Re-exploring testosterone metabolism: new insights for doping control

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Dedicat als meus pares, és gràcies a vosaltres que sóc capaç de donar un nou pas

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Abstract

The detection of endogenous anabolic androgenic steroids (EAAS) is one of the most difficult analytical challenges in the doping control field. The main problem for their detection is to distinguish between normally endogenous concentrations and those observed after the exogenous administration of an EAAS. The screening methods for EAAS are currently based on the determination of the steroid profile and the application of the athlete biological passport. The inclusion of new steroid metabolites can improve the screening capabilities of the steroid profile. Thus, the objective of the thesis is to elucidate and characterize new testosterone metabolites that can be implemented to the current steroid profile and to evaluate their usefulness for doping control analysis.

Four unreported testosterone metabolites were detected and characterized by using liquid chromatography coupled to tandem mass spectrometry approaches.

These compounds were demonstrated to come from degradation of cysteine conjugates. The formation of these conjugates implies an addition of a double bond as a phase I metabolism followed by conjugation with glutathione and the subsequent transformation to cysteine conjugates in urine.

In order to determinate the usefulness of the cysteinyl compounds for doping control purposes, a quantitative method for the indirect determination of these compounds was developed and validated. Using this method, reference population limits were established by the analysis of 174 urine samples. Additionally, different factors that can

potentially influence the excretion of these compounds were evaluated.

Finally, the usefulness of these cysteinyl metabolites for the detection of EAAS misuse was evaluated by the analysis of samples collected after different EAAS administration. The use of these metabolites seems to improve in some cases the detection capabilities of the current marker used in routine analysis.

Resum

La detecció d'esteroides androgènics anabolitzants endògens (EAAE) és un dels reptes analítics més difícils en la lluita contra el dopatge. El problema més important per a la seva detecció és distingir entre concentracions endògenes i aquelles que s'observen després de l'administració exògena d'un EAAE. Els mètodes de cribatge per a la detecció d'EAAE estan basats en la determinació del perfil esteroïdal i la introducció d'aquest en el passaport biològic de l'atleta. La inclusió de nous metabòlits d'esteroides pot ajudar a millorar les capacitats de cribatge del perfil esteroïdal. Per tant, l'objectiu d'aquesta tesis és detectar i caracteritzar nous metabòlits d'EAAE que puguin implementar-se en l'actual perfil esteroïdal i l'avaluació de la seva utilitat en la lluita contra el dopatge.

Quatre metabòlits desconeguts de la testosterona van ser detectats i caracteritzats mitjançant la utilització de la cromatografia líquida acoblada a l'espectrometria de masses en tàndem.

L'origen d'aquests compostos es va demostrar que provenia de la degradació de conjugats amb cisteïna. La formació d'aquests conjugats implica l'addició d'un doble enllaç com a reacció metabòlica de fase I acompanyat per la conjugació amb glutationa i la subseqüent degradació d'aquesta a cisteïna en orina.

Per tal de poder veure la seva aplicació en el camp del dopatge, es va desenvolupar i validar un mètode per la quantificació indirecta d'aquests compostos en orina.

Utilitzant aquest mètode es van establir límits de referència basats en l'anàlisi de 174 mostres de orina. Addicionalment, diferents factors descrits que poden afectar l'excreció en orina d'aquests compostos també van ser estudiats en detall. Finalment, es va avaluar la utilitat d'aquests metabòlits conjugats amb cisteïna per a la detecció de l'abús d'EAAE mitjançant l' anàlisis de mostres després de l'administració de diferents EAAE. L'ús d'aquests metabòlits va millorar (en alguns casos) els temps de detecció comparant-los amb els actuals marcadors utilitzats en rutina.

Preface

Testosterone is the principal endogenous androgenic anabolic steroid (EAAS) in humans. The detection of testosterone misuse is one of the most difficult challenges in the doping control field due to its endogenous nature. The screening for testosterone is usually performed by the measurement of the steroid profile and the application of the athlete biological passport. A sample is considered suspicious if a marker of the steroid profile exceeds the individual threshold calculated using the data obtained from the athlete biological passport.

Although this approach is the first step for the detection of EAAS misuse, it has some limitations. For this reason, several other strategies are being explored in order to overcome the limitations exhibited for the steroid profile.

The potentialities of LC-MS/MS represented a big change in the detection of steroids, since it presents several advantages such as faster sample preparation potentially avoiding the hydrolysis and derivatization steps. This fact allows for the detection of several metabolites which are usually unexplored by the current routine approaches.

The aim of this thesis is to demonstrate the presence of additional metabolites unnoticed until now for testosterone.

List of abbreviations

AAF: Adverse fnalytical finding

AAS: Anabolic androgenic steroids

ABP: Athlete biological passport

ACN: Acetonitrile

ADAMS: Anti-doping administration & management system

API: Atmospheric pressure ionization

AR: Androgen Receptor

ASP: Athlete Steroid Profile

CID: Collision Induced Dissociation

Cys: Cysteine

CV: Cone voltage

Ce: Collision energy

DT: Detection time

EAAS: Endogenous Anabolic Androgenic Steroids

EI: Electron ionization

EIC: Extracted Ion Chromatogram

ESI: Electrospray Ionization

GC-MS: Gas chromatography-mass spectrometry

GC/C/IRMS: Gas chromatography coupled to combustion Isotope ratio mass spectrometry

HPLC: High performance liquid chromatography

IST: Internal standard

IOC: International Olympic committee

ISL: Internal standard for laboratories

IT: Individual Threshold

LC: Liquid chromatography

LC-MS/MS: Liquid chromatography tandem mass spectrometry

LLE: Liquid-liquid extraction

LOD: Limit of detection

LOQ: Limit of quantification

MeOH: Methanol

MRPL: Minimum required performance Level

MS: Mass spectrometry

MS/MS: Tandem mass spectrometry

MSTFA: N-methyl-N-trimethylsilyltrifluoroacetamide

MW: Molecular weight

m/z: Mass to charge ratio

NAC: N-Acetyl-cysteine

NL: Neutral loss

RL: Reference limits

PI: Precursor ion

QqQ: Triple quadrupole analyser

QTOF: Hybrid quadrupole time-of flight analyse

SG: Specific gravity

SIM: Selected ion monitoring

S/N: Signal to noise ratio

SRM: Selected reaction monitoring

SPE: Solid phase extraction

T/E: Testosterone to epitestosterone ratio

TMB: *Tert*-butyl-methyl ether

TMS: Trimethylsilyl

TOF: Time of flight

WADA: World anti-doping agency

Thesis structure

This thesis is structured in 12 chapters and three main parts. The first chapter includes the introduction of the thesis, covering the main aspects of doping control analysis, endogenous anabolic androgenic steroids and analytical strategies. In the second chapter, the justification and objectives of the thesis are presented.

Part I of the thesis includes chapter 3, 4 and 5 which is addressed to study the testosterone metabolism and to elucidation of new metabolites.

Part II of the thesis covers the chapter 6, 7 and 8 which includes the development of a quantitative method, population studies and factors that may influence the excretion of the elucidate metabolites.

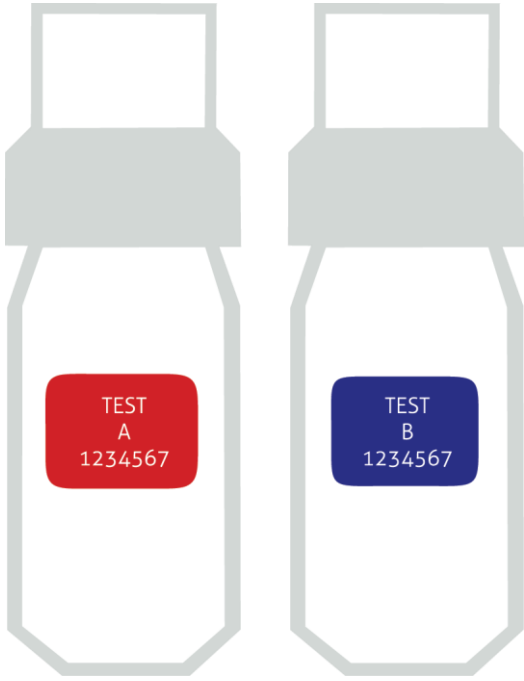
Part III of the thesis includes chapter 9 and 10 which are addressed to evaluate the usefulness of the elucidated metabolites for doping control purposes.

Chapter 11 includes the discussion of the thesis and suggestion for future works. Finally, chapter 12 covered the main conclusions extracted from the thesis results.

Three annexes are included, the first annex includes the supplementary material not included in the presented thesis, the second annex includes the scientific papers included in the thesis and third annex includes other scientific papers published by the author.

General introduction

Chapter 1



1.1. General aspects of doping

1.1.1 History of doping

Since the beginning of the human history most of the sport practice in a competitive way has been performed with all possible legal or illegal means. Hence, it can be assured that the use of doping substances is, at least, as old as humanity. Several anecdotes of substances abuse in order to enhance the performance for competitive purposes have been documented throughout the history. The ancient Greeks, for instance, who were idolized for their athletic purity, used a variety of "medical" measures to enhance their performances at the ancient Olympic games (s. VII B.C) [1]. It is also known that Nordic warriors ingested physicoactive mushrooms *amanita muscaria* prior to the fight in order to enhance their performance in the battle [2].

In the nineteenth century, as sports rose in importance, there were many known reports of drug abuse. The first one surfaced in Amsterdam in 1865, when several swimmers in a canal race were accused to use enhanced performance substances. As result of such practices, in 1886, the first known drug related death on an athlete was reported [3].

With the need of restrictions regarding drug abuse in sports, in 1967 the International Olympic Committee (IOC) published a list of "Banned Substances Classes and Methods" which includes stimulants, sympathomimetic amines, narcotics, antidepressants and tranquilizers [4], this list was periodically updated. The IOC medical commission initiated the first doping controls as a pilot project in 1968 at the winter Olympic Games in Grenoble and at the summer Olympic Games in Mexico City [4]. The systematic doping controls and analysis started at the Olympic Games in Munich in

1972. Difficulties in the implementation of the IOC code as well as several doping scandals, including the famous action of the French police during the cycling race “Tour de France” in 1998, led to the organization of the First World Conference on Doping in Sport (Lausanne, 1999) [2]. The meeting resulted in the creation of the World Anti-Doping Agency (WADA), with the objective to coordinate the fight against doping in sport. In 2004, WADA adopted the prohibited list of the IOC medical commission, since that moment, the list has been yearly updated.

1.1.2. Prohibited list

In the last version of the prohibited list (2014), the substances and methods prohibited by WADA are divided in different groups as it is shown in table 1.1. The list includes more than 200 compounds divided in nine groups of prohibited substances (non-approved substances, anabolic agents, peptide hormones, growth factors and related substances, β-2 agonists, hormone and metabolic modulators, diuretics and other masking agents, stimulants, narcotics, cannabinoids and glucocorticosteroids), three prohibited methods (manipulation of blood and blood components, chemical and physical manipulation and/or gene doping), and two groups of substances prohibited in particular sports (alcohol and β-blockers).
Doping classes from S0 to S5 and methods M1 to M3 are prohibited in all times (in- and out-competition). On the other hand, substances from S6 to S9 are prohibited only in-competition. 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 [5].

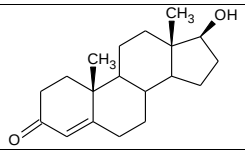
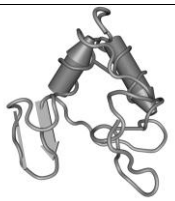
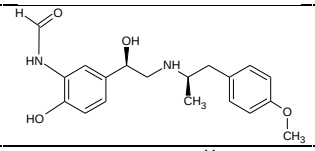
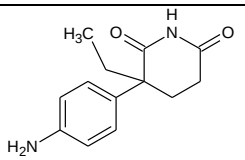
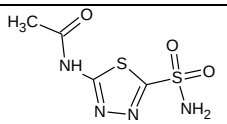
Substance Group	% of all ADAMS reported findings	Example	Name MW
Substances and methods prohibited at all times (in- and out-of-competition)			
S0. Non-approved substances	-	-	-
S1. Anabolic agents	50.6		Testosterone MW=288 Da
S2. Peptide hormones, growth factor and related substances	4.0		Insulin growth factor MW=7649 Da
S3. β-2 agonist	2.9		Formoterol MW=334 Da
S4. Hormone and metabolic modulators	1.6		Aminoglutethimide MW=232 Da
S5. Diuretics and other masking agents	7.2		Acetazolamide MW= 222 Da
M1. Enhancement of oxygen transfer	0.0		Transfusion
M2. Chemical and physical manipulation	0.02		Sample manipulation

Table 1.1. Substances and methods prohibited by WADA, % of reported ADAMS findings in 2012 and some examples of each group

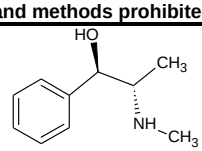
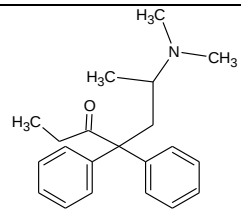
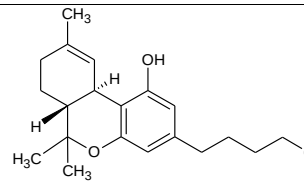
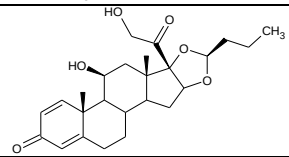
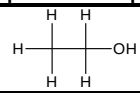
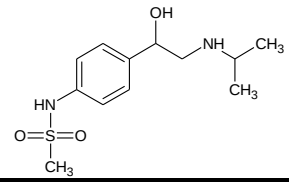
Substance Group	% of all ADAMS reported findings	Example	Name MW
M3. Gene doping	-		-
Substances and methods prohibited in-competition			
S6. stimulants	15.5		Ephedrine MW=165 Da
S7. Narcotics	0.6		Methadone MW=309 Da
S8. Cannabinoids	9.0		Tetrahydrocannabinol MW=314 Da
S9. Gluco-corticosteroids	8.1		Budesonide MW=430 Da
Substances prohibited in particular sports			
P1 Alcohol	0.1		Ethanol MW=46 Da
P2. β-Blockers	0.3		Sotalol MW=272 Da

Table 1.1 (Cont). Substances and methods prohibited by WADA, % of reported ADAMS findings in 2012 and some examples of each group

1.1.3. Analytical methods

Doping control laboratories must be ISO17025 accredited [6] and, therefore, they have to accomplish the guidelines included in the International Standard for Laboratories (ISL) [7]. WADA also publishes specific technical recommendations, addressing particular operation areas of the accredited laboratories, in technical documents (e.g, MRPL, identification criteria for qualitative assays, laboratory internal chain of custody, etc) [8].

Doping control laboratories have to develop analytical strategies to face the detection of several different substances. Analytical strategies used in doping control have to take into consideration several requirements: the high sensitivity and selectivity required in complex matrices (mostly urine and blood samples), detection of compounds with wide range of physicochemical properties and molecular weight (Table 1.1), limited samples volumes and fast analysis time. Urine is the specimen of choice for most of the prohibited substances since the collection is non-invasive, the volume available is quite large, the concentrations of drugs are higher than in blood, and since hydrophilic metabolites are also excreted in urine, the detection time window can be enlarged [9]. However, in some cases the analysis of blood samples are mandatory due to the absence of urinary markers (e.g. blood transfusions) [10].

The analytical strategy in doping control includes two steps procedures. A preliminary, fast and comprehensive screening method with a minimum sample preparation is used for the detection of a large variety of compounds and/or metabolites with similar physicochemical properties [11-14]. The screening methods are applied in all samples. The screening step provides an indication of the presence or absence of a doping agent. Once a

sample is considered suspicious, a more specifically confirmatory method optimized for the analyte in question is used for identification purposes. 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).

In order to ensure that all accredited doping control laboratories can report the presence of prohibited substances in a uniform way, a minimum detection and identification capability for testing methods has been established. This detection level is known as Minimum Required Performance Level (MRPL) [15]. The MRPL is established for each banned compound taking into account the metabolism, stability, pharmacokinetics and pharmacodynamics. Thus, substances with long-term doping effects as anabolic agents have lower MRPL values than those substances which are taken for an immediate effect (e.g. stimulants or diuretics). Whereas the MRPL for most of the AAS is set at 5 ng/mL other compounds as stimulants have a MRPL at 100 ng/mL [16].

1.1.4. Screening methods

Screening analysis are used to find suspicious samples containing the doping agent. The critical aspects for a good screening method include high throughput, sensitivity, selectivity, specificity, coverage and suitability for automation. The characteristics of the method performance are also dependent on whether non-threshold or threshold substances have to be determined [17].

For some threshold substances the concentration of the compound shall be adjusted to the specific gravity (SG) of the sample.

- **Non threshold substances**

For non-threshold compounds, the laboratory has to identify (not quantify) the compound's presence in the sample

- **Threshold substances**

Threshold substances are considered doping agents only above a threshold concentration [18] and therefore a quantitative measurement is needed.

- **Endogenous substances**

High levels of endogenous compounds, such as anabolic androgenic steroids (AAS), are quantified to estimate an AAF. Statistical thresholds levels or ratios have been established to estimate a suspicious sample (for more information see section 1.3).

1.1.5. Confirmatory method

Once a sample is considered suspicious for the presence of a forbidden substance, a confirmatory test must be performed. The confirmatory test should be at least as sensitive as the screening method and preferably more specific. Different confirmation procedures are applied depending on the substance (threshold or non-threshold). A sample is reported as an AAF when the level exceeds, with an appropriate level of confidence (95%), the threshold limits of the analyte [18].

- **Non-threshold substances**

For non-threshold substances, WADA describes in the technical document the criteria to confirm the presence of a prohibited substances in a sample [19]. A comparison of relative retention

times and relative abundances of three diagnostic ions for MS analysis or two diagnostic transitions for MS/MS analysis are needed. The results are compared with reference material and are considered as AAF if the identification criteria's are fulfilled [15]. As an example of confirmation Figure 1.1 showed the identification of triamcinolone acetonide by comparison a suspicious sample with the reference standard. Differences below 0.1 min for retention time and below 4% for the ion ratio were obtained fulfilling the criteria established for WADA [15].

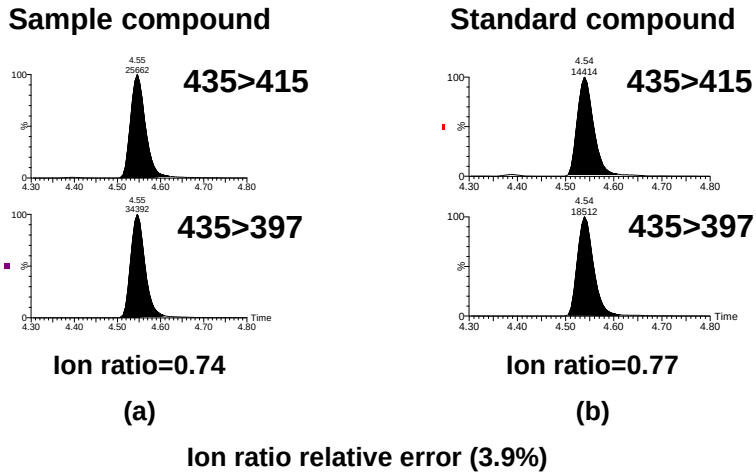


Figure 1.1. Example of identity confirmation comparing **(a)** sample suspicious for triamcinolone acetonide and **(b)** the reference standard.

- **Threshold substances**

For threshold substances, the unequivocal identification and quantification of the anlayte is mandatory [18]. The identification of the compound is performed as described for non-threshold substance. To confirm the concentrations of a threshold substances the value should be expressed as the mean concentration from triplicate determinations and the combined standard uncertainty.

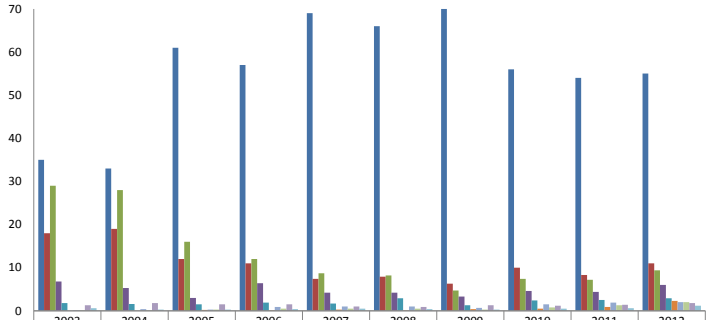
- **Endogenous substances**

For endogenous compounds, neither gas chromatography nor liquid chromatography coupled to mass spectrometry are able to differentiate endogenous substances from exogenous administration [20]. Gas chromatography coupled to combustion isotope ratio mass spectrometry (GC/C/IRMS) was introduced to distinguish between endogenous and synthetic steroids analogues (see section 1.3).

1.1.6. Adverse analytical finding reports

Based on the results obtained for all accredited anti-doping laboratories, WADA publishes yearly, the statistics summarizing the AAF. According to these statistics, anabolic agents are the most frequent reported group (50.6%) of compounds detected in the doping controls laboratories in 2012 [21]. Among anabolic agents, the endogenous anabolic androgen (EAAS) testosterone (T) is the most commonly detected across the years as showed in Figure 1.2. Particularly, in the last version of the document released in 2012 [21], T has the 55.5% of AAF and it is described as those results with a ratio of T/E>4 (see section 1.3. for further information). It has to be taken into consideration that for endogenous compounds, such as T, these findings may be merely due to the detection of concentrations outside normal reference ranges and not necessary for a doping offense [11]. In this sense, these findings summarized both the AAF and atypical findings reported in the anti-doping laboratories. Therefore, the detection of T seems to be one of the most difficult analytical challenges in the doping control laboratories.

In this thesis, the problematic of the detection of the administration of EAAS by the doping control community is presented. In particular, new insights for the detection of T will be addressed.



	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012
Testosterone	35	33	61	57	69	66	70	56	54	55
Stanozolol	18	19	12	11	7.4	7.9	6.3	10	8.3	11
Nandrolone	29	28	16	12	8.7	8.2	4.7	7.4	7.2	9.4
Methandienone	6.8	5.3	3	6.4	4.2	4.2	3.3	4.6	4.4	6
Boldenone	1.8	1.6	1.5	1.9	1.7	2.9	1.3	2.4	2.5	2.9
Dehydrochloromethyl-testosterone	0	0	0.2	0.1	0.3	0.1	0.4	0.5	0.9	2.3
Drostanolone	0.1	0.4	0.3	0.9	1	1	0.7	1.5	1.9	2
Trenbolone	0.1	0.1	0.3	0.5	0.5	0.5	0.3	0.8	1.3	2
Metenolone	1.3	1.8	1.5	1.5	1	0.9	1.3	1.2	1.4	1.8
Oxandrolone	0.6	0.3	0.3	0.4	0.5	0.4	0.3	0.5	0.6	1.2

Figure 1.2. % of the ten most frequently reported anabolic agents in anti-doping control laboratories across the years (2003-2012)

1.2. Anabolic androgenic steroids

1.2.1 General aspects

The structure of steroids is easily recognized by the cyclopentanoperhydrophenanthrene core (Figure 1.3) ring. This core is a carbon structure composed of four fused rings: three cyclohexane rings (A, B and C) and one cyclopentane ring (D). The carbon atoms are numbered as depicted in Figure 1.3. Androstanes are steroids containing 19 carbons in the core structure with typical presence of C_{18} and C_{19} methyl groups (Figure

1.3). A large variety of natural and synthetic steroids are derived from that core.

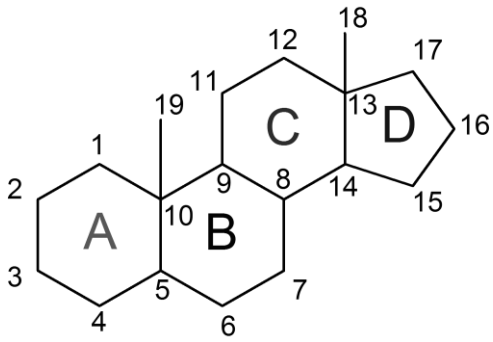


Figure 1.3. Androstane structure: core and conventional numbering of the rings and carbons

1.2.2. Biosynthesis

Steroidogenesis is the biosynthetic pathway that produces steroid hormones from cholesterol. The enzymes and intermediates involved in the steroidogenesis are depicted in Figure 1.4. Cholesterol is the common precursor for the formation of all steroid hormones which includes progestins, androgens, estrogens, glucocorticoids and mineralocorticoids (Figure 1.4).

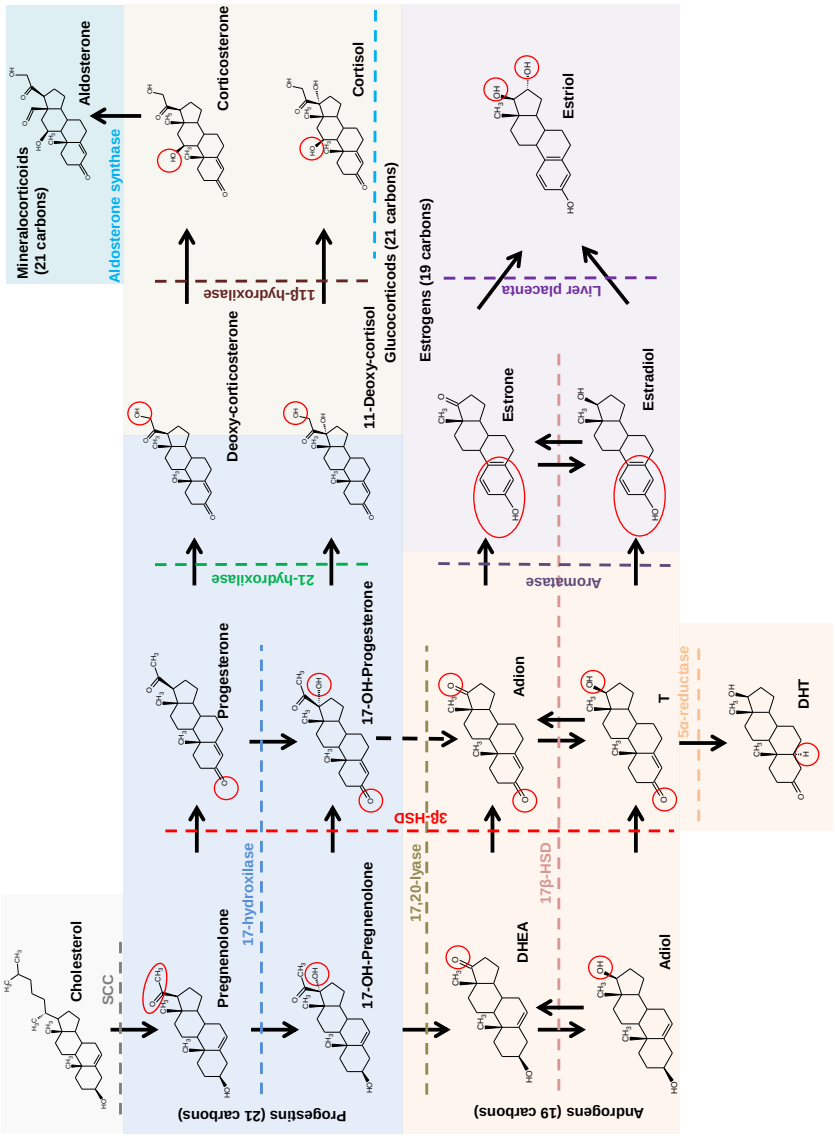


Figure 1.4. Human steroidogenesis, with major classes of steroids hormone.

Although Leydig cells are the major responsible for the generation of circulating androgenic hormones, the adrenal cortex also contributes to this production [22]. The primary source of cellular cholesterol is the serum. Cholesterol is transported to the cell via serum protein carriers. Once inside the cell, cholesterol is immediately utilized or it can be stored as lipid droplets. A second minor source of cholesterol is *de novo* synthesis, which increases following hormone stimulation of the leydig and follicle cells [23].

Enzymatic conversion of cholesterol to pregnenolone constitutes the initial step in a series of biochemical reactions that culminate in the production of the different steroid hormones [24]. This first step involves an oxidative cleavage of the side chain of cholesterol by side-chain cleavage enzyme (SCC) inside the mitochondria [25] with the loss of six carbons atoms and the formation of pregnenolone which serves as a precursor to the three other progestins (Figure 1.4).

The second enzymatic reaction results in the conversion of pregnenolone to progesterone by the enzyme 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3β-HSD). Here, the steroidogenic pathway bifurcates into Δ^5-hydroxysteroids pathway (starting with pregnenolone) and Δ^4-ketosteroid pathway (starting with progesterone) as showed in Figure 1.4. Additionally, progesterone can be converted to deoxy-corticosterone by 21-hydroxylase which in turns is converted into corticosterone. Thereafter, this compound is oxized to the end-product aldosterone (Figure 1.4)

The third enzymatic reaction involves the 17α-hydroxylase/$C_{17\text{-}20}$ lyase.(17,20 lyase) [26]. This enzyme catalyzes two chemical reactions, hydroxylation and cleavage (converts the steroid from 21-carbon to a 19-carbon molecule), and requires molecular oxygen and NADPH. Thus, for the Δ^5-hydroxysteroid pathway,

17,20 lyase initially catalyzes the conversion of pregnenolone to 17α-OH-Pregnenolone, which is then converted to DHEA by the action of 17, 20 lyase. Likewise for the Δ^4-ketosteroids, 17-hydroxylase converts progesterone to 17α-OH-Progesterone. Furthermore, this compound can be converted to 11-deoxy-cortisol by 21-hydroxylase which in turns is converted to cortisol (Figure 1.4).

The next enzymatic step involves the conversion of DHEA into androstendione by the action of 3β-HSD. Thereafter, this compound is converted to T by 17β-hydroxysteroid dehydrogenase (17β-HSD). A second possible reaction involving androstendione occurs in female, whereby androstendione is converted to estrone by aromatase [27]. In males, T is converted to dehydrotestosterone (DHT) by 5α-reductase, which is significantly more potent as an androgen than T and is also considered as an end-product hormone [25].

1.2.3. Metabolism

The metabolism of T can be discussed as a basic metabolic pathway for all AAS and has been comprehensively reviewed [28, 29]. Although most of the steroid metabolism takes place in the liver, some metabolic reactions also occur in kidney and skin [30].

In man the bulk of the AAS metabolites appear in the urine. Metabolic reactions are grouped into two types, phase I and phase II reactions, and they convert the steroid into more polar compounds in order to usually inactivate the drug and facilitate its elimination from the body. The most common structural changes after metabolism are summarized in Table 1.2.

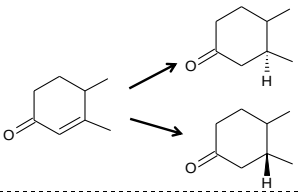
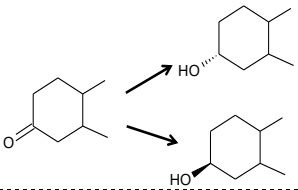
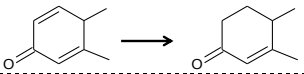
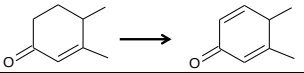
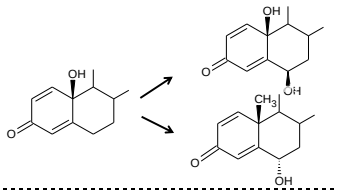
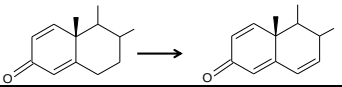
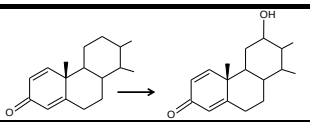
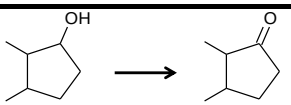
Phase I metabolism	
A-ring	
5α and 5β-reduction	
3-keto-reduction	
1,2-hydrogenation	
1,2-dehydrogenation	
B-ring	
6α and 6β-hydroxyation	
6,7-dehydrogenation	
C-ring	
12-hydroxylation	
D-ring	
17-oxidation	

Table 1.2. Common phase I and phase II reactions for steroids

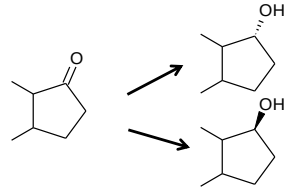
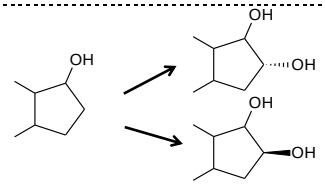
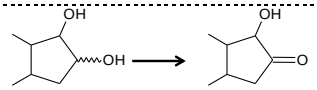
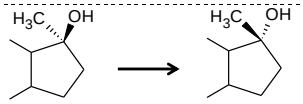
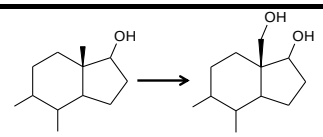
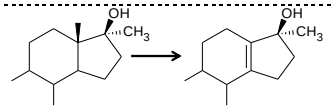
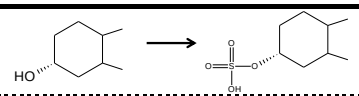
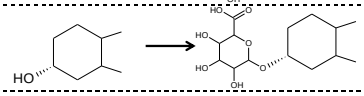
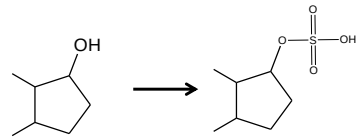
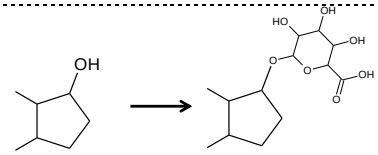
Phase I metabolism	
17-keto-reduction	
16α and 16β-hydroxylation	
16-oxidation	
17-epimerization *	
Others	
18- or 19-hydroxylation	
Dehydratation involving a 18-methyl migration *	
Phase II	
A-ring	
3-sulphonation	
3-glucuronidation	

Table 1.2. (cont.) Common phase I and phase II reactions for steroids

Phase II	
17-sulphonation	
17-glucuronidation	

*Generated through 17β-OH-Sulphate

Table 1.2. (cont.). Common phase I and phase II reactions for steroids

Phase I reactions are enzymatically catalysed; normally oxidations and reductions occurring in several positions of the steroid ring (Table 1.2). Double bond reduction towards 5α- and 5β-saturated structures, 3-keto or 17-keto reduction, 1,2-hydrogenation, 6, 12 or 16-hydroxylations, 6,7-dehydrogenation, and 17-hydroxy oxidation, are considered the main phase I metabolic reactions for AAS (Table 1.2).

Phase II reactions encompasses the conjugation of a glucuronide moiety by uridine diphospho (UDP)-glucuronosyl transferases (UGT) or the formation of sulphates by sulphatases enzymes to the free hydroxyl group of the steroid as showed in Table 1.2 [31]. This process makes the apolar steroid more soluble in water for their excretion from the body via urine. In humans, conjugation with glucuronic acid is the major conjugation reaction for AAS [32]. Other phase I and phase II reactions as the 17-Epimerization and dehydratation onvolving 18-methyl migration coming from degradation of suphate moiety [28, 33-35]. Additionally, the occurrence of 6,7-dehydro metabolites of methandienone in

alkalinized (pH>12) urine have been also described [28] as well as the existence of bis-conjugates [28, 36].

1.2.4 Mechanism of action

In human male, T is the major circulating AAS. More than 95% is secreted by the testis, which produces approximately 6-7 mg per day [37]. For a proper action of AAS sometimes is necessary the conversion of T into DHT [38] before it can fully activate the androgen receptor (AR). In absence of ligand, the AR exists in an inactive form with a complex with molecular chaperones (Figure 1.5). These proteins help in maintaining the correct conformation of the receptor necessary for efficient ligand binding [39]. Upon hormone binding, AR is translocated from the cytoplasm into the nucleus where it uses its DNA binding domain to interact as homodimer to specific DNA sequences termed androgen response elements (Figure 1.5). The attachment to DNA strands triggers a recruitment of various co-regulation proteins to form a transcription complex which activate transcription in the regulatory regions of the target gens (Figure 1.5). The co-regulators modulate the transcriptional activity and can have co-activating of co-repressing action depending on the target tissue [40]. Up-regulation of the transcriptional activity results in synthesis of messenger RNA. Ribosomal activity, in turn, translates the messenger RNA to specific proteins.

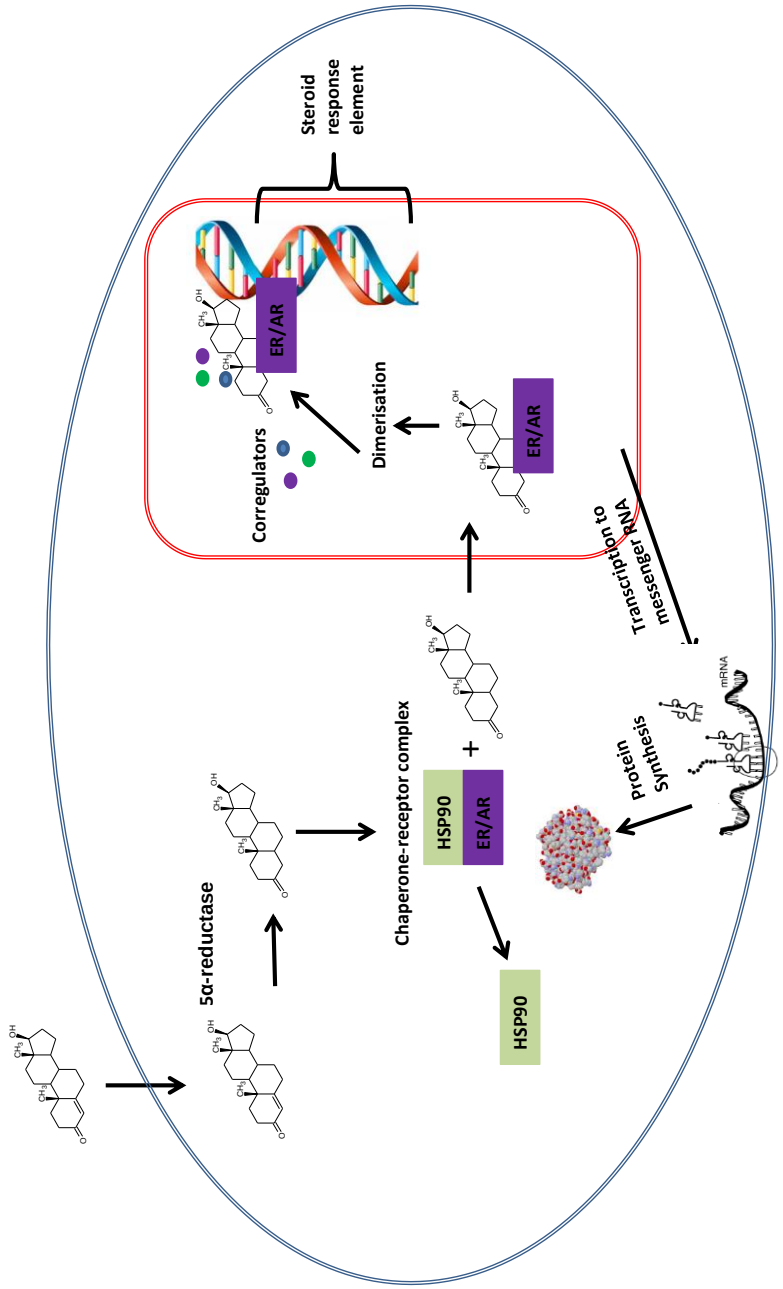


Figure 1.5. Mechanism of action of AAS at cellular level

1.2.3. Effect

In men, T plays a key role in the development of male reproductive tissues such as testis and prostate as well as promoting secondary sexual characteristics such as increased muscle, bone mass, and the growth of body hair [41]. The anabolic action effects protein metabolism by stimulating of protein synthesis and inhibition protein catabolism in skeletal muscle and bone [42]. The effects of the intake of AAS has been detailed reviewed in the literature [43]. Most of the studies presented in this review concluded that the administration of AAS increased the body dimensions mainly in the upper region of the body (neck, thorax, shoulders and upper arm) Additionally, the administration of AAS increases the lean body mass significantly, which may contribute to an increase of muscle mass. Finally, it has been demonstrated that the use of AAS enhances the effects of strength training.

