Vahermo, M., Suominen, T., Leinonen, A., Yli-Kauhaluoma, J. (2009). Synthesis and characterization of hydroxylated mesocarb metabolites for doping control. *Arch. Pharm. Chem. Life Sci.*, 342, 201-209.

[36] Handelsman, D. J. (2008). Indirect androgen doping by oestrogen blockade in sports. *British J. Pharmacol.*, 154, 598-605.

[37] Fromson, J. M., Pearson, S., Bramah, S. (1973). The metabolism of tamoxifen (I.C.I. 42,474). I. In female patients. *Xenobiotica*, 3, 711-714.

[38] Watanabe, N., Irie, T., Koyama, M., Tominaga, T., (1989). Liquid chromatographic-atmospheric pressure ionization mass spectrometric analysis of toremifene metabolites in human urine. *J. Chromatogr.*, 497, 169-180.

[39] Kim, S. Y., Suzuki, N., Santosh Laxmi, Y. R., Rieger, R., Shibutani, S. (2003). α-Hydroxylation of tamoxifen and toremifene by human and rat cytochrome P450 3A subfamily enzymes. *Chem. Res. Toxicol.*, 16, 1138-1144.

[40] Jordan, V. C. (2007). New insights into the metabolism of tamoxifen and its role in the treatment and prevention of breast cancer. *Steroids*, 72, 829-842.

[41] Claude, B., Morin, P., Bayoudh, S., de Ceaurriz, J. (2008). Interest of moleculary imprinted polymers in the fight against doping. Extraction of tamoxifen and its main metabolite from urine followed by high-performance liquid chromatography with UV detection. *J. Chromatogr. A*, 1196-1197, 81-88.

[42] Mazzarino, M., Fiacco, I., de la Torre, X., Botrè, F. (2008). A mass spectrometric approach for the study of the metabolism of clomiphene, tamoxifen and toremifene by liquid chromatography time-of-flight spectrometry. *Eur. J. Mass Spectrom.*, 14, 171-180.

[43] WADA. (2011). 2011 Laboratory Testing Figures. http://www.wada-ama.org/Documents/Resources/Testing-Figures/WADA-2011-Laboratory-Testing-Figures.pdf

[44] Massé, R., Ayotte, C., Dugal, R. (1989). Studies on anabolic steroids: I. Integrated methodological approach to the gas chromatographic-mass spectrometric analysis of anabolic steroid metabolites in urine. *J. Chromatogr. Biomed. Applic.*, 489, 23-50.

[45] Schänzer, W., Donike, M. (1993). Metabolism of anabolic steroids in man: synthesis and use of reference substances for identification of anabolic steroid metabolites. *Anal. Chim. Acta*, 275, 23-48.

[46] Ayotte, C., Goudreault, D., Charlebois, A. (1996). Testing for natural and synthetic anabolic agents in human urine. *J. Chromatogr. B*, 687, 3-25.

[47] Schänzer, W. (1996). Metabolism of anabolic androgenic steroids. *Clin. Chem.*, 42, 1001-1020.

[48] Van Eenoo, P., Delbeke, F. T. (2006). Metabolism and excretion of anabolic steroids in doping control-New steroids and new insights. *J. Steroid Biochem. Mol. Biol.*, 101, 161-178.

[49] Bi, H., Massé, R. (1992). Studies on anabolic steroids-12. Epimerization and degradation of anabolic 17β-sulfate-17α-methyl steroids in human: qualitative and quantitative GC/MS analysis. *J. Steroid Biochem. Molec. Biol.*, 42, 533-546.

[50] Schänzer, W., Opfermann, G., Donike, M. (1992). 17-Epimerization of 17α-methyl anabolic steroids in humans: metabolism and synthesis of 17α-hydroxy-17β-methyl steroids. *Steroids*, 57, 537-550.

[51] Bowers, L. D., Sanaullah. (1996). Direct measurement of steroid sulphate and glucuronide conjugates with high-performance liquid chromatography-mass spectrometry. *J. Chromatogr. B*, 687, 61-68.

[52] Schänzer, W., Donike, M. (1992). Metabolism of boldenone in man: gas chromatographic/mass spectrometric identification of urinary excreted metabolites and determination of excretion rates. *Biol. Mass Spectrom.*, 21, 3-16.

[53] Fabregat, A., Kotronoulas, A., Marcos, J., Joglar, J., Alfonso, I., Segura, J., Ventura, R., Pozo, O.J. (2013). Detection, synthesis and characterization of metabolites of steroid hormones conjugated with cysteine. *Steroids*, 78, 327-336.

[54] Pozo, O. J., Van Eenoo, P., Deventer, K., Lootens, L., Van Thuyne, W., Parr, M. K., Schänzer, W., Sancho, J. V., Hernández, F., Meuleman, P., Leroux-Roels, G., Delbeke, F. T. (2009). Detection and characterization of a new metabolite of 17α-methyltestosterone. *Drug Metab. Dispos.*, 37, 2153-2162.

[55] Parr, M. K., Fusshöler, G., Gütschow, M., Hess, C., Schänzer, W. (2010). GC-MS(/MS) investigations on long-term metabolites of 17-methyl steroids. *Recent Advances in Doping Analysis(18) Proceedings of the Manfred Donike Workshop 28th Cologne Workshop on Dope Analysis. Köln: Sportverlag Strauss*, 64-73.

[56] Schänzer, W., Geyer, H., Donike, M. (1991). Metabolism of metandienone in man: identification of conjugated excreted urinary metabolites, determination of excretion rates and gas chromatographic-mass spectrometric identification of bis-hydroxyltated metabolites. *J. Steroid Biochem. Mol. Biol.*, 38, 441-464.

[57] Rongone, E. L., Segaloff, A. (1963). In vivo metabolism of Δ1, 17α-methyltestosterone in man. *Steroids*, 1,179-184.

[58] Pozo, O. J., Lootens, L., Van Eenoo, P., Deventer, K., Meuleman, P., Leroux-Roels, G., Parr, M. K., Schänzer, W., Delbeke, F. T. (2009). Combination of liquid-chromatography tandem mass spectrometry in different scan modes with human and chimeric mouse urine for the study of steroid metabolism. *Drug Test. Anal.*, 1, 55-67.

[59] Lootens, L., Meuleman, P., Pozo, O. J., Van Eenoo, P., Leroux-Roels, G., Delbeke, F. T. (2009). uPA$^{+/+}$-SCID mouse with humanized liver as a model for in vivo metabolism of exogenous steroids: methandienone as a case study. *Clin. Chem.*, 55, 1783-1793.

[60] Maurer, H. H. (1992). Systematic toxicological analysis of drugs and their metabolites by gas chromatography-mass spectrometry. *J. Chromatogr.* 580, 3-41.

[61] Mueller, R. K., Grosse, J., Lang, R., Thieme, D. (1995). Chromatographic techniques - the basis of doping control. *J. Chromatogr. B*, 674, 1-11.

[62] Gomes, R. L., Meredith, W., Snape, C. E., Sephton, M. A. (2009). Analysis of conjugated steroid androgens: Deconjugation, derivatisation and associated issues. *J. Pharm. Biomed. Anal.*, 49, 1133-1140.

[63] Hewitt, S. A., Kearney, M., Currie, J. W., Young, P. B., Kennedy, D. G. (2002). Screening and confirmatory strategies for the surveillance of anabolic steroid abuse within Northern Ireland. *Anal. Chim. Acta*, 473, 99-109.

[64] Rodchenkov, G. M., Vedenin, V. P., Uralets, V. P., Semenov, V. A. (1991). Characterization of prednisone, prednisolone and their metabolites by gas chromatography-mass spectrometry. *J. Chromatogr.,* 565, 45-51.

[65] Shackleton, C. H. (1986). Profiling steroid hormones and urinary steroids. *J. Chromatogr.*, 379, 91-156.

[66] Hartonen, K., Riekkola, M. -L. (1996). Detection of β-blockers in urine by solid-phase extraction-supercritical fluid extraction and gas chromatography-mass spectrometry. *J. Chromatogr. B*, 676, 45-52.

[67] Damasceno, L., Ventura, R., Ortuño, J., Segura, J. (2000). Derivatization procedures for the detection of β2-agonists by gas chromatographic/mass spectrometric analysis. *J. Mass Spectrom.*, 35, 1285-1294.

[68] Solans, A., Carnicero, M., de la Torre, R., Segura, J. (1995). Comprehensive screening procedure for detection of stimulants, narcotics, adrenergic drugs, and their metabolites in human urines. *J. Anal. Toxicol.*, 19, 104-114.

[69] Niessen, W. M. A. (2003). Progress in liquid chromatography-mass spectrometry instrumentation and its impact on high-throughput screening. *J. Chromatogr. A*, 1000, 413-436.

[70] Wang, P., Stone, J. A., Chen, K. H., Gross, S. F., Haller, C. A., Wu, A. H. B. (2006). Incomplete recovery of prescription opioids in urine using enzymatic hydrolysis of glucuronide metabolites. *J. Anal. Toxicol.*, 30, 570-575.

[71] Romberg, R. W., Lee, L. (1995). Comparison of the hydrolysis rates of morphine-3-glucuronide and morphine-6-glucuronide with acid and β-glucuronidase. *J. Anal. Toxicol.*, 19, 157-162.

[72] Torrado, S., Roig, M., Farré, M., Segura, J., Ventura, R. (2008). Urinary metabolic profile of 19-norsteroids in humans: glucuronide and sulphate conjugates after oral administration of 19-nor-4-androstenediol. *Rapid Commun. Mass Spectrom.*, 22, 3035-3042.

[73] Thevis, M., Bommerich, U., Opfermann, G., Schänzer, W. (2005). Characterization of chemically modified steroids for doping control purposes by electrospray ionization tandem mass spectrometry. *J. Mass Spectrom.*, 40, 494-502.

[74] Kim, Y., Jun, M., Lee, W. (2006). Characterization of boldione and its metabolites in human urine by liquid chromatography/ electrospray ionization mass spectrometry and gas chromatography/mass spectrometry. *Rapid Commun. Mass Spectrom.*, 20, 9-20.

[75] Van Thuyne, W., Van Eenoo, P., Delbeke, F. T. (2008). Implementation of gas chromatography combined with simultaneously selected ion monitoring and full scan mass spectrometry in doping analysis. *J. Chromatogr. A*, 1210, 193-202.

[76] Deventer, K., Pozo, O. J., Van Eenoo, P., Delbeke, F. T. (2008). Detection of doping agents by LC-MS and LC-MS-MS. *LCGC North Am.*, 26, 376-389.

[77] Pozo, O. J., Van Eenoo, P., Deventer, K., Delbeke, F. T. (2008). Detection and characterization of anabolic steroids in doping analysis by LC-MS. *Trends Anal. Chem.*, 27, 657- 671.

[78] Borts, D. J., Bowers, L. D. (2000). Direct measurement of urinary testosterone and epitestosterone conjugates using high-performance liquid chromatography/tandem mass spectrometry. *J. Mass Spectrom.*, 35, 50-61.

[79] Leinonen, A., Kuuranne, T., Kostiainen, R. (2002). Liquid chromatography/mass spectrometry in anabolic steroid analysis-optimization and comparison of three ionization techniques: electrospray ionization, atmospheric pressure chemical ionization and atmospheric pressure photoionization. *J. Mass Spectrom.*, 37, 693-698.

[80] Politi, L., Groppi, A., Polettini, A. (2005). Applications of liquid chromatography-mass spectrometry in doping control. *J. Anal. Toxicol.*, 29, 1-14.

[81] Pozo, O. J., Van Eenoo, P., Deventer, K., Delbeke, F. T. (2007). Ionization of anabolic steroids by adduct formation in liquid chromatography electrospray mass spectrometry. *J. Mass Spectrom.*, 42, 497-516.

[82] Badoud, F., Guillarme, D., Boccard, J., Grata, E., Saugy, M., Rudaz, S., Veuthey, J. -L. (2011a). Analytical aspects in doping control: Challenges and perspectives. *Forensic Sci. Int.*, 213, 49-61.

[83] Thevis, M., Schänzer, W. (2007). Mass spectrometry in sports drug testing: structure characterization and analytical assays. *Mass Spectrom. Rev.*, 26, 79-107.

[84] Pozo, O. J., Van Eenoo, P., Deventer, K., Grimalt, S., Sancho, J. V., Hernández, F., Delbeke, F. T. (2008). Collision-induced dissociation of 3-keto anabolic steroids and related compounds after electrospray ionization. Considerations for structural elucidation. *Rapid Commun. Mass Spectrom.*, 22, 4009-4024.

[85] Pozo, O. J., Deventer, K., Van Eenoo, P., Delbeke, F. T. (2008). Efficient approach for the comprehensive detection of unknown anabolic steroids and metabolites in human urine by liquid chromatography-electrospray-tandem mass spectrometry. *Anal. Chem.*, 80, 1709-1720.

[86] Kazlauskas, R. (2010). Advances in sports drug testing: an overview. *Drug Test. Anal.*, 2, 523-525.

[87] Hintikka, L., Kuuranne, T., Leinonen, A., Thevis, M., Schänzer, W., Halket, J., Cowan, D., Grosse, J., Hemmersbach, P., Nielen, M. W. F., Kostiainen, R. (2008). Liquid chromatographic-mass spectrometric analysis of glucuronide-conjugated anabolic steroid metabolites: method validation and interlaboratory comparison. *J. Mass Spectrom.*, 43, 965-973.

Chapter 2

Justification and Objectives

The current prohibited list in sports includes ten groups of prohibited substances (non-approved substances, anabolic agents, peptide hormones, β-2-agonists, hormone and metabolic modulators, diuretics and masking agents, stimulants, narcotics, cannabinoids, glucocorticosteroids), three prohibited methods (manipulation of blood, chemical and physical manipulation, gene doping), and two groups of substances prohibited in particular sports (alcohol, β-blockers). The list is updated every year by the World Anti-doping Agency. The task of doping control laboratories consist of the detection of the administration of these prohibited substances or methods by the analysis of biological samples (normally, urine) from the athlete.

Most doping agents are metabolized in the body in different extent, and the parent drug and/or the metabolites excreted in urine are the markers used to detect their administration. Metabolic reactions are grouped into two types, phase I and phase II reactions. Phase I reactions are enzymatically catalyzed reactions (mainly oxidations and reductions) and usually convert the doping agent into more polar compound in order to inactivate and to facilitate its elimination from the body. Phase II or conjugation reactions conjugate the doping agents or their phase I metabolites with a polar compound to help elimination from the body. The main described phase II metabolites are conjugates with glucuronic acid or with sulphate.

Therefore, metabolic studies of doping agents are needed in order to define the best marker for the detection of the administration of each forbidden substance. In doping control, the best marker is not always

the most abundant metabolite, but the metabolite excreted for longer time after administration of the drug, especially for those forbidden substances used during training periods (e.g., anabolic androgenic steroids). Therefore, even minor metabolites may be useful and need to be studied. For this reason, in addition to the detection and characterization of new metabolites, the study of the excretion profiles of each metabolite needs to be performed to evaluate the detection time.

Metabolic studies of doping agents have been traditionally performed using gas chromatography-mass spectrometry (GC-MS) with electron ionization, and the detection of most doping agents has been based for many years on the analysis of metabolites elucidated by GC-MS methods. In the last decade, liquid chromatography-mass spectrometry (LC-MS) methods have also been used. GC-MS has some limitations for the study of the metabolism of drugs, such as the impossibility of analysis of thermolabile compounds, the difficulties on the analysis of polar compounds that need previous derivatization and the impossibility to directly analyse phase II metabolites (conjugated metabolites). In addition, for some compounds the use of electron ionization, an universal hard ionization technique, results in few diagnostic ions limiting the usefulness of GC-MS for identification and characterization of new metabolites.

Due to the limitations to directly analyse conjugated metabolites by GC-MS, phase II metabolic reactions of doping agents have been normally studied using specific hydrolysis of the conjugates to release the phase I metabolites that were then studied by GC-MS and also by

LC-MS, in the last decade. Most metabolic studies used hydrolysis with enzymes with β-glucuronidase activity, thus mainly conjugates with glucuronic acid have been studied, even when using LC-MS analysis. Except for specific compounds, other phase II metabolites, such as sulphate conjugate metabolites, have not been comprehensively studied mainly due to the difficulties in the hydrolysis of sulphates to phase I metabolites.