The secondary sexual characteristics are mainly associated with androgenic changes: enlargement of the larynx causes deepening of the voice, terminal hair is stimulated to grow in pubic, axillary and facial regions, sebaceous glands activity is promoted which can result in acne and the influence on the central nervous system causes a sense of well-being, increased libido and sometimes more aggressive behaviour.

Obviously, athletes are primarily interested in the anabolic effect of AAS rather than androgenic action which is undesirable for them. Therefore, big efforts have been made in order to separate both effects. With structural modifications of T, scientists attempted to achieve optimal anabolic-androgenic dissociation, the anabolic effect can be enhanced but, they cannot be entirely separated from androgenic effect since they all bind to the same AR [31, 38].

1.3 EAAS and doping

1.3.1. First perspectives for the detection of EAAS. Introduction of T/E

In doping control laboratories, testing for exogenous AAS in urine was implemented during the Olympic Montreal games in 1976, when adequate detection methods became possible [4]. However, the detection of the misuse of EAAS remained imposible until Donike *et.al.* introduced the ratio of T glucuronide and its 17α-Epimer epitestosterone (E) glucuronide in 1983 [44]. E is 17α-epimer of T and remain practically constant after T intake [45]. Thus, the resulting effect is an increase in the ratio between T and E excreted as glucuronide (T/E) after the administration. Based upon 2700 urine samples specimens, an average value for the T/E was found to be around 1 [46]. The T/E values showed a logarithmic normal distribution with an upper limit value lower than 6 [47]. Using this data, the IOC banned the use of EAAS in 1983 and steated that T/E ratio above 6 was sufficient proof for EAAS abuse. However, due to naturally occurring cases of T/E above 6 [47, 48] some years later IOC stablished that a follow up investigation was needed for T/E values between 6 and 10 meanwhile any sample with T/E higher than 10 was automatically set positive. Additionally, due to a deletion in the gene responsible for the excretion of T glucuronide, T/E distribution showed two sub-populations with respective mean T/E of 0.1 and 1 [49].

1.3.2. Changing the prespectives for the detection of EAAS. Introduction of GC/C/IRMS

A first step forward in the fight against EAAS abuse was the development, introduction and optimization of GC/C/IRMS [50-52].

Whereas a T/E>6 is an excellent indicator of T administration, it is not a definitive test. The use of GC/C/IRMS allowed for the differentiation between the exogenously administered steroids to those produced naturally in the body by their relative content of carbon isotopes. Endogenous steroids are formed via cholesterols as explained in section 1.2.2, whereas pharmaceutical T is synthesized from soy. Both origins are characterized by different fixation of the carbon isotope ^{13}C [53]. As a consequence, soy derived steroids has a low ^{13}C content which is significantly different from the endogenous steroids which reflects naturally occurring ^{13}C fraction. The isotopic abundance of the analyte is measured with great precision and accuracy and it is compared to that of an international reference compound (Pee Dee Belemnite or a reference gas):

$$\delta^{13} = \left[\frac{(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{sample}}{(^{13}C/^{12}C)_{reference}} \right] \times 1000$$

Despite its discriminating power, the use of GC/C/IRMS is only used for confirmatory purposes. The large amount of urine required, the time-consuming methods needed for this analysis prevent against the use of this approach as a screening method.

1.3.3. New perspectives for the detection of EAAS. Introduction of the steroid profile and athlete biological passport

Due to the introduction of a confirmatory method for EAAS, in 2004, WADA decreased the threshold for the T/E to 4 and

recommended a confirmatory procedure with GC/C/IRMS if one of following criteria was found:

- T/E > 4
- T or E glucuronides > 200 ng/mL
- Androsterone or Etiocholanolone glucuronides > 10000 ng/mL
- DHEA glucuronide > 100 ng/mL

The reference ranges [54] and intra-individual stability of these metabolites were studied and became well-established steroid profile parameters [49, 55].
In the last version of the technical document for the detection of EAAS (released in 2014), WADA includes the quantification of the steroid profile [56] and the determination of the so-called athlete biological passport (ABP) [56]. The steroid profile includes the quantification of the following compounds:

- T
- E
- Androsterone
- Etiocholanolone
- 5α-androstane-3α,17β-diol
- 5β-androstane-3α,17β-diol
- T/E

The term athlete biological passport (ABP) was first proposed in early 2000s when monitoring of select haematological variables was identified as a means to define an individual's haematological profile.

More recently this concept has taken for the detection of EAAS misuse which is called Athlete Steroidal Passport (ASP). This approach allows the continuous and systematic storage and evaluation of the parameters of the steroid profile. Several aspects of the ASP have been developed and validated in recent years as the introduction of the adaptive Bayesian model to evaluate longitudinal biological profiles. The model progressively adapts the calculated upper and lower reference levels evolving from a population-based to a subjected-based approach when the number of recorded measurements per athlete grows as showed in Figure 1.6. The implementation of the ASP was enabled by coupling the athlete's information with the laboratory test results, both collected and reported via Anti-Doping Administration & Management System (ADAMS), a recently installed reporting system by WADA that combines doping related information of the laboratories, athlete and testing organisation [57].

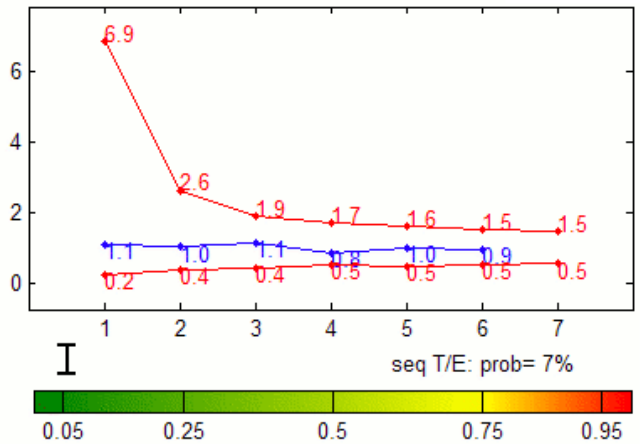


Figure 1.6. Longitudinal T/E profiles and corresponding individual limits assessed with the adaptative bayesian model

The laboratory shall confirm the relevant steroid profile marker or ratio (e.g. the T/E ratio) measured when, upon reporting the results in ADAMS and following the application of the adaptive model of the ASP to the "longitudinal steroid profile" of the athlete, the laboratory is informed through ADAMS of an adverse analytical finding. In the case when the adaptative model in ADAMS cannot process the "longitudinal steroid profile" of the sample, the laboratory shall confirm the sample when one of the following criteria is met:

- T/E > 4
- T or E glucuronides > 200 ng/mL in males or >50 ng/mL for females
- Androsterone or Etiocholanolone glucuronides > 10000 ng/mL combined with ratio Androsterone/Etiocholanolone lower than 0.4 in males or greater than 4 in either sex.

When the parameter of the steroid profile indicates the need to further investigations according to WADAS's criteria [56], the measurement by GC/C/IRMS of the ratio between stable carbons isotopes $^{13}C/^{12}C$ is mandatory.

The application of ASP is able to detect small but noticeable changes in the markers of the steroid profile. This characteristic also makes that the ASP is largely sensitive to any source of individual variability associated to the marker. Thus, an in deep knowledge about the factors that can influence the steroid profile excretion is of central importance. These factors are further discussed in the chapter 8.

1.4. Analytical strategies for the detection of EAAS

1.4.1 Routine analytical method

In doping control analysis, the detection of AAS in urine gained tremendous efficiency with the introduction of mass spectrometric detection coupled to separating techniques in the early eighties. This technique combined the required analytical sensitivity and specificity in order to differentiate between different steroid compounds in urine. Therefore, the use of GC-MS based detection methods for steroids are widely applied in most of the doping control laboratories [49, 55, 58-60].

The method involves several analytical steps in order to optimize the gas chromatographic behaviour of the steroid molecule. Since the bulk of the excreted steroids are conjugated with glucuronides, the initial step of the method includes an hydrolysis step in order to remove this moiety by an enzymatic hydrolysis at pH=7 [61]. Thereafter, the neutral steroids are extracted for the urine matrix using an apolar solvent normally diethyl or *tert*-butyl-methyl ether by a liquid-liquid extraction (LLE) (Figure 1.7). Once the steroids are in the organic phase, the solvent is evaporated.

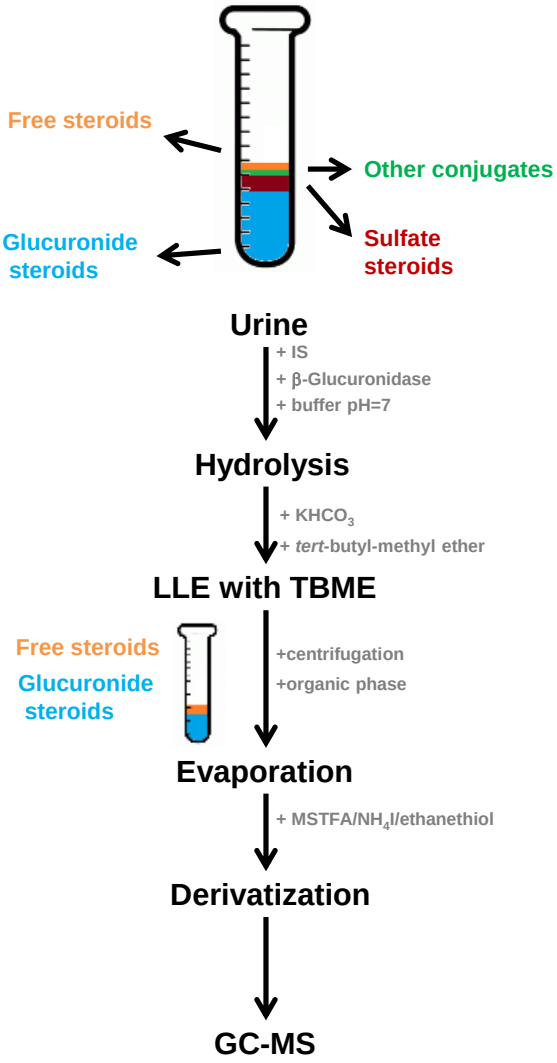


Figure 1.7. Flown chart of the sample treatment commonly used for the detection of steroids by GC-MS

AAS must be derivatized to become amenable for the GC-MS analysis [62]. Most of the current routine methods developed for GC-MS are based on the detection of the trimethylsiliated steroid derivatives [49, 55, 58, 59, 62, 63]. The common protocol for trimethylsiliation was developed by the group of Prof. Donike and

co-workers [64, 65], this approach remains as the reference method in the anti-doping community, consists in the addition of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) as derivatization reagent to achieve the protected TMS derivatives which maintain stability during separation in gas chromatography [66]. In order to use the derivatisation step also for the more stable keto functions, iodotrimethylsilane (TMSI), the most reactive trimethylsilyating reagent, serves as catalyst which is formed *in situ* when ammonium iodide is added. Decomposition of TMSI leads to the formation of iodine which reacts with the analyte to form steroid-iodide-adducts. Therefore, a reducing agent (e.g. ethanethiol) is added to inhibit the iodine formatio (Figure 1.8.). The derivatisation step is performed at higher temperatures in order to enolise the more stable keto functions of the molecule. The failure of some steroids to provide a single reaction product [67], together with the chemical rearrangement of others [68] are limitations associated with the derivatization process.

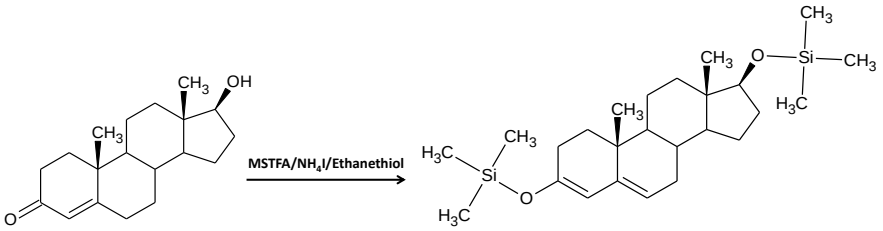


Figure 1.8. TMS derivatization process for testosterone

After the derivatization step, the sample is analysed by GC-MS, usually operating in selected ion monitoring (SIM, see figure 1.9) mode including three ions for each steroid metabolite. By selecting the ions by mass over charge (*m/z*) with the quadrupole of the mass spectrometer, SIM mode detection allows to increase the

numbers of scans per second. The use of SIM mode allowed to improve analytical sensitivity and specificity.

Based upon the gas chromatographic retention time relative to the internal standard and their mass spectra, the different EAAS included in the steroid profile can be separated, identified and quantified with corresponding reference standards.

1.4.2. Other analytical methods (LC-MS/MS)

Although, the use of the GC-MS for the detection of steroid abuse is still the gold standard for doping control laboratories, the irruption of the liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) in doping control analysis has minimized several problems related to traditional GC-MS methods including sample preparation, improvement in limits of detection and the possibility to detect thermolabile compounds [62]. LC-MS/MS instruments have the possibility to directly detect phase II metabolites (without hydrolysis) and very polar compounds avoiding derivatization steps [35, 69]. Additionally, LC-MS/MS provides more sensitivity, the analytical time is generally shorter in comparison with GC-MS procedures and necessities less tedious sample preparation procedures due to the high analyte selectivity of LC-MS/MS [69-71]. Furthermore, LC-MS/MS permits the determination of thermally labile compounds, chemically unstable and/or volatile drugs [69, 70, 72, 73].

- **Cromatography**

LC-MS/MS allows for the combination of the separation capabilities of the LC and the sensitivity and identification power of MS/MS as detector. A liquid mobile phase consisting out of a mixture of solvents with different polarity is used [74]. The separation critically

depends on the selection of both the stationary and the mobile phase. The LC-MS/MS methods are normally based on reverse phase columns (C_{18}, C_8…) with water and methanol (or acetonitrile) for the mobile phase. Several mobile phase modifiers can be used to modify the chromatographic separation.

- **Ionization**

Ionization is probably the bottleneck of LC-MS/MS detection of AAS. Due to low proton affinity of the chemical functions normally observed in the steroid structure (hydroxyl, alkens and/or keto moieties) several AAS exhibited poor ionizations. In fact, only AAS containing conjugated keto functions as T or nitrogen as stanzolol shows an abundant $[M+H]^+$ ion after ESI+ [75]. Thus, the formation of adducts is necessary for the ionization of AAS without these functions. Adducts like $[M+NH_4]^+$, $[M+Na]^+$, $[M+Ag]^+$, $[M+H+CH_3OH]^+$, $[M+F]^-$ or $[M+HCOO]^-$ have been described for the ionization of AAS [75-77].

- **Analyzers**

Contrary to GC-MS, the implementation of analytical methods with LC-MS equipped with single quadrupole analyzers was not practical due to the lack of fragmentation in API interfaces and to the complexity of matrices used in doping control analysis. The development of electrospray ionization (ESI) interfaces in the 1990s was critical in the irruption of LC-MS/MS instruments in doping control tests [78]. A detailed description of the function of this interface and their application can be found in several books [79, 80]. Therefore, the application of LC-MS in the doping field started directly with the analyzers with MS/MS capabilities, such triple quadrupole (QqQ). The important role of QqQ instruments in

doping control analysis can be extracted from several reviews about this topic [20, 74, 81-83]. These analyzers include two additional quadrupoles (one for mass filtering and the other one which is a hexapole or octapole for collision-induced dissociation (CID) called collision cell).

In the last years, the implementation of robust high resolution (HR) instruments such time-of-flight (TOF) and Orbitraps analyzers have shown new alternatives for the detection of steroids due to their sensitivities obtained in scan mode [84]. The *modus operandi* of these instruments is the acquisition of the full scan raw data and, therefore, the detection of all compounds ionizables by the source. Thereafter, extracted ion chromatograms with narrow mass window (<10 ppm) are evaluated for desired doping agents [85].

- **Scan modes**

Due to the special configuration of QqQ, different scan modes can be used as showed in Figure 1.9:

- **Full scan:** The full scan mode allows for the detection of all ionized molecules in the interface. For this purpose, neither the collision cell nor the 3rd quadrupole are used (Figure 1.9). In QqQ instruments each m/z is scanned separately and the consecutive scans produced the full scan spectrum. This result in a lower sensitivity when applying this mode.

- **SIM:** By this mode only one ion (*m/z*) of interest is selected in the first quadrupole (Figure 1.9). SIM is the method of choice when a single quadrupole instrument is used.

- **SRM:** In selected reaction monitoring, an ion (*m/z*) is selected in the first quadrupole (precursor ion), it is fragmented in the collision cell at optimized collision energy and the *m/z* of a fragment (product ion) is selected in the third quadrupole (Figure 1.9). SRM is the method of choice when the structure of the analyte is known and high sensitivity is required.

- **Product ion scan:** In this mode, a precursor ion is selected in the first quadrupole and it is fragmented in the collision cell at adequate collision energy and a full scan is performed in the 3rd quadrupole (Figure 1.9). This scan mode is very useful to obtain structural information about the molecule.

- **Precursor ion scan:** By this mode, the first quadrupole scans all the ions coming from the interface, the collision cell fragments all these ions at fixed collision energy and one ion is selected in the 3rd quadrupole (Figure 1.9). This mode allows for the detection of compounds sharing a common structure.

- **Precursor ion scan:** By this mode, the first quadrupole scans all the ions coming from the interface, the collision cell fragments all these ions at fixed collision energy and one ion is selected in the 3rd quadrupole (Figure 1.9). This mode allows for the detection of compounds sharing a common structure.

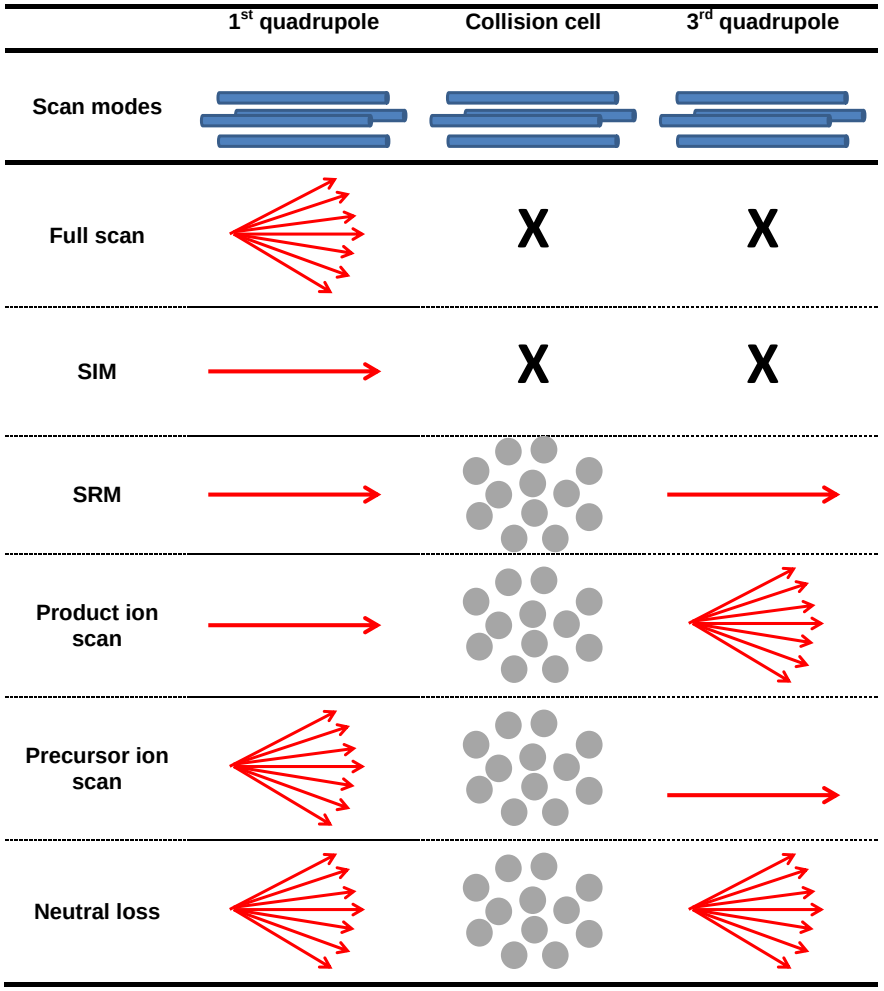


Figure 1.9. Scheme of the different scan modes available in QqQ instruments

- **Neutral loss scan:** In this mode, both the 1st and 3rd quadrupole scan with a phase for a fixed mass value and at the collision cell fragment at a fixed collision energy (Figure 1.9). Using this scan mode only analytes having a specific (neutral) loss in their fragmentation will be detected.

- **LC-MS/MS and steroids**

The emergence of commercially available LC-MS/MS instruments represented one of the biggest changes in routine analysis in doping control laboratories. The first application of the LC-MS/MS for steroids analysis was reported in 1996 in our laboratory [78]. The importance of the LC-MS/MS in the detection of target AAS in the last years has been widely reviewed [86-88].

Several, LC-MS/MS methods have been published both for qualitative detection of exogenous AAS and the quantitative detection of EAAS:

- The qualitative detection methods are especially developed for those exogenous AAS which the detection by GC-MS is problematic (e.g. stanozolol, tetrahydrogestrinone, fluxymesterone and oxandrolone) [86, 87, 89].

- The quantitative detection methods are mainly focused in the detection of EAAS in human urine mainly for the determination of the phase II metabolites conjugates with glucuronide or sulphate. Glucuronides can be ionized in both positive and negative ionizations modes and their fragmentation pathways have been in-depth studied [90]. The low detection limits required for these analytes (around 1 ng/mL for testosterone and epitestosterone in urine) makes the use of SRM mode almost mandatory. Several papers can be found using SRM methods for the detection of steroids glucuronides either in positive [91-93] or negative [93-95] ionization modes.

- **Open detection methods**

An additional special feature of QqQ instruments is the capability of performing alternative scans modes, such as neutral loss (NL) or precursor ion (PI) scans (Figure 1.9). The use of these modes allows the open detection of compounds sharing a specific chemical feature. For this, reason they are a valuable tool for the detection of unknown compounds belonging to a specific family. This characteristic has been used for doping control field [96, 97] or metabolic studies [98-100].

Additionally, the development of full acquisition methods based on HR instruments is a promising alternative for the open detection for AAS. The main application of HR methods for the open detection of AAS has been successfully applied in illegal cocktails [101] and nutritional supplements [102].

1.4.3 New analytical tools for the detection of EAAS

The aim of the screening methods for anabolic steroids in doping control laboratories is to detect the misuse of these substances in the most specific way and for the longest period possible. In this way, the best marker for the detection of an endogenous steroid abuse is not always the most abundant metabolites but the one which can be distinguished from normal concentrations for the longest period (the so-called long-term metabolite) [36].

Different new approaches have been developed during the years in order to improve the detection capabilities of the current methods for the detection of steroid profile such the application of subjected-based reference limit, or the development and implementation of the ABP [103, 104].

In the last decade, a growing use of computer aided approaches has been used in fundamental research as well as in forensic

science [105] and doping [106, 107]. Particularly “omic”-technologies are being used in a boarding range of applications in health and life science. Based on this technologies, a powerful machine learning technique called support vector machine approach has been proposed as discriminative tool for the evaluation of steroid profile [108].

On the other hand, the characterization and elucidation of minor metabolites has been shown as a powerful approach in order to increase the diagnostic specificity of the steroid profiling [36, 99, 109, 110]. The use of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has allowed for the detection of several long-term metabolites for some exogenous AAS by the use of metabolic studies [99, 100, 111, 112].

1.5 Metabolic studies by MS based methods

As previously stated, the detection of new metabolites can be detected for a longer period than those commonly monitored. For these reasons, in depth metabolic studies are of outmost importance in order to identify as many metabolites as possible and to select the best marker.

1.5.1. General strategies

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 [113] as well as cell cultures [114] have been successfully employed for the study of human metabolism of some AAS.

Most of the metabolic studies based on MS strategies are conducted by comparing samples collected before and after the administration of the drug to healthy volunteers. Depending on the instrument used, different strategies can be applied:

- Most of the studies are performed with single quadrupole instruments, the most logical strategy is to evaluate the peaks appearing only in the full scan chromatogram of post-administration urine samples [113, 114]. This untargeted approach has the main advantage of detecting metabolites generated by unforeseen metabolic biotransformations. On the other hand, the relative poor sensitivity of the scanning mode can prevent the detection of minor metabolites.

- A second approach is to evaluate extracted ion chromatograms (EIC) corresponding to common ions of the compound or to perform target analysis using SIM or SRM methods. As an example, two 16-hydroxylated metabolites of methylstenbolone were recently reported by extracting the ions *m/z* 218 and *m/z* 231 [115]. The use of this approach improves significantly the sensitivity, however it requires an *ex ante* prediction of the metabolism and precludes the determination of unexpected metabolites.

- A third approach is to perform the synthesis of the potential metabolites and to check their presence by a target analysis in post-administration samples using SIM or SRM methods [116]. The main advantage of this approach is its high sensitivity.

1.5.2. GC-MS based strategies

Metabolic studies of AAS have been traditionally performed using gas chromatography coupled to mass spectrometry (GC-MS) by detecting the phase I metabolites after the hydrolysis of the urine using the analytical strategy described in section 1.4.1.

The knowledge about the mass spectrometric behaviour of EAAS is an essential tool for the identification of metabolites as it allows both the prediction of ions characteristic of theoretical metabolites and for the proposal of potential structures for unknown metabolites.

The detection of exogenous AAS, both underivatized and derivatized by GC-MS after EI and the determination of the fragmentation rules have been extensively investigated for more than fifty years [62, 117, 118]. Due to the fact that EI is a universal hard ionization technique, it is possible to establish relationships between structure and the fragment ions. Thus, mass spectra of TMS derivates commonly show ions at M^+-15 (loss of a methyl radical), M+-90 (loss of a trimethylsilanol), and M^+-15-90. In addition to that, some features in the structure generate specific ions like the ones represented in Figure 1.10a.

1.5.3. LC-MS/MS based strategies

As stated in section 1.4.1, GC-MS has some limitations for the execution of metabolic studies, among them; the need of derivatization of polar compounds and the impossibility to directly detect conjugated metabolites are the most significant. Over the last years, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has shown several novel possibilities for the detection and identification of new phase I and phase II metabolites.

The CID behaviour of AAS has been studied dividing the AAS into groups based on chemical similarities [119, 120]. Relationships between the structure and product ions obtained can be established as it has been recently reviewed [62, 74]. Thus, several ions or losses are common to specific chemical features like those depicted in Figure 1.10b. At high collision energy, most of AAS shows three common ions at m/z 77, 91 and 105 [97] (Figure 1.10c).

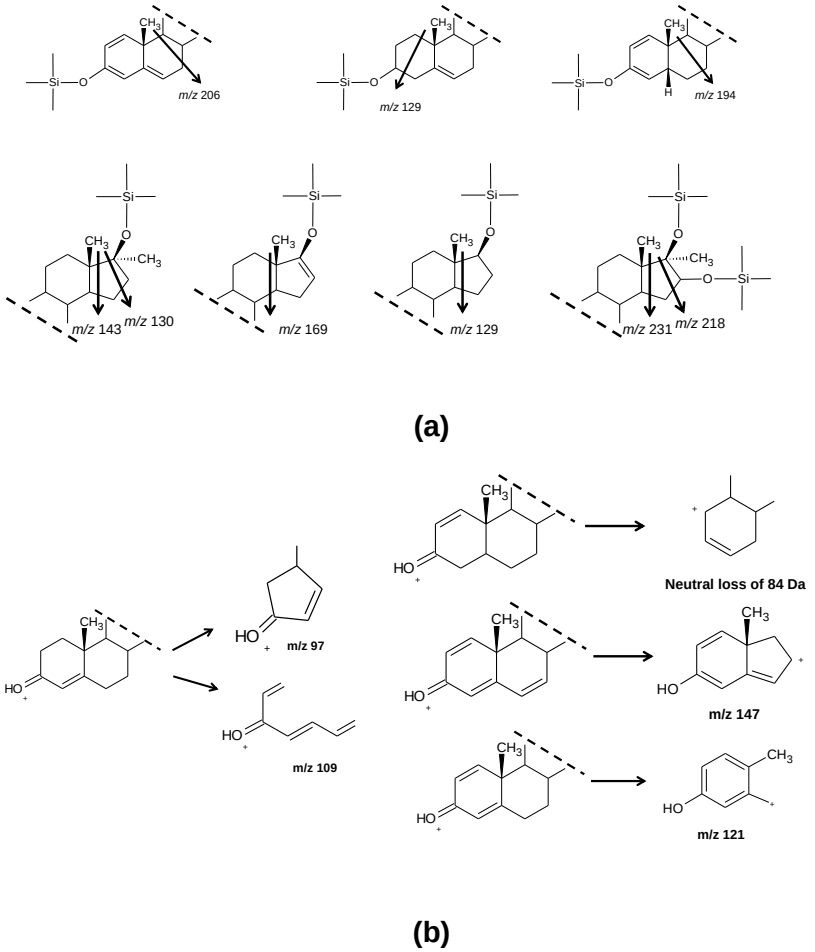
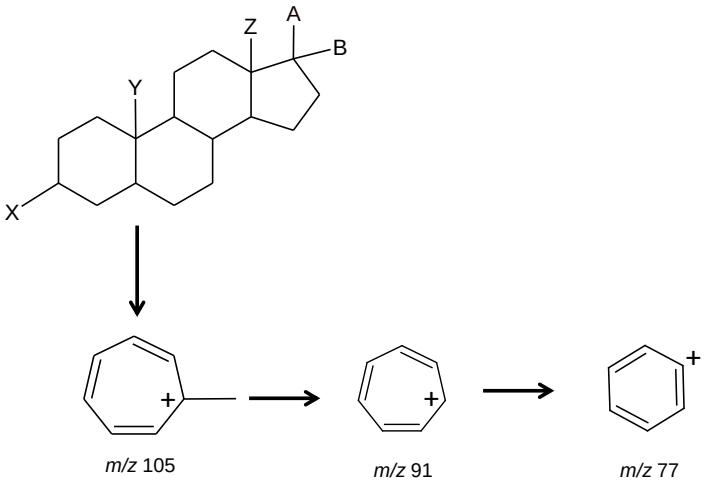


Figure 1.10. (a) Common MS fragmentation of enol-TMS derivatives of AAS after EI, **(c)** common MS CID of AAS after ESI ionization and **(d)** common ions for all steroids



(c)

Figure 1.10. (cont.) (a) Common MS fragmentation of enol-TMS derivatives of AAS after EI, **(b)** common MS CID of AAS after ESI ionization and **(c)** common ions for all steroids

1.5.4. HR based strategies

The use of different HR analyzers provides alternative strategies for the open detection of unknown metabolites. For instance, three main metabolites of boldione were detected using single quadrupole analyzers [121] whereas up to fifteen were detected by using strategies based on triple quadrupole analyzers [110].

Strategies based on accurate mass measurements normally compare scan chromatograms obtained for a negative sample and a positive sample. The positive samples may be a post-administration sample, an *in vitro* incubated sample or a real positive case. Since ionization depends on the structure, it is feasible to predict the main ion formed for each potential metabolite. Therefore, the *m/z* of the main ion formed can be extracted from the scan chromatogram with a narrow mass window increasing the sensitivity and the specificity of the detection. This

strategy has been used for the detection of many steroids in several matrices like equine, canine or human urine [122-124].

A promising strategy using accurate mass measurements is to compare the complete set of data obtained from negative and positive samples by means of statistical software. This process is based on preprocessing, alignment and multivariate statistics [125]. The main advantage of this approach is that every single peak is evaluated as a potential biomarker for the administration of the AAS. The main limitation for metabolic studies is that a potential biomarker is not necessarily associated with a metabolite.

1.5.5. Open detection based strategies

The high versatility of QqQ instrument allows for several scan modes including PI and NL scan (see section 1.4.2., scan modes). Due to the presence of common ions and fragments depending on the AAS structure, the development of methods based on these modes of operation provides alternative tools for the study of AAS metabolism. Thus, several methods have been proposed for the detection of AAS depending on the structure [97, 120] and their application to urine samples allowed for the discovery of unreported metabolites for exogenous AAS like stanozolol, fluoxymesterone, methandienone or methyltestosterone [99, 100, 126-128]. This approach shows a relative high sensitivity and specificity, which make the determination of differences between negative and positive samples relatively simple. However, part of the AAS structure is normally predefined (the one producing the selected ions or loss) and therefore, metabolites without that part will not be detected by these methods.

Although the search of new metabolites has been shown as a powerful technic for exogenous AAS, their applicability has not

been demonstrated for EAAS. Therefore, LC-MS/MS has several features that can be effectively exploited for the detection of new EAAS metabolites. Based on the common ions and neutral losses (Figure 1.10c) observed in the analysis of the AAS fragmentation, the development of methods based on these *modus operandi* provides an invaluable analytical tool for the study of EAAS metabolism.

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Justification and objectives

Chapter 2

2.1. Justification

The detection of the misuse of endogenous anabolic androgenic steroids (EAAS) is one of the most difficult analytical challenges in the doping control field. The main problem for their detection is to distinguish endogenous concentrations from the exogenous administration.

EAAS, as testosterone, are the most widely reported substances by doping control laboratories. The most informative and comprehensive tool to suspect about an EAAS doping offense is the determination of the steroid profile. Nowadays, the trend in doping control analysis is the establishment of the steroid profile in the so-called athlete biological passport. This approach consists in the determination of an individual threshold value for each athlete for the different compounds included in the steroid profile. A sample is suspicious when this athlete threshold value is exceeded. The use of new biomarkers can increase the capabilities of detection of the steroid profile.

In order to elucidate new biomarkers, metabolic studies are of outmost importance. Traditionally, these studies have been performed after hydrolysis of the phase II metabolites excreted in urine. For this purpose, an enzymatic hydrolysis of the phase II conjugate prior to a liquid-liquid extraction has been commonly used. Thereafter, the organic layer is separated for their subsequent analysis; meanwhile the aqueous phase is discarded. Since most of the metabolic studies used hydrolysis with β-glucuronidase enzymes, only metabolites conjugated with glucuronide acid or excreted as free compounds have been systematically studied for EEAS. The in-depth study of the

discarded fraction might reveal the presence of more diagnostic biomarkers for the screening of EAAS misuse.

The detection of most of the doping agents has been based for many years on the detection of metabolites elucidated by GC-MS methods. In the last decades, the irruptions of the LC-MS/MS have shown several new possibilities against the traditionally used GC-MS methods. Among them, the use of LC-MS/MS allows for the direct detection of phase II metabolites and the direct detection of polar compounds, avoiding hydrolysis and derivatization steps. In addition the use of QqQ allowed for the sensitive detection and structural information. Additionally, the use of QqQ instruments offers the possibility to apply methods for the open detection of compounds sharing a common structure. These methods make the QqQ a valuable tool for the detection and elucidation of metabolites.

2.2. Objectives

The main objective of this thesis is to evaluate the presence of new testosterone metabolites that can complement the current steroid profile and the evaluation of the usefulness of these new markers to detect the administration of T and other EAAS. For this purpose, the normally discarded aqueous fraction is explored by different LC-MS/MS methods.

In order to reach this goal, several specific objectives are proposed:

1. Investigation of the potential occurrence of T metabolites after alkaline treatment of the urine.

2. Elucidation and characterization of the structure of the detected metabolites.

3. Study of the metabolism responsible for the occurrence of these new metabolites in urine.

4. Development of a method for the quantification of these compounds in urine.

5. Determination of the population distribution.

6. Study of the factors that may influence the excretion of these new markers.

7. Evaluation of the usefulness of these new metabolites as markers for the detection of oral and gel testosterone administration.

8. Evaluation the usefulness of these new markers for the detection of other EEAS.

For a better understanding of these objectives, the thesis has been divided in three parts:

- In part I, the specifics objectives 1, 2 and 3 are addressed. The T metabolism was re-explored by focusing in the normally discarded fraction in routine methods. Based in previous results reported for exogenous AAS, alkaline treatment was selected as extraction method. Several new T metabolites released under these conditions are elucidated and characterized by LC-MS/MS approaches.

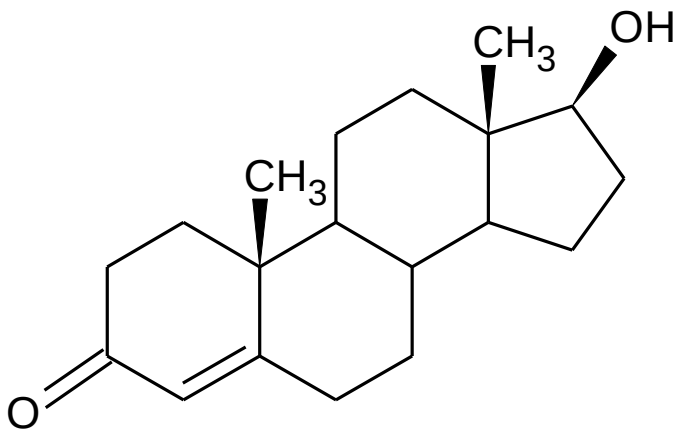
Thereafter, an in deep study of the metabolism responsible for the occurrence of these compounds in urine is performed.

- In part II, the specific objectives 4, 5 and 6 are addressed. A method for the quantitative determination of the compounds released after alkaline treatment is developed and validated. Using the developed method population studies are conducted by the analysis of 173 urine samples in order to obtain reference population values for the metabolites released in alkaline media. Additionally, the effect on these metabolites of different factors that commonly affect the steroid profile such sample preservation, endogenous factors and exogenous factors are evaluated.