LC-MS offers several advantages with respect to GC-MS. It allows the direct detection of phase II metabolites and the direct detection of polar compounds, avoiding hydrolysis and derivatization steps. In addition, LC coupled to tandem mass spectrometry (MS/MS) offer different possibilities for the sensitive detection and structural elucidation of unknown compounds such as new metabolites. Methods able to detect compounds based on a specific structural characteristic can be developed by either precursor ion scan or neutral loss scan acquisitions. The main limitation of LC-MS may be the poor ionization obtained for some metabolites.

The aim of this thesis was to evaluate the metabolism of different doping agents using LC-MS technology in order to improve the detection capabilities of these compounds. Compounds where GC-MS show relevant limitations have been selected.

The specific objectives were:

- To study the metabolism of mesocarb, a thermolabile stimulant that decomposes in the injection-port of the GC.

- To study the metabolism of toremifene, an anti-estrogenic drug with poor electron ionization and GC properties.

- To study sulphate metabolites of anabolic androgenic steroids that have not been comprehensively studied up to now , to improve the retospectivity of the detection of these compounds.

At the same time, the usefulness of LC-MS methods to study metabolism of drugs will be evaluated and limitations of the different approaches will be assessed.

Chapter 3

Identification of free and conjugated metabolites of mesocarb in human urine by LC-MS/MS

This chapter has been published as:

Gómez, C., Segura, J., Monfort, N., Suominen, T., Leinonen, A., Vahermo, M., Yli-Kauhaluoma, J., Ventura, R. Identification of free and conjugated metabolites of mesocarb in human urine by LC-MS/MS. *Analytical and Bioanalytical Chemistry. 2010;* 397(7): 2903-2916.

Chapter 4

Mass spectrometric characterization of urinary toremifene metabolites for doping control analyses

This chapter has been published as:

Gómez, C., Pozo, O. J., Diaz, R., Sancho, J. V., Vilaroca, E., Salvador, J. P., Marco, M. P., Hernandez, F., Segura, J., Ventura, R. Mass spectrometric characterization of urinary toremifene metabolites for doping control analyses. *Journal of Chromatography A. 2011;* 1218(29): 4727-4737.

Chapter 5

Study of hydrolysis and MS/MS behaviour of AAS sulphates

1. Introduction

For many AAS most of the metabolic profile remains unknown as only a small part of the dose administered is recovered in urines as known metabolites [1].

Metabolism of AAS is normally studied by GC/MS after hydrolysis, mainly glucuronides have been studied. Sulphate conjugate metabolites have not been comprehensively studied mainly due to the difficulties in the hydrolysis of sulphates to phase I metabolites [2]. LC-MS/MS is the most suitable approach for the reliable detection of steroid sulphates [2,3] because they can be analysed directly without a previous hydrolysis step.

The global objective of the study was the evaluation of the phase II metabolism of AAS to look for sulphate conjugates of steroid metabolites that could be used as long-term markers of AAS misuse. In this chapter, the study of ionization and collision induced dissociation (CID) behaviour of model compounds has been performed. The compounds studied are described in Figure 1. The knowledge of this behaviour is important in the structure proposal of unreported metabolites. In addition, hydrolysis efficiency of sulphate conjugate using different hydrolysis protocols has been evaluated.

Figure 1. Structures of selected sulphated steroids.

2. Experimental

2.1. Standard solutions

Noretiocholanolone sulphate, 17α-nandrolone sulphate, 17β-boldenone sulphate, epitestosterone sulphate, testosterone sulphate, androsterone sulphate, ethiocholanolone sulphate, 5α-dihydrotestosterone sulphate, 17α-nandrolone, 17β-nandrolone, 17β-boldenone, epiboldenone and 6β-hydroxyandrostendione were obtained from NMI (Pymble, Australia). 17β-nandrolone sulphate, 5-androsten-3α-ol-17one sulphate, DHEA sulphate, epiandrosterone sulphate, 11-ketoethiocholanolone sulphate and 6β-hydroxyandrostendione sulphate were purchased from Steraloids Inc. (Newport, USA). Epitestosterone, testosterone, and 5α-dihydrotestosterone were obtained from Sigma-Aldrich (Steinheim, Germany). 7-propylteophylline, synthesized at IMIM-Hospital del Mar (Barcelona, Spain), was used as internal standard.

Tert-butyl methyl ether (TBME, HPLC grade), ethyl acetate (HPLC grade), acetonitrile and methanol (LC gradient grade), formic acid (LC/MS grade), potassium carbonate, sulphuric acid, sodium hydroxide, potassium hydroxide, di-sodium hydrogen phosphate, sodium hydrogen phosphate, sodium chloride, sodium acetate trihydride, acetic acid, hydrochloric acid, L-cysteine, ammonia hydroxyde, and ammonium chloride (all analytical grade) were purchased from Merck (Darmstadt, Germany). Ammonium formate (HPLC grade), sulfatase type H1, β-Glucuronidase-sulfatase type H2 from *Helix pomatia* from was obtained from Sigma-Aldrich (Steinheim,

Germany). Milli Q water was obtained by a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).

Stock standard solutions were prepared by dissolving the compounds in methanol (1 mg mL^{-1}). Working standard solutions were prepared by 1:10 and 1:100 dilutions of the stock standard solutions with methanol. All standard solutions were stored at -20 ºC.

2.2. Sample preparation for the extraction of steroid sulphates

Sample preparation for the extraction of steroid sulphates was based on a previously described procedure [4]. Briefly, 100 ng mL^{-1} of 7-propylteophylline was added to 5 mL aliquots of urine samples and the pH was made alkaline by addition of 100 µL of 5.3 M ammonium chloride solution (adjusted to pH 9.5 with ammonia). Then, sodium chloride (1 g) was added to promote salting-out effect and the samples were extracted with 8 mL of ethyl acetate by shaking at 40 mpm for 20 min. After centrifugation (3500 rpm, 5 min), the organic layers were evaporated to dryness under a nitrogen stream in a water bath at 40 °C. The extracts were reconstituted with 100 µL of a mixture of deionized water: acetonitrile (90:10, v/v) and aliquots of 5 µL were analyzed by LC-MS/MS

2.3. Hydrolysis procedures

Four hydrolysis procedures were studied:

2.3.1. Solvolysis

50 μL of working standard solution of 10 μg mL^{-1} of the steroid sulphate were evaporated to dryness under a stream of nitrogen, reconstituted with 4 mL of ethyl acetate/methanol/sulphuric acid (80:20:0.06, v|v|v) and incubated at 55 ºC for 2 h. The samples were neutralized with 60 μL of 1 M NaOH and evaporated to dryness under a stream of nitrogen [5]. The residues were reconstituted in 1 mL of sodium phosphate buffer (0.2 M, pH 7) and 250 μL of 5 % K_2CO_3 solution were added. The extraction was performed with 5 mL of TBME by shacking at 40 mpm for 20 min. After centrifugation (3500 rpm, 5 min), the organic layers were separated and evaporated to dryness under a stream of nitrogen in a water bath at 40 ºC. The dry extracts were reconstituted with 100 μL of a mixture of deionised water:acetonitrile (50:50, v|v) and 5 μL were analyzed by LC-MS/MS.

2.3.2. Hydrolysis sulphatase H1

50 μL of working standard solution of 10 μg mL^{-1} of the steroid sulphate were evaporated to dryness under a stream of nitrogen, reconstituted with 3 mL of acetate buffer 0.1 M pH 5.2 and 10 mg of sulfatase type H1 and incubated at 55 ºC for 3 h. 250 μL of 5 % K_2CO_3 solution were added. The extraction of the free steroid was performed as described (section 2.3.1).

2.3.3. Hydrolysis sulphatase H2

50 μL of working standard solution of 10 μg mL^{-1} of the steroid sulphate were evaporated to dryness under a stream of nitrogen, reconstituted with 3 mL of acetate buffer 0.1 M pH 5.2 and 15 μL of sulfatase type H2 and incubated at 55 ºC for 3 h. 250 μL of 5 % K_2CO_3 solution were added. The extraction was performed as described (section 2.3.1).

2.3.4. Acidic hydrolysis

50 μL of working standard solution of 10 μg mL^{-1} of the steroid sulphate were evaporated to dryness under a stream of nitrogen, reconstituted with 2 mL of HCl 6 M and 100 mg of cysteine, and incubated at 120 ºC for 90 min. 2 mL of KOH 6 M and 70 μL ammonium chloride solution pH 9.5 were added. The extraction was performed as described (section 2.3.1).