- In part III, the specific objectives 7 and 8 are treated. The usefulness of the new compounds released after alkaline treatment of the urine for the detection of oral and gel testosterone as well as other EAAS administration are evaluated. The results are compared with those obtained using the actual markers of the steroid profile.

Re-exploring testosterone metabolism

Part I



The main objective of the first part of the thesis was to re-explore the testosterone metabolism looking for new unreported metabolites. For this purpose, the normally discarded urine fraction in routine analysis was analysed. Different LC-MS/MS modes were used for the detection and characterization of the potential metabolites.

In chapter three, the metabolism of testosterone was revisited. Four previously unreported metabolites were detected after the alkaline treatment of the urine and the application of a precursor ion scan mode by LC-MS/MS of the ions *m/z* 77, 91 and 105. The metabolites were characterized by studying their fragmentation pattern. 4,6-Androstadien-3,17-dione (Δ^6-AED), 1,4-androstadien-3,17-dione (Δ^1-AED), 4,6-androstadien-17β-ol-3-one (Δ^6-T) and 15-androsten-3,17-dione (Δ^{15}-AD) were proposed as feasible structures for these metabolites on the basis of the mass spectrometry data. The proposed structures were confirmed by analysis of synthetic reference compounds. Only Δ^{15}-AD was not confirmed, owing to the lack of a commercially available standard. Since, the metabolite Δ^6-AED has a structure analogue to that of the exogenous anabolic steroid boldenone, specific transitions for boldenone and its metabolite 5β-androst-1-en-17β-ol-3-one were also monitored. Both compounds were also detected after KOH treatment, suggesting that this metabolic pathway can be involved in the occurrence of endogenous boldenone reported by several authors.

In the chapter four, the origin of the occurrence of Δ^1-AED, Δ^6-AED, Δ^6-T and Δ^{15}-AD in urine was revealed. Although several experiments seem to indicate that these compounds are

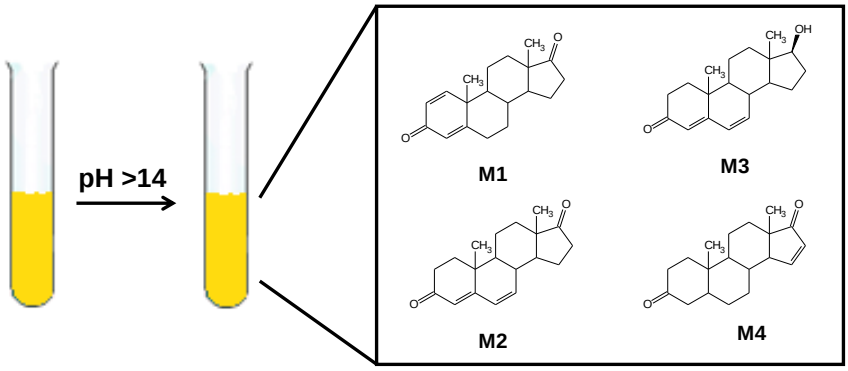
testosterone metabolites, their origin is still unknown. In this chapter, it was demonstrated that these metabolites are produced from the degradation of cysteine conjugates. Several testosterone metabolites conjugated with cysteine were synthesized and characterized by NMR techniques. Their detection in human urine was performed by LC–MS/MS. The acquisition of several transitions in SRM mode and the comparison between ion ratios and retention times allowed for the unequivocal confirmation of the presence of cysteine conjugates in urine. The analysis of urine samples collected after testosterone administration confirmed that synthesized cysteine conjugates are testosterone metabolites. The fact that these conjugates result in polyunsaturated compounds in urine after alkaline treatment were demonstrated by fraction collection and alkaline treatment of each fraction. Besides, the presences of these metabolites were also confirmed in human plasma. The formation of these metabolites would implies an unreported metabolic biotransformation: 6,7-dehydrogenation as phase I metabolism followed by conjugation with glutathione and subsequent transformation to cysteine conjugates. Finally, the existence of similar metabolites for cortisol and progesterone was also confirmed by LC–MS/MS indicating that the presented metabolic pathway is not exclusively active in androgens, but common to progestagens and glucocorticoids.

In the chapter five, the phase I metabolism responsible of the occurrence of this compounds was studied. In chapter 4, the formation of a ring double bond by a phase I metabolic transformation and the subsequent nucleophilic conjugation with glutathione was proposed as a putative metabolic pathway for the occurrence of Δ^1-AED, Δ^6-AED, Δ^6-T and Δ^{15}-AD in urine. The main

goal of this chapter is to confirm the first step of the postulated pathway. For that purpose, human hepatocyte cells systems were incubated with a pure T standard. The cell culture supernatants were analyzed by LC-MS/MS using a selected reaction monitoring method. Major T metabolites such as androsterone and 4-androstene-3,17-dione, together with the recently reported Δ^1 and Δ^6 metabolites were simultaneously quantified. The formation of Δ^1-AED, Δ^6-AED, Δ^6-T and Δ^1-T (boldenone) after incubation of T in hepatocyte cell cultures were demonstrated by comparing the retention times and the ion ratios of the metabolites with those obtained by analysis of commercial standards. Thus, the formation of double bonds Δ^1 and Δ^6 by hepatic phase I metabolism of T were confirmed.

Testosterone metabolism revisited

Chapter 3



This chapter has been adapted from:

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3.1. Introduction

Testosterone (T) is the principal androgenic anabolic steroid in humans. It is produced mainly in the testis and it is involved in the development of several tissues and processes [1].

T metabolism helps to balance the production rate to maintain the androgen concentrations at appropriate levels [1]. The main site of T metabolism is the liver (owing to the presence of steroid catabolic enzymes), although metabolism is also present in other tissues, such as the skin [2]. In-depth knowledge of T metabolites can play an essential role in several fields, such as doping control analysis, where the alteration of one of more of them can help in the detection of the misuse of T or related compounds.

T undergoes an extensive phase I metabolism. The main metabolism includes the 17-oxidation, A-ring reduction and 3-reduction. The combination of these metabolic pathways generates most of the known metabolites, e.g. androsterone, etiocholanolone, 5α-dihydrotestosterone, 5β-dihydrotestosterone, androstenedione, 5α-androstane-3α,17β-diol and 5β-androstane-3α,17β-diol [2-4]. Other minor metabolites involving different metabolic pathways such as hydroxylation in several sites and cyclation have been found in human urine [5-7].

In phase II metabolism, T and its metabolites are mainly conjugated with glucuronic acid [2, 8, 9]. Therefore, analytical methods for the detection of T and metabolites normally involve an enzymatic hydrolysis step [10-13]. In spite of being less abundant than glucuronidation, other phase II metabolic pathways are important for some T metabolites and other related compounds. Sulphonation is the most important pathway for 3β-hydroxy steroids (such as DHEA) and it is responsible for the presence of 3α-5cyclo-

5α-androstane-6β-ol-17-one in human urine [5]. Sulphonation of 17-alkylated steroids is also responsible of the presence of 18-nor-17,17-dimethyl steroid metabolites in urine after steroid administration [9, 14].

The characterization of minor steroid metabolites can be of great importance in the doping control field as their inclusion in screening methods may improve the detection of steroid misuse. Thus, the characterization of a minor metabolite of methandienone improved the time in which the misuse of this steroid could be detected [15]. Additionally, 6-ene-epimethyltestosterone was recently characterized as a long-term metabolite for the detection of methyltestosterone misuse [16]. Some metabolites containing also a double bond in position 6 have been characterized for other steroids such as methandienone [9, 17]. A 6-ene metabolite for T was also found after incubation experiments of rat live homogenates with T [18]. The occurrence of a 6,7-dehydro metabolite of methandienone in alkalinized (pH>12) urine was also described [9]. However, to our knowledge, no T metabolites have been described after alkaline treatment of the urine.

The goal of this study was to investigate the potential occurrence of urinary T metabolites released after alkaline treatment of urine. For this purpose, a previously reported strategy based on the integration of different mass spectrometric scan modes was applied [19].

3.2. Experimental

3.2.1. Chemicals and reagents

4,6-androstadien-3,17-dione and 4,6-androstadien-17β-ol-3-one were obtained from Steraloids (Newport, USA). 1,4-Androstadien-

3,17-dione and 1-androsten-3,17-dione were purchased from NMI (Pymble, Australia). Boldenone and boldenone metabolite (17β-hydroxy-5β-androsta-1-en-3-one) were provided by Steraloids (Newport, USA) and National Measurement Institute (Pymble, Australia), respectively. Methandienone, 4-androsten-3,17-dione, 5α-androsta-3,17-dione and 5β-androsta-3,17-dione were obtained from Sigma-Aldrich (St Louis, MO, USA)

The β-glucuronidase preparation (type Escherichia coli K12) was purchased from Roche Diagnostics (Mannheim, Germany). Analytical-grade potassium carbonate, potassium hydroxide pellets, disodium hydrogen phosphate, sodium hydrogen phosphate and tert-butyl methyl ether were obtained from Merck (Darmstadt, Germany). Pyridine, methoxyamine hydrochloride and trimethylsilylimidazole were purchased from Sigma-Aldrich (St Louis, MO, USA).

Acetonitrile and methanol (LC-gradient), formic acid (LC/MS grade) and cyclohexane were purchased from Merck (Darmstadt, Germany). Milli-Q water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain). Detectabuse® columns were obtained from Biochemical Diagnostics (Edgewood, NY, USA).

3.2.2. LC-MS/MS instrumentation

A precursor ion scan was performed with a triple quadrupole (6410 Triple Quad LC-MS) mass spectrometer equipped with an electrospray interface (Agilent, Palo Alto, CA, USA) interfaced to a 1200 binary pump (Agilent) for the chromatographic separation. The fragmentor was set to 125 V and the optimal collision energy for each ion was selected. The gas temperature was set at 350 °C and the capillary voltage at 4,000 V. A scan time of 0.2 s was used

to have enough points per peak without increasing the noise. The first quadrupole was selected to scan between 250 and 400.

The LC separation was performed using an Eclipse Plus C_{18} column (50 mm×2.1 mm inner diameter, 1.8 µm) (Agilent), at a flow rate of 300 µL/min. Water and methanol both with formic acid (0.01%) and ammonium formate (1 mM) were selected as mobile phase solvents. A gradient programme was used; the percentage of organic solvent was linearly changed as follows: 0 min, 30%; 1.5 min, 30%; 8 min, 45%; 15 min, 55%; 29.5 min, 95%; 30.5 min, 95%; 31 min, 30%, 34 min, 30%.

Selected reaction monitoring (SRM) was carried out using a triple quadrupole (Quattro Premier XE) mass spectrometer equipped with an orthogonal Z-spray electrospray interface (Waters Associates, Milford, MA, USA) interfaced to an Acquity (Waters Associates) UPLC system for the chromatographic separation. The drying gas as wellas the nebulizing gas was nitrogen. The desolvation gas flow rate was set to approximately 1,200 L/h and the cone gas flow rate was set to 50 L/h. A cone voltage of 25 V and capillary voltages of 3.0 kV were used in positive ionization mode. The nitrogen desolvation temperature was set to 450 ºC and the source temperature was set to 120 °C.

The LC separation for SRM purposes was performed using the same column, flow rates and mobile phases as for the precursor ion scan. A gradient programme was used; the percentage of organic solvent was linearly changed as follows: 0 min, 45%; 1 min, 45%; 3.5 min, 65%; 4.5 min, 95%; 5 min, 95%; 5.5 min, 45%; 8 min, 45%.

3.2.3. Gas chromatography (GC)-MS instrumentation

Gas chromatography (GC)–MS was carried out using a 6890N gas chromatograph coupled to a 5975 MSD (Agilent Technologies, Palo Alto, CA, USA). The steroids were separated on an HP-Ultra1 cross-linked methyl silicone column, 16.5 m×0.2 mm inner diameter, film thickness 0.11 µm (J&W Scientific, Folsom, CA, USA). Helium was used as the carrier gas at a constant pressure of 5 psi. A 2 µL aliquot of the final derivatized extract was injected into the system operated in splitless mode (valve opened at 2 min). The gas chromatograph temperature was ramped as follows: initially 50 °C, held for 3 min, increased to 180 °C at 30 °C/min, and thereafter increased to 250 °C at 3 °C/min. The injector and transfer lines were kept at 280 °C. The mass range scanned was from 100 to 600 amu.

3.2.4. Accurate mass measurements

- **Electrospray ionization accurate mass measurements (LC–quadrupole time of flight)**

Accurate mass experiments were carried out using a hybrid quadrupole time-of-flight (TOF) mass spectrometer (QTOF Premier) equipped with an orthogonal Z-spray electrospray interface (Waters) interfaced to an Acquity (Waters) UPLC system for the chromatographic separation. The drying gas as well as the nebulizing gas was nitrogen. The desolvation gas flow was set to approximately 600 L/h and the cone gas flow was set to 50 L/h. A cone voltage of 40 V and a capillary voltage of 3.0 kV were used in positive ionization mode. The nitrogen desolvation temperature was set at 350 °C and the source temperature was set to 120 °C. The TOF-MS resolution was approximately 10,000 (full width at half maximum) at *m/z* 556. MS and MS/MS spectra were acquired over

an *m/z* range of 50–1,000. For both MS and MS/MS, argon (99.995%) was used as the collision gas with a pressure of approximately 8×10-3 mbar in the collision cell. The microchannel plate detector potential was set to 1,700 V. A scan time of 0.2 s per spectrum was chosen.

Calibration of the *m/z* axis was performed using the built-in single syringe pump, directly connected to the interface. Calibration was conducted from *m/z* 50 to *m/z* 1,000 with a 1:1 mixture of 0.1 M sodium hydroxide and 10% formic acid diluted (1:25) with acetonitrile/water (80:20), at a flow rate of 10 µL/min. For automated accurate mass measurement, the lockspray probe was used, using as the lock mass a solution of leucine encephalin (2 µg/mL) in acetonitrile/water (50:50) pumped at 30 µL/min through the lockspray needle using a reagent delivery module from Waters. The protonated molecule of leucine enkephalin at *m/z* 556.2771 was used for recalibrating the mass axis and ensuring a robust accurate mass measurement with time.

- **Electron ionization accurate mass measurements (GC–TOF)**

An Agilent 6890N GC system was coupled to a GC-TOF mass spectrometer (Waters Corporation, Manchester, UK), operating in electron ionization mode. The GC separation was performed using a fused-silica HP-5MS capillary column of 30 m×0.25 mm inner diameter and film thickness of 0.25 µm (J&W Scientific, Folson, CA, USA). Helium was used as the carrier gas at constant flow rate of 1 mL/min. The initial oven temperature was 50 °C. After 3 min the temperature was increased at 30 °C/min to 180 °C, then at 3 °C/min to 300 °C. One microlitre of the derivative was injected in splitless mode (valve opened at 1 min). The injector was kept at

280 °C, and the interface and source temperatures were set to 250 °C. The TOF mass spectrometer was operated at an acquisition rate of 1 spectrum per second over the mass range *m/z* 50–650, using a multichannel plate voltage of 2,500 V. The TOF-MS resolution was approximately 7,000 (full width at half maximum). Heptacose (ion monitored *m/z* 218.9856) used for the daily mass calibration and as the lock mass, was injected via a syringe in the reference reservoir at 30 °C.

3.2.5. Sample preparation

Urine samples (5 mL) were passed through a Detectabuse® column, previously conditioned with 2 mL methanol and 2 mL water. The column was then washed with 2 mL water and finally the analytes were eluted with 2 mL methanol.

The methanolic eluate was evaporated under nitrogen at 50 °C and reconstituted in 1 mL sodium phosphate buffer (0.2 M, pH 7). After addition of 30 µL β-glucuronidase solution, the sample was hydrolysed for 1 h at 55 °C. After the hydrolysed sample had been cooled to room temperature, 250 µL of a 5% potassium carbonate solution was added to the sample (pH 9.5). Liquid–liquid extraction was performed twice by addition of 6 mL *tert*-butyl methyl ether. After centrifugation, 750 µL of the aqueous layer was separated.

After addition of the internal standard (methandienone 1 µg/mL, 50 µL), the sample was alkalinized by addition of 100 µL of 6 M KOH. Hydrolysis was performed for 15 min at 60 °C. After the mixture has been cooled to room temperature, a liquid–liquid extraction was performed by addition of 2 mL *tert*-butyl methyl ether. The sample was centrifuged and the organic layer was separated and evaporated.

For LC-MS/MS analysis, the residue was dissolved in 150 µL of a mixture of water and acetonitrile (50:50, v/v) and 10 µL was directly injected into the system.

For GC-MS, the residue was dissolved in 50 µL cyclohexane for analysis of the underivatized compound. For derivatization, 100 µL of a 2% (w/v) methoxyamine solution in pyridine was added to the residue. After the mixture had been heated for 1 h at 60 °C, pyridine was evaporated off, 100 µL trimethylsilylimidazole was added and the mixture was heated for 3 h at 80 °C. The non-volatile components were then removed by adding 1 mL cyclohenane and 3 mL water. After the vortex mixing and centrifugation, the organic layer containing the methoxime–trimethylsilyl derivatives was transferred to a new tube, concentrated to 50 µL and transferred to a microvial. One microlitre of this extract was injected onto the GC–TOF system in splitless mode (valve opened at 1 min).

3.2.6. Administration study samples

Six healthy male volunteers (age, 27.2±2.1 years; weight, 73.4±4.0 kg; height, 1.75±0.03 m; mean ± standard deviation) were given one oral dose of 120 mg T undecanoate (Androxon™, three 40 mg capsules; Organon).

Ethical approval for the study had been granted by Comité Ètic d'Investigació Clínica of our institute (CEICIMAS no. 94/467) and the Spanish Health Ministry (DGFPS no. 95/75). All of the subjects participating in the study gave their written informed consent. Urine samples were collected as described elsewhere [20]. Samples collected before T undecanoate administration and at 0–4 h after administration were used for this study. Aliquots of 50 mL urine were frozen at –20 °C until analysis.

3.3. Results and discussion

3.3.1. Precursor ion scan

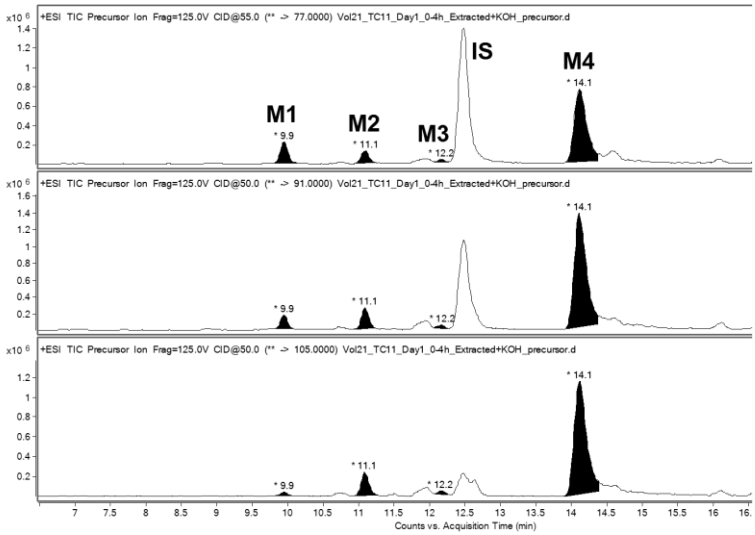
The first hydrolysis step performed in the sample preparation allowed for the deconjugation of glucuronides. After liquid–liquid extraction, glucuronides and free excreted steroids were removed from the sample. Therefore, these compounds were not present in the final extract, where only conjugates released after KOH treatment were present.

A precursor ion scan method based on the detection of the ions at *m/z* 77, 91, and 105 [21] was applied for the open detection of testosterone metabolites. The application of this method to urinary samples collected before and after intake of testosterone undecanoate showed the presence of four peaks, the intensities of which increased after testosterone undecanoate administration (Figure 3.1). These peaks (M1, M2, M3 and M4) could be associated with T metabolites.

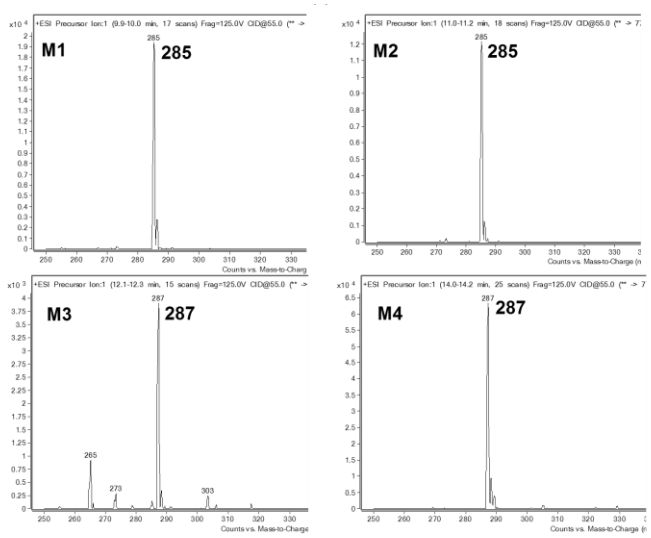
According to the data obtained by the precursor ion scan, M1 and M2 exhibit an $[M+H]^+$ at *m/z* 285, while M3 and M4 have an $[M+H]^+$ at *m/z* 287.

3.3.2. Full scan

Owing to the possible occurrence of species different from $[M+H]^+$ (e.g. $[M+H-nH_2O]^+$) in the ionization of steroids, an in-depth study of the adducts formed is necessary to obtain the molecular weight of an unknown steroid [19].



(a)



(b)

Figure 3.1. (a) Precursor ion scan chromatograms for the urine sample collected 4 h after testosterone undecanoate administration. **(b)** Spectra obtained for the peak corresponding to each metabolite.

Several species, including $[M+Na]^+$ and $[M+H-H_2O]^+$, were obtained for each metabolite (Table 3.1), confirming their molecular weights (284 for M1 and M2 and 286 for M3 and M4). Additionally, the accurate mass measurements allowed for the establishment of the molecular formula of the metabolites (mass differences lower than 2 mDa). M1 and M2 have a formula of $C_{19}H_{24}O_2$, corresponding to four hydrogen atoms fewer than T, whereas M3 and M4 have a formula of $C_{19}H_{26}O_2$, which corresponds to two hydrogen atoms fewer than the parent drug (Table 3.1).

Metabolite	Formula	specie	*m/z*	Formula	Mass difference (mDa)
M1	$C_{19}H_{24}O_2$	$[M+H-H_2O]^+$	267.1755	$C_{19}H_{23}O$	0.5
		$[M+H]^+$	285.1863	$C_{19}H_{25}O_2$	0.8
		$[M+Na]^+$	307.1683	$C_{19}H_{24}O_2Na$	0.9
M2	$C_{19}H_{24}O_2$	$[M+H-H_2O]^+$	267.1753	$C_{19}H_{23}O$	0.3
		$[M+H]^+$	285.1545	$C_{19}H_{25}O_2$	1.0
		$[M+Na]^+$	307.1681	$C_{19}H_{24}O_2Na$	0.7
M3	$C_{19}H_{26}O_2$	$[M+H-H_2O]^+$	269.1916	$C_{19}H_{25}O$	1.1
		$[M+H]^+$	287.2017	$C_{19}H_{27}O_2$	0.6
		$[M+Na]^+$	309.1840	$C_{19}H_{26}O_2Na$	0.9
M4	$C_{19}H_{26}O_2$	$[M+H-H_2O]^+$	269.1912	$C_{19}H_{25}O$	0.7
		$[M+H]^+$	287.2014	$C_{19}H_{27}O_2$	0.3
		$[M+Na]^+$	309.4841	$C_{19}H_{26}O_2Na$	1.0

Table 3.1. Accurate mass measurements for the ions obtained in liquid chromatography (LC)-time-of-flight (TOF) full-scan analysis for testosterone metabolites M1, M2, M3 and M4.

3.3.3. Product ion scan

The main product ions obtained for each metabolite are shown in Table 3.2. M1, M2 and M3 exhibited one neutral loss of water, whereas M4 showed two losses. Excluding the common losses of water, very different product ion spectra were obtained despite the similarity of the molecular formulas of the metabolites. This is in agreement with previous studies on the fragmentation of steroids

where it was shown that a small difference in the steroid structure can result in significantly different fragmentation pathways [22-24].

Metabolite	Formula	Product ion (*m/z*)	Formula	Error (mDa)
M1	$C_{19}H_{24}O_2$	267.1766	$C_{19}H_{23}O$	1.7
		151.1125	$C_{10}H_{15}O$	0.2
		147.1178	$C_{11}H_{15}$	0.4
		121.0654[a]	C_8H_9O	0.1
M2	$C_{19}H_{24}O_2$	267.1759	$C_{19}H_{23}O$	1.0
		227.1450	$C_{16}H_{19}O$	1.4
		149.0975[a]	$C_{10}H_{13}O$	0.9
		97.0662	C_6H_9O	0.9
M3	$C_{19}H_{26}O_2$	269.1900	$C_{19}H_{25}O$	0.5
		151.1133[a]	$C_{10}H_{15}O$	1.0
		133.1032	$C_{10}H_{13}$	1.5
		97.0664	C_6H_9O	1.1
M4	$C_{19}H_{26}O_2$	269.1913	$C_{19}H_{25}O$	0.8
		251.1813	$C_{19}H_{23}$	1.3
		123.0809	$C_8H_{11}O$	0.1
		95.0858[a]	C_7H_{11}	0.3

[a] Most abundant product ion

Table 3.2. Abundant product ions obtained in LC-quadrupole TOF analysis for the fragmentation of testosterone metabolites M1, M2, M3 and M4.

3.3.4. Proposal of structures for the metabolites

To make a proposal for the structures of the metabolites, previously published information about collision induced dissociation of steroids was evaluated [22–24]. For this purpose, two zones of the spectra were studied: (1) the product ions with *m/z* between the $[M+H]^+$ and 100 Da lower than the $[M+H]^+$ obtained at 20 eV collision energy and (2) the most abundant ions at 30 eV [24].

- **M1**

M1 has two oxygen atoms and its product ion spectrum at 20 eV exhibited one neutral loss of water. That is common for these

anabolic steroids containing a highly conjugated keto function [24]. Additionally, the most abundant product ion was observed at *m/z* 121.0654 (Table 3.2). This ion is characteristic for 1,4-diene-3-keto steroids [22-24]. On the basis of these parameters and the absence of other abundant ions, 1,4-androstadiene-3,17-dione was suggested as the structure for M1. The sporadic occurrence of this compound in human urine was recently reported [25].

- **M2**

Similarly to M1, the product ion scan of M2 at low collision energy exhibited only a loss of water at 20 eV, indicating the conjugation of the keto moiety in the structure. The most abundant product ion at 30 eV collision energy was at *m/z* 149.0975, corresponding to the formula $C_{10}H_{13}O$. This ion can be related to a fragment containing C and D rings, which is common for those steroids having double bonds in both A and B rings [24].
Besides, metabolism including the formation of a double bond in position 6 has been proposed [15-17]. Therefore, 4,6-androstadiene-3,17-dione was suggested as the structure for M2.

- **M3**

Similar to M2, the product ion spectrum of M3 showed one loss of water at 20 eV and ions corresponding to C and D rings (*m/z* 151.1133 and 133.1032) at 30 eV. This could be due to the occurrence of a double bond in position 6. Thus, 17-hydroxy-4,6-androstadiene-3-one could be established as a feasible structure for M3. The orientation (α and β) of the hydroxyl group in the 17 position could not be established as stereoisomers produce similar product ion spectra [24].

- **M4**

Two losses of water were observed in the collision-induced dissociation spectrum of M4 at 20 eV, suggesting the absence of a large conjugation in the keto group [23]. The most abundant ions at 30 eV corresponded to fragments containing only one ring (*m/z* 123.0809 and 95.0858). This could indicate the presence of double bonds in only one ring. Additionally, product ions common for specific positions such as 1, 4 and 5 were not present in the spectrum.

To have more data on this metabolite, the GC-TOF spectrum after formation of the methoxime-trimethylsilyl derivative was acquired (Figure 3.2b). The molecular ion at *m/z* 344.2522 ($C_{21}H_{32}N_2O_2$, 3.8 mDa error) showed the presence of two keto functions. Therefore, the M4 structure contains two keto groups (possibly at C3 and C17 because it is a T metabolite) and an additional double bond.

Owing to the absence of hydroxyl functions in M4 and its high response, it could also be analysed by GC-MS without derivatization (Figure 3.2c). The results were compared with those obtained for commercially available steroids containing two keto groups at C3 and C17 (1-androsten-3,17-dione, 4-androsten-3,17-dione, 5α-androstan-3,17-dione and 5β-androstan-3,17-dione) and the spectra obtained with accurate mass measurements were evaluated. 1-Androsten-3,17-dione and 4-androsten-3,17-dione had a loss of 42 Da (42.0106 Da using GC-TOF), coming from the fragmentation of the A ring [22]. M4 did not exhibit this loss (Figure 3.2c and supplementary information S-3.1), suggesting that the double bond is not located in the A ring. Additionally, the spectra for all four reference standards evaluated exhibited a loss of 44 Da (44.0262 Da by GC-TOF), corresponding to a loss of CH_2CHOH coming from the D ring [22]. The GC-TOF spectrum of M4 did not

show any ion at *m/z* 242.1671, suggesting a change in the D ring. Similarly to metabolites M1, M2 and M3, the generation of a conjugated double bond seems to be the most feasible alternative in the formation of M4. Therefore, 15-androstene-3,17-dione was proposed as the structure for M4.

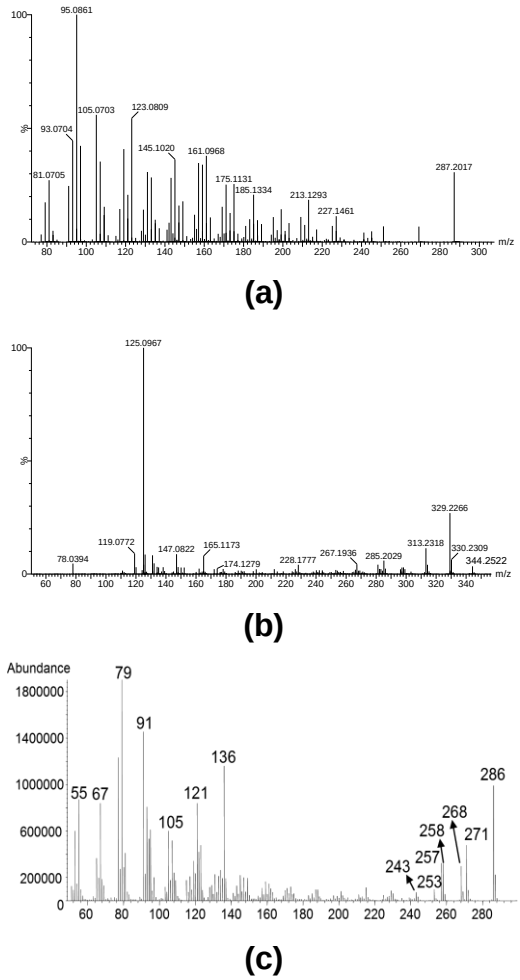


Figure 3.2. Mass spectra for M4: **(a)** liquid chromatography-quadrupole time of flight analysis, **(b)** gas chromatography (GC)-time of flight analysis of the methoxime-trimethylsilyl derivative and **(c)** GC-MS of the underivatizated compound

3.3.5. Comparison with reference material

The comparison between the metabolite and the reference material is necessary for the final confirmation of the metabolite structure [26]. Three of the proposed compounds (1,4-androstadiene-3,17-dione, 4,6-androstadiene-3,17-dione and 17β-hydroxy-4,6-androstadiene-3-one) were commercially available. Therefore, this comparison was feasible for M1, M2 and M3.

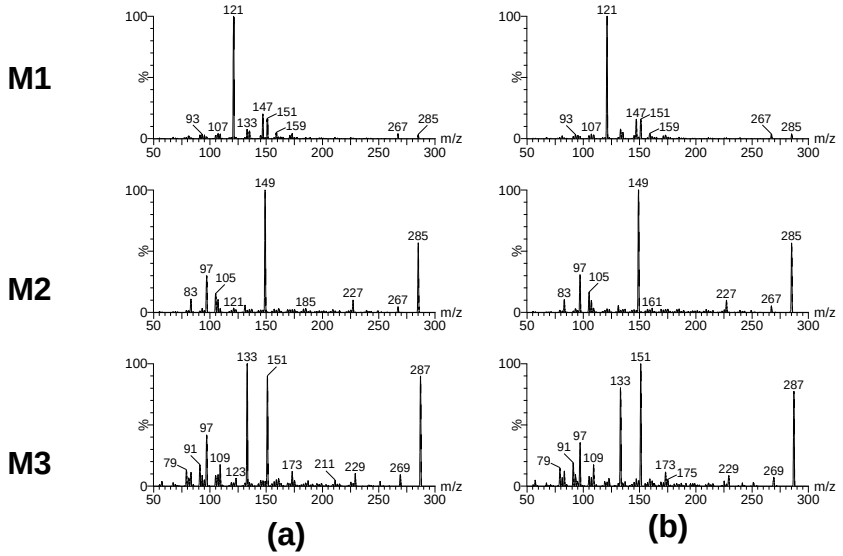


Figure 3.3. Product ion scans at 20 eV for **(a)** reference material 1,4-androstendiene-3,17-dione, 4,6-androstendiene-3,17-dione and 17β-hydroxy-4,6-androstadiene-3-one and **(b)** M1, M2 and M3 in a post-administration samples

The product ion spectra and the retention times of these metabolites were identical to those obtained for the reference material (Figure 3.3), confirming the proposed structures for M1, M2 and M3. The comparison with reference material also allowed for the confirmation of the 17-hydroxyl group configuration in M3. The commercially available 17β-hydroxy-4,6-androstadiene-3-one

shared with M3 both the retention time and the product ion spectrum. Since the separation of 17α-hydroxyl and 17β-hydroxyl steroids is not difficult by LC and the product ion spectra of both analogues present different relative abundances, it can be concluded that the 17-hydroxyl of M3 has a β orientation.
Unfortunately, 15-androstene-3,17-dione was not commercially available and, therefore, the synthesis of this substance seems to be necessary to confirm the real structure of M4.

3.3.6. Application to post-administration urine samples

After confirmation of the metabolite structures, an SRM method was developed for the qualitative detection of these compounds in urine samples. The optimized parameters for each metabolite are shown in Table 3.3 Two transitions were selected for each metabolite.

	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)
1,4-androstadiene-3,17-dione (M1)	285	121	20
		147	15
4,6-androstadiene-3,17-dione (M2)	285	149	25
		97	35
17-hydroxy-4,6-androstadiene-3-one (M3)	287	151	20
		133	25
15-androstene-3,17-dione (M4)[a]	287	95	25
		123	20
boldenone	287	121	30
		135	20
boldenone metabolite	289	187	20
		205	20

[a] Proposed structure

Table 3.3. Selected reaction monitoring method for the detection of testosterone metabolites

The occurrence of naturally produced boldenone has been discussed during the last few years [9, 25, 27-30] and recently the natural origin of this steroid in some human urine samples was proved [31]. However, the metabolic mechanism which generates this steroid still remains unclear. Owing to the similarity between M1 (1,4-androstadiene-3,17-dione) and boldenone (17-hydroxy-1,4-androstadiene-3-one), the presence of boldenone as a T metabolite released after alkaline treatment cannot be discarded. For this reason, monitoring of selected transitions for the detection of boldenone and boldenone metabolite (17β-hydroxy-5β-androst-1-en-3-one) was added to the SRM method (Table 3.3).

The SRM method was applied in the analysis of samples collected before and after T undecanoate administration. The results showed the occurrence of M1, M2, M3 and M4 in all samples collected before administration (Figure 3.4a). The response obtained after T undecanoate administration increased by a factor of around 20 for M2 and M3 and more than 100 times for M1 and M4. A similar increase for all metabolites was obtained in the analysis of the samples collected from the six volunteers. These results confirmed the metabolic nature of all four compounds (Figure 3.4).

In the analysis of preadministration samples, boldenone and boldenone metabolite were normally not detected, although in some cases a peak close to the limit of detection of the method was found (Figure 3.4a). However both peaks appeared in the samples after administration of T undecanoate, although their intensities were clearly lower than for the other metabolites (Figure 3.4b). These results seemed to indicate that boldenone is formed after hydrolysis of a phase II T metabolite.

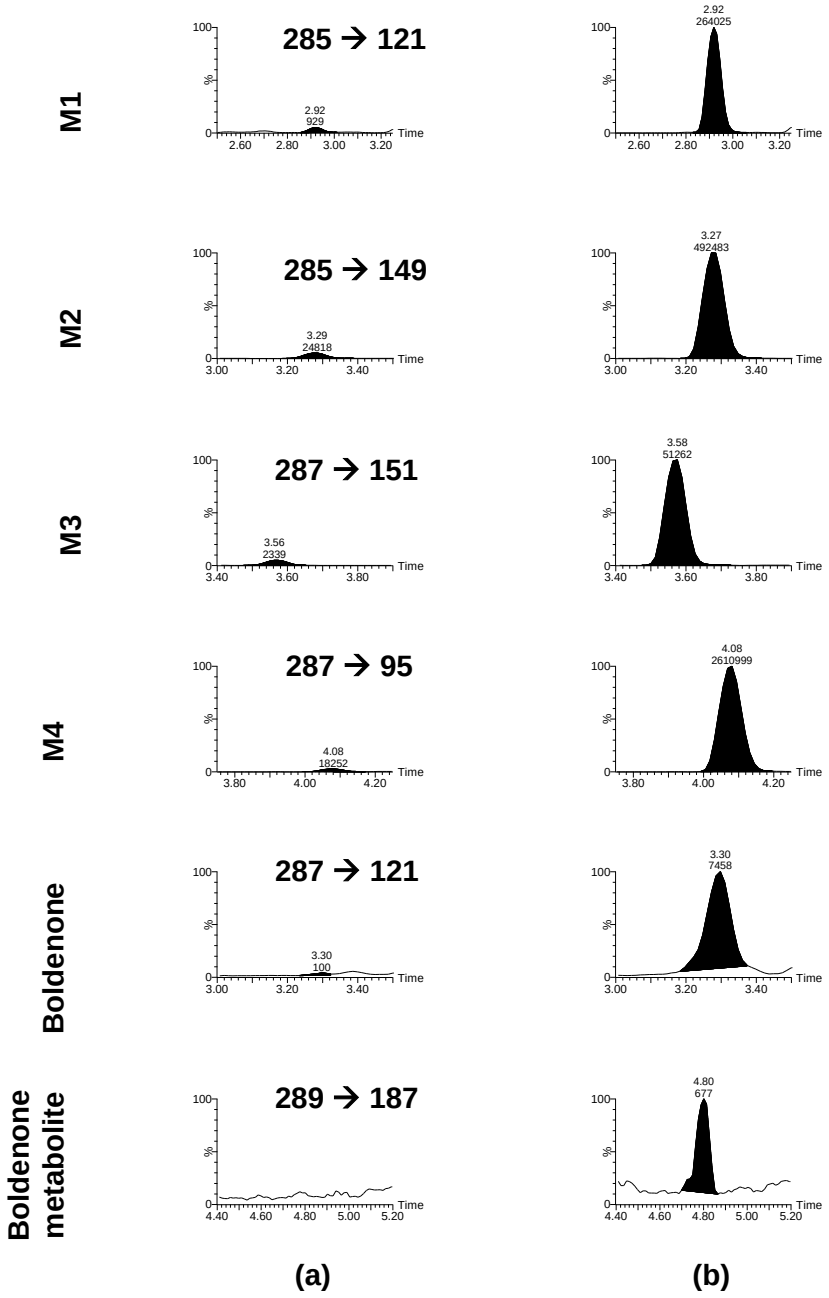


Figure 3.4. SRM chromatograms for urine sample collected (a) before and (b) after testosterone undecanoate administration

The possibility that the occurrence of these metabolites was due to a reaction in alkaline conditions of one component of the urine was also evaluated. For this purpose, a urinary extract taken before the alkaline hydrolysis was injected into the C_{18} LC column using a slow gradient (the methanolic solvent changed linearly from 10 to 70% in 30 min). Fractions were collected every 30 s and alkaline hydrolysis was performed for each fraction. The fractions were then analysed by the qualitative method. Every analyte was obtained in a different fraction. Since the matrix components should differ from one fraction to another, this indicated that the metabolites were obtained independently of the matrix components and therefore the reaction between the steroid and some matrix components could be discarded as the source of these metabolites.

3.4. Conclusion

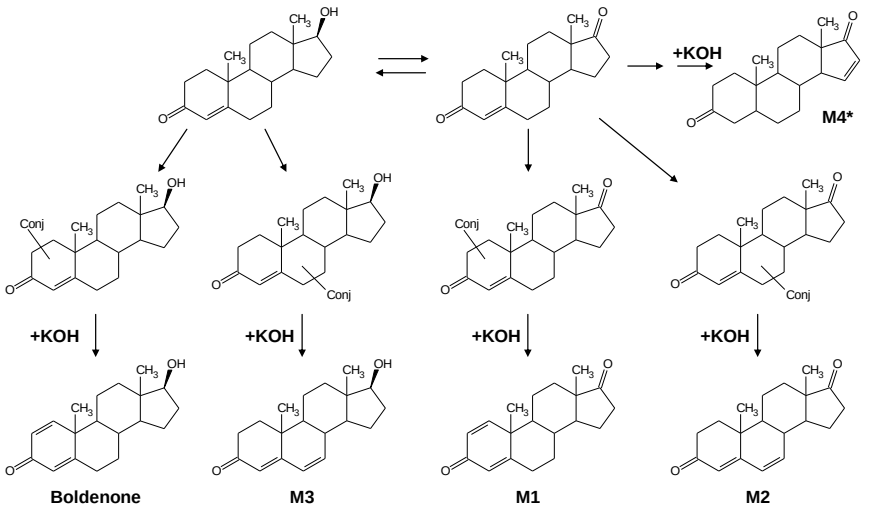
Four testosterone metabolites have been characterized. Owing to their commercial availability, the structures of three of them (1,4-androstadiene-3,17-dione, 4,6-androstadiene-3,17-dione and 17-hydroxy-4,6-androstadiene-3-one) could be confirmed by comparison with the reference material. The synthesis of 15-androstene-3,17-dione seems to be necessary to corroborate that this is the real structure of M4.

All four metabolites are present in urine after alkaline treatment. The analysis of different fractions collected from the urine indicated that these compounds are not produced by reaction of urinary components. Thus, the most feasible explanation is the presence of an alkali-labile conjugate possibly at C1 or C2 for M1 and at C6 or C7 for M2 and M3 (Figure. 3.5). Although similar behaviour has been observed for some exogenous steroids [9], to our knowledge,

the presence of these compounds in urine after alkaline treatment has not been reported yet for T. The elucidation of the cause of the occurrence of these metabolites could help in the understanding of T metabolism. Besides, if the cause is the presence of an unknown conjugate, its direct detection by LC-MS/MS could be a promising option for the detection of T misuse.

Boldenone and boldenone metabolite are also present after alkaline treatment of T in postadministration urine samples. Therefore, the elucidation of the cause of this occurrence would be valuable in the understanding of the presence of endogenous boldenone detected in several samples.

As T metabolites, the inclusion of these compounds in the steroid profile could help in the detection of T misuse and body disposition.



* Proposed structure.

Figure 3.5. Proposed testosterone metabolism involving the metabolites described in this study

3.5. Reference

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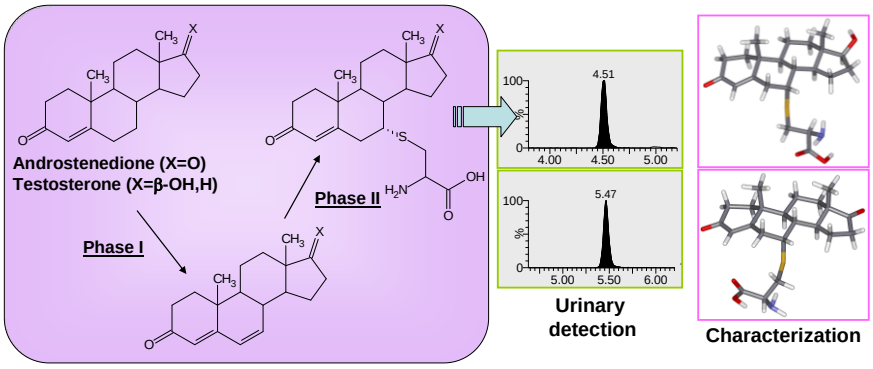
Steroid metabolites conjugated with cysteine

Chapter 4



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4.1. Introduction

Steroid hormones are lipophilic, low molecular weight compounds that play a number of important physiological roles [1]. According to their structure and activity, steroid hormones are divided in five major groups: androgens, estrogens, glucocorticoids, mineralocorticoids, and progestagens. They are synthesized mostly by endocrine glands such as the gonads, the adrenals, and during gestation by the fetoplacental unit. After being released into the bloodstream, they act on both peripheral target tissues and the central nervous system. Despite their relatively simple chemical structure, due to an extensive phase I and phase II metabolism, steroids occur in a wide variety of biologically active forms [2].

The advances in the knowledge of steroid metabolism have been essential in several fields. For example, the occurrence of a metabolic imbalance or the presence of specific steroid metabolites has been described as biomarkers for many pathological conditions [3], or as a proof of hormone abuse [4]. Steroid hormone metabolites (both phase I and phase II) play important roles in many cellular processes through both genomic and non-genomic mechanisms [5, 6]. In addition to that, the link between steroids and the development and proliferation of several types of cancer is well documented [7, 8].

The major phase I metabolic transformations include reductions and oxidations [2]. Hormonal steroids with a 3-oxo-4-ene group are primarily metabolized to 3α-hydroxy-5βH and 3α-hydroxy-5αH [9]. In phase II metabolism, steroid hormones are mainly excreted as glucuronide or sulfate conjugates [10, 11]. Other phase II conjugates have also been described, such as disulphates, diglucuronides or conjugates with N-acetylglucosamine [12-14].

Analytical strategies for the detection of steroid metabolites are normally based on hydrolysis of the phase II metabolites, derivatization and detection by gas chromatography coupled to mass spectrometry (GC–MS) [15]. Liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) complements GC–MS results since hydrolysis and derivatization steps can be circumvented [16]. Thus, the use of LC–MS/MS allowed for the detection of previously unreported steroid metabolites [17-20] and for the direct detection of phase II metabolites [16, 20-22]. Additionally, the advent of LC–MS/MS opened new alternatives for the detection of steroid metabolites like the use of analytical strategies based on precursor ion scan [23-25] or neutral loss scan [25, 26].

In this thesis, the use of a precursor ion scan method allowed the detection of some androgens, including Δ^6-AED and Δ^6-T, after alkaline treatment of human urine samples (see chapter 3). Their unequivocal identification was performed by MS and comparison with reference material. Metabolites released after alkaline treatment of the urine were also reported for some exogenous steroids [4, 27]. Experimental results indicated that they are not excreted as classical phase II metabolites (glucuronides or sulfates) and the origin of these compounds is still unknown (see chapter 3). One of the feasible hypotheses is the existence of an additional phase II metabolism responsible for the release of these metabolites in alkaline media.

Chemical structures of Δ^6-AED and Δ^6-T share a polyunsaturated carbonyl group which is an ideal Michael's acceptor. The mercapturic acid pathway is one of the most studied xenobiotic conjugation reactions. The nucleophilic attack of the thiol coming from the glutathione on an electrophilic center results in the

formation of a more stable compound and prevents the covalent attachment of the electrophilic to nucleophiles on critical macromolecular targets. The pathway is initiated by the formation of a thioether of glutathione inside the cell and culminates in the biosynthesis of N-acetylcysteine conjugates through cysteine conjugates [28]. Therefore, we considered the nucleophilic species involved in the mercapturic pathway as suitable candidates for the conjugation reaction.

The goal of this study was to determine if the occurrence of urinary testosterone metabolites after basic treatment of the urine is related with the presence of cysteine or N-acetylcysteine conjugates. For this purpose, metabolites were synthesized and characterized by NMR and MS techniques. The qualitative presence of these metabolites in urine was confirmed by LC–MS/MS in SRM mode. Additionally, the suitability of direct and indirect approaches for the urinary detection of these metabolites has been evaluated. Finally, the potential occurrence of this metabolic pathway to other steroid hormones like progesterone and cortisol was also verified.

4.2. Experimental

4.2.1 Chemical and reagents

Δ^6-AED, Δ^6-T, 4,6-pregnadiene-11β,17,21-triol-3,20-dione (Δ^6-Cort) and 4,6-pregnadien-3,20-dione (Δ^6-Prog) were obtained from Steraloids Inc. (Newport, USA). Cysteine (Cys), N-acetylcysteine (NAC), trifluoroacetic acid (TFA) and all other chemicals were purchased from Sigma–Aldrich Química S.A. (Madrid, Spain). Unless otherwise stated, all the chemicals were used without further purification.

Acetonitrile (ACN) and methanol (MeOH) (LC gradient grade), formic acid, ammonium formate and sodium sulfate (LC/MS grade) were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain). The Sep-Pak® Vac RC (500 mg) C_{18} cartridges were purchased from Waters (Milford, Massachusetts, USA). L-Cysteine was purchased from Sigma–Aldrich Química S.A. (Madrid, Spain). *Tert*-butylmethyl ether (TBME) and potassium hydroxide were obtained from Merck (Darmstadt, Germany).

4.2.2. General procedure for the synthesis, purification and characterization of cystein-S-yl- and N-acetylcystein-S-yl-adducts of the steroids

The general scheme of the synthesis is shown in Figure 4.1a. Each steroid (1 equiv) is dissolved in 3 volumes of MeOH and is added to 1 volume of cysteine or N-acetylcysteine (100 equiv) dissolved in 6 M KOH. The mixture is left under vigorous stirring for 2 h. After that, the organic solvent is removed under vacuum at 27 ºC and the remaining aqueous solution is neutralized with 6 M HCl, filtered and purified.

Product purification was performed by semi-preparative HPLC with a Waters Prep LC 4000 system using an Atlantis® dC_{18} OBD™ column of 5 µm and 19 x 150 mm. The neutralized mixture was injected to the HPLC system (A: H_2O/0.1% TFA and B: 50% ACN/50% H_2O/0.1% TFA with a gradient of 0% B for 10 min, 0% B to 100% B in 100 min). In the case of the N-acetylcystein-S-yl-adducts, a second HPLC purification is needed (A: H_2O and B: 50% ACN/50% H_2O with a gradient of 0% B to 90% B in 45 min).

4.2.3. NMR analysis

NMR spectra were acquired on a Varian (Palo Alto, CA) Mercury 400 MHz spectrometer equipped with an OneNMR with the ProTune system probe. Samples were dissolved in 700 µL of methanol-d4 and placed in a standard 5 mm NMR tube. NMR chemical shifts are reported in ppm downfield relative to the internal solvent peak, and *J* values are in Hz. Proton and carbon assignments were made using a combination of 1D-^{1}H and ^{13}C spectra and 2D DQCOSY, gNOESY, gHSQC, and gHMBC experiments.

4.2.4. Synthetic procedure

- **7α-Cystein-*S*-yl-4-androsten-17β-ol-3-one (Δ^6-T-Cys).**

Following the general procedure, 50 mg (0.17 mmol) of Δ^6-T dissolved in 15 mL of MeOH were added to a solution of cysteine (2.1 g, 17.35 mmol) dissolved in 5 mL of 6 M KOH. After HPLC purification (70 min r. t.) 32 mg (0.065 mmol, 37% yield) of white solid were obtained as the trifluoroacetate (TFA) salt. ^{1}H NMR (400 MHz, CD_3OD) δ 5.78 (s, 1H, H4), 4.18 (dd, *J* = 6.9, 4.2 Hz, 1H, H21), 3.59 (t, *J* = 8.6 Hz, 1H, H19), 3.24 (q, *J* = 3.35 Hz, 1H, H7), 3.15 (dd, *J* = 14.0, 4.2 Hz, 1H, H20), 3.06 (dd, *J* = 14.0, 7.0 Hz, 1H, H20'), 2.90 (ddd, *J* = 14.8, 3.7, 1.7 Hz, 1H, H6β), 2.62 (dd, *J* = 14.8, 2.6, 2.6 Hz, 1H, H6α), 2.51 (ddd, *J* = 17.0, 15.0, 5.1 Hz, 1H, H2β), 2.30 (dt, *J* = 17.0, 3.1 Hz, 1H, H2α), 2.11 (m, 1H, H1β), 2.08 (m, 1H, H8β), 2.04 (m, 1H, H16α), 1.86 (dt, *J* = 12.6, 3.2, 3.2 Hz, 1H, H12β), 1.74 (m, 1H, H15α), 1.70 (m, 1H, H1α), 1.63 (m, 1H, H11α), 1.53 (m, 1H, H16β), 1.52 (m, 1H, H11β), 1.46 (m, 1H, H14α), 1.37 (m, 1H, H15β), 1.31 (m, 1H, H9α), 1.28 (s, 3H, Me19), 1.04 (td, *J* = 12.7, 12.7, 4.2 Hz, 1H, H12α), 0.81 (s, 3H, Me18).^{13}C

NMR (101 MHz, CD_3OD) δ 201.78 (C3), 170.88 (C5), 170.28 (C22), 127.25 (C4), 82.13 (C17), 54.03 (C21), 49.28 (C7), 49.11 (C9), 47.92 (C14), 43.94 (C13), 40.85 (C8), 39.95 (C10), 39.61 (C6), 37.24 (C12), 36.66 (C1), 34.74 (C2), 32.52 (C20), 30.44 (C16), 23.73 (C15), 21.72 (C11), 18.11 (C19), 11.56 (C18). The complete set of NMR experiments can be seen in supplementary information (see http://dx.doi.org/10.1016/j.steroids.2012.11.017).

- **7α-*N*-acetylcystein-*S*-yl-4-androsten-17β-ol-3-one (Δ^6-T-NAC).**

The reaction was carried out with 30 mg (0.10 mmol) of Δ^6-T dissolved in 9 mL of MeOH and 1.7 g (10,42 mmol) of *N*-acetylcysteine dissolved in 3 mL of 6M KOH. After HPLC purification (82 min r. t. for the first purification and 36 min r.t. for the second one) 22.1 mg (0.05 mmol, 50% yield) of white solid were obtained. ^{1}H NMR (400 MHzCD_3OD) δ 5.78 (s, 1H, H4), 4.54 (dd, *J* = 8.9, 4.5 Hz, 1H, H21), 3.59 (t, *J* = 8.6 Hz, 1H, H17), 3.17 (q, *J* = 3.1 Hz, 1H, H7), 3.05 (dd, *J* = 13.6, 4.5 Hz, 1H, H20), 2.86 (ddd, *J* = 14.7, 3.7, 1.7 Hz, 1H, H6β), 2.79 (dd, *J* = 13.6, 8.9 Hz, 1H, H20'), 2.63 (dd, *J* = 14.8, 2.6 Hz, 1H, H6α), 2.50 (ddd, *J* = 17.0, 15.0, 5.1 Hz, 1H, H2β), 2.30 (dt, *J* = 17.0, 3.1, 3.1 Hz, 1H, H2α), 2.10 (m, 1H, H1β), 2.05 (m, 1H, H8β), 2.02 (m, 1H, H16α), 2.00 (s, 3H, Me24), 1.84 (dt, *J* = 12.6, 3.0, 3.0 Hz, 1H, H12β), 1.74 (m, 1H, H15α), 1.72 (m, 1H, H1α), 1.62 (m, 1H, H11α), 1.51 (m, 1H, H16β), 1.50 (m, 1H, H11β), 1.41 (m, 1H, H14α), 1.34 (m, 2H, H15β, H9α), 1.27 (s, 3H, Me19), 1.03 (td, *J* = 12.8, 12.7, 4.2 Hz, 1H), 0.80 (s, 3H, Me18). ^{13}C NMR (101 MHz,CD_3OD) δ 201.79 (C3), 173.82 (C22), 173.29 (C23), 171.57 (C5), 127.11 (C4), 82.20 (C17), 53.47 (C21), 49.00 (C9), 47.93 (C14), 47.34 (C7), 43.89 (C13), 40.73 (C8), 39.94 (C10), 39.22 (C6), 37.35 (C12), 36.67 (C1), 34.76 (C2),

33.09 (C20), 30.48 (C16), 23.52 (C15), 22.43 (C24), 21.79 (C11), 18.16 (C19), 11.57 (C18). The complete set of NMR experiments can be seen in supplementary information (see http://dx.doi.org/10.1016/j.steroids.2012.11.017).

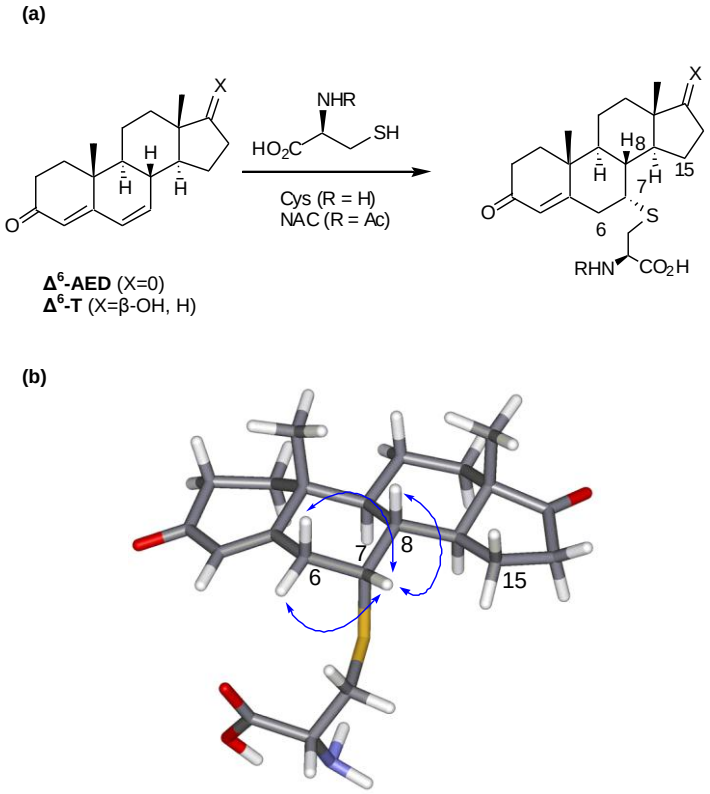

Figure 4.1. (a) Conjugated addition of cysteine and *N*-acetylcysteine to the discussed steroids. **(b)** Three-dimensional structure of Δ^6-AED -Cys, selected nOes are shown with double headed arrows.

- **7α-Cystein-*S*-yl-4-androsten-3,17-dione (Δ^6-AED -Cys)**.

20 mg (0.07 mmol) of Δ^6-AED dissolved in 6 mL of MeOH and 852 mg (7.03 mmol) of cysteine dissolved in 2 mL of 6M KOH were used. After purification by HPLC (63 min r. t.) 13.7 mg of white solid (0.03mmol, 40% yield) were obtained as a TFA salt. ^{1}H NMR (500 MHz,CD_3OD) δ = 5.80 (br s, 1H, H4), 4.22 (dd, *J* = 6.9, 4.2 Hz, 1H,

H21), 3.38 (q, *J* = 3.1 Hz, 1H, H7), 3.19 (dd, *J* = 14.0, 4.2 Hz, 1H, H20), 3.11 (dd, *J* = 14.0, 7.0 Hz, 1H, H20'), 2.95 (ddd, *J* = 14.9, 3.9, 1.8 Hz, 1H, H6β), 2.67 (dd, *J* = 15.0, 2.7 Hz, 1H, H6α), 2.51 (m, 1H, H2β), 2.50 (m, 1H, H16α), 2.31 (dt, *J* = 17.0, 3.2, 3.2 Hz, 1H, H2α), 2.29 (td, *J* = 11.2, 11.2, 3.5 Hz, 1H, H8β), 2.15 (m, 1H, H1β), 2.11 (m, 1H, H16β), 2.08 (m, 1H, H15α), 1.79 (m, 1H, H12β), 1.78 (m, 1H, H14α), 1.71 (m, 1H, H11α), 1.70 (m, 1H, H1α), 1.69 (m, 1H, H15β), 1.55 (dq, *J* = 13.2, 13.2, 13.2, 4.2, 1H, H11β), 1.37 (td, *J* = 11.9, 11.9, 4.4 Hz, 1H, H9α), 1.30 (s, 3H, Me19), 1.23 (td, *J* = 13.1, 13.1, 4.2 Hz, 1H, H12α), 0.96 (s, 3H, Me18). ^{13}C NMR (101 MHz, CD_3OD) δ 222.65 (C17), 201.63 (C3), 171.21 (C22), 170.41 (C5), 127.47 (C4), 54.82 (C21), 49.03 (C9), 48.74 (C13), 48.35 (C14), 48.06 (C7), 40.22 (C8), 39.98 (C10), 39.44 (C6), 36.63 (C1), 36.37 (C16), 34.74 (C2), 32.84 (C20), 32.15 (C12), 22.30 (C15), 21.36 (C11), 18.06 (C19), 14.04 (C18). The complete set of NMR experiments can be seen in supplementary information (see http://dx.doi.org/10.1016/j.steroids.2012.11.017)

- **7α-*N*-acetylcystein-*S*-yl-4-androsten-3,17-dione (Δ^6-AED-NAC).**

As above, 10 mg (0.035 mmol) of Δ^6-AED dissolved in 3 mL of MeOH and 570 mg (3.49 mmol) of *N*-acetylcysteine dissolved in 1 mL of 6M KOH were used. After HPLC purification (75min r. t. for the first purification and 37 min r.t. for the second one) 7.4 mg (0.02 mmol, 50% yield) of white solid were obtained. ^{1}H NMR (400 MHz, CD_3OD) δ 5.80 (br s, 1H, H4), 4.57 (dd, *J* = 8.9, 4.5 Hz, 1H, H21), 3.31 (1H, H7, overlapped with solvent signal), 3.10 (dd, *J* = 13.7, 4.5 Hz, 1H, H20), 2.92 (ddd, *J* = 14.9, 3.8, 1.8 Hz, 1H, H6β), 2.83 (dd, *J* = 13.6, 9.0 Hz, 1H, H20'), 2.68 (dd, *J* = 14.9, 2.7 Hz, 1H, H6α), 2.51 (m, 1H, H16α), 2.49 (m, 1H, H2β), 2.31 (dt, *J* = 17.3,

3.3, 3.3 Hz, 1H, H2α), 2.25 (td, *J* = 11.2, 11.2, 3.5 Hz, 1H, H8β), 2.11 (m, 1H, H1β), 2.10 (m, 1H, H16β), 2.09 (m, 1H, H15α), 2.00 (s, 3H, Me24), 1.78 (m, 1H, H12β), 1.77 (m, 1H, H14α), 1.72 (m, 1H, H1α), 1.71 (m, 1H, H11α), 1.64 (m, 1H, H15β), 1.54 (dq, *J* = 13.1, 13.1, 13.0, 4.0 Hz, 1H, H11β), 1.40 (td, *J* = 11.5, 11.5, 4.3 Hz, 1H, H9α), 1.29 (s, 3H, Me19), 1.21 (td, *J* = 13.0, 13.0, 4.3 Hz, 1H, H12α), 0.94 (s, 3H, Me18). ^{13}C NMR (101 MHz, CD_3OD) δ 222.87 (C17), 201.65 (C3), 173.91 (C22), 173.28 (C23), 170.98(C5), 127.32 (C4), 53.58 (C21), 48.95 (C9), 48.71 (C13), 48.50 (C14), 46.52 (C7), 40.10 (C8), 39.97 (C10), 39.07 (C6), 36.62 (C1), 36.44 (C16), 34.74 (C2), 33.22 (C20), 32.24 (C12), 22.43 (C24), 22.07 (C15), 21.43 (C11), 18.12 (C19), 14.01 (C19). The complete set of NMR experiments can be seen in supplementary information (see http://dx.doi.org/10.1016/j.steroids.2012.11.017)

4.2.5. Molecular modeling calculations

All the molecular modeling calculations were carried out using Spartan '06 software working on a DELL workstation, and they were performed as follows. The corresponding α and β Cys and NAC conjugates at C7 atom of the steroids were constructed. A full conformational analysis using a Monte Carlo conformational search without restrictions was applied for each compound. In this way, 10000 different conformations were stochastically generated and minimized using the Merck Molecular Force Field (MMFF). With this protocol we are confident that we were able to map all the conformational space of the systems. The 100 lowest energy geometries were analyzed and ordered following the MMFF energies (see http://dx.doi.org/10.1016/j.steroids.2012.11.017). The corresponding Boltzmann distribution at 298 K was calculated using the relative energy of each conformation. For the

visualization of the existing conformers, the superimposition of the energetically accessible conformations (typically >99% of the conformers from the Boltzmann distribution at 298 K) was carried out by overlying the carbon atoms of the steroid ring. See supplementary information (see http://dx.doi.org/10.1016/j.steroids.2012.11.017)

4.2.6. LC-MS/MS conditions

For the detection and characterization of the compounds, methods of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) were applied. The analyses were carried out using a triple quadrupole (Quattro Premier XE) mass spectrometer equipped with an orthogonal Z-spray-electrospray ionization source (ESI) (Waters Associates, Milford, MA, USA) interfaced to an UPLC system, Acquity (Waters Associates) for the chromatographic separation. Drying gas as well as nebulising gas was nitrogen. The desolvation gas flow was set to approximately 1200 L/h and the cone gas flow to 50 L/h. A capillary voltage of 3.0 kV was used in both positive and negative ionization mode. Cone voltages of 25 V and 20 V were selected for positive and negative ionization mode, respectively. The nitrogen desolvation temperature was set to 450 ºC and the source temperature to 120 ºC. The LC separation was performed using an Eclipse Plus C_{18} column (50 x 2.1 mm i.d., 1.8 μm) (Agilent, Palo Alto, CA, USA). The column temperature was set to 55 ºC. Water and MeOH both with formic acid (0.01%) and ammonium formate (1 mM) were selected as mobile phase solvents. Gradient elution and flow-rate used is described in each section.

4.2.7. Mass spectrometry characterization of synthesized compounds

For the characterization of the compounds conjugated with Cys and NAC, synthesized conjugates were dissolved in a mixture of H_2O:ACN (9:1,v/v) and 10 µL were injected into the system. Full scan methods acquired in both positive and negative ionization modes were applied. The *m/z* range was selected from 200 to 500 in order to acquire the potential adducts and in-source fragments. After that, the collision-induced dissociation behavior of the target analytes was evaluated at different collision energies (from 10 to 40 eV). A gradient program was used at a flow-rate of 300 µL/min; the percentage of organic solvent was linearly changed as follows: 0 min, 30%; 1 min, 30%; 16 min, 60%; 17 min, 90%; 18 min, 90%; 18.5 min, 30%; 20 min, 30%.

4.2.8. Analysis of metabolites in biological matrixes

- **Urine**

For the direct detection of the Cys and NAC conjugated compounds, urine samples (5 mL) were passed through a C18 column, previously conditioned with 2 mL of MeOH and 2 mL of H_2O. The column was then washed with 2 mL of H_2O and, finally, the analytes were eluted with 2 mL of MeOH. The methanolic extracts were evaporated under nitrogen stream in a bath at 50 ºC. Finally, for the LC-MS/MS analysis, the residue was dissolved in 150 µL of a mixture of H_2O:ACN (9:1,v/v) and a volume of 10 µL was directly injected into the system. A gradient program was used at a flow-rate of 300 µL/min; the percentage of organic solvent was linearly changed as follows: 0 min, 30%; 1 min, 30%; 16 min, 60%; 17 min, 90%; 18 min, 90%; 18.5 min, 30%; 20 min, 30%. The

detection of Δ^6-AED-Cys, Δ^6-T-Cys, Δ^6-AED-NAC and Δ^6-T-NAC was performed by the SRM method described in Table 4.1.

The same conditions were used for the extraction and detection of 6-Prog-Cys and 6-Cort-Cys. However, due to the polarity of 6-Cort-Cys the gradient was changed and the percentage of organic solvent was linearly modified as follows: 0 min, 15%; 1 min, 15%; 16 min, 60%; 17 min, 90%; 18 min, 90%; 18.5 min, 15%; 20 min, 15%. The detection of Δ^6-Prog-Cys and Δ^6-Cort-Cys was performed by the SRM methods described in Table 4.1.

For the indirect detection, the release of the compounds conjugated with Cys and NAC was achieved using the method described in chapter 3. Briefly, 5 mL of urine were alkalinized with 300 µL of KOH 6 M and after 15 min of incubation at 60 ºC, a liquid-liquid extraction was performed by addition of 6 mL of TBME. The sample was centrifuged and the organic layer separated and evaporated. The residue was dissolved into 150 µL of a mixture of H_2O:ACN (1:1, v/v) and 10 µL were directly injected into the LC-MS/MS system. A gradient program was used at a flow-rate of 400 µL/min; the percentage of organic solvent was linearly changed as follows: 0 min, 25%; 0.2 min, 25%; 5 min, 75%; 6 min, 90%; 7 min, 90%; 7.2 min, 25%; 9 min, 25%. Δ^6-AED and Δ^6-T were determined by the SRM method described in Table 4.1.

Analyte	Precursor ion (m/z)	Cone (V)	Product ion (m/z)	Collision energy (eV)
Δ^6-AED-Cys	406	20	285	20
			149	35
			107	45
			105	45
			97	40
			83	45
Δ^6-AED-NAC	448	20	285	20
			149	35
			107	45
			105	45
			97	40
			83	45
Δ^6-T-Cys	408	20	287	20
			151	30
			133	35
			109	45
			107	45
			97	40
Δ^6-T-NAC	450	20	287	20
			151	30
			133	35
			109	45
			107	45
			97	40
Δ^6-Cort-Cys	482	15	361	20
			307	30
			266	40
			251	45
			239	40
			121	50
Δ^6-Prog-Cys	434	15	313	20
			159	30
			97	40
			85	35

Table 4.1. SRM method for the direct detection and confirmation of Δ^6-AED -Cys, Δ^6-AED -NAC, Δ^6-T-Cys, Δ^6-T-NAC, Δ^6-Prog-Cys and Δ^6-Cort-Cys in urine.

- **Plasma**

Plasma (10 mL) was extracted with 12 mL of TBME. After addition of sodium sulphate and centrifugation, both aqueous and organic phases were clearly separated. The organic layer was transferred and evaporated under nitrogen stream at 50 ºC. The residue was dissolved into 150 µL of a mixture of H_2O:ACN (1:1, v/v) and 10 µL were directly injected into the LC-MS/MS system. In this extract, the presence of the free metabolites in plasma was evaluated.

The aqueous layer was treated with 300 µL of KOH 6 M and after 15 min of incubation at 60 ºC, extraction was performed by addition of 6 mL of TBME. The sample was centrifuged and the organic layer separated and evaporated. The residue was dissolved into 150 µL of a mixture of H_2O:ACN (1:1, v/v) and 10 µL were directly injected into the LC-MS/MS system. In this extract, the presence of the metabolites excreted as conjugates was evaluated.

LC-MS/MS methods previously described for the indirect detection of the analytes was used for both extracts.

4.2.9. Fraction collection

A urine sample collected from 0 to 4 h after the administration of testosterone undecanoate was treated as described above for the direct detection of Cys and NAC conjugates. In order to clarify the origin of the metabolites released after alkaline treatment, LC fractionation of the urine extracts was performed. For this purpose, fractions were collected every 0.5 min. Every fraction was treated in alkalin conditions and analyzed by the indirect method.

4.2.10. Origin of biological samples

Urine samples obtained after administration of 120 mg of testosterone undecanoate (Androxon^{TM}, three 40 mg capsules; Organon) to 5 healthy volunteers were studied. Ethical approval for the study was granted by Comité Ètic d'Investigació Clínica of our institute (CEIC-IMAS no.94/467) and the Spanish Health Ministry (DGFPS no. 95/75). All of the subjects participating in the study gave their written informed consent.

Urine samples with elevated cortisol and progesterone concentrations were obtained from one volunteer with Cushing's syndrome and four pregnant volunteers, respectively.

A plasma sample collected from an anonymous volunteer was used for the confirmation of the presence of the analytes in plasma.

4.3. Results and discussion

4.3.1 Synthesis and NMR characterization of cysteine and *N*-acetylcysteine conjugates

Cys and NAC derivatives of Δ^6-AED and Δ^6-T have been synthesized. The cysteinyl compound 7-cystein-*S*-yl-4-androsten-3,17-dione (Δ^6-AED-Cys) was obtained by treating Δ^6-AED with a large excess (100-fold) of cysteine in alkaline conditions and subsequent purification by preparative reverse-phase HPLC (Figure 4.1a). Its structure was fully characterized by a complete set of bidimensional NMR experiments, which allowed the assignation of all the corresponding proton and carbon NMR signals. For instance, the long-range $^{1}H/^{13}C$ correlations (gHMBC) showed a cross peak between C7 and the protons of the SCH_2 group, which unambiguously demonstrates the attachment of the cysteine at C7 of the steroid and through the sulfur atom. The careful analysis of the NMR data also gave important information about the stereochemistry of Δ^6-AED-Cys. The proton signal at C7 appears as a quartet with a coupling constant ($^{3}J_{HH}$) of 3.38 Hz with all the vicinal protons (the proton at C8 and those at C6). This value corresponds to a dihedral angle of about 60°, which implies that H7 is equatorial. Moreover, the NOESY experiment showed cross-peaks between H7 and H8, H6axial and H6equatorial, all of

them with a comparable intensity. This experimental evidence implies a similar distance between H7 and the corresponding protons at C8 and C6. Overall, all the spectroscopic data confirmed the α configuration of the cysteinyl residue (Figure 4.1b). Very similar situation was observed for the 17β-OH derivative (Δ^6-T-Cys) or when the reactions were performed with *N*-Ac-Cys, leading to Δ^6-AED-NAC and Δ^6-T-NAC. For additional information, the complete set of NMR experiments can be seen in the supplementary information (see http://dx.doi.org/10.1016/j.steroids.2012.11.017).

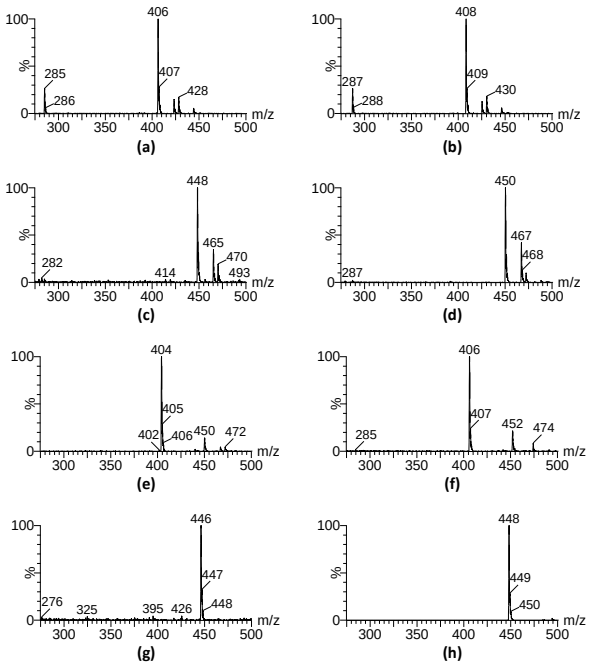

Figure 4.2. Full scan spectra in ESI+ **(a)** Δ^6-AED–Cys, **(b)** Δ^6-T-Cys, **(c)** Δ^6-AED–NAC and **(d)** Δ^6-T-NAC and in ESI- for **(e)** Δ^6-AED–Cys, **(f)** Δ^6-T-Cys, **(g)** Δ^6-AED–NAC and **(h)** Δ^6-T-NAC.

4.3.2 Mass spectrometric behavior of cysteine and *N*-acetylcisteine conjugates

The mass spectrometric behavior of Cys and NAC conjugates was studied using the synthetized compounds. Both conjugates exhibited an abundant $[M+H]^+$ ion as base peak of the ESI-MS spectrum in positive ionization mode (Figure 4.2a-4.2d). Besides the protonated molecule, other adducts like $[M+NH_4]^+$ and $[M+Na]^+$ were also found. For Cys conjugates, both adduct exhibited an abundance of 10-20%. In the case of NAC conjugates, the $[M+NH_4]^+$ was found to be more prominent with an abundance ranging from 35-50%. Additionally, in-source fragmentation was also observed in Cys conjugates producing the $[M+H-Cys]^+$ ion. In negative ionization mode, both Cys and NAC conjugates showed an abundant $[M-H]^-$ ion as base peak (Figure 4.2e-4.2h). Additionally, Cys conjugates exhibited also a formate adduct ($[M+HCOO]^-$) with abundances around 10%. Collision induced dissociation (CID) of Cys conjugates, in positive ionization mode; at low collision energy (10 eV) was dominated by a loss of 121 Da corresponding to the neutral loss of Cys. Similarly, CID for NAC conjugates at low collision energy exhibited a loss of 163 Da corresponding to the neutral loss of the NAC moiety. When increasing the collision energy, the fragmentation of both Cys and NAC conjugates were identical to the observed for the free ene-steroids (Figure 4.3 and 4.4). This fact suggests that the neutral loss of the Cys is formed in the collision cell by a retro-Michael's reaction generating the free ene-steroid. Once formed, this steroid followed the CID behavior usually observed for 3-keto steroids [33-34]. Thus, Δ^6-AED-Cys and Δ^6-T-Cys generated ions at *m/z* 149

and *m/z* 151/133 respectively, containing all of them the C and D rings of the steroid structure.