2.4. Instrumental Analysis

Chromatographic separation was carried out on a Waters Acquity UPLC™ system (Waters Corporation, Milford, MA) using an ZORBAX Eclipse Plus C_{18} column (2.1 mm × 50 mm, 1.8 μm particle size, Agilent). The column temperature was set to 55 °C. The mobile phase consisted of methanol with 1 mM ammonium formate and 0.01 % formic acid (solvent A) and deionized water with 1mM ammonium formate and 0.01 % formic acid (solvent B). For the study of ionization and collision induced dissociation (CID) separation was

performed at a flow rate of 0.3 mL min^{-1} and using a gradient pattern: from 0 to 0.5 min, 10 % A; from 0.5 to 5 min, to 60 % A; from 5 to 7 min, to 90 % A, during 0.2 min, 90 % A; from 7.2 to 7.4 min, to 10 % A; from 7.4 to 10 min, 10 % A. When applying neutral loss scan and precursor ion scan methods, the following gradient pattern was used at flow rate of 0.2 mL min^{-1}: from 0 to 0.5 min, 15 % A; from 0.5 to 9 min, to 50 % A; from 9 to 14 min, 50 % A, from 14 to 16.4 min, to 90 % A, during 0.6 min, 90 % A; from 17 to 17.5 min, to 15 % A; from 17.5 to 20 min, 15 % A. Injection volume was 10 μL.

The LC instrument was coupled to a Quattro Premier XE triple quadrupole mass spectrometer (Micromass, Waters Corporation, Milford, MA.) with an electrospray (Z-spray) ionization source. Source conditions were fixed as follows: lens voltage, 0.2 V; source temperature 120 °C; desolvation temperature, 450 °C; cone gas flow rate, 50 L/h; desolvation gas flow rate, 1,200 L/h. In positive ionization mode capillary voltage was set at 3 kV whereas in negative ionization mode capillary voltage was set at 2.5 kV. High-purity nitrogen was used as desolvation gas and argon was used as collision gas.

To study ionization and for the evaluation of the hydrolysis procedures, acquisition was performed in scan mode (*m/z* 250 to *m/z* 600). To study CID fragmentation, acquisition was performed in product ion scan mode of the $[M+H]^+$ or $[M-H]^-$ ions, using different collision energies (20, 30 and 40 eV). Different precursor ion and neutral loss scan methods were applied (*m/z* 350 to *m/z* 475).

3. Results and discussion

3.1. Study of ionization and CID fragmentation using electrospray source of steroids conjugated with sulphate

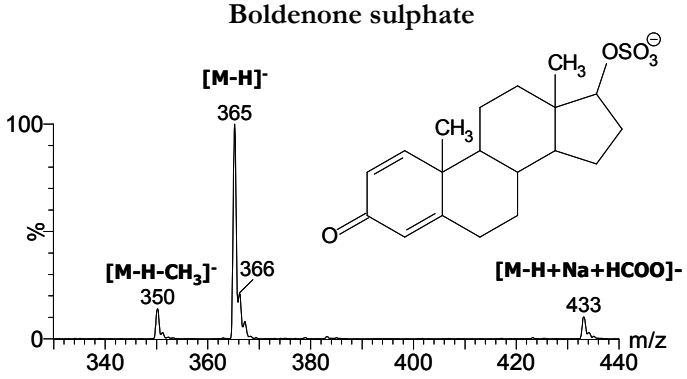
3.1.1. Ionization of sulphated steroids

The first step of the study was to evaluate the ionization of sulphated steroids available as reference materials. The following sulphated steroids were selected (Figure 1): norethiocholanolone sulphate, 17α-nandrolone sulphate, 17β-nandrolone sulphate, 17β-boldenone sulphate, epitestosterone sulphate, testosterone sulphate, 5-androsten-3α-ol-17-one sulphate, DHEA sulphate, androsterone sulphate, ethiocholanolone sulphate, epiandrosterone sulphate, 5α-dihydrotestosterone sulphate, 11-ketoethiocholanolone sulphate, 6β-hydroxyandrostendione sulphate. Sulphated steroid standards were selected with the aim to cover the most structural differences, including as many positions as possible of sulphate group, hydroxylation, and reductions (Figure 1).

Electrospray ionization (ESI) in positive and negative modes were tested. A relation between the formation of some adducts and structural characteristics of the steroids sulphates were observed (Table 1). All sulphate conjugates exhibited an abundant $[M-H]^-$ in ESI negative mode due to the deprotonation of the sulphate moiety as described by other authors [2,6-9]. Formation of minor adducts corresponding to $[M-H+Na+HCOO]^-$ was observed in most of cases (Table 1). Additionally, in case of boldenone sulphate a neutral loss of

15 Da corresponding to $[M\text{-}H\text{-}CH_3]^-$ with m/z 350 was also observed (Figure 2).

Figure 2. MS Scan of boldenone sulphate standard in ESI negative mode.

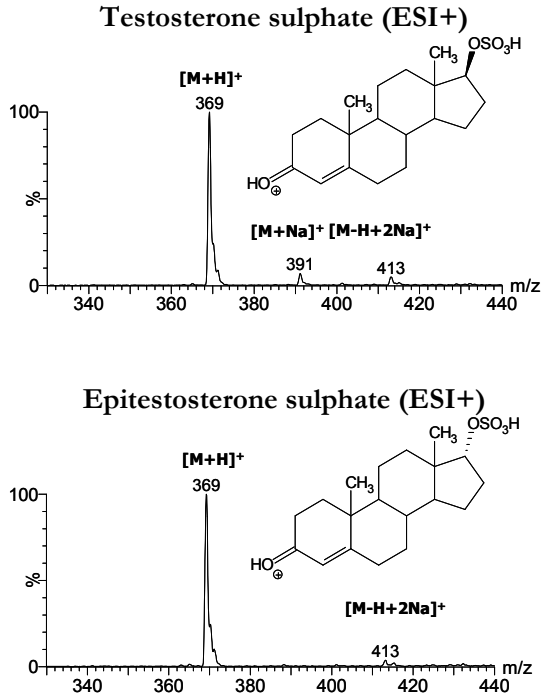


Working in ESI positive mode, only steroid sulphates with a 3-keto group exhibited an abundant $[M+H]^+$ (Table 1). Formation of characteristic adducts in 17-sulphate steroids were observed; $[M+Na]^+$ and $[M\text{-}H+2Na]^+$ were observed in 17β sulphates whereas 17α sulphates only presented $[M\text{-}H+2Na]^+$ (Table 2). As an example, in Figure 3 the MS scan of a 17β-sulphate (Testosterone sulphate) and a 17α-sulphate (Epitestosterone sulphate) are presented. In case of 11-ketoethiocholanolone sulphate, 5-androsten-3α-ol-17one sulphate and 6β-hydroxyandrostendione sulphate, different ammonium adducts and also neutral losses of H_2O and sulphate group from an in source fragmentation have been observed.

Table 1. Ionization of steroids conjugated with sulphate. Electrospray ionitzation in negative and positive mode.