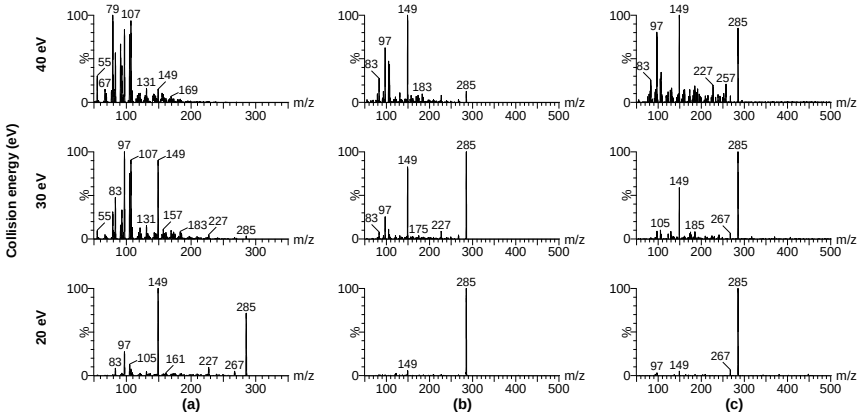

Figure 4.3. Product ion spectra in ESI+ for **(a)** Δ^6-AED (precursor ion *m/z* 285), **(b)** Δ^6-AED-Cys (precursor ion *m/z* 406), **(c)** Δ^6-AED-NAC (precursor ion *m/z* 448) acquired at 20 eV (bottom), 30 eV (medium) and 40 eV (top)

In negative ionization mode, the product ion spectra of Cys conjugates was dominated by the ion at *m/z* 120 corresponding to the Cys residue (Figures 4.5). In the NAC conjugates, the equivalent ion at *m/z* 162 corresponding to the NAC moiety was observed (Figures 4.5). Both conjugates exhibited also an ion at *m/z* 317 for Δ^6-AED derivatives and at *m/z* 319 for Δ^6-T derivatives. These ions can be explained after the fragmentation of the bond between the sulfur atom and the Cβ of the aminoacid residue.

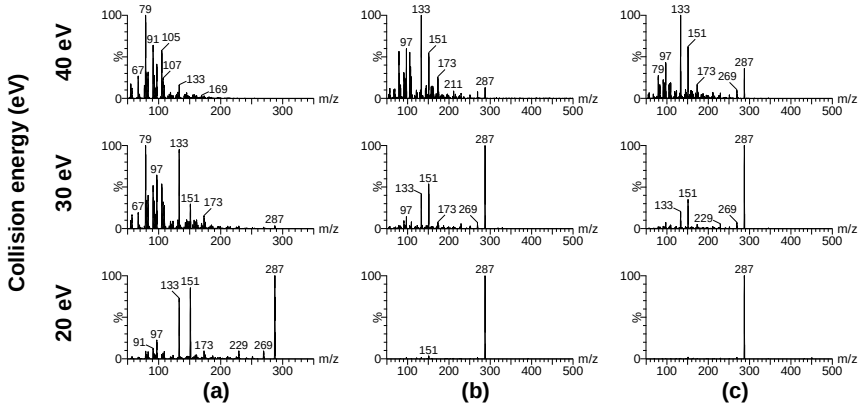

Figure 4.4. Product ion spectra in ESI+ for **(a)** Δ^6-T (precursor ion m/z 287), **(b)** Δ^6-T-Cys (precursor ion m/z 408), **(c)** Δ^6-T-NAC (precursor ion m/z 450) acquired at 20 eV (bottom), 30 eV (medium), and 40 eV (top).

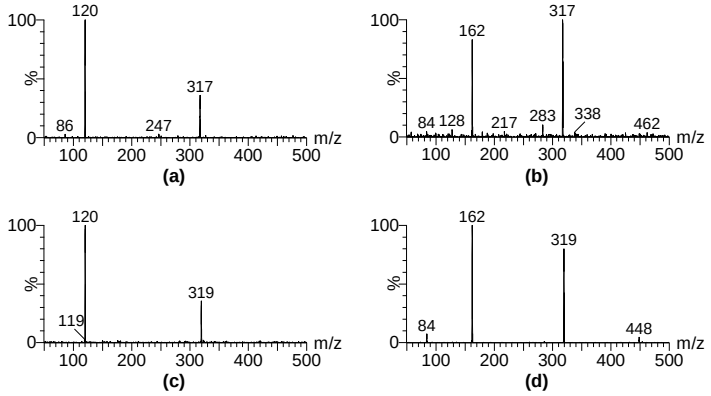

Figure 4.5. Product ion spectra in ESI- for **(a)** Δ^6-AED-Cys, **(b)** Δ^6-AED-NAC, **(c)** Δ^6-T-Cys and **(d)** Δ^6-T-NAC acquired at 20 eV.

4.3.3. Molecular modeling calculations

According to our spectroscopic data, the corresponding Cys and NAC adducts were formed by a conjugated addition of the thiol functionality to the C7 position of the steroids by the α face, setting the sulfur residue in axial disposition. To further support the

observation of only 7α-adducts, we performed molecular modeling calculations of all the discussed adducts, in both the α and the β configurations The theoretical calculations rendered the α configuration as the most stable isomers in all the cases by more than 3.7 kcal/mol, corresponding to a predicted α/β proportion >99%, in an excellent agreement with the experimental observation (see http://dx.doi.org/10.1016/j.steroids.2012.11.017). Besides, inspection of the optimized geometries suggested that the higher stability of the α isomers is due to steric hindrance in the β forms between the S atom of the cysteine and the nearby methylenes at C15 (Figure 4.1b).

4.3.4. Alkaline treatment of synthesized conjugated metabolites

Δ^6-AED and Δ^6-T were detected in urine after alkaline treatment of the urine sample [27]. The concentration of these analytes increased after testosterone administration suggesting that they are testosterone metabolites. The origin of these compounds was still unknown. Synthesized material (both Cys and NAC conjugates) was treated in alkaline conditions. After the alkaline treatment of the synthesized Cys and NAC metabolites, no conjugate could be detected and the free steroid (Δ^6-AED or Δ^6-T) was the only species observed. This suggests that these conjugates are responsible for the formation of Δ^6-AED and Δ^6-T in alkaline conditions.

A comparison in terms of sensitivity between direct detection and indirect detection (alkaline treatment) for the metabolites was also performed. The indirect detection was found to be more sensitive

than the direct for both Cys and NAC conjugates (Figure 4.6). This fact is due to better ionization and fragmentation efficiency. Additionally, in real samples a cleaner extract, minimizing the matrix effect, is obtained after applying the procedure for the indirect detection of the analytes. Hence, indirect detection is preferable for the detection of these metabolites mainly in those cases where they are present at low concentrations.

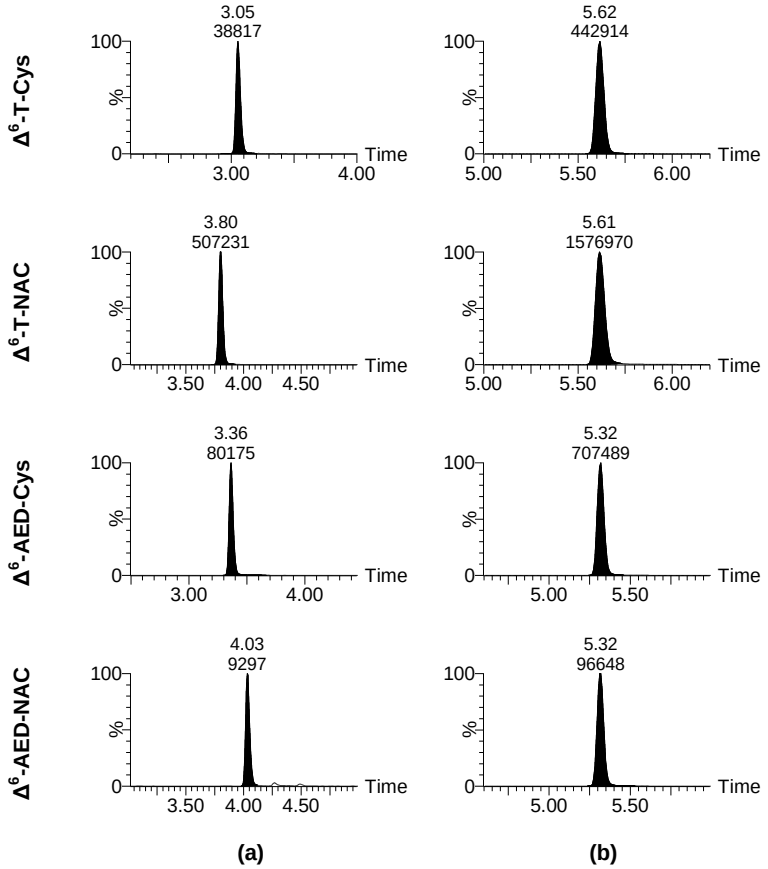

Figure 4.6. Comparison on sensitivity obtained for synthesized reference material by the SRM method using **(a)** direct detection of the conjugated, and **(b)** indirect detection of the conjugate (detection of the steroid released after alkaline treatment).

4.3.5. Confirmation of the presence of Cys conjugates of testosterone metabolites in human urine

Comparison between the analytes and reference materials is necessary in order to unequivocally confirm the structure of the target compound. For this purpose, several transitions were selected for every target analyte in the SRM method (Table 4.1). The ratios between the different transitions were evaluated for each target compound. Finally, the ion ratios obtained for the target compound in urine (samples collected before and after oral administration of testosterone undecanoate to healthy volunteers) and the corresponding reference standard material were compared.

For Δ^6-AED-Cys a peak eluting at 5.46 min was observed for all selected transition (Figure 4.7) in urine samples collected after testosterone administration. The relative abundance ratios between all detected transitions were similar to those obtained for the synthesized standard. Additionally, identical retention times were obtained for both the urinary species and the authentic compound, thus confirming the presence of Δ^6-AED -Cys in human urine. For Δ^6-T-Cys, a peak was observed at 4.50 min for all selected transitions in the post-administration sample (Figure 4.7). The relative abundances calculated for all selected transitions and the retention times were also in agreement with the synthesized material confirming also the presence of Δ^6-T-Cys in human urine.

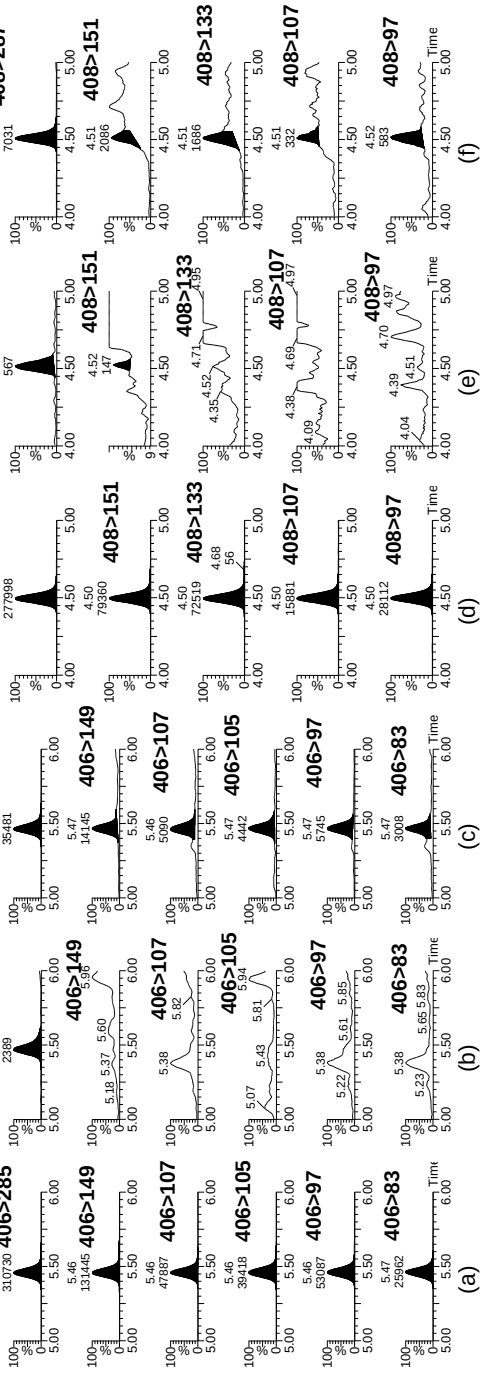

Figure 4.7. SRM confirmation of the presence of Δ^6-AED-Cys in urine. **(a)** Synthesized material, **(b)** extracted urine collected before administration, and **(c)** extracted urine collected 4 h after testosterone undecanoate administration. SRM confirmation of the presence of Δ^6-T-Cys in urine. **(d)** Synthesized material, **(e)** extracted urine collected before administration, and **(f)** extracted urine collected 4 h after testosterone undecanoate administration

The response abundance for these compounds, already present in the urine of non-treated subjects, increased in the samples collected after testosterone administration confirming that Δ^6-AED-Cys and Δ^6-T-Cys are testosterone metabolites. However, only the most abundant transition was detected in the samples collected before administration. No peak of Δ^6-AED-NAC and Δ^6-T-NAC eluting at the expected retention time predicted for the synthetized material was detected in urine samples after testosterone administration.

4.3.6 Detection of the free metabolites after isolation of the conjugates by LC fractionation

After the alkaline treatment of the synthesized Cys and NAC metabolites, no conjugate could be detected and the free steroid (Δ^6-AED or Δ^6-T) was the only species observed. This suggests that these conjugates are responsible for the formation of Δ^6-AED and Δ^6-T in alkaline conditions.

This point was clarified by HPLC fractionation of post-testosterone administration urine extracts and subsequent alkaline treatment of the fractions.

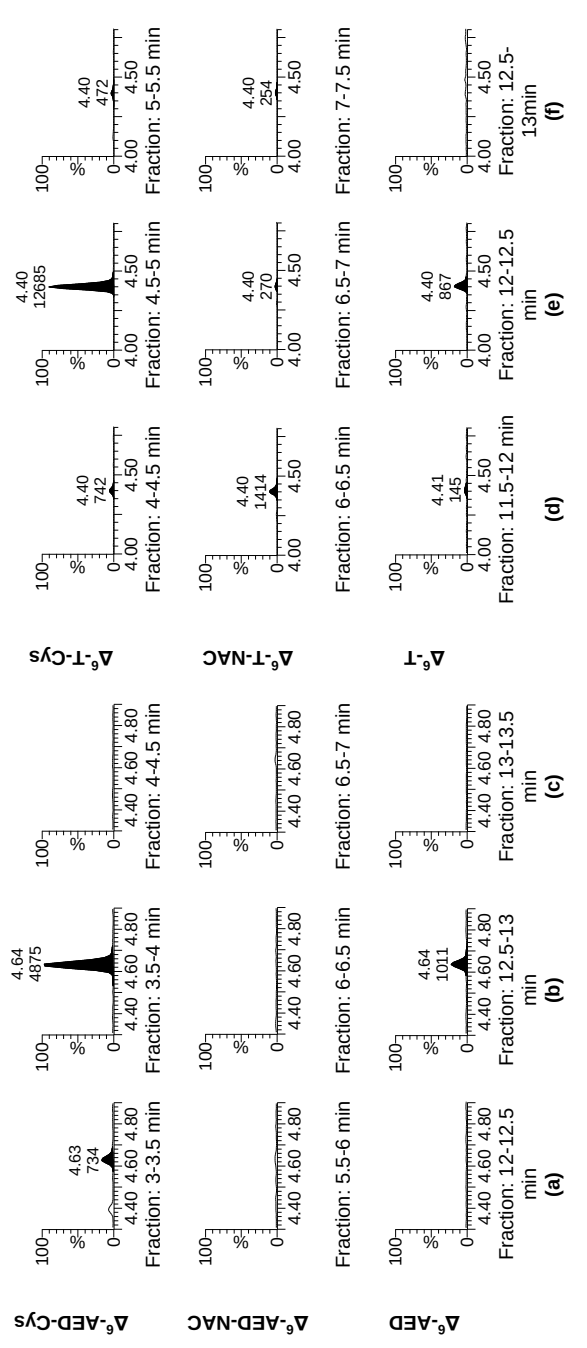

Figure 4.8. SRM chromatograms obtained for Δ^6-AED and Δ^6-T in the treatment of the fractions collected from a urine sample 4 h after testosterone undecanoate administration. **(a)** Fraction collected before the theoretical retention time for **(a)** Δ^6-AED and **(d)** Δ^6-T, sample collected at the theoretical retention time for **(b)** Δ^6-AED and **(e)** Δ^6-T and fraction collected after theoretical retention time for **(c)** Δ^6-AED and **(f)** Δ^6-T. Top: fractions collected for Δ^6-T-Cys and Δ^6-AED-Cys, medium: fractions collected for. Δ^6-T-NAC and Δ^6-AED-NAC and bottom: fractions collected for Δ^6-T and Δ^6-AED

A urine sample collected after testosterone undecanoate administration (0-4 h) was subjected to LC fractionation. Fractions were collected every 0.5 min. Every fraction was treated in alkaline conditions and analyzed by the indirect method. The peak corresponding to Δ^6-T was found mainly in the fraction collected between 3.5 and 4 min and Δ^6-AED was detected after treating the fraction collected between 4.5 and 5 min (Figure 4.8). These fraction times match with the experimental retention times for the Cys conjugates of Δ^6-T and Δ^6-AED, respectively. No free steroids were observed after the alkaline treatment of the fractions collected at the expected retention times for the NAC conjugates, indicating that these conjugates are not present in urine. Small amounts of the metabolites were detected in the fractions collected at the retention times of the free steroids. These results indicated that conjugation with Cys is the main source for the presence of these metabolites.

4.3.7. Detection of the new testosterone metabolites in plasma samples in free and conjugated form

The presence of Δ^6-AED and Δ^6-T in plasma of healthy volunteers was also confirmed. Both metabolites, Δ^6-AED and Δ^6-T, were detected unconjugated in plasma although at low concentrations (estimated concentrations of 15 pg/mL and 1 pg/mL for Δ^6-AED and Δ^6-T respectively). Due to these low concentrations, confirmation of the identity of Δ^6-AED in plasma could be done with three transitions (supplementary information S-4.2) while only the two most abundant transitions could be detected for Δ^6-T (supplementary information S-4.3).

The presence of Cys conjugates in plasma was confirmed by the indirect method. After alkaline treatment the concentrations of Δ^6-AED and Δ6-T increased around 4 times (estimated concentrations 50 pg/mL for Δ^6-AED and 5 pg/mL for Δ^6-T). This increase in concentrations allowed for the detection of more transitions in the confirmation of their identity (supplementary information S-4.4 and S-4.5).

The increase of concentration for both analytes seems to indicate that both compounds are predominantly conjugated in plasma. However, the impossibility to directly detect the conjugates in plasma makes to confirm the identity of the conjugates impossible. Cys and glutathione conjugates are the most feasible options for this species.

4.3.8. Confirmation of the presence of progesterone and cortisol metabolites conjugated with cysteine in human urine

In order to confirm that the metabolic pathway reported for testosterone and androstenedione is common to other steroid hormones, analogous Cys metabolites for progesterone (7α-cystein-*S*-yl-progesterone; Δ^6-Prog-Cys) and cortisol (7α-cystein-*S*-yl-cortisol; Δ^6-Cort-Cys) were synthesized by treating Δ^6-Prog (6,7-dehydroprogesterone) and Δ^6-Cort (6,7-dehydrocortisol) respectively with an excess of cysteine in alkaline conditions. The identity of the synthesized compounds was checked by LC-MS/MS. Following the behavior of other Cys conjugates, the $[M+H]^+$ was the most abundant ion in both cases in positive ionization mode. Other ions like $[M+Na]^+$ or $[M+NH_4]^+$ or those formed by in-source loss of the cysteine moiety were also observed (supplementary

information S-4.6). In negative ionization mode, the full scan spectra of both compounds were dominated by the $[M-H]^-$. In the case of Δ^6-Prog-Cys, the ion $[M+HCOO]^-$ was also observed (supplementary information S-4.6).

Transition	Synthesized material	Sample 1	Sample 2	Sample 3	Sample 4
434 → 313	100 %	100 %	100 %	100 %	100 %
434 → 159	22 %	24 %	20 %	21 %	21 %
434 → 97	7 %	8 %	6 %	7 %	6 %
434 → 85	13 %	13 %	11 %	15 %	12 %

Table 4.2. Relative abundances of the transitions acquired for Δ^6-Prog-Cys in the synthesized material and in the urine samples collected from pregnant women

The CID results for the synthesized material also showed the expected behavior for Cys conjugates. Thus, in positive ionization mode, the product ion spectra of both compounds at low collision energy was dominated by the neutral loss of cysteine ($[M+H-121Da]^+$) producing the ions at *m/z* 313 and 361 for Δ^6-Prog-Cys and Δ^6-Cort-Cys respectively. At higher collision energy, the product ion spectra of both the Cys, conjugated and the free steroid showed the same ions. Thus, the product ion spectrum of both Δ^6-Prog-Cys and 6,7-dehydroprogesterone was dominated for the ion 159 whereas for cortisol several ions including *m/z* 173 and *m/z* 121 were obtained (supplementary information S-4.7). In negative ionization mode, both compounds exhibited the ion at *m/z* 120 which can be explained by the Cys residue. The ions at *m/z* 345 for Δ^6-Prog-Cys and *m/z* 393 for Δ^6-Cort-Cys can be explained after the fragmentation of the bond between the sulfur atom and the Cβ of the aminoacid residue which was also observed for the

testosterone related compounds (supplementary information S-4.8).

Based on this information, SRM methods for the confirmation of the presence of Δ^6-Prog-Cys and Δ^6-Cort-Cys in urine were developed (Table 4.1). The method for progesterone metabolites was applied to samples collected from 4 pregnant volunteers (gestational age: 32-40 weeks). The comparison of retention times and ion ratios between the selected transitions allowed for the confirmation of the presence of this conjugate in urine (Table 4.2 and supplementary information S-4.9).

In the case of cortisol metabolite, the product ion spectrum showed a large number of product ions, most of them with an abundance lower than 10% of the main ion (*m/z* 361). This fact made the confirmation of the presence of this compound difficult. For this reason, we analyzed a urine sample from a patient affected by Cushing's syndrome with elevated urinary concentrations of cortisol. In this sample, despite the low relative abundance of several of the ions, the concentration of the cortisol metabolite was high enough to allow the confirmation of Δ^6-Cort-Cys (Table 4.3 and supplementary information S-4.10).

Transition	Synthesized material	Sample
482 → 361	100%	100%
482 → 307	2%	3%
482 → 266	6%	6%
482 → 251	8%	8%
482 → 239	9%	9%

Table 4.3. Relative abundances of the transitions acquired for Δ^6-Cort-Cys in the synthesized material and in the urine sample collected from Cushing's syndrome patient

4.3.9. Proposed metabolic pathway

A metabolic pathway can be postulated based on the information obtained for these compounds conjugated with Cys . The presence of 7α-Cys conjugates in urine indicates a phase II metabolic reaction between the thiol moiety (either coming from cysteine or from glutathione residues) and an electrophilic substrate. For testosterone, the most rational substrates are Δ^6-AED and Δ^6-T and therefore, it can be inferred that these substances are already present in the cell. The presence of Δ^6-T was reported in incubation experiments of liver homogenates of rats with testosterone [35], but to the best of our knowledge our results are the first proof of their existence in humans. Besides, 6,7-dehydrogenation has been reported in the metabolism of some exogenous steroids in humans like methandienone or 17α-methyltestosterone [4-18]. Thus, androstenedione and testosterone could be metabolized to Δ^6-AED and Δ^6-T, respectively, by a 6,7-dehydrogenation reaction, suggesting that this reaction is a phase I metabolic biotransformation for androgens (Figure 4.7).

On the other hand, although glutathione conjugates have not been reported in humans for steroids, cellular glutathione levels of isolated hepatocytes decreased during incubation with Δ^6-T [36], suggesting this conjugation as the most feasible phase II metabolic reaction for this compound. Therefore, a 6,7-dehydrogenation as a phase I biotransformation , followed by a intracellular conjugation with glutathione as a phase II reaction can be postulated as an active metabolic pathway for testosterone and androstenedione. Analogously to what happens to glutathione conjugates of other xenobiotics, after extracellular degradation of the glutathione

moiety, the Cys conjugates would be excreted in urine [37] (Figure 4.7).

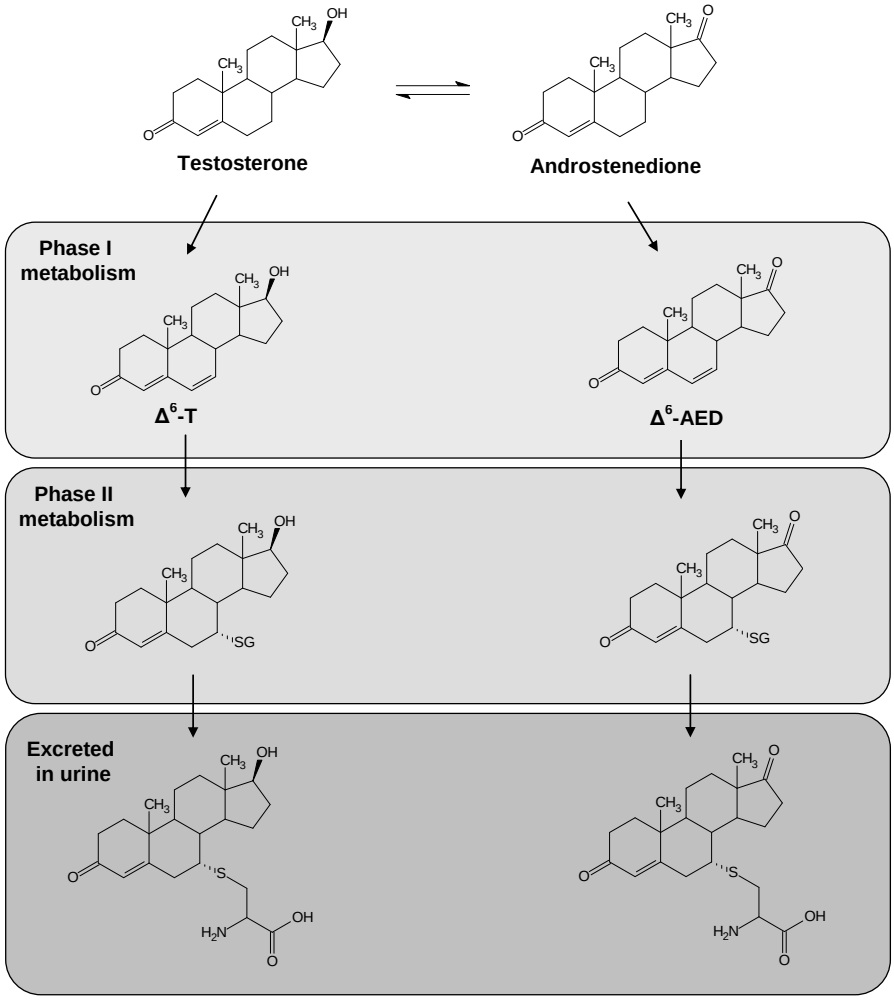

Figure 4.7. Proposed metabolic pathway for testosterone (SG: glutathione).

Similar metabolites have been found for cortisol and progesterone. This fact confirmed that the metabolic pathway postulated in this study, i.e. 6,7-dehydrogenation as phase I metabolism followed by conjugation with glutathione and subsequent transformation in Cys

conjugates, is not restricted to androgens, but common to other classes of steroids such as glucocorticoids and progestagens. These results open new analytical options for investigating the biological processes involving a wide range of steroid hormones, and might lead to the discovery of alternative markers for disorders related to their biosynthesis. Additionally, it is expected that this pathway is also present in the metabolism of exogenous steroids opening the possibility to detect alternative markers which can improve the detection of the misuse of these compounds.

4.4. Conclusion

Δ^{6}-AED and Δ^{6}-T conjugated both with Cys and NAC were successfully synthesized and characterized by NMR analysis confirming that Cys and NAC were added in position C7 with α-configuration. The presence of Cys conjugates of testosterone metabolites in urine has been unequivocally confirmed by their LC-MS/MS detection and comparison with synthesized material. However, no NAC conjugates of Δ^{6}-AED and Δ^{6}-T could be detected in urine samples.

The formation of this conjugates with Cys implies a potential unreported metabolic pathway where after the intercellular conjugation with glutathione as phase II metabolism, the glutathione moiety is degraded to Cys and excreted in urine. This postulated metabolic pathway has been also found in other steroids hormones like progesterone and cortisol by the synthesis and detection of the 7α-cystein-S-yl-progesterone and 7α-cystein-S-yl-cortisol respectively. Similarly, the presence of Δ^{1}-AED-Cys in urine after alkaline treatment was also demonstrated to come from 1α-

cysteine-S-yl-2-androsten-3,17-dione after the administration of boldione for our research group [29].

Although for Δ^{15}-AD no confirmation of the conjugated with cysteine was possible due to the absence of commercial standard, this compound was only observed after the alkaline treatment of the urine (see chapter 3). Therefore, similarly to Δ^{6}-AED and Δ^{6}-T, is expected that the Δ^{15}-AD come from a conjugated with cysteine. Therefore, in order to make the thesis more understandable, Δ^{15}-AD is considered as cysteinyl compound.

According to our results, the indirect detection (i.e. alkaline treatment of the sample, extraction, and analysis by LC-MS/MS) possibly is the most adequate analytical strategy for the screening of these conjugates due to the higher sensitivity achieved when compared to the direct detection.

The fragmentation pathways in both positive and negative ionization modes observed for the synthesized Cys and NAC conjugates seemed to indicate a common behaviour. This behaviour can be used in the future for the development of analytical methods for the open detection of this kind of conjugates, which in turn might lead to the detection of new metabolites for endogenous hormones and drugs.

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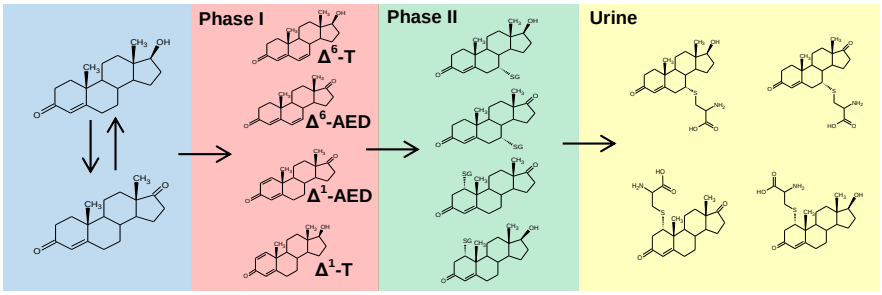
Hepatocyte studies

Chapter 5



This chapter has been adapted from:

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5.1. Introduction

Testosterone (T) is the principal anabolic androgenic steroid (AAS) in humans and its metabolism is considered the basic metabolic pathway for synthetic AAS. The metabolism of T has been extensively investigated *in vivo* and *in vitro* studies in several animal models and clinical studies in humans [1, 2].

Metabolic reactions are classified in two groups, phase I and phase II reactions [1, 3]. In the case of AAS, phase I metabolism includes, among others, oxidations, reductions and hydroxylation, whereas phase II metabolism or conjugation reactions couple the steroid molecules mainly with glucuronic acid [4] or sulphate [5], although other conjugate reactions have been also described [6-8].

Several T metabolites Δ^1-AED, Δ^6-AED and Δ^6-T released after alkaline treatment of the urine were detected and elucidated in samples collected after an oral administration of T (see chapter 3). Minor amounts of boldenone (Δ^1-T) were also found under these conditions (see chapter 3) suggesting that this metabolic pathway is involved in the endogenous detection of boldenone previously reported. Additionally, the occurrence of Δ^1-T has been related as a product of the microbial gut present in human body [1, 9].

The presence of Δ^6-AED and Δ^6-T in urine after basic treatment was demonstrated to arise from the release of the cysteine moiety of 7α-Cystein-S-yl-4-androsten-3,17-dione (Δ^6-AED-Cys) and 7α-Cystein-S-yl-4-androsten-17β-ol-3-one (Δ^6-T-Cys), respectively (see chapter 4). In the same way, the presence of Δ^1-AED and Δ^1-T in urine after alkaline treatment was also demonstrated to come from 1α-Cystein-S-yl-2-androsten-3,17-dione (Δ1-AED-Cys) and 1α-Cystein-S-yl-2-androsten-17β-ol-3 one after boldione administration [10].

Based on this information, a putative metabolic pathway was postulated for the occurrence of these compounds in urine (Figure 5.1). Briefly, after a phase I reaction consisting of dehydrogenation either in position 1 for Δ^1-AED and Δ^1-T or in position 6 for Δ^6-AED and Δ^6-T, these compounds are intracellularly conjugated with glutathione as a phase II metabolic reaction and, after an extracellular degradation of the glutathione moiety, these compounds are excreted in urine as conjugated with cysteine (see chapter 4 for further information). This proposed metabolic pathway needs to be confirmed.

The use of cell cultures is a rational way to study the phase I metabolism occurring into de cell system [11, 12]. In fact, the use of in vitro cell system for the study of androgen metabolism is well established [13, 14]. In particular, the occurrence of Δ^6-T in animal models was confirmed by incubating microsomal liver cells from rats with T [15]. However, no evidences for the occurrence Δ^1-T, Δ^1-AED and Δ^6-AED in human hepatocyte cells have been reported up to now.

The aim of the present study is to confirm the first step of the metabolic pathway shown in Figure 5.1. For this purpose, the formation of Δ^1-AED, Δ^6-AED, Δ^6-T and Δ^1-T after incubation of human hepatocytes cells cultures with T was evaluated. The results regarding the formation rate for these metabolites have been compared with the behaviour of major T metabolites like androsterone, etiocholanolone and 4-androstene-3,17-dione.

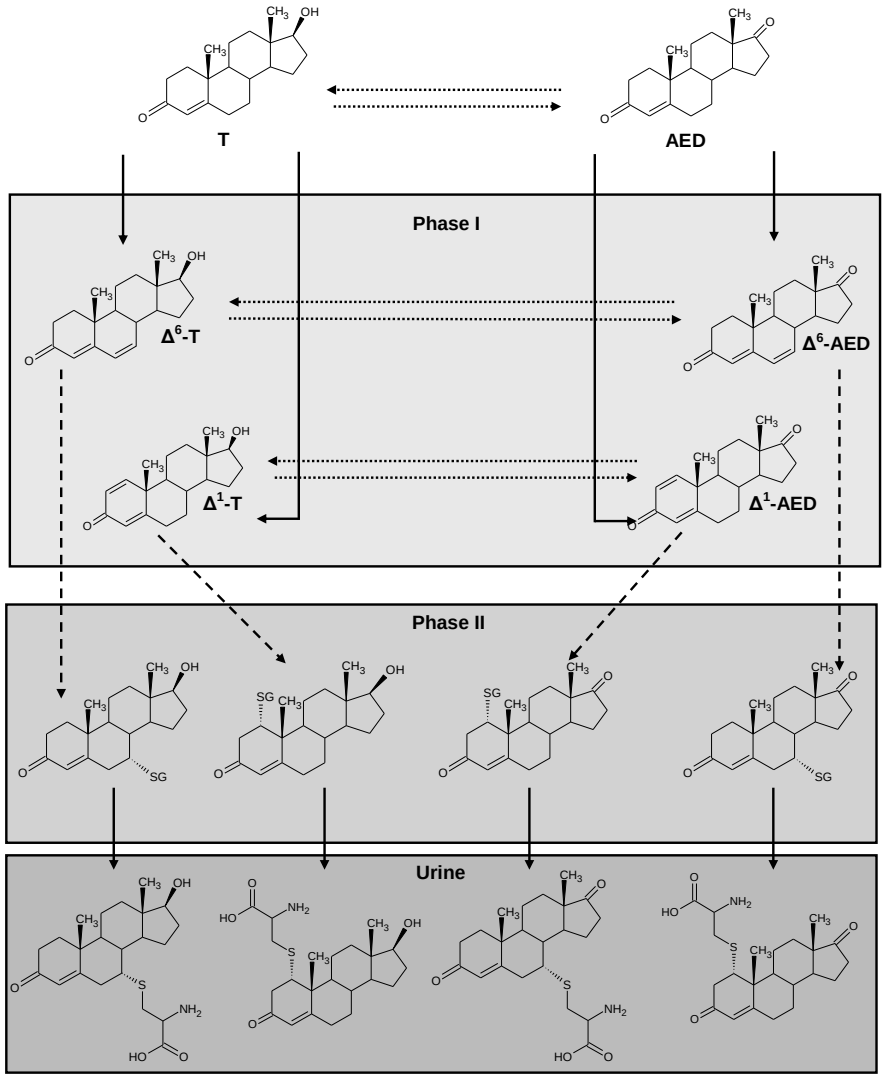

Figure 5.1. Proposed metabolic pathway for the occurrence of cysteinyl conjugates in urine. (SG:Glutatione)

5.2. Experimental

5.2.1. Chemical and Reagents

Δ^6-AED, Δ^6-T, boldenone (Δ^1-T) were obtained from Steraloids Inc. (Newport, USA). Δ^1-AED was purchased from NMI (Pymble, Australia). Metandienone used as internal standard (ISTD), testosterone (T), dihydrotestosterone (DHT), androstenedione (AED), androsterone (Andros) and etiocholanolone (Etio) were obtained from Sigma-Aldrich (St Louis, MO, USA).

Acetonitrile, methanol (LC gradient grade), formic acid, ammonium formate and sodium sulphate (LC/MS grade) were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain). Tert-butylmethyl ether (TBME) was obtained from Merck (Darmstadt, Germany).

Dlubecco's modified Eagle's medium (DMEM), DMEM F12, penicillin, streptomycin and L-glutamine were purchased from Life Technologies (Alcobendas, Madrid). Fetal bovine serum (FBS) medium and hank's balanced salt solution (HBSS) were purchased from PAA laboratories (Houdstone Business Park, UK). HepG2 cells were obtained from the American Type Culture Collection (Manassas, VA, USA)

5.2.2. LC-MS/MS conditions

Mass spectrometric analyses were carried out using a triple quadrupole (Quattro Premier XE) mass spectrometer equipped with an orthogonal Z-spray-electrospray ionization source (ESI) (Waters Associates, Milford, MA, USA) interfaced to an UHPLC system, Acquity (Waters Associates) for the chromatographic separation. Drying gas as well as nebulising gas was nitrogen. The

desolvation gas flow was set to approximately 1200 L/h and the cone gas flow to 50 L/h. Different cone voltages were used (table 5.1), and a capillary voltage of 3.0 kV were used in positive ionization mode. The nitrogen desolvation temperature was set to 450 ºC and the source temperature to 120 ºC.

The LC separation was performed using an Acquity UPLC BEH C_{18} column (100 x 2.1 mm i.d., 1.8 μm) (Waters Associates, Milford, MA, USA), at a flow rate of 300 μL/min. Water and methanol both with formic acid (0.01%) and ammonium formate (1 mM) were selected as mobile phase solvents. A gradient program was used; the percentage of organic solvent was linearly changed as follows: at 0 min, 45%; at 0.5 min, 45%; at 10 min, 65%; at 11.5 min, 90%; at 12 min, 45%; at 13 min, 45%.

Mass spectrometric analyses were performed in selected reaction monitoring (SRM) mode by monitoring two ion transitions for each analyte as described in Table 5.1.

Analyte	Precursor ion	Product ion		
	m/z	m/z	Collision energy (eV)	Cone voltage (V)
Δ^1-AED	285	121[a]	20	20
		147	15	20
Δ^6-AED	285	149[a]	25	25
		97	35	25
Δ^1-T	287	121[a]	30	20
		135	15	15
Δ^6-T	287	151[a]	20	25
		133	25	25
T	289	97[a]	30	25
		109	20	25
Etio/Andros	308	255[a]	20	20
		273	20	20
DHT	291	255[a]	15	25
		273	10	25
AED	287	97[a]	20	25
		109	20	25

[a]Ion selected for quantitative purposes

Table 5.1. SRM parameters for selected steroids

5.2.3. Testosterone purification

Due to the presence of traces of Δ^6-T observed in commercial T standards, a purification step was required. A HP1090 liquid chromatograph with automatic injection system and diode array detection (Hewlett-Packard, Waldbronn, Germany) was used for purification. The LC column was a XBridge C_{18} column (250 x 4.6 mm i.d., 5 µm) (Waters Associates, Milford, MA, USA). The mobile phase was water and methanol at a flow rate of 1 mL/min. Gradient elution was as follows: at 0 min 25%; at 10 min 25%; at 11 min 75%; at 11.10 min 25%; at 15 min 25%. The column effluent was monitored at 244 nm, where T exhibits a maximum absorption. An aliquot of 20 µL of a 5 mg/mL solution standard of T in methanol was injected and the peak of T was isolated by collecting a 0.5 min fraction corresponding to its retention time ± 0.25 min. In order to increase the concentration of the T purified, this process was repeated 10 times, and the individual fractions were combined and reconstituted in ten millilitres of methanol for the final standard of T purified. Data evaluation was performed by HP Chemstation software Rev. A.06.03 (Hewlett-Packard).

The absence of any of the metabolites in the purified T solution was tested by LC-MS/MS using the method previously described in section 5.2.2.

The concentration of the final T purified solution was calculated by measuring the absorbance at 240 nm in an Agilent 8453 spectrophotometer (Agilent Technologies, Palo Alto, California, USA), and comparing with the reference molar absorptivity for T [16] .

5.2.4. Hepatocytes preparation and incubation studies

HepG2 cells were seeded (1x106 cells) in T75 flasks and grown to confluence in DMEM, supplemented with 10% fetal bovine serum, 50 U/mL penicillin and 50 µg/mL streptomycin. Thereafter, cells were switched to serum-free medium for 16 h and subsequently incubated (37 °C, 5% CO_2) at variable time intervals in HBSS in a water-jacketed CO_2 incubator (Nuaire, USA). Cells were treated with T or vehicle (acetonitrile 0.5%) for 2, 4, 8, 12 or 24 h. Following cell culture, supernatants were obtained on completing the incubation periods. Samples were centrifuged at 2.000 rpm (4 °C) and frozen at -20 °C until further analysis.

5.2.5. Sample treatment

After the addition of 0.8 mL of water and 50 µL of ISTD solution (metandienone, 1 µg/mL) to 0.2 mL of the medium cell culture from the hepatocyte studies, a liquid-liquid extraction with 3 mL of TMBE was performed. The sample was centrifuged and the organic layer was separated and evaporated. The residue was dissolved into 150 µL of a mixture of water:acetonitrile (1:1, v/v). Finally, 10 µL were injected into the LC-MS/MS system.

5.2.6. Identification and quantification of the selected T metabolites

Comparison between the analytes and reference materials is necessary in order to unequivocally confirm the structure of the target compound. For this purpose, two different transitions were selected for every target analyte in the SRM method (Table 5.1). The ratio between the two selected transitions and the retention time (RT) were evaluated for each compound. Also, the ion ratio obtained for a target compound in both the sample and the

reference material were compared. For the unequivocal confirmation of the target analytes the criteria acceptance of the technical document of the World Anti-Doping Agency was followed [17]. Differences below ± 2% or ± 0.1 minutes for RT and a relative error (RE) below than ± 20% for the ion ratio were considered for confirmation of the identity.

For quantification purposes, the most abundant ion transition for each analyte was selected (Table 5.1). The concentrations for major T metabolites (DHT, AED, Etio and Andros) were quantified by using an approach based on the method described by Surowiec et al [18]. For the metabolites recently reported as potential phase I metabolites (Δ^1-AED, Δ^6-AED, Δ^6-T, Δ^1-T) the quantification was performed by a modification of the method developed in chapter 6 for the urinary quantification of these compounds. Calibration standards at three concentrations levels (0.03 µM, 0.3 µM and 3.5 µM) for all compounds were prepared in ultrapure water and used for quantification. MassLynx software was used for data management and quantification.

5.3. Results and discussion

5.3.1. Reliability of the hepatocyte results

Several experiments were performed in order to unequivocally confirm that the identified steroids were produced exclusively by hepatocytes after incubation with T.

In a first step, the existence of traces of the target metabolites in the commercial T standard was evaluated by using LC-MS/MS in the SRM mode described in Table 5.1. Traces of Δ^6-T and AED (around 0.01%) were detected in the commercial standard of T (data not shown). The presence of these traces of Δ^6-T would

impede from discerning the actual origin of the metabolites and, therefore, would hamper for the correct interpretation of the results. For this reason, a purification step was applied to the T standard using UHPLC fractionation. The absence of the target analytes in the purified T standard was confirmed by analysis with the SRM mode (Table 5.1). Only T was present in the solution used for the incubation experiments.

In a second step, different growth cell mediums were tested in order to check potential degradation effects of the medium and experimental conditions on the purified T standard. For this purpose, T at the same concentration used in cell incubation studies was added into the medium and maintained ,without hepatocytes, at 37 ºC during 24 h. Two commonly cell culture media (DMEM and DMEM F12) were tested. These cell culture media are widely used for growing different types of mammalian cells [19]. The analysis of these culture media showed the occurrence of traces of Δ^6-T and AED generated by degradation of T after incubation for 24 h in the media without hepatocyte cells. Similarly to the T standard impurities, these degradation effects could disturb the interpretation of the results. Some of the supplements present in the growth media might be responsible for T degradation. Therefore, other less complex cell culture media were tested.

Among the large quantity of growth media, the physiological buffer HBSS was shown to be the simplest medium used for cell cultures. This buffer is isotonic and non-toxic for the cells and is able to maintain the osmolarity of the cells. Using HBSS, no traces of Δ^6-T or AED were detected in the analysis of the media incubated at 37 ºC during 24 h with T and without hepatocyte cells.

Therefore, the use of the purified T standard and HBSS growth media provided the appropriated cell culture experimental conditions to ensure that the T metabolites detected after incubation were exclusively produced by hepatocyte cell cultures without any contribution of external sources.

5.3.2. Identification of T metabolites

The occurrence of all selected major T metabolites was observed after 24 h of incubation with T (Figure 5.2). As expected, AED, DHT, Andros and Etio (eluting at 5.8 min, 8.6 min, 10.4 min and 10.2 min, respectively were observed in the hepatocyte cell culture supernatants collected after 24 h incubation (Figure 5.2). Similar ion ratios (RE below 10%) and retention times (differences below 0.02 min, RE below 0.5 %) were observed for the major T metabolites (Table 5.2) confirming the identity of these compounds. The presence of these metabolites, which are normally reported in the literature for the incubation of T with cell samples [13, 20], suggested that the use of a restrictive growth method such as HBSS, was not directly affecting the normal intercellular metabolism of T.

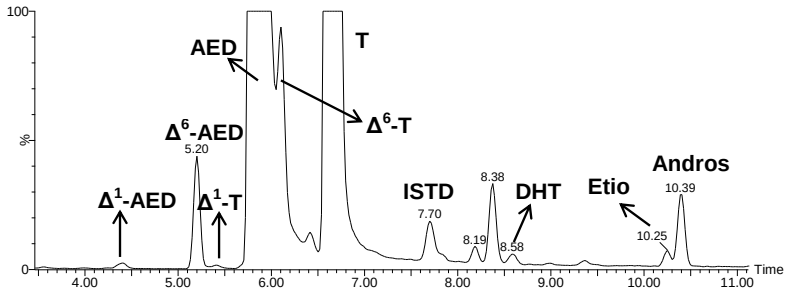

Figure 5.2. LC-MS/MS chromatogram for the analysis of the hepatocyte cultures incubated with T for 24 h.

Regarding the postulated phase I T metabolites, peaks for Δ^1-AED, Δ^6-AED, Δ^1-T and Δ^6-T (eluting a 4.4 min, 5.2 min, 5.3 min and 6.0 min, respectively) were observed in the supernatant of the hepatocyte cell cultures collected after 24 h incubation in HBSS medium (Figure 5.2). The ratios between all selected transitions were similar to those obtained for the reference standards with RE for each target compound below 10 % (Table 5.2). Additionally, identical RTs (RE below 0.5%) were reported for all analytes when comparing with the reference standards (Table 5.2), confirming the formation of these compounds by the hepatocytes.

	Hepatocyte studies		Standard		RE (%)	
Compound	Ion ratio	Retention time (min)	Ion ratio	Retention time (min)	Ion ratio	Retention time (min)
Δ^1- AED	3.72	4.42	4.08	4.40	8.9	0.5
Δ^6- AED	1.89	5.20	1.78	5.20	6.5	0
Δ^1- T	9.57	5.34	1.05	5.37	4.9	0.5
Δ^6- T	0.76	6.00	0.84	6.00	9.1	0
AED	1.03	5.84	1.07	5.84	3.3	0
DHT	4.23	8.58	4.68	8.58	9.5	0
Etio	5.36	10.25	5.13	10.23	4.4	0.2
Andros	1.29	10.39	1.38	10.39	6.8	0

Table 5.2. Retention times, ion ratios and relative errors (RE) observed for the target analytes in the hepatocyte studies and the standard compounds.

Thus, the unequivocal identification of Δ^1-AED, Δ^6-AED, Δ^1-T, Δ^6-T into the hepatic cells system confirmed the alternative phase I metabolism postulated in chapter 4.

5.3.3. Time-course production of the target metabolites

For this evaluation, the concentration of the target metabolites in the hepatocyte cell culture at 0 h, 2 h, 4 h, 8 h, 12 h and 24 h after the incubation with T was calculated.

The initial concentration of T (0 h of incubation) was 4.3 µM. As expected, the T concentrations decreased, reaching a minimum of 1.9 µM after 24 h of incubation (Figure 5.3). This fact indicates that almost the 50% of the initial T was metabolized to different compounds.

The major metabolite observed after the incubation of T with hepatocytes cells was AED. As observed in Figure 5.3, T is rapidly transformed to AED with concentrations of 2.2 µM at 4h of incubation. After that point, the increase was less pronounced reaching concentrations of 2.8 µM at 24 h. A similar behaviour was observed for DHT (Figure 5.3), a rapid increase was observed for the concentrations of DHT at 2 h (16.8 nM), and thereafter, only a moderate increase, reaching concentrations around 18.3 nM at 24 h, is observed. The results for Etio and Andros substantially differed for those obtained for AED and DHT. A constant increase in the studied collection period was observed for Andros and Etio (Figure 5.3) reaching a maximum in the last collected sample (24 h). Concentrations ten times lower were observed for Andros compared to those concentrations observed for Etio after 24 h of incubation (212 nM and 28.1 nM respectively).

The behaviour observed for AED and DHT suggests that T is rapidly metabolized to these compounds (mainly to AED) which are gradually converted to other metabolites. On the other hand, the behaviour exhibited for Etio and Andros showed that these metabolites are generated in a slower way.

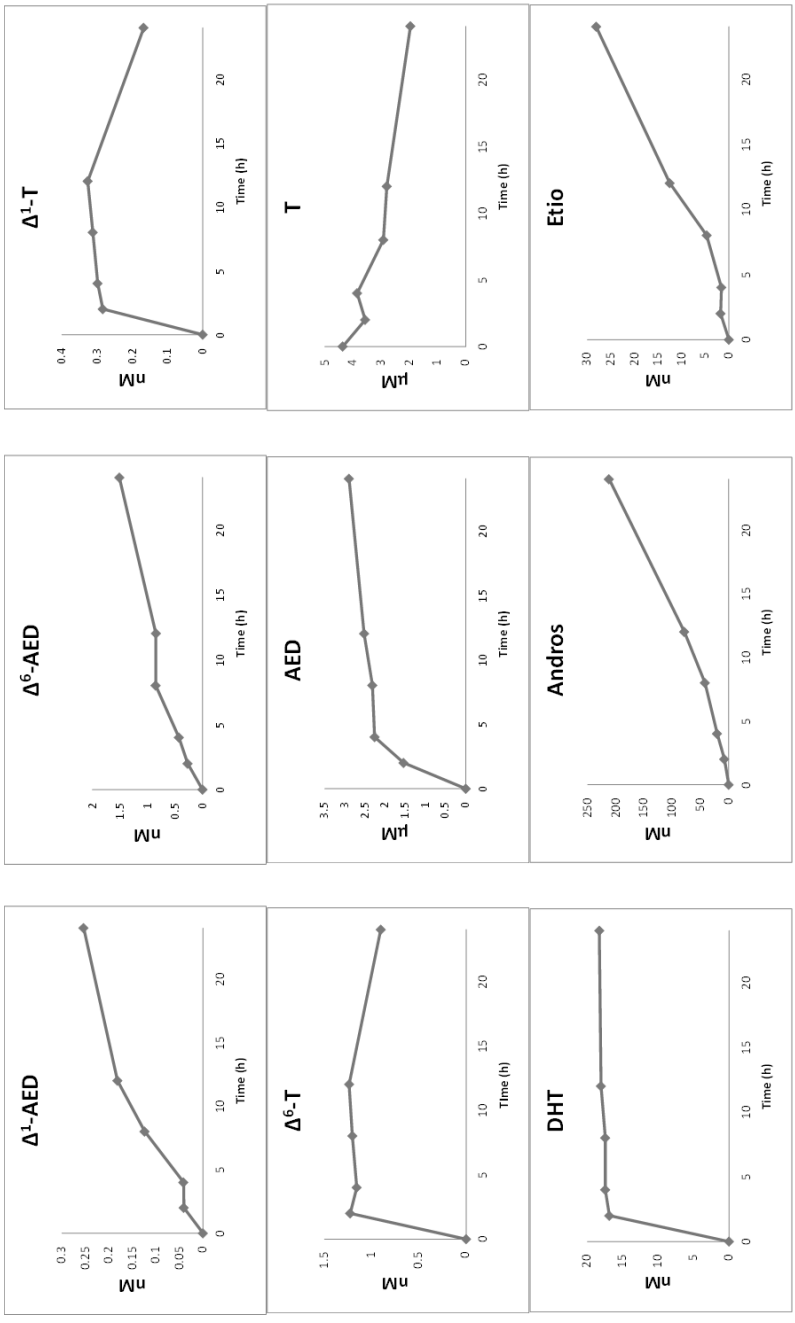

Figure 5.3. Time-course production of the target analytes in human hepatocyte cell cultures incubated with T.

Regarding the postulated T phase I metabolites implying the addition of double bond (Δ^1-AED, Δ^6-AED, Δ^1-T and Δ^6-T), lower concentrations than those obtained for the major T metabolites were obtained (Figure 5.3).

After incubation, similar behaviour was observed for Δ^1-T and Δ^6-T (Figure 5.3). A rapid increase was observed at 2 h of incubation (0.28 nM for Δ^1-T and 1.2 nM for Δ^6-T). Thereafter, these concentrations remained almost unaltered obtaining concentrations of Δ1-T around 0.17 nM and of Δ^6-T around 0.91 nM at 24 h of incubation. On the other hand, a constant increase was observed for Δ^1-AED and Δ^6-AED during the collection time. Maximum concentrations (0.25 nM for Δ^1-AED and 1.5 nM for Δ^6-AED) were obtained after 24 h of incubation.

Similarly to AED and DHT, the behaviour exhibited for Δ^1-T and Δ^6-T suggested that T is rapidly metabolized to produce these metabolites. Thereafter, it seems that both compounds are converted to other metabolites. Regarding Δ^1-AED and Δ^6-AED the progressive increase observed in their concentrations is analogous to the behaviour observed for Etio and Andros, suggesting that these compounds are generated in a slower way than AED, DHT, Δ^1-T and Δ^6-T.

5.3.4. Metabolic considerations

The occurrence of Δ^6-T and Δ^6-AED after the incubation of human hepatocyte cells with T, confirmed that T suffered a 6,7-dehydrogenation as a phase I metabolism pathway (Figure 5.1). This fact is in accordance with the occurrence of Δ^6-T after the incubation of liver microsomes from rat with T [15]. Additionally, the presence of Δ^1-T and Δ^1-AED also confirmed the 1,2-dehydrogenation as phase I metabolic pathway.

As explained above, T is rapidly metabolized to AED and thereafter this compound seems to be slowly converted to Andros and Etio. In the same way, AED might be slowly converted to Δ^1-AED and Δ^6-AED by a dehydrogenation in position 1 and 6, respectively. This hypothesis would explain the results of these metabolites during incubation times. Additionally, the close correlation observed for these compounds (Figure 5.3) supports the fact that these compounds have a common origin.

Similarly to AED and DHT, Δ^1-T and Δ^6-T seemed to be rapidly formed suggesting that they are directly generated from T. The close correlation observed for Δ^1-T and Δ^6-T supports a common origin for both compounds (Figure 5.3). Additionally, once formed, it is likely that these compounds can be also converted into Δ^1-AED and Δ^6-AED respectively (Figure 5.1).

5.4. Conclusions

The occurrence of Δ^1-AED, Δ^6-AED, Δ^1-T and Δ^6-T after the incubation of T with human hepatocyte cells has been successfully demonstrated. This fact confirmed that T suffered 1, 2 and 6, 7-dehydrogenations as hepatic phase I metabolic pathways. Therefore, the first step of the previously proposed metabolic pathway for the occurrence of cysteinyl metabolites in urine (Figure 5.1) has been proved. Results also suggested that T can be converted to Δ^1-T and Δ^6-T and these metabolites together with AED can be converted to Δ^1-AED and Δ^6-AED. In order to confirm the whole metabolic pathway proposed, glutathione conjugates should be unequivocally detected and identified.

Although the occurrence of naturally produced Δ^1-T has been widely discussed during the last years [1, 21-24], and it has been

reported in some individuals [9]. The metabolic mechanism which generates this compound was unclear. The data obtained from the hepatic incubation of T showed the formation of cysteinyl conjugates as one of the potential pathways for the occurrence of Δ^1-T in urine.

Since the metabolism of T can be seen as a basic metabolic pathway for many steroids, the confirmation of the existence of this alternative pathway for T would help in both the understanding of the formation of similar metabolites for other steroids [15, 25] and in the discovery of new metabolites of exogenous steroids. Therefore, several new biomarkers can be found by focusing on this alternative pathway of the steroid metabolism.

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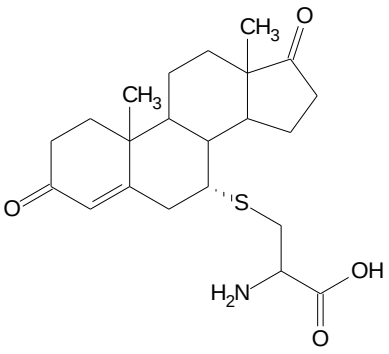
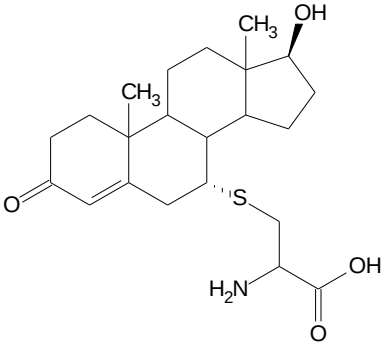
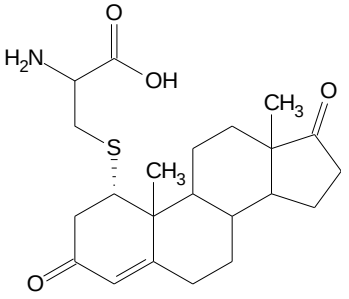
Part II:

Studies on cysteinyl metabolites

Abstract







The main objective of the II part of the thesis was to study the population distribution of the cysteinyl compounds and to establish reference limits for each marker. Additionally, in this part, an in deep study of factors that can influence the excretion of the cysteine conjugated compounds was performed. For these purposes, a quantitative method for the determination of cysteinyl compounds in urine was developed.

In chapter six, a rapid, accurate and sensitive method based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the quantification of the T and metabolites released under basic conditions (Δ^1-AED, Δ^6-AED, Δ^6-T and Δ^{15}-AD) were developed and validated. Urine samples (5 mL) were alkalinized and the released analytes were extracted by liquid-liquid extraction. The chromatographic separation was performed in 8 minutes. MS/MS determination was performed under selected reaction monitoring mode using electrospray ionization in positive mode. The method was shown to be linear from 0.05 to 50 ng/mL for Δ^1-AED, Δ^6-T and T while for Δ^6-AED the linearity from 0.5 to 500 ng/mL was demonstrated ($r>0.99$). Limits of detection below 0.05 ng/mL were obtained for all analytes. Intra-assay precision and accuracies, evaluated at three concentrations levels (0, 1, 1 and 20 ng/mL) were below 20%. The applicability of the analytical method was confirmed by analysis of several samples collected from healthy volunteers and samples collected after oral testosterone undecanoate administration. The analytical method was found to be able to quantify the metabolites either at the low concentrations levels presents in the urine from untreated volunteers and at the high concentrations found after T administration. Short analysis time, simple sample preparation and satisfactory quantitative

parameters make this method potentially useful for anti-doping control purposes.

In chapter seven, 173 urine samples collected from healthy volunteers were analysed by the method developed for the quantification of Δ^1-AED, Δ^6-AED, Δ^6-T and Δ^{15}-AD. The results obtained were used for the determination of the 97.5% and 99% of reference limit for each compound. Additionally, reference limits for different ratios including the cysteynil compounds were also determinated.

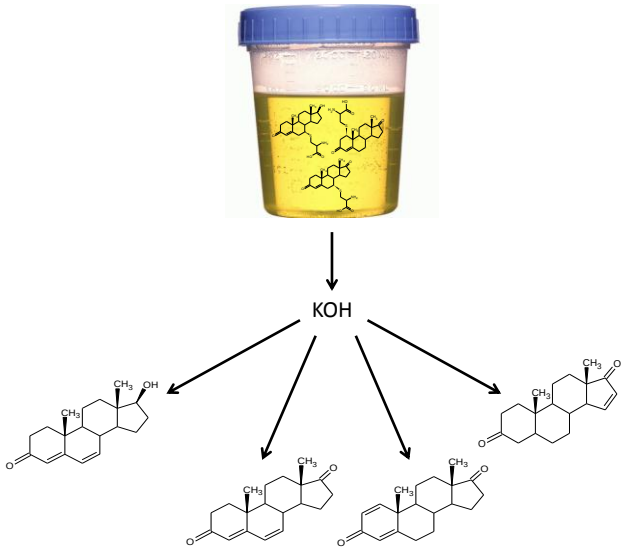
In chapter eight, an in deep evaluation of the factors that can influence the excretion of the cysteinyl compounds in urine were performed. The incorporation of the steroidal module of the athlete biological passport in doping control analysis makes the detection of small changes possible. These changes in markers could indicate the administration of endogenous anabolic steroids. For this reason, the study of intrinsic variations associated to each marker is of outmost importance. In this chapter, an in deep evaluation of the factors that can influence the excretion of the recently reported testosterone metabolites conjugated with cysteine (Δ^1-AED, Δ^6-AED, Δ^6-T and Δ^{15}-AD) was performed. The results were compared with those observed when using markers of the current steroid profile like the T/E ratio. A high stability was observed for these metabolites in front of factors influencing the sample preservation such as microbial degradation and freeze/thaw cycles. Similar to the T/E ratio, in addition to the gender related differences, a moderate ultradian and circadian rhythms were observed for the cysteine conjugated compounds.

Additionally, notable alterations in the excretion of these compounds were observed in the earliest stages of pregnancy. However, contrary to the observed for the T/E ratio, the UGT2B17 polymorphism, responsible for the low T/E ratio in Asian populations, did not influence the excretion of the cysteinyl compounds. Finally, the use of exogenous substances such as alcohol or 5α-reductase inhibitors dramatically affects the excretion of these cysteinyl compounds.

Overall, the presented data described the stability of the urinary cysteinyl steroids under the influence of many factors, proving their potential as adequate parameters to be included in the steroidal module of the athlete's biological passport.

Quantification of cysteinyl metabolites

Chapter 6



This chapter has been adapted from:

Fabregat A, Pozo OJ, Marcos J, Segura J, Ventura R. Quantification of testosterone and metabolites released after alkaline treatment in human urine. Drug Test Anal. 2010. 2(11-12):630-6. DOI: 10.1002/dta.227

6.1. Introduction

Anabolic androgenic steroids are the most commonly detected substances prohibited in humans sports. Testosterone (T) is the principal androgenic anabolic steroid in human and it is the most frequently reported adverse analytical finding among anti-doping laboratories [1]. T is available in a wide range of forms and dosages, e.g. it is available as gels, as an ester in oil by intra-muscular injections and oral preparations.

T has been used as a performance-enhancing substance in sport for decades [2] , and the performance benefits produced by T have been clearly demonstrated [3]**.** However, due to its endogenous nature, the detection of T misuse is an analytical challenge for doping control laboratories. The Testosterone/ Epitestosterone ratio (T/E) is the most well established reference marker for T misuse [4]. T/E ratios greater than 4 are considered indicative of T misuse and need further investigations [5]. The T/E ratio can be determined either by gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS) [5-7]. Despite this, the T/E ratio has several limitations: genetic aspects can have significant effects on urinary T/E excretion [8], values lower than 4 can be found in samples with a low basal T/E even after T administration [9, 10] . In addition, low retrospectivity in T misuse detection can be found depending on the route of administration (e.g., main changes in steroid profiles were reported during the 0-12 hours after oral administration [11]). Nowadays, the quantification of the steroid profile and the application of the athlete biological passport are used to minimize the drawbacks exhibited for the T/E. However, the measurement of the T/E ratio by GC/MS

is still the most sensitive marker for the screening testosterone misuse in sports.

The detection of additional metabolites can improve the screening for anabolic steroids by increasing retrospectivity and sensitivity. This fact has been demonstrated in the screening of some exogenous steroids, such as methandienone [12], stanozolol [13] and methyltestosterone [14], where the discovery of long-term metabolites allowed for an improvement in the detection of the misuse to these steroids.

In chapter 3, four additional T metabolites (Δ^1-AED, Δ^6-AED, Δ^6-T and Δ^{15}-AD) have been described in urine after the treatment with KOH and analysis by LC-MS/MS**.** The structures of three of them have been confirmed by analysis of reference compounds while Δ^{15}-AD has been proposed as feasible structure (see chapter 3). The cysteinyl origin of these compounds was also confirmed as a phase II metabolism for these compounds as demonstrated in chapter 4. As T metabolites, these compounds could help in the detection of T misuse in the same way that recently reported metabolites improved the detection of exogenous steroids [12-14]. In the case of exogenous steroids, the sole identification of the metabolite is enough to declare an adverse analytical finding and qualitative methods are normally applied for this purpose. Contrarily, due to their endogenous nature, T metabolites are present in every sample and screening methods for detection of T misuse should differentiate between basal concentration levels and the concentrations found after T administration. Moreover, the tendency in the anti-doping control field is to establish an individual biological passport where an accurate quantification is compulsory. Therefore, appropriate methods for the quantification of

endogenous metabolites in urine are required for their application in the doping control field.

The aim of the present work study was to develop and validate a quantitative method for the determination of these new cysteinyl T metabolites based on LC-MS/MS. The application of the developed method was tested by quantifying these metabolites in samples collected from healthy persons and samples collected after intake of a single oral dose of testosterone undecanoate.

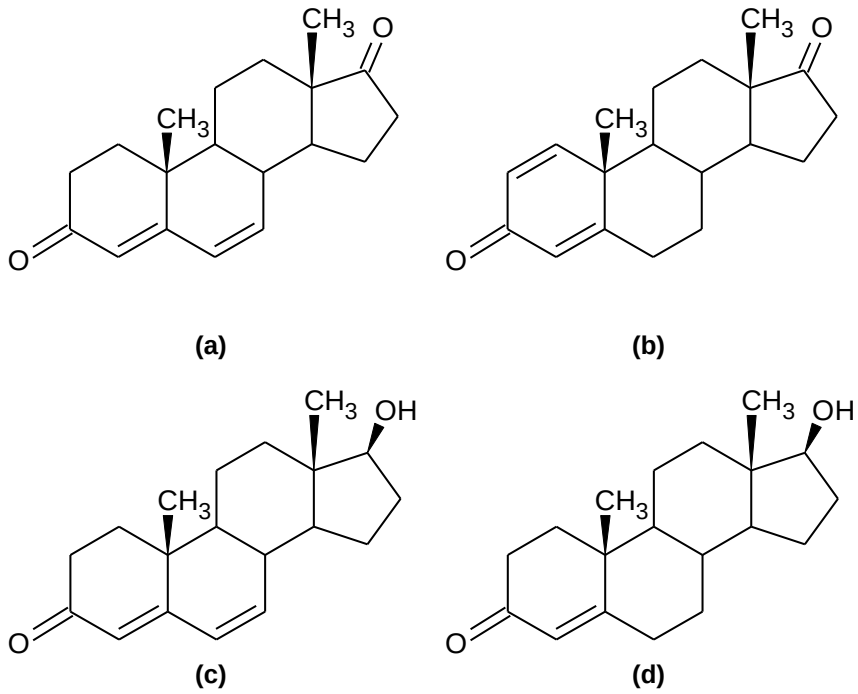


Figure 6.1. Structure for selected analytes **(a)** Δ^6-AED, **(b)** Δ^1-AED, **(c)** Δ^6-T and **(d)** Testosterone

6.2. Experimental

6.2.1 Chemicals and reagents

Δ^6-AED and Δ^6-T were obtained from Steraloids Inc. (Newport, USA). Δ^1-AED was purchased from NMI (Pymble, Australia). Methandienone, used as an internal standard (ISTD) and testosterone (T) were obtained from Sigma-Aldrich (St Louis, MO, USA). For structures of the analytes see Figure 6.1.

Citric acid, sodium bicarbonate, urea, calcium chloride, sodium chloride, iron II sulphate, magnesium sulphate, sodium sulphate, potassium dihydrogen phosphate, ammonium chloride, analytical grade potassium carbonate, potassium hydroxide pellets, di-sodium hydrogen phosphate, sodium hydrogen phosphate and *tert*-butyl-methyl ether were obtained from Merck (Darmstadt, Germany). Lactic acid, creatinine and urea were purchased from Sigma-Aldrich (St Louis, MO, USA).

Acetonitrile and methanol (LC gradient grade), formic acid (LC/MS grade) (Merck, Darmstadt, Germany). Ultrapure water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain). Detectabuse® columns were obtained from Biochemical Diagnostics Inc (Edgewood, New York, USA).

6.2.2 LC-MS/MS

Selected Reaction Monitoring (SRM) method was carried out using a triple quadrupole (Quattro Premier XE) mass spectrometer equipped with an orthogonal Z-spray-electrospray ionization source (ESI) (Waters Associates, Milford, MA, USA) interfaced to an UPLC system, Acquity (Waters Associates) for the chromatographic

separation. Drying gas as well as nebulising gas was nitrogen. The desolvation gas flow was set to approximately 1200 L/h and the cone gas flow to 50 L/h. A cone voltage of 25 V, and a capillary voltage of 3.0 kV were used in positive ionization mode. The nitrogen desolvation temperature was set to 450 ºC and the source temperature to 120 ºC.

The LC separation was performed using an Acquity UPLC BEH C_{18} column (100 x 2.1 mm i.d., 1.8 μm) (Waters Associates, Milford, MA, USA), at a flow rate of 300 μL/min. Water and methanol both with formic acid (0.01%) and ammonium formate (1 mM) were selected as mobile phase solvents. pH was not monitored, despite of this all steroids showed stable retention times. A gradient program was used; the percentage of organic solvent was linearly changed as follows: 0 min., 45%; 1 min., 45%; 3.5 min., 65%; 4.5 min., 95%; 5 min., 95%; 5.5 min., 45%; 8 min, 45%.

Analytes were determined by a Selected Reaction Monitoring method including two transitions for each compound (Table 6.1). The most abundant and specific was selected for quantitative purposes.

6.2.3. Sample preparation

After addition of the ISTD (methandienone 1 µg/mL, 50 µL), urine samples (5 mL) were directly alkalinized by addition of 300 µL of KOH 6 M (no previous enzymatic hydrolysis was performed). Incubation was performed during 15 minutes at 60 ºC. After cooling to room temperature, a liquid-liquid extraction was performed by addition of 6 mL *tert*-butylmethylether. The sample was centrifuged and the organic layer separated and evaporated.

The residue was dissolved into 150 µL of a mixture of water:acetonitrile (50:50, v/v) and 10 µL were directly injected into the LC-MS/MS system.

Analyte	Precursor ion		Product ion	
	m/z		m/z	Collision energy (eV)
Δ^1-AED	285	$[M+H]^+$	121[a]	20
			147	15
Δ^6-AED	285	$[M+H]^+$	149[a]	25
			97	35
Δ^6-T	287	$[M+H]^+$	151[a]	20
			133	25
T	289	$[M+H]^+$	97[a]	30
			109	20
Methandienone	301	$[M+H]^+$	121	20

[a] Ion selected for quantitative purposes

Table 6.1. MS/MS conditions for selected analytes

6.2.4. Artificial urine preparation

The artificial urine was prepared based on a protocol described elsewhere [15]**.** 0.1 g of lactic acid, 0.4 g of citric acid, 2.1 g of sodium bicarbonate, 10 g of urea, 0.07 g of uric acid, 0.8g of creatinine, 0.37 g of calcium chloride*$2H_2O$, 5.2 g of sodium chloride, 0.0012 g of iron II sulphate*$7H_2O$**,** 0.49 g of magnesium sulphate*$7H_2O$, 3.2 g of sodium sulphate*$10H_2O$, 0.95g of potassium dihyrogen phosphate, 1.2 g of di-potassium hydrogen phosphate, and 1.3 g of ammonium chloride were dissolved in one liter of ultrapure water. The artificial urine presented a pH of 6.8 and a specific gravity of 1.0126.

6.3. Method validation

6.3.1. Linearity

Calibration standards at ten concentration levels (0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20 and 50 ng/mL for Δ^1-AED, Δ^6-T and T and at 0.5, 1, 2, 5, 10, 20, 50, 100, 200 and 500 ng/mL for Δ^6-AED) were prepared in ultrapure water and analysed. Calibration curves were calculated by least-squares linear regression. Method was considered linear if a correlation coefficient (r) higher than 0.99 was obtained.

6.3.2. Intra-assay accuracy and precision

Quality control samples were prepared to check for precision and accuracy of the method. Since T and its metabolites are endogenous compounds ubiquitously present in human urine, quality control samples were prepared by spiking appropriate amounts of methanolic solutions of each compound to artificial urine. Three concentration levels were chosen along the calibration ranges. For Δ^1-AED, Δ^6-T and T, control samples were prepared at 0.1, 1 and 20 ng/mL. For Δ^6-AED the control samples were prepared at 1, 10 and 200 ng/mL.

Intra-assay precision of the method is expressed as the relative standard deviation (%) of the estimated concentrations obtained for four replicates of the quality control samples analyzed in one assay, at each concentration level. Intra-assay accuracy is expressed as the relative error (%) in the estimation of each concentration for the quality control samples.

6.3.3. Inter-assay accuracy and precision

Inter-assay precision is given as the relative standard deviation (%) of the estimated concentrations obtained for all replicates analyzed along the three validation assays and by two different operators (n=10, at each concentration level). Inter-assay accuracy is obtained as described for intra-assay accuracy (n=10, at each concentration level).

6.3.4. Limit of detection (LOD)

The LOD, defined as the lowest concentrations with a value of the signal/noise ratio (S/N) of 3, was calculated by estimating the S/N from the chromatogram at the lowest concentration assayed.

6.3.5. Limit of quantification (LOQ)

The LOQ was defined as the lowest concentration assayed, which gave acceptable recoveries and precision.

6.3.6. Stability

Freeze/thaw stability was studied during a week by performing three cycles of freezing at -20 ºC and thawing at room temperature. Concentrations calculated after these cycles were compared with those obtained in the analysis of the fresh urine.

6.3.7. Matrix effect

Due to the unavailability of truly blank urine samples, the matrix effect was evaluated by standard additions. In a first step, the concentrations levels of the analytes in different urine samples (n=6) were calculated by mean of an external calibration. Once the external calibration concentrations were established, standard additions were carried out in every sample by addition of four concentration levels (0.5, 1, 2 and 3 times the external calibration concentration). All samples were then analysed and the areas obtained were plotted against de added concentration. The standard addition concentration value was calculated for each samples using a least squares linear regression by extrapolation to the x axis.

The matrix effect was calculated for each analyte as the difference between the external calibration concentration and standard addition concentration.

6.3.8. Application to real samples

In order to evaluate the applicability of the developed method, 16 samples collected from healthy volunteers (males, 20-35 years) were analysed. Furthermore, post-administration samples collected from four volunteers after oral administration (from 0 to 4 h) of a single dose of 120 mg of testosterone undecanoate (TU) (Androxon^{TM}, three 40 mg capsules; Organon) were analysed. These samples were stored at -20 ºC until analysis. Ethical approval for the study was granted by Comité Ètic d'Investigació Clínica of our institute (CEIC-IMAS no.94/467) and the Spanish

Health Ministry (DGFPS no.95/75). All of the subjects participating in the study gave their written informed consent.

6.5. Results and discussion

6.5.1. Method optimization

The Δ^1-AED, Δ^6-AED and Δ^6-T were recently reported as T metabolites released after alkalinization of the sample (see chapter 3). The presence of these compounds in urine after basic treatment was demonstrated to arise from a cysteinyl conjugate as a phase II metabolism (see chapter 4).