Compound	MW	ESI-		ESI+	
		m/z	Specie	*m/z*	Specie
Noretiocholanolone 3α-sulphate	**356**	355	$[M-H]^-$	-	-
		423	$[M-H+Na+HCOO]^-$		
17α-nandrolone 17α-sulphate	**354**	353	$[M-H]^-$	355	$[M+H]^+$
		383	$[M-H+30]^-$	385	$[M+H+30]^+$
		421	$[M-H+Na+HCOO]^-$	399	$[M-H+2Na]^+$
17β-nandrolone 17β-sulphate	**354**	353	$[M-H]^-$	355	$[M+H]^+$
		421	$[M-H+Na+HCOO]^-$	377	$[M+Na]^+$
				399	$[M-H+2Na]^+$
17β-boldenone 17β-sulphate	**366**	365	$[M-H]^-$	367	$[M+H]^+$
		433	$[M-H+Na+HCOO]^-$	389	$[M+Na]^+$
		350	$[M-H-CH_3]^-$	411	$[M-H+2Na]^+$
Epitestosterone 17α-sulphate	**368**	367	$[M-H]^-$	369	$[M+H]^+$
		435	$[M-H+Na+HCOO]^-$	413	$[M-H+2Na]^+$
Testosterone 17β-sulphate	**368**	367	$[M-H]^-$	369	$[M+H]^+$
		435	$[M-H+Na+HCOO]^-$	391	$[M+Na]^+$
				413	$[M-H+2Na]^+$
5-androsten-3α-ol-17one 3α-sulphate	**368**	367	$[M-H]^-$	271	$[M+H-SO_3-H_2O]^+$
				288	$[M+NH_4-SO_3-H_2O]^+$
				293	$[M+Na-SO_3-H_2O]^+$
				306	$[M+NH_4-SO_3]^+$
				386	$[M+NH_4]^+$
DHEA 3β-sulphate	**368**	367	$[M-H]^-$	-	-
		435	$[M-H+Na+HCOO]^-$		
Androsterone 3α-sulphate	**370**	369	$[M-H]^-$	-	-
		437	$[M-H+Na+HCOO]^-$		
Ethiocholanolone 3α-sulphate	**370**	369	$[M-H]^-$	-	-
		437	$[M-H+Na+HCOO]^-$		
Epiandrosterone 3β-sulphate	**370**	369	$[M-H]^-$	-	-
		437	$[M-H+Na+HCOO]^-$		
5α-dihydrotestosterone 17β-sulphate	**370**	369	$[M-H]^-$	371	$[M+H]^+$
		437	$[M-H+Na+HCOO]^-$	393	$[M+Na]^+$
				415	$[M-H+2Na]^+$
11-ketoethiocholanolone 3α-sulphate	**384**	383	$[M-H]^-$	287	$[M+H-SO_3-H_2O]^+$
				304	$[M+NH_4-SO_3-H_2O]^+$
				309	$[M+Na-SO_3-H_2O]^+$
				322	$[M+NH_4-SO_3]^+$
				402	$[M+NH_4]^+$
6β-hydroxyandrostendione 6β-sulphate	**382**	381	$[M-H]^-$	383	$[M+H]^+$
				285	$[M+H-SO_3-H_2O]^+$
				307	$[M+Na-SO_3-H_2O]^+$
		449	$[M-H+Na+HCOO]^-$	365	$[M+H-H_2O]^+$
				427	$[M-H+2Na]^+$

Figure 3. MS Scan of testosterone sulphate and epitestosterone sulphate standards in ESI positive mode.



3.1.2. CID fragmentation of sulphated steroids

CID study was performed at different collision energies. Product ions observed in negative ionization mode and neutral losses observed in positive ionization mode were common to all model compounds.

In ESI negative mode, the sulphoconjugated steroids had a poor fragmentation pattern. As examples, product ion scan spectra of testosterone sulphate and epitestosterone sulphate are shown in Figure 4. As it can be seen, product ion spectra were characterized by only one abundant product ion at *m/z* 97 (Table 2), corresponding to

hydrogensulphate ion (HSO_4^-) that was observed in all sulphated steroids at 30 eV. Product ions at *m/z* 96 (corresponding to SO_4^-, resulting from an homolytic cleavage) and *m/z* 80 (corresponding to SO_3^-, resulting from an homolytic cleavage) were also observed in 17β-sulphate steroids at collision energies higher than 40 eV whereas these product ions were not observed in steroid sulphates with 17α-hydroxy configuration. This behaviour was previously described by other authors for testosterone, epitestosterone, nandrolone and DHEA sulphates [2,6-8]. Nevertheless, all product ions observed in negative ionization mode correspond to sulphate group and structural information about unconjugated steroid can not be obtained. In some cases, specific neutral losses can be observed, e.g. MS/MS spectra of boldenone sulphate exhibited a neutral loss of a methyl group [M-H-$CH_3^{\bullet}$]$^-$. The specificity of this loss can be useful to increase the selectivity of the detection when looking for steroid sulphates in urine matrix, as described in chapter 5 of this thesis.

In positive ESI mode, the most commonly observed fragments in the MS/MS mass spectra of steroid sulphates are the neutral loss of 80 Da corresponding to the sulphate moiety ($[M+H-SO_3]^+$) and sequential losses of water from the steroid nucleus ($[M+H-SO_3-nH_2O]^+$). This behaviour was previously described by other authors for testosterone and epitestosterone sulphate [6,8]. After losses of sulphate group, product ion mass spectra obtained could be compared with those in free form, i.e. the fragmentation obtained was the same as free steroid As examples, product ion scan mass spectra of testosterone sulphate and unconjugated testosterone are shown in Figure 5. Therefore, structural information about unconjugated steroid can be obtained by

comparison of mass spectra of sulphate steroids with those in free form. A summary of the CID fragmentation behaviour is described in Table 2.

Figure 4. Product ion mass spectra of testosterone sulphate and epitestosterone sulphate standards in ESI negative mode at 40 eV.

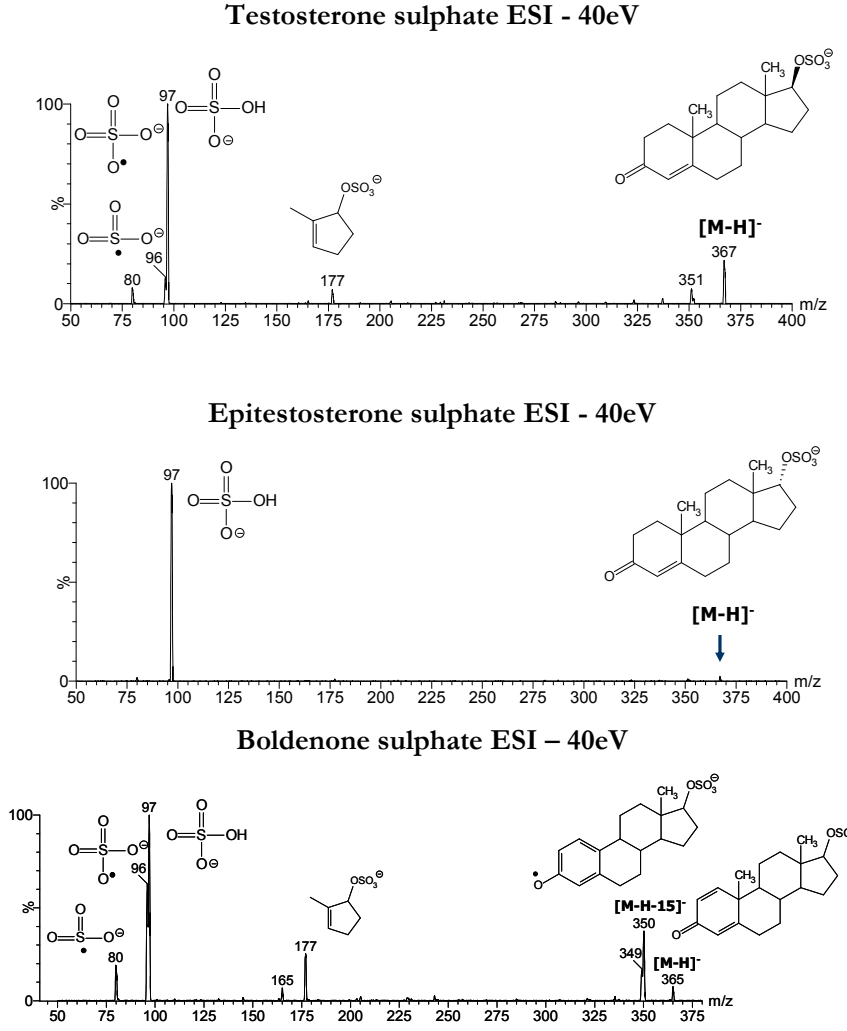


Figure 5. Product ion mass spectra of testosterone sulphate, testosterone, boldenone sulphate and boldenone standards in ESI positive mode at 20 eV.