Due to the low sensitivity exhibited for the direct detection of cysteinyl compounds (see chapter 4), an indirect method based on the qualitative approach used in chapter 3 was used. Briefly, urine were pre-concentrated in a Detectabuse® column, then, after enzymatic hydrolysis, the glucuronides and the free steroids were removed. Finally, a basic treatment (100 µL of KOH 6 M, 15 minutes to 60 ºC) was performed in order to extract the above mentioned metabolites. Although this method was found to be useful for qualitative purposes, some of the steps performed in the method exhibited uncontrolled losses of analytes which would hamper its quantitative application. For this reason, the method was revised in order to avoid these drawbacks.

Firstly, the efficacy of the column (a XAD-2 Detectabuse®) for the retention of the conjugated form of these metabolites was questioned. For this purpose, four urine samples were passed through the columns, previously conditioned with methanol and water. The column was then washed with water and both the

fraction corresponding to the loading of the sample and the water washing step, were mixed and analyzed as non-retained fraction. Finally, the column was eluted with methanol. This eluate was analysed as retained fraction.

The results for both fractions were compared. A significant percentage of the metabolites was detected in the unretained fraction indicating loses during this step which can affect the quantitative parameters of the method. For Δ^1-AED, Δ^6-AED and Δ^6-T the unretained fraction ranged from 8% to 71% while T was the highest retained compound and almost quantitative retention was achieved in most of samples**.** The percentage of analyte lost in the pre-concentration step was not constant, showing that the retention efficiency of these metabolites may depend in part of the urine matrix or the variability within the columns hampering the quantification of the analytes. For this reason, the pre-concentration step was eliminated.

A complete release of the analytes is crucial for the quantitative determination of the metabolites. In order to optimize incubation step, different concentrations of KOH and different times of incubation were tested using blank samples from healthy volunteers. The addition of different volumes of KOH 6 M was studied. Adding 300 µL of KOH 6 M allowed for the complete release of the cysteine conjugated phase II metabolites.

The incubation time was also studied. To optimize this step, three samples were analyzed using different times (5 min, 15 min, 30 min, 1 hour and overnight) with 300 µL of KOH 6 M at 60 ºC. The concentration for each sample was calculated (Table 6.2). The concentrations did not change between 5 minutes to overnight. Only T showed variations in the concentration obtained after

overnight incubation, probably due to the fact that higher matrix effects are obtained when increasing the incubation time. 15 minutes was selected to assure the complete release of the cysteinyl metabolites.

	Concentration (ng/mL)				
Analyte	**5'**	**15'**	**30'**	**1 h**	**Overnight**
Δ^1-AED	0.5	0.5	0.6	0.6	0.5
Δ^6-AED	25.7	25.9	28.3	25.2	27.9
Δ^6-T	2.8	2.5	2.9	2.6	3.2
T	0.8	0.8	0.9	0.8	0.5

Table 6.2. Concentrations obtained for each metabolite and the parent compound as a function of the incubation time in a sample from a healthy volunteer: 5 minutes (5'), 15 minutes (15'), 30 minutes (30'), 1 hour(1h) and overnight

The need of the hydrolysis step was also evaluated. Avoiding the enzymatic hydrolysis reduces the total analytical time but the partial degradation of cysteinyl compounds after basic treatment can affect the final concentration of the analytes. In order to check that common phase II metabolites were not degraded under basic conditions, testosterone glucuronide and sulphate standards were treated as described above. Neither testosterone nor other analytes were found in the extract confirming the stability of common phase II metabolites under the studied conditions.

Therefore, 5 mL of urine, 300 µL of KOH 6 M and 15 minutes at 60ºC were used for the indirect quantification of the cysteinyl compounds.

6.5.2. Method validation

The assay was shown to be linear over the range 0.05-50 ng/mL for Δ^1-AED, Δ^6-T and T, and over the range 0.5-500 ng/mL for Δ^6-AED reproducible linear relationship between concentration and response was found over the measured concentrations range. The correlation coefficients for all metabolites were higher than 0.99 (Table 6.3).

Analyte	Range (ng/mL)	Corr. coeff. (r)	Spiked conc. (ng/mL)	Error (%)	RSD intra-assay (%) (n=4)	RSD inter-assay (%) (n=10)	LOD (ng/mL)
Δ^1-AED	0.05-50	0.993	0.1	1.3%	16	11	0.006
			1	1.6%	7	10	
			20	-2.6%	5	11	
Δ^6-AED	0.5-500	0.992	1	-10%	18	19	0.001
			10	9.8%	4	10	
			200	-12.9%	6	8	
Δ^6-T	0.05-50	0.997	0.1	0%	10	12	0.047
			1	-4.2%	7	11	
			20	-11.7%	2	12	
T	0.05-500	0.994	0.1	-1.9%	23	28	0.006
			1	-1.7%	9	7	
			20	-13.4%	9	10	

Table 6.3. Linearity, precision, accuracy and LOD for each metabolite and the parent compound

Results for accuracy and precision are summarized in Table 6.3. Both, intra-assay RSD and inter-assay RSD were normally below 20%. These data indicates that the method provides adequate accuracy and precision for the detection of these three metabolites in urine samples. Only for T, RSD values exceeding 20% (23% for intra-assay and 28% for inter-assay) were obtained at the lowest assayed concentration.

The established LOQ for both Δ^1-AED and Δ^6-T was 0.05 ng/mL, whereas for Δ^6-AED a LOQ of 0.5 ng/mL was achieved. For T, the

LOQ was estimated at 1 ng/mL due to the poor precision obtained at 0.1 ng/mL. On the other hand, the estimated LOD ranged from about 1 pg/mL for Δ^6-AED to 47 pg/mL for Δ^6-T (Table 6.3). Typical chromatograms obtained for a blank artificial urine and an artificial urine spiked at 0.1 ng/mL are presented in Figure 6.2 showing the sensitivity of the method.

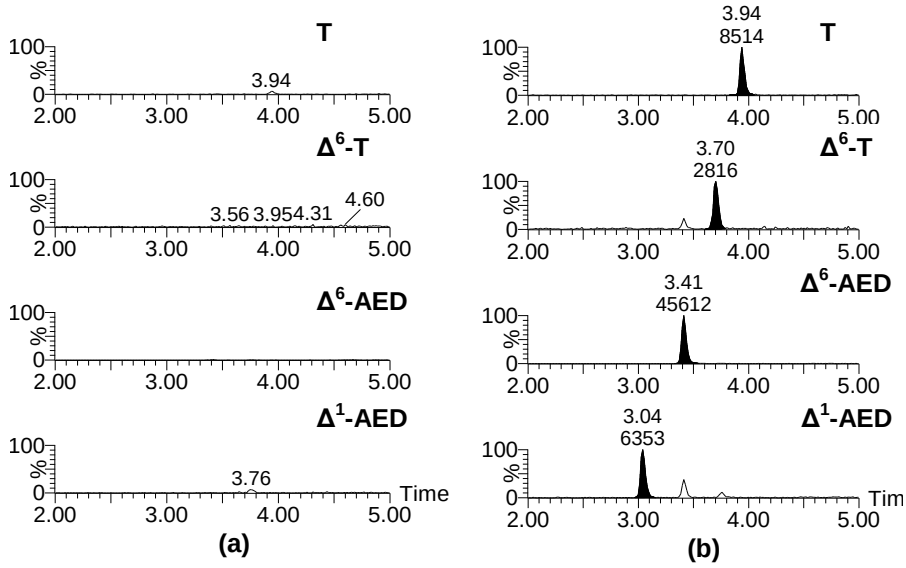


Figure 6.2. Chromatograms of **(a)** artificial urine and **(b)** artificial urine spiked to 0.1 ng/mL of T and metabolites

Urine samples were found to be stable during a week at –20 ºC and after three freeze/thaw cycles. Concentrations with deviations lower than 12% with respect to initial concentrations were found after these storage conditions.

Ion suppression or enhancement can affect the urinary quantification of anabolic steroids [16, 17]. The common approach

to quantify this matrix effect is the comparison between a blank sample spiked at a known concentration after extraction and a solvent spiked at the same concentration. Due to the endogenous nature of these compounds, the use of this approach was not possible. Isotope labelled compounds can be used to minimize this limitation [6]. However, isotopes labelled analogues for most of the selected compounds were not commercially available. Therefore, the matrix effect was evaluated by standard additions (Table 6.4).

The results for Δ^6-AED, Δ^6-T and T showed no differences between the concentrations obtained by external calibration and by standard additions indicating that no matrix effect was observed for these compounds. In the case of the earliest eluting compound (Δ^1-AED), an enhancement of around a 40% was observed probably due to some components of the matrix. The RSD of the 6 urine tested was also evaluated in order to check if the matrix effect was dependent on the sample. For Δ^1-AED, Δ^6-AED and Δ^6-T low RSD were obtained indicating that the matrix effect did not depend on the sample. The higher RSD obtained for T (26%) could be associated either with a higher variability of the matrix effect depending on the sample or with the poor precision obtained for T (see Table 6.3).

Concentration (ng/mL)	Δ^1-AED			Δ^6-AED			Δ^6-T			T		
	Std. Add[a]	Ext. cal[b]	Matrix effect (%)	Std. Add[a]	Ext. cal[b]	Matrix effect (%)	Std. Add[a]	Ext. cal[b]	Matrix effect (%)	Std. Add[a]	Ext. cal[b]	Matrix effect (%)
Vol.1	0.31	0.48	53%	35.1	34.7	-1%	3.36	3.37	0%	0.72	0.43	-40%
Vol.2	0.15	0.20	31%	18.9	16.9	-11%	3.04	2.91	4%	0.05	0.06	8%
Vol.3	0.23	0.31	35%	24.1	25.1	4%	2.19	2.11	4%	0.14	0.20	43%
Vol.4	0.10	0.14	34%	24.2	22.3	-8%	1.71	1.50	-13%	0.02	0.02	-8%
Vol.5	0.47	0.72	53%	44.1	41.4	-6%	3.52.	3.80	8%	0.14	0.17	15%
Vol.6	0.55	0.71	30%	96.0	70.3	-27%	6.43	5.98	7%	0.09	0.10	11%
Matrix effect[c] (mean)			+39%			-7%			-2%			+5%
RSD			8%			12%			7%			26%

[a]Concentration (ng/mL) obtained using standard addition approach.
[b]Concentration (ng/mL) obtained using external calibration.

Table 6.4. Matrix effect calculated with standard addition and external calibration for each metabolite and the parent compound.

6.5.3. Method application

The developed method was applied to quantify Δ^1-AED, Δ^6-AED, Δ^6-T and T in urine samples from volunteers who have declared not taking any forbidden substance (n=16) and samples collected after the intake of a single oral dose of TU (n=4). These metabolites were detected in all samples. For samples of untreated volunteers, the concentrations ranged from 0.06 to 0.5 ng/mL for Δ^1-AED, from 5.3 to 52.6 ng/mL for Δ^6-AED and from 0.6 to 7.9 ng/mL for Δ^6-T. In the case of T, concentrations between 0.02 and 0.4 ng/mL were obtained. However, since these values are below the LOQ established during validation, they should be taken as estimative.

For the samples collected between 0 and 4 hours after TU intake, higher concentrations were obtained for all T metabolites. Concentrations from 27.7 to 69.9 ng/mL for Δ^1-AED, from 73.5 to 195.4 ng/mL for Δ^6-AED, from 2.92 to 22.5 ng/mL for Δ^6-T and from 1.1 to 3.5 ng/mL for T were observed.

Therefore, the validated method is able to quantify both low concentrations obtained in non-treated volunteers and high concentrations obtained after oral testosterone administration. A moderate increase was observed for T, Δ^6-AED and Δ^6-T concentrations after T administration. However, in the case of Δ^1-AED, the concentrations after T administrations were more than 50 times higher than the obtained in basal conditions. This fact seems to indicate that Δ^1-AED can be an adequate marker for the detection of T misuse. The analysis of samples collected for longer periods after T administration would be necessary in order to confirm this suitability.

Representative chromatograms of a urine sample with a low concentration (untreated subject) and a urine sample with high

concentration (sample collected after TU intake) are shown in Figure 6.3. As it can be seen, the sensitivity of the method allowed for the correct determination of the metabolites at the low basal concentrations present in urine.

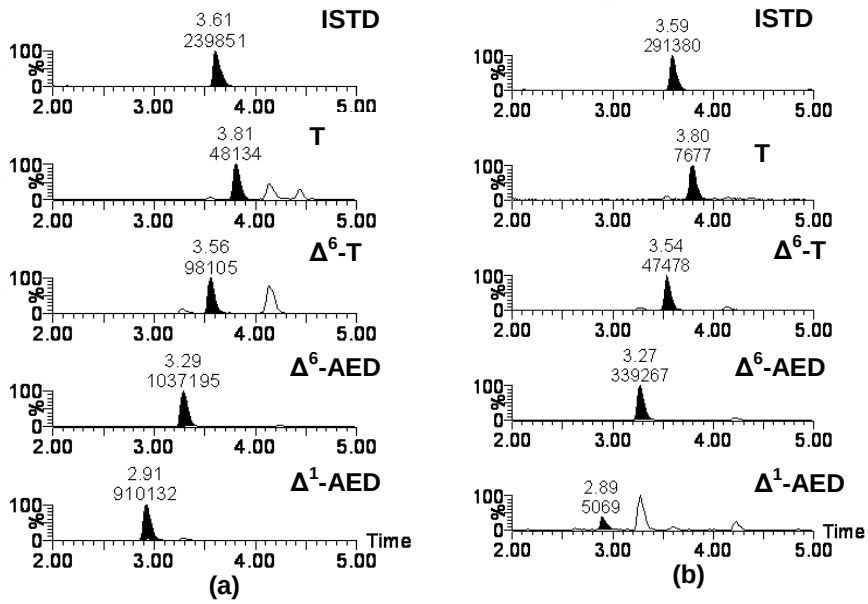


Figure 6.3. Chromatograms of actual urine samples: **(a)** sample collected from 0 to 4h after TU intake (Δ^1-AED: 43.7 ng/mL, Δ^6-AED: 104.7 ng/mL, Δ^6-T: 16.6 ng/mL, T: 4.5 ng/mL) and **(b)** sample from a untreated volunteer (Δ^1-AED: 0.14 ng/mL, , Δ^6-AED: 14.5 ng/mL, Δ^6-T: 1.9 ng/mL, T: 0.17 ng/mL)

6.6. Conclusions

A rapid, sensitive, accurate and precise quantitative method based on LC-MS/MS has been developed and validated for the indirect determination of Δ^1-AED, Δ^6-AED, Δ^6-T and T in urine after basic treatment. Up to our knowledge it is the first method for the urinary quantification of these metabolites.

The method allowed for the quantification of these analytes at both the concentrations found in untreated samples and the concentrations observed after T intake. Only in the case of T the poor precision obtained hampered the quantification at the basal concentration levels. However, the developed method allowed for the correct quantification of T at the levels detected in samples collected after T administration.

The satisfactory quantitative data obtained combined with the reduced analysis time resulting from a simple sample preparation and fast instrumental analysis makes this method appropriate for doping control analyses. The short overall analysis time is of special interest in the doping testing field, where a large number of samples have to be analysed in a short period of time.

The application of this method to samples collected before and after testosterone administration would be useful to evaluate the usefulness of these metabolites in the detection of testosterone misuse mainly in those cases in which the measurement of T/E shows several limitations.

6.7. References

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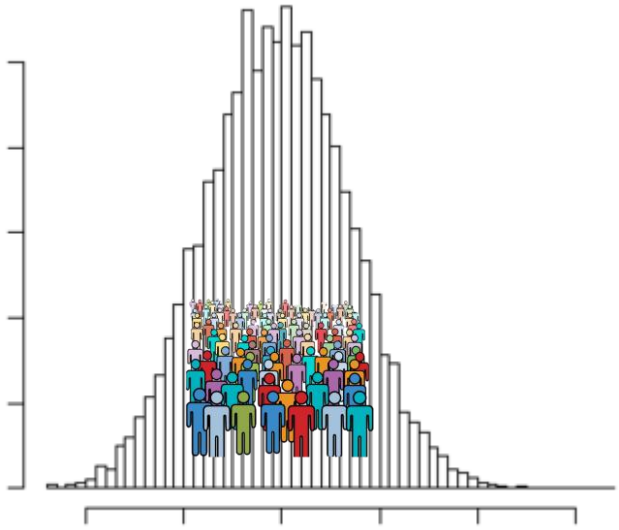
Population studies

Chapter 7



This chapter has been adapted from:
Fabregat A, Pozo O.J, Marcos J, Segura J, Ventura R. Alternative markers for the long-term detection of oral testosterone misuse. Steroids. 2011. 76(12):1367-1376. DOI: 10.1016/j.steroids.2011.07.005

7.1. Introduction

Androgenic anabolic steroids (AAS) can increase lean body mass and strength [1] and can cause several adverse effects for the athlete's health [2]. For these reasons, AAS are included in the list of prohibited substances by the World Anti-Doping Agency (WADA) [3]. According to the statistics published by WADA, the category of anabolic agents represents 50.6% of the reported cases by accredited doping control laboratories. Among those, 55.5% correspond to T, either as adverse analytical findings or as atypical findings reported [4]. Therefore, the quest for novel analytical approaches for the detection of T misuse is still one of the most important challenges in the doping control field.

Obviously, the main difficulty for the screening of T use is its endogenous nature. Doping control laboratories need to distinguish between T exogenously administered and that endogenously produced. For this purpose, the ratio of urinary testosterone to its isomer epitestosterone (T/E) excreted as glucuronides was proposed in 1983 as the first marker for T misuse [5].

Steroid profiling of the urine offered the most versatile and informative screening tool for the detection of T abuse in doping control [6]. This technique implies the quantitative determination of concentrations and ratios of different endogenously produced steroids, precursors and metabolites [7]. Currently the steroid profile includes several steroids which are quantified by GC-MS [8]. The ratio between T/E is considered as the most sensitive and robust parameter for the detection of AAS [5, 9]. In order to differentiate between a normal value of the steroid profile and altered value caused by doping with endogenous AAS, thresholds levels are mandatory as decision limits. In 2004, WADA establishes

a threshold for the T/E of 4 and recommended a confirmatory procedure with GC/C/IRMS if one of following criteria is found:

- T/E ≥ 4
- T or E glucuronides > 200 ng/mL
- Andros or Etio glucuronides > 10000 ng/mL
- DHEA glucuronide > 100 ng/mL

In the last version of the technical document for the detection of endogenous AAS (released in 2014), WADA recommended the application of the so-called athlete biological passport (ABP) and the application of the Bayesian model [10]. When a sample cannot be processed by the ABP, the laboratory shall confirm the sample if one of the above stated parameter is met.

In this thesis, four T metabolites: Δ^1-AED, Δ^6-AED, Δ^6-T and Δ^{15}-AD have been characterized (see chapter 3), and an adequate method for its quantitation in urine samples have been developed and validated in chapter 6. These metabolites are released after basic treatment of the urine from a cysteine conjugate (see chapter 4). Since they are T metabolites, these compounds can be used as markers for the detection of T misuse. In order to study the usefulness of these compounds for the doping control, the determination of threshold population limits are needed.

Therefore, the aim of this study is to determine reference limits for the cysteinyl testosterone metabolites and ratios between them based on the analysis of 173 urine samples collected from a healthy population.

7.2. Experimental

7.2.1. Chemicals and reagents

Δ^6-AED and Δ^6-T were obtained from Steraloids Inc. (Newport, USA). Δ^1-AED was purchased from NMI (Pymble, Australia). Methandienone, and methyltestosterone used as internal standard (ISTD) for LC-MS/MS and for GC-MS procedures respectively and testosterone and epitestosterone were obtained from Sigma-Aldrich (St Louis, MO, USA). The β-glucuronidase preparation (from Escherichia coli type K12) was purchased from Roche Diagnostics (Mannheim, Germany).

Analytical grade potassium carbonate, potassium hydroxide pellets, di-sodium hydrogen phosphate, sodium hydrogen phosphate, tert-butyl-methyl ether, and ammonium iodide were obtained from Merck (Darmstadt, Germany).

Acetonitrile and methanol (LC gradient grade), formic acid, ammonium formate (LC/MS grade) and cyclohexane were purchased from Merck (Darmstadt, Germany). Milli Q water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain). Detectabuse® columns were obtained from Biochemical Diagnostics Inc (Edgewood, New York, USA).

7.2.2. LC-MS/MS instrumentation

Selected reaction monitoring (SRM) method was carried out using a triple quadrupole (Quattro Premier XE) mass spectrometer equipped with an orthogonal Z-spray-electrospray ionization source (ESI) (Waters Associates, Milford, MA, USA) interfaced to an UPLC system, Acquity (Waters Associates) for the chromatographic separation. Drying gas as well as nebulising gas was nitrogen. The desolvation gas flow was set to approximately 1200 L/h and the

cone gas flow to 50 L/h. A cone voltage of 25 V, and a capillary voltage of 3.0 kV were used in positive ionization mode. The nitrogen desolvation temperature was set to 450 ºC and the source temperature to 120 ºC.

The LC separation was performed using an Acquity UPLC BEH C_{18} column (100 x 2.1 mm i.d., 1.8 μm) (Waters Associates, Milford, MA, USA), at a flow rate of 300 μL/min. Water and methanol both with formic acid (0.01%) and ammonium formate (1 mM) were selected as mobile phase solvents. A gradient program was used; the percentage of organic solvent was linearly changed as follows: at 0 min., 45%; at 1 min., 45%; at 3.5 min., 65%; at 4.5 min., 95%; at 5 min., 95%; at 5.5 min., 45%; at 8 min, 45%.

Analytes were determined by a SRM method including two transitions for each compound (see chapter 6). The most specific transitions (285>121 for Δ^1-AED, 285>149 for Δ^{6-}AED, 287>151 for Δ^6-T and 287>95 for Δ^{15}-AD) were selected for quantitative purposes.

7.2.3. GC-MS instrumentation

GC/MS was carried out on a 6890N gas chromatograph coupled with a 5975 MSD (Agilent technologies, Palo Alto, CA, USA). The steroids were separated on a HP-Ultra1 cross-linked methyl-silicone column, 16.5 m x 0.2 mm i.d., film thickness 0.11 μm (J&W Scientific, Folsom, CA, USA). Helium was used as the carrier gas at a constant pressure of 5 psi. A 2 μL aliquot of the final derivatized extract was injected into the system operated in split mode (split ratio 1:15). The GC temperature is ramped as follows: .at 0 min, 180 °C; then 3 ºC/ min up to 230 ºC; then 40 ºC/min up to 310 ºC; then 3 min to 310 ºC.

T and E were determined in SIM mode by monitoring *m/z* 432 at 13.2 min and 12.3 min respectively. Methyltestosterone used as ISTD was determined by measuring *m/z* 446 at 14.9 min.

7.2.4. Sample preparation

The method used for LC-MS/MS determination was based upon the method described and validated elsewhere in chapter 6. Briefly, after addition of 50 µL of ISTD (methandienone at 1 µg/mL), 5 mL of urine were basified by addition of 300 µL of KOH (6 M). The mixture was heated at 60 ºC for 15 min, followed by a liquid-liquid extraction with 6 mL of *tert*-butylmethylether. The sample was centrifuged and the organic layer separated and evaporated. The residue was dissolved into 150 µL of a mixture of water:acetonitrile (50:50, v/v). Finally, 10 µL were directly injected into the LC-MS/MS system.

For GC-MS, after addition of 50 µL of ISTD (methyltestoterone 50 ng/ml), 5 mL of urine were passed through a Detectabuse® column, previously conditioned with 2 mL methanol and 2 mL water. The column was then washed with 2 mL water and finally the analytes were eluted with 2 mL methanol. The methanolic eluate was evaporated under nitrogen stream at 50 °C, reconstituted in 1 mL of sodium phosphate buffer (0.2 M, pH 7), and hydrolysed with 30 µL of β-glucuronidase. The mixture was incubated at 55 °C for 1 h. After cooling to room temperature, 250 µL of a 5% potassium carbonate solution were added to the hydrolysate (pH = 9.5). Liquid-liquid extraction was performed by addition of 6 mL tert-butylmethylether. The sample was centrifuged at 3500 rpm, and the organic layer separated, evaporated to dryness under nitrogen stream and dried in a vacuum desiccator over phosphorus pentoxide. The extracts were reconstituted and derivatised with 50

µL of MSTFA:NH_4I:2-Mercaptoethanol (1000:2:6, v/w/v) for 20 min at 60 °C.

7.2.5. Urine samples

The population reference limits were established by the analysis of 173 urine samples (134 males and 39 females). The individuals providing these samples declared not to have taken any prohibited substance.

7.2.6. Data analysis

The urinary concentrations of the metabolites were corrected to a specific gravity of 1.020 according to WADAS's technical document on endogenous steroids [8] to reduce variability due to urine dilution.

Urinary concentrations of Δ^1-AED, Δ^6-AED, Δ^6-T and Δ^{15}-AD after alkaline treatment were obtained by comparing with a calibration curve. Due to the fact that authentic Δ^{15}-AD is not commercially available, this steroid was semi-quantified by assuming equal response to Δ^6-T (the analyte with the closest retention time). Ratios between the different metabolites were studied.

The program REFVAL was used for the determination of the upper RL [29]. This statically approach was recommended by the Expert Panel on Theory of Reference Values of the International Federation of Clinical Chemistry (IFCC). RL of 97.5% and 99% both with a 95% of confidence interval (CI) were chosen. The descriptive data for the distributions were calculated using the 500 iterative bootstrap method implemented in the REFVAL program. The option in the software for the detection of outliers was not activated.

7.3. Results

7.3.1. Concentrations

- **Testosterone**

Urinary concentrations of T after glucuronide hydrolysis were quantified by the conventional GC-MS method applied in routine doping control. The maximum and minimum concentrations obtained in the population study are summarized in Table 7.1. The 97.5% and 99% RL obtained for T concentration were 89 and 116 ng/mL respectively.

Compounds	Cmax (ng/mL)	Cmin (ng/mL)	97.5% RL	95% CI	99.0% RL	95% CI
T*	129	1	89	70-111	116	90-129
E*	123	0.5	102	71-124	110	102-123
Δ^1-AED	1.1	0.01	0.7	0.5-0.8	0.9	0.7-1.1
Δ^6-AED	60	0.15	56	38-60	59.4	54-60
Δ^6-T	10.6	0.02	7.9	6.9-10.3	10.4	7.9-10.6
Δ^{15}-AD	4.2	0.01	2.7	2.1-3.8	3.8	2.9-4.2

Table 7.1. The 97.5% and 99% RL with a 95% confidence intervals (95% CI) and maximum (Cmax) and minimum (Cmin) concentration obtained for T and E after glucuronide hydrolysis and cysteinyl metabolitest in population urine samples (n=173).

- **Cysteinyl metabolites**

The frequency distributions for Δ^1-AED, Δ^6-AED, Δ^6-T and Δ^{15}-AD concentrations of the population samples are shown in Figure 7.1. The minimum and maximum concentrations obtained for each metabolite and their 97.5% and 99% RLs with a 95% of CI are summarized in Table 7.1. Concentrations above to the limit of

detection of the method [28] (0.006 ng/mL for Δ^1-AED, 0.001 ng/mL for Δ^6-AED, 0.047 ng/mL for Δ^6-T) were obtained in all samples for all compounds. However, few samples (three for Δ^1-AED and one for Δ^6-AED, Δ^6-T and Δ^{15}-AD) were below the limit of quantification of the method i.e. 0.05 ng/mL for Δ^1-AED, Δ^{15}-AD and Δ^6-T and 0.5 ng/mL for Δ^6-AED. Concentration values in the sub-ng/mL range (between 0.01 and 1.1 ng/mL) were found for Δ^1-AED, while higher concentrations were obtained for Δ^6-T (between 0.20 to 10 ng/mL), Δ^{15}-AD (between 0.06 to 4.2 ng/mL) and Δ^6-AED (between 0.15 to 60 ng/mL).

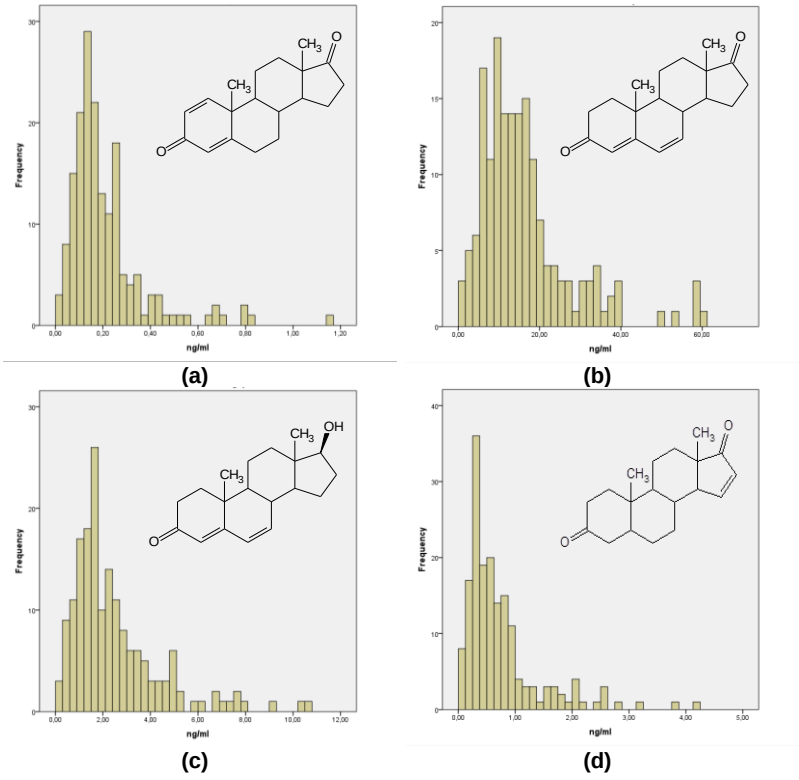


Figure 7.1. Histograms obtained for **(a)** Δ^1-AED, **(b)** Δ^6-AED, **(c)** Δ^6-T and **(d)** Δ^{15}-AD in the population study

7.3.2. Ratios

- **T/E**

The maximum and minimum values found for T/E in the analysis of the population samples were 0.05 and 7.0 respectively. The 97.5% and 99% RLs obtained with this data were 3.5 and 4.6 respectively (Table 7.2).The results obtained in the population study for T/E ratio started to show the commonly observed bimodal distribution [30, 31]. The two maximums modes for the T/E were observed around 0.13 and 1.16 (Figure 7.2).

- **Cysteinyl metabolites**

Twelve ratios can be performed by relation between the four cysteine conjugated metabolites. The population RLs of each ratio are summarized in Table 7.2. In order to have more intuitive data, a multiplication factor was applied to some of the ratios (Table 7.2). Monomodal distributions were obtained for all tested ratios (Figure 7.2). The RSD of the population data for all these ratios was also calculated. All ratios have a RSD between 50 and 80%. However, ratios performed between Δ^6-T and Δ^6-AED were found to be more constant among the population with a RSD of 25%.

- **Cysteinyl metabolites and E**

The four ratios between cysteine conjugated metabolites and E were studied. The population RLs of each ratio are summarized in Table 7.2. Monomodal distributions were observed for all four ratios (see supplementary material S-7.11). RSDs between 132 and 176% were obtained.

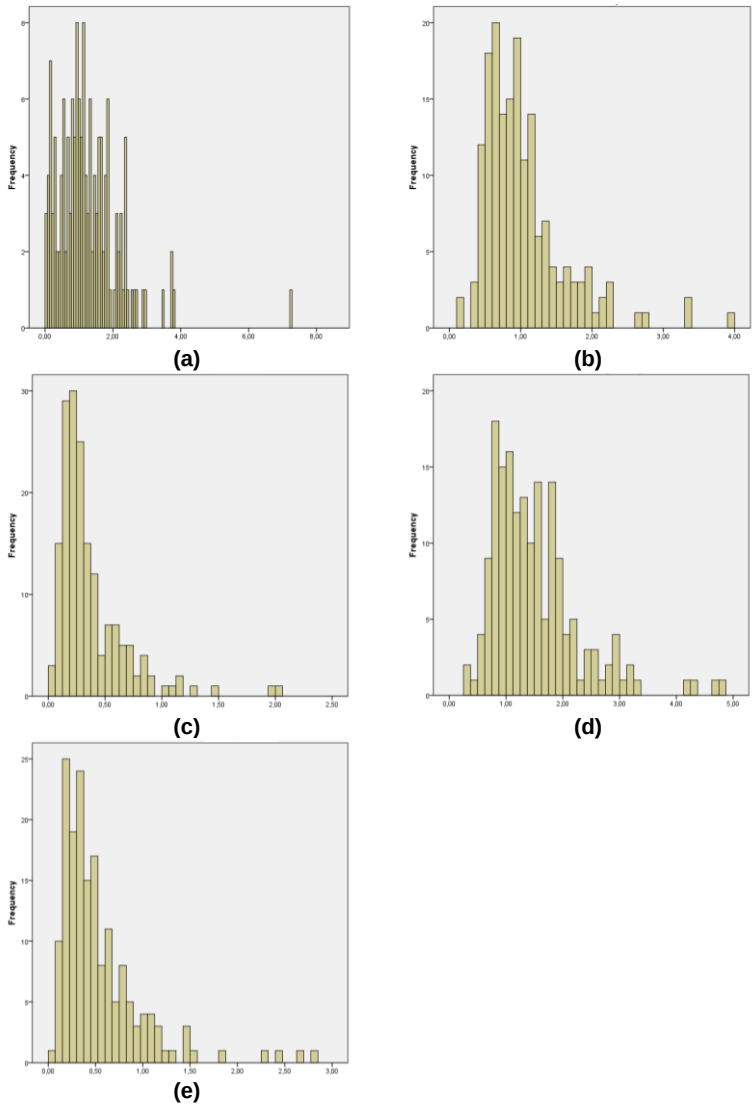


Figure 7.2. Histograms obtained for **(a)** T/E after glucuronide hydrolysis and **(b)** Δ^1-AED/Δ^6-T, **(c)** Δ^{15}-AED/Δ^6-T, **(d)** Δ^1-AED/Δ^6-AED and **(e)** Δ^{15}-AD/Δ^6-AED

Ratios	Min	Max	97.5% RL	95% CI	99.0% RL	95% CI
T/E	0.05	7.0	3.5	2.5-3.7	4.6	3.5-7.2
Δ^1-AED/Δ^6-AED (x100)	0.2	4.7	3.8	2.9-4.6	4.6	3.4-4.7
Δ^{15}-AD/Δ^6-AED (x10)	0.05	2.8	1.8	1.2-2.6	2.6	1.6-2.8
Δ^6-T/Δ^6-AED	0.07	0.3	0.2	0.2-0.3	0.3	0.2-0.3
Δ^1-AED/Δ^6-T (x10)	0.1	3.9	2.6	2.1-3.3	3.4	2.3-3.9
Δ^6-AED/Δ^6-T	3.6	14.7	10.9	10.3-14.7	12.4	10.8-14.7
Δ^{15}-AED/Δ^6-T	0.04	2.0	1.1	0.9-1.9	1.9	1.1-2.0
Δ^6-AED/Δ^1-AED (/100)	0.2	3.8	1.8	1.5-3.4	3.4	1.8-3.8
Δ^6-T/Δ^1-AED (/10)	0.2	6.4	2.9	2.1-6.2	6.2	2.6-6.4
Δ^{15}-AD/Δ^1-AED	0.4	21.7	10.0	7.8-14.6	14.6	9.3-21.7
Δ^1-AED/Δ^{15}-AED (x10)	0.4	24.1	14.1	8.8-19.6	19.66	24.1
Δ^6-T/Δ^{15}-AD	0.4	22.8	15.6	12.0-17.2	17.3	15.2-22.8
Δ^6-AED/Δ^{15}-AD (/10)	0.3	20.9	7.7	7.2-11.7	12.3	7.9-20.9
Δ^1-AED/E (x100)	0.03	23.5	8.9	5.2-19.9	20.9	9.3-23.5
Δ^6-AED/E	0.02	17.2	4.5	4.1-4.8	8.2	4.5-17.2
Δ^6-T/E (x10)	0.03	26.5	6.6	5.6-7.9	13.3	6.3-26.5
Δ^{15}-AD/E (x10)	0.02	11.0	2.8	2.1-4.0	5.3	2.7-11.0

Table 7.2. The 97.5% and 99% RL with a 95% confidence intervals (95%CI) and maximum and minimum values obtained for each ratio: T/E measured after glucuronide hydrolysis, and ratios within cysteinyl metabolites in population study (n=137)

7.4. Discussion

Population basal levels (n=173) were used for the establishment of 97.5% and 99% RL with 95% CI of the different markers. The 97.5% RL were chosen in order to establish the RL for doping control purposes. Although a larger number of samples will provide more precise RLs, the T/E threshold of 4 established by WADA for T misuse is very close to the 97.5% and 99% RLs obtained in the population study described in this paper (3.5 and 4.6, respectively). Therefore, it could be extrapolated that the RLs established in the present study samples are adequate. The RLs obtained for T concentration (89 ng/mL for the 97.5% RL and 116 ng/mL for the

99% RL) are quite far to the RL established by WADA (200 ng/mL). Nevertheless, these values are very similar to those previously reported with the analysis of 3000 samples [31] where a value close of 200 ng/mL was obtained for the 99,9 % RL.

In agreement with previously published data [30,31], T/E exhibited a bimodal distribution. Contrarily, all cysteine conjugated metabolites followed a monomodal distribution (Figure 7.1). An additional difference between T and the metabolites conjugated with cysteine is the low basal concentrations found for most of the compounds (typically below 10 ng/mL and in some cases like Δ^1-AED below 1 ng/mL). Thus, if it is intended to use these metabolites as markers for TU misuse, high sensitive methods are required.

7.5. Conclusion

The established reference limits can be useful to establish a threshold limit value for these compounds and ratios for doping control purposes. These thresholds limits can be used to distinguish between naturally occurring concentrations to those observed after the administration of testosterone for cysteine conjugated compounds.

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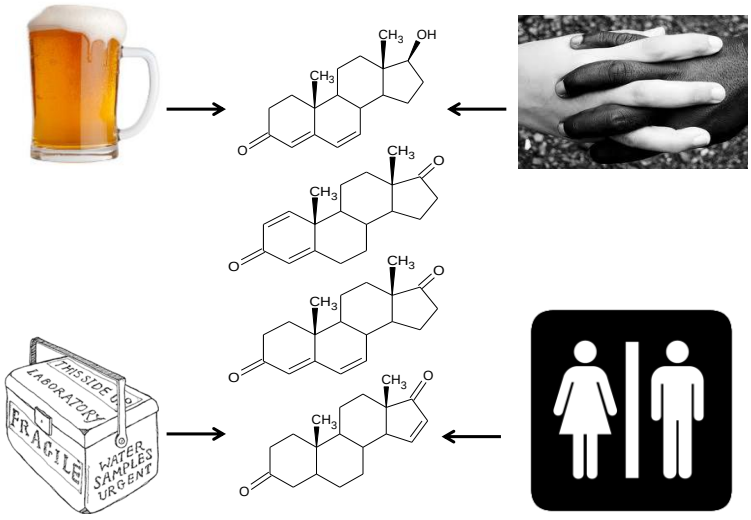
Factors affecting urinary excretion of cysteinyl metabolites

Chapter 8



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8.1. Introduction

Anabolic androgenic steroids (AAS) are the most frequently reported substances by doping control laboratories. Among them, administration of testosterone (T) is the most detected one with a 55.5% of the adverse analytical findings in 2012 [1]. Since T is naturally produced in the body, the mere analytical detection of T is not sufficient to report an adverse analytical finding. Thus, anti-doping control laboratories have to face the challenge of differentiating between endogenous production and exogenous administration.