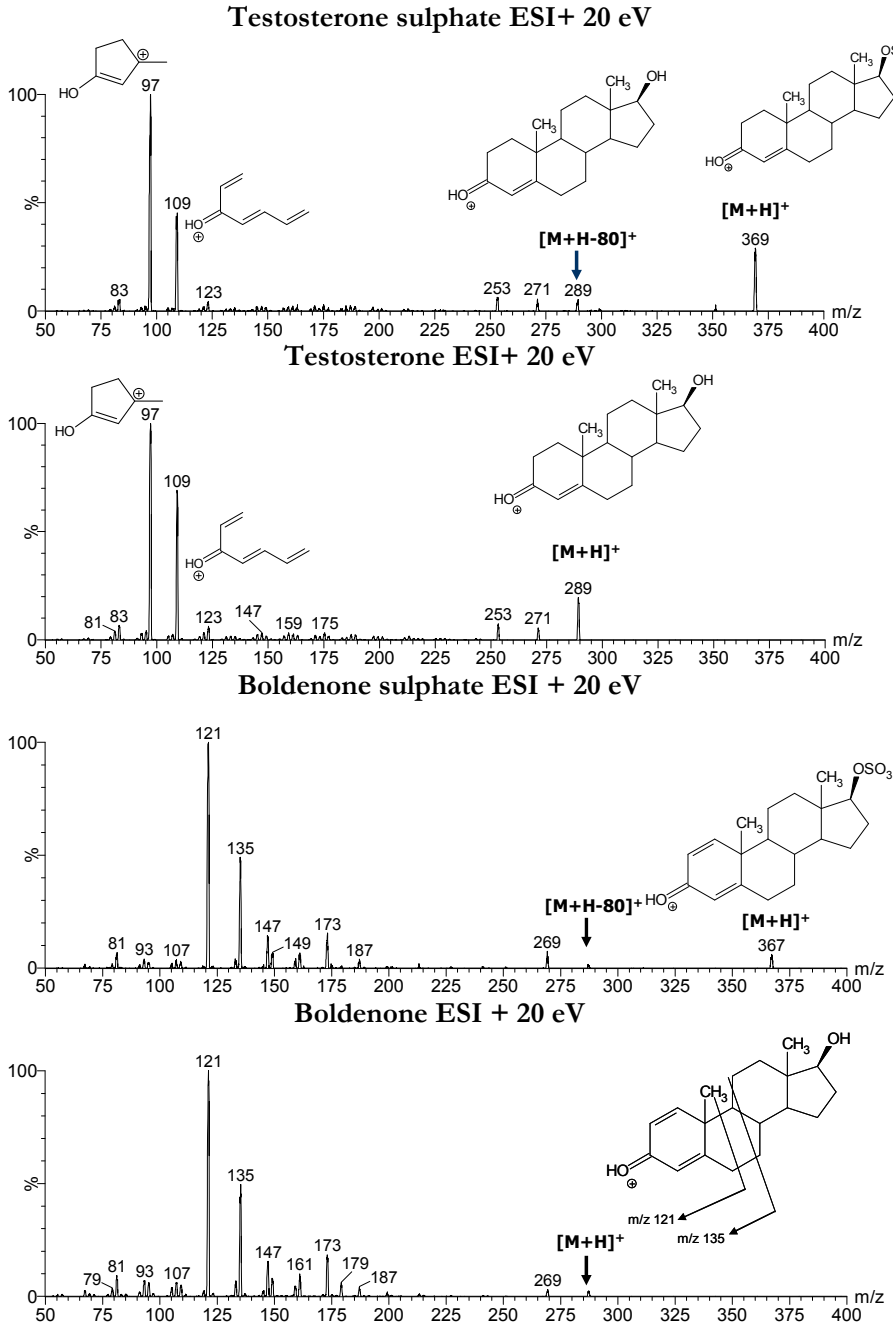


Table 2. Ionization and fragmentation behaviour of sulphate AAS.

ESI -			
Steroid structure		**Ionization**	**CID**
17-CO		$[M-H]^-$	m/z 97
17α-OH		[M-H]-	m/z 97
17β-OH		[M-H]-	m/z 97, 96, 80
ESI +			
Steroid structure		**Ionization**	**CID**
3-CO	17α-OH	$[M+H]^+$, $[M-H+2Na]^+$	$[M+H-SO_3]^+$, $[M+H-SO_3-H_2O]^+$, ions of free steroid
	17β-OH	$[M+H]^+$, $[M+Na]^+$, $[M-H+2Na]^+$	
3-OH		n. d.	n. d.

3.2. LC-MS/MS strategies for the detection of unknown sulphate AAS metabolites

Based on the fragmentation of sulphate conjugates two strategies could be applied in order to directly find sulphate conjugated AAS metabolites in urine samples. On the one hand, for the identification of unknown sulphate metabolites, when the AAS sulphate is ionized in ESI positive mode, a neutral loss scan method, based on the losses of 80 Da ($[M+H-SO_3]^+$) and 98 Da ($[M+H-SO_3-H_2O]^+$), could be applied to urine samples collected before and after administration of an AAS. This method can be used for the detection of sulphate steroid metabolites with a keto group in C3.

On the other hand, as stated previously, a common product ion, *m/z* 97, was observed for all sulphate metabolites in negative ionization mode. Based on this result, a precursor ion scan method of *m/z* 97

can be used for the detection of unknown sulphate steroid metabolites.

The methods were tested using standard mixtures of the steroids sulphates. In Figures 6 and 7, results obtained after application of the scan methods to a mixture of steroid sulphates are shown. In Figure 6, some examples of chromatograms obtained after application of the precursor ion scan method of m/z 97 in ESI – mode are shown. In Figure 7, examples of chromatograms obtained after application of neutral loss scan method of 80 and 98 Da in ESI + mode are presented.

Figure 6. Precursor ion scan of m/z 97 tested in different steroid sulphate standards (standard solutions at 2.5 μg mL^{-1}).

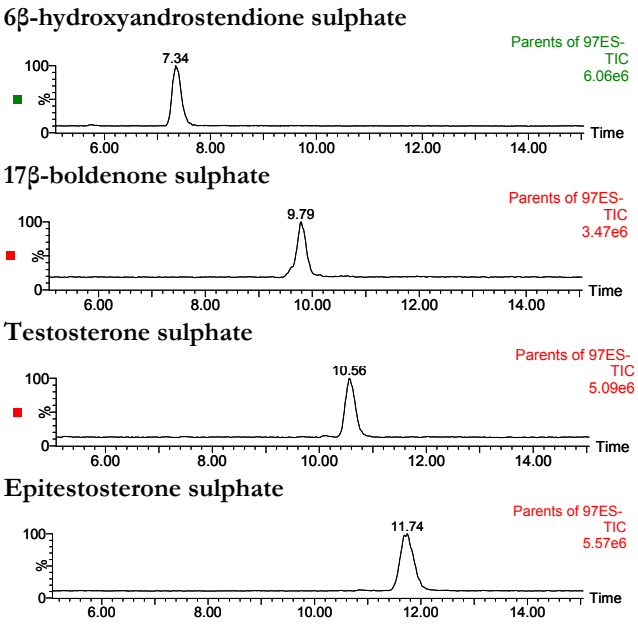
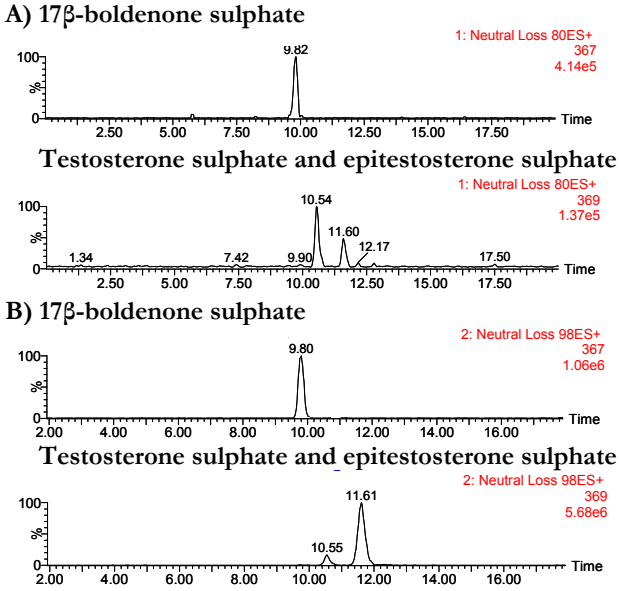
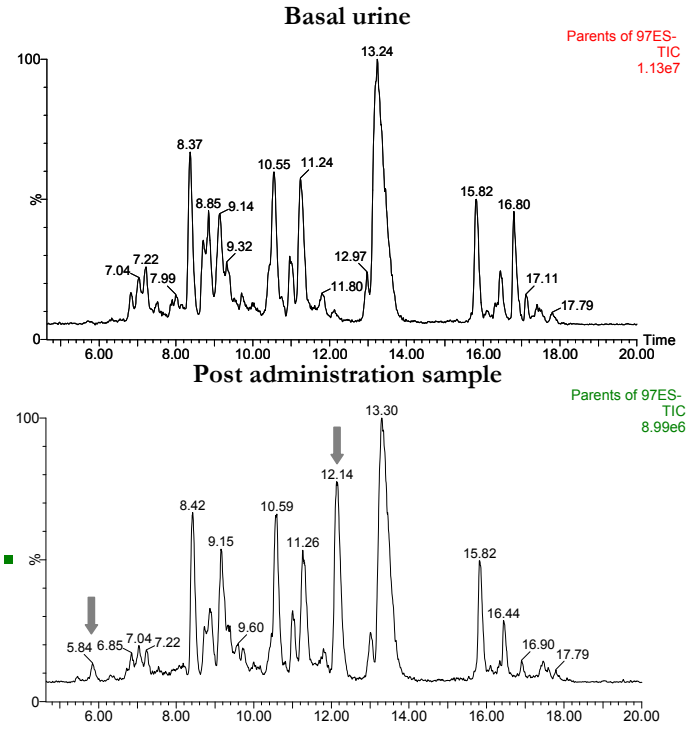


Figure 7. Neutral loss scan methods tested in different steroid sulphate standards (standard solutions at 2.5 μg mL^{-1}): A) Neutral Loss of 80 Da, and B) Neutral Loss of 98 Da.



In Figure 8, an example of the application of the precursor ion scan method of *m/z* 97 to urines obtained before and after administration of methylstestosterone is shown. As can be seen, additional peaks, corresponding to potential sulphate metabolites are detected in the urine obtained after administration of the drug (retention times 5.84 and 12.14 min). Furthermore, when the theoretical mass of the sulphate metabolite is known, an extracted ion can be done.

Figure 8. Precursor ion scan of *m/z* 97. Basal urine and sample collected after administration of methyltestosterone.



Alternatively, a SRM method using theoretical transitions of the potential metabolites can be used to detect sulphated metabolites. The transitions can be calculated from $[M-H]^-$ of the potential metabolite to the characteristic fragment ion at *m/z* 97. This approach has more sensitivity when compared with precursor ion scan. Applying this strategy it is necessary to know the mass of the potential sulphate metabolite and unexpected metabolites can not be detected.

3.3. Hydrolysis study

For the hydrolysis of sulphate conjugates, enzymatic or chemical hydrolysis methods can be applied. Different enzymes with sulfatase activity have been reported [10]. The preparations from *Patella vulgata* and *Helix pomatia* (type H1 and H2), which contain both β-glucuronidase and arylsulfatase activity, are the most used. Arylsulfatase enzymes desulphate steroids with an aromatic A ring, such as estrogen sulphates. Chemical hydrolysis can be achieved using hot acid (hydrochloric or sulphuric). The cleavage of the conjugate is strongly influenced by the choice of acid, temperature and duration of the reaction [10].

A study of hydrolysis of steroid sulphates was performed using reference standards. Most common procedures for the cleavage of conjugated steroids were studied. Enzymatic hydrolysis using sulfatase type H1 and type H2 from *H. pomatia,* solvolysis, and acidic hydrolysis with hydrochloric acid were performed. In Table 3, the recoveries of the different procedures tested are shown.

The results show that the efficacy of the hydrolysis of sulphates can be affected by the position of the sulphate moiety and the α/β configuration. As it can be seen in Table 3, using sulfatase type H1 from *H. pomatia* only steroids with 3β sulphates were hydrolyzed with recoveries around 30 %, the other sulphate steroids were not affected by the enzyme. On the other hand, similar results were obtained using sulfatase type H2. 3β-sulphated steroids were also hydrolyzed with recoveries between 30 to 40 %, 17β-sulphated steroids were weakly

hydrolysed, with recoveries around 4 %, and 3α-sulphates were not hydrolyzed in any of the enzymatic conditions studied. As can be seen in Figure 9, epiandrosterone sulphate (3β-sulphate) is hydrolyzed after enzymatic hydrolysis with sulphatase type H2, whereas androsterone sulphate (3α-sulphate) is not hydrolyzed. The effiency of the hydrolysis depends on the position of the sulphate, as it was described by previous authors [10].

Table 3. Recoveries of the free steroids sulphates after application of different hydrolysis procedures to the steroid sulphates.

Compound	Solvolysis	Sulfatase type H1	Sulfatase type H2	Acidic hydrolysis
Noretiocholanolone 3α-sulphate	69 %	-	-	37 %
17α-nandrolone 17α-sulphate	33 %	-	-	-
17β-boldenone 17β-sulphate	68 %	-	4 %	-
Epitestosterone 17α-sulphate	91 %	-	-	-
Testosterone 17β-sulphate	34 %	-	4 %	76 %
DHEA 3β-sulphate	78 %	29 %	28 %	-
Androsterone 3α-sulphate	137 %	-	-	-
Ethiocholanolone 3α-sulphate	86 %	-	-	36 %
Epiandrosterone 3β-sulphate	80 %	33 %	38 %	54 %
5α-dihydrotestosterone 17β-sulphate	109 %	-	2 %	13 %
6β-hydroxyandrostendione 6β-sulphate	5 %	-	-	-

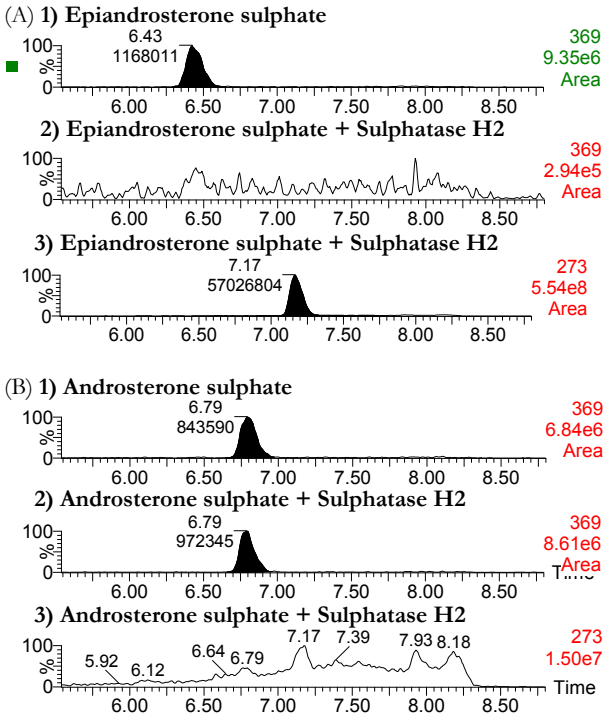
All sulphate conjugates can not be cleaved using enzymes. An alternative to enzymatic hydrolysis is chemical hydrolysis. Using a solvolysis procedure all sulphated steroids tested were hydrolysed. In most cases the recoveries of the solvolysis were between 70 and 90 %. Additionally, it covers a wider number of AAS sulphates than enzymatic hydrolysis.

Regarding acidic hydrolysis with hydrochloric acid, it was not possible to establish a pattern of behaviour of sulphated steroids. Some of the tested sulphated steroids were not hydrolysed whereas other sulphate

steroids were hydrolysed with recoveries between 10 to 80 %, depending on the sulphate.

Figure 9. Hydrolysis with sulphatase type H2 of epiandrosterone sulphate (A) and androsterone sulphate (B):

1. Chromatogram obtained in ESI negative of *m/z* 369 (specific for epiandrosterone sulphate and androsterone sulphate) in the sample before hydrolysis.
2. Chromatogram obtained in ESI negative of *m/z* 369 (specific for epiandrosterone sulphate and androsterone sulphate) in the sample after hydrolysis.
3. Chromatogram obtained in ESI positive of *m/z* 273 (specific for epiandrosterone and androsterone) in the sample after hydrolysis.



Conclusions

A study of hydrolysis and MS/MS behaviour (ionization and CID) of AAS sulphates has been performed.

All AAS conjugated with sulphate were ionized in ESI negative mode and only AAS with C3 keto group were ionized in ESI positive mode. Regarding CID, all AAS sulphates exhibited an ion at m/z 97 in ESI negative mode corresponding to HSO_4^-. Slightly differences were observed between 17α and 17β-sulphates; product ions at m/z 96 and m/z 80 were observed in 17β-sulphate steroids at higher collision energies. In ESI positive mode, the most common observed fragments were the neutral loss of 80 Da and sequential losses of water from the steroid nucleus. After the loss of the sulphate group, product ion mass spectra obtained could be compared with those obtained in free form, i.e. the fragmentation obtained was the same as free steroid. Therefore, structural information about the steroid structure can be obtained. Based on the fragmentation of sulphate conjugates different strategies have been proposed to directly find sulphate conjugated AAS metabolites.

After testing different hydrolysis approaches it can be concluded that there is no gold standard method for the cleavage of AAS sulphates; enzymatic hydrolysis efficiency depends on the structure of AAS. Solvolysis cleaves almost the majority of AAS sulphates, but with recoveries between 5 to 100 %.

References

[1] Schänzer, W. (1996). Metabolism of anabolic androgenic steroids. *Clin. Chem.*, 42, 1001-1020.

[2] Antignac, J. -P., Brosseaud, A., Gaudin-Hirret, I., André, F., Le Bizec, B. (2005). Analytical strategies for the direct mass spectrometric analysis of steroid and corticosteroid phase II metabolites. *Steroids*, 70, 205-216.

[3] Segura, J. Ventura, R., Marcos, J., Gutiérrez-Gallego, R. (2008). Doping substances in human and animal Sport. Chapter 21. Boguz, M. J. (Ed.) Forensic Science. *Handbook of analytical separations*, 6, 649-744.

[4] Ventura, R., Roig, M., Monfort, N., Sáez, P., Bergés, R., Segura, J. (2008). High-throughput and sensitive screening by ultra-performance liquid chromatography tandem mass spectrometry of diuretics and other doping agents. *Eur. J. Mass Spectrom.*, 14, 191-200.

[5] Torrado, S., Roig, M., Farré, M., Segura, J., Ventura, R. (2008). Urinary metabolic profile of 19-norsteroids in humans: glucuronide and sulphate conjugates after oral administration of 19-nor-4-androstenediol. *Rapid Commun. Mass Spectrom.*, 22, 3035-3042.

[6] Borts, D. J., Bowers, L. D. (2000). Direct measurement of urinary testosterone and epitestosterone conjugates using high-performance liquid chromatography/tandem mass spectrometry. *J. Mass Spectrom.*, 35, 50-61.

[7] Buiarelli, F., Giannetti, L., Jasionowska, R., Cruciani, C., Neri, B. (2010). Determination of nandrolone metabolites in human urine: comparison between liquid chromatography/tandem mass spectrometry and gas chromatography/mass spectrometry. *Rapid Commun. Mass Spectrom.*, 24, 1881-1894.

[8] Bowers, L. D., Sanaullah. (1996). Direct measurement of steroid sulphate and glucuronide conjugates with high-performance liquid chromatography-mass spectrometry. *J. Chromatogr. B*, 687, 61-68.

[9] Yi, L., Dratter, J., Wang, C., Tunge, J. A., Desaire, H. (2006). Identification of sulfation sites of metabolites and prediction of the compounds' biological effects. *Anal. Bioanal. Chem.*, 386, 666-674.

[10] Gomes, R. L., Meredith, W., Snape, C. E., Sephton, M. A. (2009). Analysis of conjugated steroid androgens: Deconjugation, derivatisation and associated issues. *J. Pharm. Biomed. Anal.*, 49, 1133-1140.

Chapter 6

New potential markers for the detection of boldenone misuse

This chapter has been published as:

Gómez, C, Pozo, O J, Geyer, H, Marcos, J., Thevis, M, Schänzer, W, Segura, J, Ventura, R. New potential markers for the detection of boldenone misuse. *Journal of Steroid Biochemistry and Molecular Biology. 2012*; 132(3-5): 239-246.

Chapter 7

Alternative long-term markers for the detection of methyltestosterone misuse

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Chapter 8

A new sulphate metabolite as a long-term marker of metandienone misuse

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Chapter 9

Conclusions

The metabolism of different doping agents has been evaluated using liquid chromatography-mass spectrometry (LC-MS) techniques in order to solve different aspects of the detectability of the compounds.

Mesocarb:

1. For mesocarb, a thermolabile stimulant, the parent drug and 19 metabolites have been detected in urine including mono-, di-, and trihydroxylated metabolites. Nine of the metabolites are excreted in free form, five of them as conjugates with sulphate and the remaining five as conjugates with glucuronic acid. Structures of most of the metabolites have been proposed. All of the metabolites were detected in urine up to 48 h after administration, and the most abundant metabolites were p-hydroxymesocarb and p-hydroxymesocarb conjugated with sulphate.

Toremifene:

2. For toremifene, an anti-estrogenic drug with poor electron impact and gas chromatographic behaviour, the parent compound and 20 metabolites have been detected in urine after administration of the drug. Structures of the most abundant metabolites have been proposed with the assistance of accurate mass measurements. Different metabolic pathways have been proposed, including: mono- and di-hydrolxylations, N-desmethylation, methylation, reduction, reduction, dehalogenation and combinations of them. Metabolites are excreted free and/or conjugated with glucuronic

acid or sulphate. All metabolites were detected up to one month after administration; the most abundant metabolites were detected in the free fraction and they resulted from dehalogenation of the drug.

Sulphate conjugated metabolites of anabolic androgenic steroids (AAS):

3. A study of hydrolysis and MS/MS behaviour (ionization and collision induced dissociation) of AAS metabolites conjugated with sulphate has been performed. There is no gold standard method to cleave AAS sulphates. Enzymatic hydrolysis efficiency depends on the structure of AAS sulphate. Solvolysis cleaves almost all AAS sulphates studied, but with recoveries between 5 to 100 %.

4. A common ionization and fragmentation behaviour has been observed among steroid sulphates and based on it; general methods for the detection of unknown sulphate metabolites of AAS have been defined. Specific neutral losses have been observed for some steroid sulphates that increase the sensitivity and specificity of the detection.

   To detect unknown metabolites neutral loss and precursor ion scan methods, or SRM methods including theoretical transitions based on these neutral losses or product ions can be used. SRM methods offer a more sensitive detection although unexpected metabolites cannot be detected.

5. Boldenone sulphate and epiboldenone sulphate have been identified and characterized as boldenone metabolites. Epiboldenone sulphate could be detected in urine up to 56 h afer administration (the last sample collected of the excretion study), although it accounts for less than 1% of the administered dose.
   Boldenone and epiboldenone sulphates can be used as markers of exogenous boldenone administration before performing IRMS analysis.

6. For methyltestosterone, three new sulphate metabolites have been detected and structures have been proposed: S1 (17α-methyl-5β-androstan-3α,17β-diol 3α-sulphate); S2 (17β-methyl-5α-androstan-3α,17α-diol 3α-sulphate) and S3 (17β-methyl-5β-androstan-3α,17α-diol 3α-sulphate). S2 and S3 are result of the initial formation of a disulphate. S2 was detected in urine up to 21 days after methyltestosterone administration, improving three times the retrospectivity of the detection with respect to other previously reported long-term metabolites.

7. Several new sulphate metabolites have been detected after metandienone administration. One of them was characterized as 18-nor-17β-hydroxymethyl-17α-methylandrost-1,4,13-triene-3-one conjugated with sulphate. That metabolite was detected up to 26 days after administration of a single oral dose of the drug, improving the retrospectivity of the detection with respect to other long-term metabolites described.

8. The results demonstrate the importance of sulphatation as a phase II metabolic pathway for AAS, and the interest of sulphate conjugates as long-term metabolites of these doping agents.

General conclusions:

9. The usefulness of LC-MS for the detection and characterization of metabolites of doping agents has been demonstrated, especially for the study of new phase II metabolites and for metabolic studies of thermolabile compounds and polar compounds with poor electron ionization or gas chromatographic properties.

10. The metabolism of doping agents needs to be re-evaluated using LC-MS technology to look for new metabolites that improve the detection capabilities of doping controls in sports.

Annexes

Related publications

Gómez, C., Fabregat, A., Pozo, O. J., Marcos, J., Segura, J., Ventura, R. Analytical strategies based on mass spectrometric techniques for the study of steroids metabolism. *Trends in Analytical Chemistry. 2014; 53: 106-116.*

Gómez, C., Pozo, O. J., Fabregat, A., Marcos, J., Deventer, K., Van Eenoo, P., Segura, J., Ventura, R. Detection and characterization of urinary metabolites of boldione by LC-MS/MS. Part I: Phase I metabolites excreted free, as glucuronide and sulphate conjugates, and released after alkaline treatment of the urine. *Drug Testing and Analysis. 2012;* 4(10): 775-785.

Pozo, O. J., Gómez, C., Marcos, J., Segura, J., Ventura, R. Detection and characterization of urinary metabolites of boldione by LC-MS/MS. Part II: Conjugates with cysteine and *N*-acetylcysteine. *Drug Testing and Analysis. 2012;* 4(10), 786-797.