The screening of T misuse remained impossible until Donike *et. al*. introduced the ratio between T and its 17α-epimer epitestosterone (E) as an indication of T intake [2]. Since then, the use of the T/E ratio has been the gold standard to distinguish between endogenous concentrations and those obtained after the exogenous administration of T. A sample is considered suspicious if an abnormal value of T/E above the reference limit established by the World Anti-Doping Agency, is detected [3]. However, for the ultimately confirmation the use of Gas Chromatography coupled to Combustion Isotope Ratio Mass Spectrometry (GC/C/IRMS) is mandatory in order to discern between exogenously administered steroids to those produced naturally in the body by their relative content of carbon isotopes [4-6].

In January 2014, an step forward was taken in the detection of T intake with the incorporation of the steroid profile in the so-called athlete biological passport (ABP) [3]. The ABP allows for the continuous and systematic storage and evaluation of the athlete's parameters of the steroid profile in a longitudinal way. This

approach uses individual reference limits for each athlete based on the data obtained throughout all the tests conducted worldwide for this subject [7]. Owing to these individual reference limits, the ABP is sensitive to detect endogenous steroid administrations by small but noticeable changes in the markers of the steroid profile. However, this characteristic also makes ABP largely sensitive to any source of intra-individual variability associated to the markers. For this reason, studies of the different factors that can alter the steroid profile which in turn may lead to a false suspicion of doping are of outmost importance [8, 9].

Currently, the steroid profile includes the quantification of the following compounds: T, E, Androsterone (Andros), Etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5α-Adiol) and 5β-androstane-3α,17β-diol (5β-Adiol) [3]. The inclusion of other minor T metabolites in the steroid profile has been presented as a powerful approach in order to increase the diagnostic specificity of ABP [10-12]. In this way, several T metabolites released after an alkaline treatment of the urine sample (Δ^1-AED, Δ^6-AED, Δ^6-T and Δ^{15}-AD) have been reported and characterized in chapter 3. These metabolites were shown to originate from a phase II cysteinyl conjugate (see chapter 4).

The aim of this study is to evaluate how the concentrations and ratios of the T metabolites conjugated with cysteine are effected by the factors which are known to influence the steroid profile. In order to make the study more understandable, the evaluated parameters are divided in three different categories: sample preservation (section 8.3), endogenous factors (section 8.4) and exogenous factors (section 8.5).

8.2. Experimental

8.2.1. Chemicals and reagents

Δ^6-AED and Δ^6-T, 5α-androstanedione (5α-AD), and 5β-androstanedione (5β–AD) were obtained from Steraloids Inc. (Newport, USA). Δ^1-AED was purchased from NMI (Pymble, Australia). Testosterone (T), epitestosterone (E), androsterone (Andros), etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5α-Adiol), 5β-androstane-3α,17β-diol (5β-Adiol), as well as the internal standards methandienone (MD) and methyltestosterone (MT) were obtained from Sigma-Aldrich (St Louis, MO, USA). The β-glucuronidase preparation (from Escherichia coli, type K12) was purchased from Roche Diagnostics (Mannheim, Germany). The DRI® EtG enzyme immunoassay was obtained from Thermo Fisher Scientific (Waltham, MA, USA).

Analytical grade potassium carbonate, potassium hydroxide pellets, di-sodium hydrogen phosphate, sodium hydrogen phosphate, *tert*-butyl-methyl ether, and ammonium iodide were obtained from Merck (Darmstadt, Germany). The derivatization reagent *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) was from Karl Bucher Chemische Fabrik GmbH (Waldstetten, Germany) and 2-mercaptoethanol was from Sigma-Aldrich (St Louis, MO, USA).

Acetonitrile and methanol (LC gradient grade), formic acid and ammonium formate (LC/MS grade) were purchased from Merck (Darmstadt, Germany). Milli Q water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).

8.2.2. Instrumentation

- **LC-MS/MS**

Selected reaction monitoring (SRM) method was carried out using a triple quadrupole (Quattro Premier XE) mass spectrometer equipped with an orthogonal Z-spray-electrospray ionization source (ESI) (Waters Associates, Milford, MA, USA) interfaced to an UHPLC system, Acquity (Waters Associates) for the chromatographic separation. Drying gas as well as nebulizing gas was nitrogen. The desolvation gas flow was set to approximately 1200 L/h and the cone gas flow to 50 L/h. A cone voltage of 25 V, and a capillary voltage of 3.0 kV were used in positive ionization mode. The nitrogen desolvation temperature was set to 450 ºC and the source temperature to 120 ºC. The LC separation was performed using an Acquity UHPLC BEH C_{18} column (100mm x 2.1 mm i.d., 1.7 μm particle size) (Waters Associates), at flow rate of 300 μL/min. Water and methanol both with formic acid (0.01% v/v) and ammonium formate (1 mM) were selected as mobile phase solvents. Additional details such as detailed gradient and SRM method used can be found in chapter 6.

- **GC-MS**

The GC–MS analysis was performed on an Agilent 6890 GC system coupled to a 5975B Vl MSD mass spectrometer from Agilent Technologies (Palo Alto, USA). The instrument was equipped with a 100% dimethylpolysiloxane Ultra1 column (17 m x 0.2 mm i.d., 0.11 μm film thickness) from J&W (Palo Alto, CA, USA). The temperatures of the instrument parts were 280 ºC for the injector, 280 ºC for the transfer line, 230 ºC for the ion source

and 150 ºC for the quadrupole. The GC temperature program was: at 0 min, 180 ºC; then 3 ºC/min up to 230 ºC, then 40 ºC/min up to 310 ºC, then 3 min at 310 ºC.

Helium was used as a carrier gas which was under constant pressure of 19 psi. For the steroid quantification Selective Ion Monitoring (SIM) was used with a dwell time of 20 ms for all monitored ions. Additionally, *m/z* 272 was monitored to screen for the possible presence of the mono-trimethylsilyl derivatives of androsterone and etiocholanolone which is an indication of incomplete derivatization.

8.2.3 Sample preparation procedures to measure steroid metabolites

- **Testosterone metabolites conjugated with cysteine**

Due to the low sensitivity achieved for the direct detection of the cysteynil compounds in urine samples (see chapter 4), the quantification of cysteinyl compounds (Δ^1-AED , Δ^6-AED, Δ^6-T and Δ^{15}-AD) was performed by an indirect method after the cleavage of the cysteine moiety based on the LC-MS/MS approach described in section 6. Briefly, after addition of 50 µL of the ISTD solution (MD, 1 µg/mL) 2.5 mL of urine were basified with 300 µL of a 6 M KOH solution. The mixture was heated at 60 ºC for 15 min, followed by a liquid-liquid extraction (LLE) with 6 mL of *tert*-butylmethylether. The sample was centrifuged and the organic layer separated and evaporated. The residue was dissolved into 150 µL of a mixture of water:acetonitrile (1:1, v/v). Finally, 10 µL were injected into the LC-MS/MS system.

For the microbial degradation studies (see section 2.5.1 and 3.1.1), an additional LLE with 6 mL *tert*-butylmethylether was performed prior to the alkaline treatment in order to separate the analytes present in the free faction. Thereafter, the sample was treated as stated above.

- **Conventional testosterone metabolites**

According to the WADA technical document, laboratories have to report the concentrations corresponding to the six steroids included in the steroid module of the ABP. These steroids are: T, E, Andros, Etio, 5α-Adiol and 5β-Adiol, and have to be quantified by GC-MS after the formation of the corresponding enol-TMS derivatives [3]. In the present study, these quantifications were performed by using a GC-MS procedure adapted from a method developed elsewhere [10]. Fifty µL of an ISTD solution (MT at 2 µg/mL) and 1 mL of phosphate buffer (1 M, pH 7) were added to 2.5 mL of urine. The mixture was hydrolyzed with 50 µL of β-glucuronidase during 1 h at 55 ºC. After cooling, the sample was basified with 250 µL of K_2CO_3 (25%, w/v). A LLE was performed by addition of 6 ml of *tert*-butylmethylether. The tubes were centrifuged, the organic layer separated, and evaporated under a nitrogen stream. The dry residue was derivatised with 50 µL of a MSTFA/NH_4I/2-mercaptoethanol (1000:2:6, v/w/v) mixture for 20 min at 60 ºC. Finally, 1 µL was injected into the GC-MS system working in split mode (20:1).

- **Ethyl glucuronide detection**

For the detection of Ethyl glucuronide (EtG) the DRI® EtG enzyme immunoassay was used. This immunoassay provides a semi-quantitative determination of EtG in human urine. The sample procedure for the detection of EtG was based on the method described elsewhere [13].

8.2.4 Correction of the urine dilution

In order to reduce the variability due to urine dilution, the urinary concentrations were corrected to a specific gravity of 1.020 according to WADA's technical document on endogenous steroids [14].

8.2.5 Ethical approval

Ethical approvals for all these studies was granted by the clinical research ethical committee of our institute (CEIC-Parc de Salut Mar, Barcelona, Spain). All subjects participant in these studies gave their written informed consent.

8.2.6. Statistical analysis

Statistical analyses were performed using SPSS version 18 (Jul 30, 2009). For the statistical analysis, a t-Student and its non-parametric analogous Mann-Whitney U tests were used. The normality of the different markers evaluated in this study was evaluated by using a Kolmogorov-Smirnov test. Statistical significance was taken as $p<0.05$.

8.3. Sample preservation

8.3.1 Introduction

Since in most of the cases, the analysis of urine samples for drug testing is not performed immediately after their collection, it is very important to evaluate the effect of storage conditions [15].

The fact that the urine samples are collected in non-sterile conditions offers the microorganisms the opportunity to grow, especially when samples are stored for a long time [9]. It is known that microorganisms can cause urinary alteration by hydrolysis of glucuronide and sulphate and can cause oxireductase reactions of endogenous steroids [16-18]. Therefore, bacterial activities in urine may cause significant changes in the measured steroid concentrations. The primary reaction that occurs is the deconjugation of glucuronides and sulphates [18, 19]. This fact seems not to be a major problem in doping control tests because both the conjugated and unconjugated fraction is analysed together, and therefore, no alteration are expected by the bacterial degradation [20]. However, this deconjugation is normally associated to other transformations like uncontrolled oxidoreduction or hydrolysis reactions, which might lead to the alteration of several steroid profile parameters. For instance, Mareck *et al*. reported an increased T/E from 5.3 to 9.8, determined in a combined fraction of conjugated and unconjugated steroids [8].

Markers of bacterial urine degradation are the accumulation of 5α-androstan-3,17-dione (5α-AD) and 5β-androstan-3,17-dione (5β-AD) which are the product of Andros and Etio deconjugation respectively, followed by a bacterial 3-hydroxysteroid-dehydrogenase activity. [21].

Additionally, in anti-doping controls, the urine sample is collected and divided into two aliquots (A and B). While aliquot A is analysed immediately upon arrival to the laboratory and may suffer several freeze/thaw cycles for conducting different tests, the B aliquot is stored at -20 ºC waiting for a possible counter analysis [22]. Knowledge about the stability of the steroids to different freeze/thaw cycles is critical for a proper interpretation of the results. In the case of the urinary T/E ratio, a high stability has been observed [20].

8.3.1 Studies on sample preservation

- **Microbial degradation**

Urine samples from 3 male and 3 female volunteers (age 39±11 years; weight, 72±14 kg) were collected in non-sterile containers. Each urine sample was divided into 6 aliquots of 5 ml each for the subsequent incubation at 37 ºC. Aliquots were incubated during 0, 1, 2, 3, 4 and 7 days, respectively. Every selected day, the aliquot was divided into two different sub-aliquots of 2.5 mL and analysed.

- **Freeze/thaw stability**

The effect of different freeze/thaw cycles were studied using urine samples from 3 male and 3 female volunteers (age 32±7 years; weight, 73±12 kg). For this purpose, 4 aliquots of each urine sample collected underwent 0, 1, 2 or 3 freeze (at -20 ºC) and thawing (at room temperature) consecutive cycles, respectively.

8.3.2 Results and discussion

- **Microbial degradation**

The microbial degradation of the urine sample was evaluated by monitoring the concentration of the commonly used markers for sample degradation, i.e. 5α-AD and 5β-AD (Figure 8.1a and 8.1b) [48]. As expected, the concentrations of these steroids increased with the incubation time of the urine sample at 37 ºC (Figure 8.1a and 8.1b). Whereas the concentrations of 5α-AD showed a rapid increase exhibiting a maximum at the third day of incubation (around 5 μg/mL), the concentrations of 5β-AD exhibited a progressive increase, reaching the value of 5 μg/mL at the seventh and last day of incubation (Figure 8.1b and 8.1b). The calculated concentrations of these compounds exceeded by far the values proposed as an indication limit for degradation (10 ng/mL for 5α-AD and 20 ng/mL 5β-AD) even in the first collected urine (after 2 days of incubation at 37 ºC) [23]. The maximum values observed for these markers exceeded more than 100 fold these values (Figure 8.1a and 8.1b) proving the intense microbial action in the incubated samples.

The results observed for the T/E ratio (Figure 8.1c) in this study were similar to those found in a previous study, where no significant variations (less than 30%) in the T/E values were observed [20, 24].

The effect of the microbial degradation in the selected cysteinyl steroids was evaluated by calculating the percentage of the free fraction and the percentage of the conjugated fraction in each volunteer. The mean and standard deviation (SD) for all analyzed volunteers were represented along the days of incubation.

Additionally, the total amount which is the sum of the two analysed fractions (conjugated and free) was monitored (Figure 8.1).

A similar behaviour after different days of incubation at 37 ºC was observed for the urinary concentrations of Δ^1-AED, Δ^6-AED, Δ^6-T and Δ^{15}-AD. An important increase in the percentage of the free compound in harmony with a decrease of the percentage of the conjugated compound was observed (Figures 8.1d-8.1g). For Δ^{15}-AD, a higher variation in the percentage of the conjugated form and the percentage of the free form was observed during the incubation (Figure 8.1g). These results are in agreement with the release of the cysteine moiety. A similar release behaviour was observed for T glucuronide after the incubation for 72 h at 37 ºC [16, 20]. Since the cleavage of the cysteine moiety is the first step of the analytical method (see chapter 6), the fact that the degradation process of these compounds mainly leads mainly to the cleavage of the cysteine conjugates minimizes its influence on the quantification of these steroid.

According to the quantitative results, the ratios Δ^1-AED/Δ^6-AED, Δ^1-AED/Δ^6-T showed variations below 30% during the incubation experiments (Figures 8.1h and 8.1i). These results are similar than those observed for the T/E ratio. However, due to the large variations observed for Δ^{15}-AD, higher variations (from 40% to 300%) were observed for the ratios involving Δ^{15}-AD (Figure 8.1j and 8.1l).

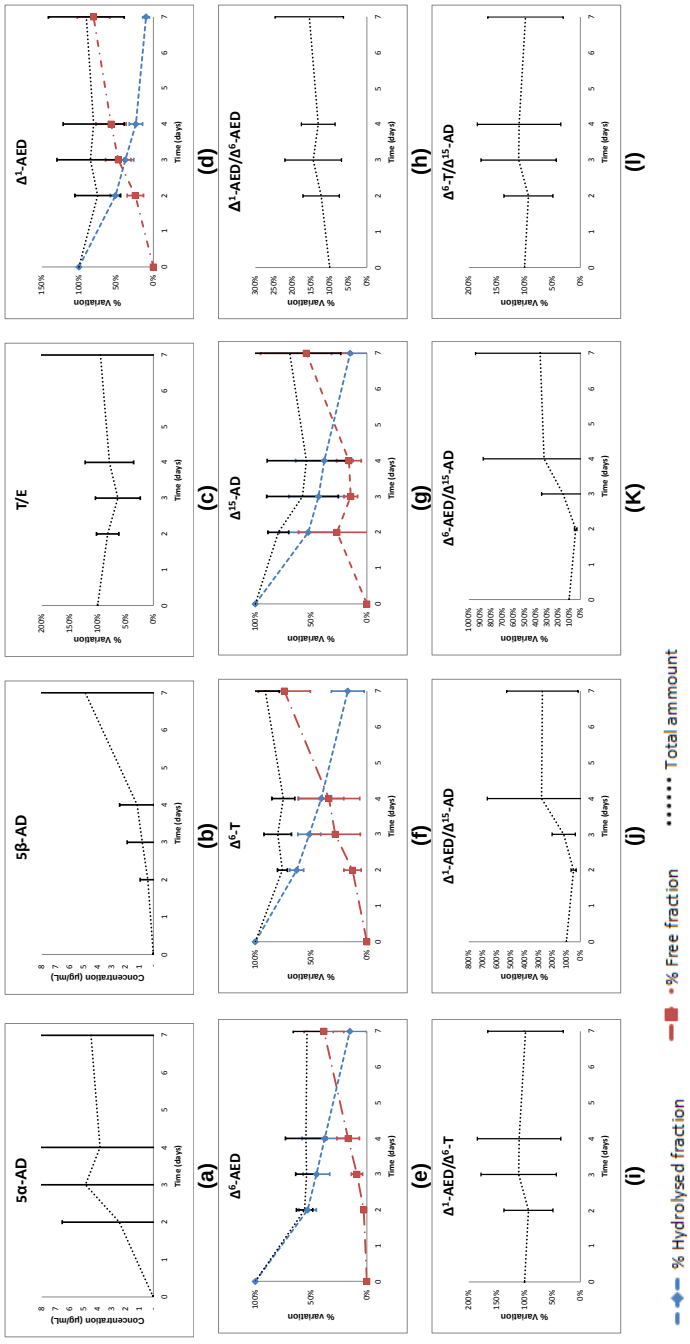

Figure 8.1. Representation along the time of the concentration (µg/mL) of the common markers of microbial degradation **(a)** 5α-AD and **(b)** 5β-AD, the % of variation of the hydrolyzed fraction, % of free fraction and % of total amount for the common marker **(c)** T/E and the selected markers among compounds conjugated with cysteine **(d)** Δ^1-AED, **(e)** Δ^6-AED, **(f)** Δ^6-T, **(g)** Δ^{15}-AD **(h)** Δ^1-AED/Δ^6-AED, **(i)** Δ^1-AED/Δ^6-T, **(j)** Δ^1-AED/Δ^{15}-AD, **(k)** Δ^6-AED/Δ^{15}-AD and **(l)** Δ^6-T/Δ^{15}-AD.

- **Freeze/thaw cycles**

The stability of the cysteinyl compounds and the ratios between them during freeze/thaw conditions were evaluated by calculating the individual relative standard deviation (RSD) for each marker after 3 freeze/thaw cycles in samples from 6 volunteers.

All compounds and tested ratios showed RSDs values below 15% after the different freeze/thaw cycles applied to the samples (Table 8.1). These results are comparable to those previously described for the T/E ratio, where variations less than 10% was observed after 3 cycles [20].

	Freeze/thaw
Compound	**RSD mean±SD**
Δ^1-AED (ng/mL)	14±4.9
Δ^6-AED (ng/mL)	11±4.8
Δ^6-T (ng/mL)	3±2.2
Δ^{15}-AD (ng/mL)	6±3.5
Δ^1-AED /Δ^6-T	12±4.8
Δ^1-AED/Δ^6-AED	5±2.0
Δ^1-AED/Δ^{15}-AD	12±4.0
Δ^6-AED/Δ^{15}-AD	10±4.2
Δ^6-T/Δ^{15}-AD	5±3.2

Table 8.1. Effect of freeze/thaw cycles for the cysteinyl compounds and the ratios selected between these compounds

8.4. Endogenous factors

8.4.1 Introduction

Due to the homeostasis of biosynthesis and metabolism of endogenous steroids [25], the amounts of steroids in 24 h periods are rather constant for a given individual [26]. However, the concentrations of the eliminated endogenous steroids may vary according to the urinary flow. The use of ratios between steroid compounds minimize this fluctuation [27]. Despite of this, T/E ratio showed variations greater than 30% in longitudinal studies [28, 29]. Besides, due to the influence of the daily circadian rhythm in the steroid excretion, plasma variations between 20-40% for T have been reported [30]. This circadian rhythmicity in the production of T is reflected in a moderate circadian rhythm in the urinary T excretion.

Some small differences were reported between genders. For instance, while the intra-individual variations in the T/E ratio in males is expected to be less than 30% [31], the observed variations in females are higher due to the menstrual cycle, and also in some extent to a higher analytical imprecision derived from the lower T and E concentrations [32]. Variation between genders were reported in the concentrations of androgens exhibiting lower concentration in female urine samples [28]. However, no significant changes were observed for the T/E ratio between genders [31, 32]. Other factors like extensive exercise can also influence the steroid excretion [33].

Pregnancy dramatically affects the maternal excretion of steroids. The changes observed in the steroid excretion are mainly due to the fact that products of the feto-placental unit have no other exit

than the maternal serum and urine [34]. Significant differences were observed in the T/E values in the first trimester of pregnancy when comparing with basal values. Whereas T remained almost unaltered during gestation, a significant rise was observed for E during the first weeks of pregnancy leading to a decrease in the T/E ratio [35]. At present, pregnancy is the only influencing factor that has been evaluated for the conjugated steroids with cysteine, and different behaviours were revealed [35]. A substantial rise of Δ^1-AED and a decrease of Δ^6-T concentrations were observed during the first trimester of pregnancy. Subsequently, all the ratios involving these compounds were affected. However, the Δ^{15}-AD and the ratios involving this compound were not monitored in that study.

Finally, large differences in the steroid excretion between Asian and Caucasian populations have been widely reported [36]. These differences are derived from a deletion in the gene UGT2B17 which encodes the enzyme responsible for the glucuronidation process of the T but not for the E [37]. This deletion was found to be more common in Asian ethnic descents. Since T is excreted mainly as a glucuronide conjugate, populations showing a deletion in this gene have lower low T/E values than the rest [38]. For this reason T/E exhibited a bimodal distribution with two subpopulation with respective means T/E around 0.1 and 1.0 [21, 39]. Consequentially, if a single cut-off value for the T/E ratio is applied to the whole population, athletes with the deletion will have an unfair advantage [40]. Therefore, the risk of a false negative result is higher in athletes carrying the UGT2B17 gene deletion. The application of the ABP will drastically minimized this problem since a subjected-based reference threshold is used.

8.4.2 Studies on endogenous factors

- **Ultradian variations and circadian variations**

For the study of ultradian variations, urine samples from 8 male and 8 female volunteers (age 39±10 years; weight, 65±16 kg) were collected weekly during two consecutive months.

Regarding the circadian variations, urine samples from the same 16 volunteers were collected during one day at four different times: 9 a.m., 1 p.m., 9 p.m. and 12 p.m.

- **Gender variations**

For the gender variations, the first morning urine from 8 males and 8 female volunteers (age 39±10 years; weight, 65±16 kg) were analyzed.

- **Ethnic variations**

Three urine samples from 6 Asian volunteers (age 51±7 years; weight 75±12 kg; T/E values below 0.2) and 6 Caucasian volunteers (age 27±5 years; weight 71±10 kg; T/E between 1 and 3) were investigated.

- **Pregnancy**

The effect of the pregnancy was evaluated by analyzing spot urine samples from a pregnant woman (age 35 years; weight, 45 kg) collected weekly before (n=9) pregnancy and during (n=8) the first trimester of gestation.

8.4.3 Results and discussion

- **Ultradian variations**

The ultradian variations of the steroids conjugated with cysteine, and the ratios among them, were evaluated by calculating the individual RSDs values for each marker in weekly collected samples (8 consecutive weeks) for 8 male and 8 female volunteers. The results stratified by gender are summarized in Table 8.2.

Variations below 36% were usually observed for the markers Δ^1-AED, Δ^6-AED Δ^6-T and Δ^{15}-AD in male volunteers (Table 8.2). Regarding female samples, higher RSDs values (between 33% and 40%) were observed for the urinary concentrations of these cysteinyl compounds (Table 8.2). This fact can be attributed to hormonal changes derived from the menstrual cycle as it was reported for the excretion of androgens in female samples [32]. The use of the ratios allowed minimizing the urinary variations for both male and female volunteers (Table 8.2). Nevertheless, the use of the ratios involving Δ^{15}-AD showed higher RSDs values. The high variability observed for these ratios may be caused by the fact that for Δ^{15}-AD a semi-quantification using Δ^6-T as reference compound was performed due to the lack of a reference material (see chapter 6).

In summary, variations observed for cysteinyl markers were usually lower than those observed for the T/E ratio in longitudinal studies, where a variations greater than 30 % were reported [28, 29].

- **Circadian variations**

Similarly to the ultradian variations, daily variations of male and female volunteers for the studied markers were evaluated by calculating the individual RSDs values in samples collected at

different times during the same day. Results are summarized in Table 8.2.

Variations around 30% were in general observed for the urine concentrations of Δ^1-AED, Δ^6-AED, Δ^6-T and Δ^{15}-AD for male volunteers. Higher variations (around 40%) were reported for the urine concentrations of these compounds in female volunteers (Table 8.2). Different behaviours were observed in the excretion of these compounds during the day depending on the gender. These data suggested the presence of a circadian rhythm in the excretion of these steroids conjugated with cysteine. The use of the ratios Δ^1-AED/Δ^6-T and Δ^1-AED/Δ^6-AED allowed minimizing the urinary variations of the circadian rhythm with RSDs around 15% and 20% for male and female volunteers, respectively (Table 8.2). In agreement with those results observed for the ultradian variations, higher RSDs were observed for those ratios involving Δ^{15}-AD.

Daily circadian variations observed for these compounds were similar to those results observed for the excretion of androgens both in male [31] and female [32] during the day.

- **Gender variations**

Genders differences were evaluated by calculating the mean and SD of male and female samples, respectively (Table 8.2).

The concentrations of Δ^6-AED, Δ^6-T and Δ^{15}-AD showed significant differences (t-test; $p<0.01$, $p<0.05$ and $p<0.01$, respectively) between genders (Table 8.2). Whereas higher values were found in female samples for Δ^6-AED (25.7 ng/mL vs. 16.8 ng/mL in male samples) and Δ^6-T (4.3 ng/mL vs. 3.7 ng/mL in male samples), the urinary concentrations of Δ^{15}-AD were shown to be higher in male samples (1.1 ng/mL vs 0.58 ng/mL in female samples). In contrast, no significant differences were observed for the urinary

concentrations of Δ^1-AED (Table 8.2). Results for Δ^6-AED and Δ^6-T are not in agreement with the observed for the excretion of androgens where lower concentrations are generally observed in female samples [26].

Regarding the selected ratios among these compounds, no significant differences were observed for the ratios Δ^1-AED/Δ^6-T and Δ^1-AED/Δ^6-AED (Table 8.2). This is in agreement with results observed for the T/E ratio, where no differences between genders have been observed [31, 32]. However, due to the large variations observed in the concentrations of Δ^{15}-AD between genders, notably differences were observed when using those ratios involving this compound (Table 8.2).

- **Genetic variations**

For the study of potential genetic variations the mean and SD values calculated for each marker were calculated for both Asian (T/E below 0.3) and Caucasian (T/E between 1 and 3) volunteers. The possible differences between these two ethnics groups were studied by using a t-student test with a $p<0.05$. The data observed for these two groups are summarized in Table 8.2.

The expected difference between Asian and Caucasian volunteers for the T/E ratio was confirmed: values of 0.16±0.05 and 1.7±0.97 were respectively obtained for the T/E ratio in Asian and Caucasian volunteers respectively.

	RSD mean±SD				Concentration mean±SD			
	Ultradian		Circadian		Gender variations		Genetic variations	
Compound	Male	Female	Male	Female	Male	Female	Caucasiac	Asiatic
Δ^1-AED (ng/mL)	26±10	40±13	27±10	41±26	0.26±0.11	0.31±0.37	0.24±0.17	0.24±0.14
Δ^6-AED (ng/mL)	23±10	36±11	31±26	39±23	16.8±10.1**	25.7±17.6**	19.6±14.2	20.6±17
Δ^6-T (ng/mL)	24±11	33±12	30±22	42±24	3.7±1.9*	4.3±2.9*	3.3±2.3	2.6±2.2
Δ^{15}-AD (ng/mL)	36±11	34±12	27±9	43±15	1.1±0.78**	0.58±0.57**	1.6±1.3	1.5±1.3
Δ^1-AED /Δ^6-T	17±7	24±12	15±9	18±12	0.96±0.60	0.78±0.57	0.9±0.67	1.2±0.9
Δ^1-AED/Δ^6-AED	18±4	22±7	15±12	16±10	1.8±0.77	1.2±0.76	0.17±0.03	0.13±0.02
Δ^1-AED/Δ^{15}-AD	37±15	32±11	25±18	32±7	3.2±1.8**	6.3±5.1**	2.3±4.1	3.1±4.5
Δ^6-AED/Δ^{15}-AD	35±19	24±4	31±19	29±4	2.1±1.5**	5.4±2.7**	1.7±1.6	1.7±1.2
Δ^6-T/Δ^{15}-AD	46±24	30±11	31±17	28±4	4.7±3.6**	9.6±5.6**	2.9±2.3	2.2±1.5

Table 8.2. Effect of ultradian, circadian, gender and genetic variations for the cysteinyl compounds and the ratios selected between these compounds.

The expected difference between Asian and Caucasian volunteers for the T/E ratio was confirmed: values of 0.16±0.05 and 1.7±0.97 were respectively obtained for the T/E ratio in Asian and Caucasian volunteers respectively.

Moreover, no significant differences were observed for the concentrations of Δ^1-AED, Δ^6-AED, Δ^6-T and Δ^{15}-AD among the two groups (Table 8.2). Similarly, no significant differences were observed when applying the selected ratios among cysteinyl steroids (Table 8.2). This fact contrasts with the results reported for the T/E marker in this study and previous works [38, 41] where significant differences (around 10 folds) for the T/E marker were found when comparing the values of Caucasian and Asian individuals. Thus, these data shows that the polymorphism in the UGT2B17 (observed in the 81% of the Asian population) [38] has no effect on the excretion of the cysteinyl metabolites. This behaviour for the cysteinyl metabolites was expected since UGT2B17 is related to the glucuronidation process.

- **Pregnancy**

In order to evaluate the effect of pregnancy in the excretion of cysteinyl markers during the first stages of gestation, the mean and SD of the quantification of the basal samples and samples collected during the first trimester of pregnancy was calculated (Table 8.3). The differences were compared by using a t-student test.

Whereas the concentrations of Δ^6-AED remained almost unaltered during the first trimester of pregnancy, a substantial increase in Δ^1-AED excretion followed by a slightly decrease in Δ^6-T concentrations were reported (Table 8.3). Due to these changes, a significant increase in the ratios Δ^1-AED/Δ^6-T and Δ^1-AED/Δ^6-AED

was also observed (for more details see [35]). The urinary concentrations of Δ^{15}-AD exhibited a significance increase (around 10 times) in the first trimester of the pregnancy as it can be seen in Table 8.3. Due to the large increases observed for Δ^{15}-AD, the ratios involving this compound showed a significant variation in their values during the first weeks of pregnancy. This behaviour follows somehow the results observed for the classical T/E ratio, where a decrease during the first trimester of pregnancy was observed (Table 8.3). The effect is however more pronounced in the cysteinyl compounds.

	Compounds				
	Δ^1-AED	**Δ^6-AED**	**Δ^6-T**	**Δ^{15}-AD**	**T/E**
Basal	0.21±0.08	36±11	4.1±0.5	0.085± 0.05	0.54±0.16
1st	0.40 ±0.16	40±12	3.9±0.88	1.0±0.4	0.32±0.03
p	0.018*	0.480	0.865	0.0001**	0.001**
	Ratios				
	Δ^1-AED/ Δ^6-T	**Δ^1-AED/ Δ^6-AED**	**Δ^1-AED/ Δ^{15}-AD**	**Δ^6-AED/ Δ^{15}-AD**	**Δ^6-T/ Δ^{15}-AD**
Basal	0.51±0.09	0.57±0.08	11.2±7.5	20.6±15.4	28.5±21.01
1st	1.0±0.46	0.97±0.25	3.9±1.4	4.0±0.88	3.8±0.4
p	0.000008**	0.000002**	0.019*	0.009**	0.005*

*p<0.05 **p<0.01

Table 8.3. Comparison of concentrations in basal samples and in samples obtained during the first trimester of pregnancy for metabolites Δ^1-AED, Δ^6-AED, Δ^6-T and Δ^{15}-AD, and selected ratios of these compounds as previously reported [35] and the ratios involving Δ^{15}-AD.

8.4 Exogenous factors

8.4.1 Introduction

It is known that several exogenous factors can influence the steroid pattern in urine. For instance, ethanol consumption may imply a direct change in the steroid production. Several hypothesis have been postulated in the literature for this behaviour [42, 43]. The most accepted one is that ethanol metabolism increases the ratio of NADH/NAD^+, which results in a supressed steroid oxidation [42]. The effects of the ethanol in the T/E ratios have been extensively studied [43-45]. The intake of a cute alcohol intake doses has been demonstrated to rise urinary T concentration, while E levels remain unaltered [44, 46]. Consequently, the oral intake of ethanol leads to a significant increase in the T/E ratio. The determination of ethyl glucuronide (EtG) and ethyl sulphate (EtS) are well-established markers for the ethanol consumption in clinical toxicology [47, 48]. Therefore, in order to avoid false positive results, ethanol markers have to be monitored in the suspicious urine specimens [49].

On the other hand, the use of 5α-reductase inhibitors such as dutasteride and finasteride can lead to important modifications in the steroid profile. These kind of drugs are used therapeutically to treat benign prostatic hyperplasia [50] as well as male baldness [51] by decreasing the 5α-reductase activity, and consequently the circulating levels of the potent endogenous androgen DHT. These drugs affects the production of 5α-steroids such as Andros or 5α-Adiol [52]. After the intake of a single 5 mg dose of finasteride the Andros/Etio ratio was reduced to a 10% of its original value in 24 hours, and the alteration was still present 8 days after the intake [48].

8.4.2 Studies on exogenous factors

- **Ethanol consumption**

For the ethanol (EtOH) consumption studies, 18 male volunteers (age 27±5 years and weight 75±5 kg) were selected. Three different studies (Placebo, sample dose 24 g of EtOH and sample dose of 42 g EtOH) were conducted. The ethanol studies were performed using a pharmaceutical preparation of absolute EtOH diluted with lemon flavored water (200 mL) at the required concentration. For the placebo study 200 mL of water with lemon flavor was given to the volunteers. The patients were randomized to treatment with placebo, 24 g of EtOH or 42 g EtOH (six volunteers for each treatment).

Different urine samples were collected, one sample before the treatment (0 h) and samples at 2 h, 4 h, 6 h 12 h and 24 h after the administration.

- **5α-reductase inhibitor studies**

For the 5α-reductase inhibitor studies, a single dose of 0.5 mg of dutasteride (one capsule of Avodart®) was given to one male volunteer (age 49 years; weight 87 kg). Urine samples before the administration (0 h) and at 3 h, 9 h, 15 h, 39 h and 72 h after the administration were collected.

8.4.3 Results and dicussion

- **Ethanol**

The effect of the EtOH consumption in the selected markers was evaluated by calculating the variations observed after the ingestion of placebo, 24 g and 42 g of EtOH. For this purpose, the relative variations of the markers after the administration were calculated. Figure 8.2 represents the mean and SD for all analysed volunteers, together with the concentrations of ethylglucuronide (EtG) in all urine samples.

As expected, a dose dependent increase in the EtG concentrations was observed after the EtOH ingestion (Figure 8.2a). In the same way, T/E values showed a two folds increase after the intake of 42 g of EtOH (Figure 8.2b). Basal values were recovered 12 h post-administration. These results are in agreement with previously published studies on the effect of the EtOH in the steroid profile [43-45].

The variation on the concentrations of the cysteine conjugated steroids and the ratios among them after the ingestion of EtOH are depicted in Figures 8.2c-8.2k.

Similarly to the results observed for the circadian rhythm (see section 3.2.2), placebo samples showed variations less than 40% for all selected concentrations and ratios within cysteinyl compounds (data not showed).

As it can be observed in Figures 2c-2f, no substantial dose dependent differences were observed for the concentrations of Δ^1-AED, Δ^6-AED, Δ^6-T and Δ^{15}-AD. However different behaviours were observed for the urinary concentrations of these compounds after the ingestion of EtOH. The urinary concentrations of Δ^1-AED, Δ^6-AED, and Δ^{15}-AED exhibited a decrease in their concentrations

(below 50%) after the EtOH administration; thereafter these compounds recovered the normal values (Figures 2c, 2d and 2f, respectively). However, the urinary concentrations of Δ^6-T showed a slight decrease in the first hours of the administration and, thereafter, an increase of around 100% was produced (Figure 2e). The decrease observed for Δ^1-AED, Δ^6-AED and Δ^{15}-AD is in agreement with the hypothesis that after EtOH ingestion the T metabolism is depressed [42]. However, the increase observed for Δ^6-T concentrations after the ethanol ingestion is similar to the behaviour observed for T concentrations. This fact supports the hypothesis that while Δ^6-T is a direct product of T, whereas Δ^1-AED and Δ^6-AED most likely are metabolites of androstendione (see chapter 5).

Since similar behaviour was observed for Δ^1-AED, Δ^6-AED and Δ^{15}-AD, the use of ratios involving these steroids allowed to minimize the variations (below 40%) after the EtOH ingestion (Figures 8.2g, 8.2i and 8.2j). Due to its increase, the ratios involving Δ^6-T exhibited higher variations (Figures 8.2h and 8.2k).

Thus, the use of the ratios Δ^1-AED/Δ^6-AED, Δ^1-AED/Δ^{15}-AD and Δ^6-AED/Δ^{15}-AED exhibited lower variations than those observed for the T/E ratio after EtOH ingestion.

- **5α-reductase inhibitors**

The effect of the use of 5α-reductase inhibitors was evaluated by monitoring the variation of the concentrations of the cysteinyl compounds and the variation of the selected ratios is illustrated in Figure 8.3.

The ratio between Andros and Etio (Figure 8.3a) was used for the evaluation of the 5α-reductase inhibitory effect. As expected, large decreases (around 7 times) were observed for the Andros/Etio

values after the administration of dutasteride (Figure 8.3a). These results are in agreement with the inhibition of the biosynthesis of Andros by the effect of the 5α-reductase inhibitor and the concomitant build-up in the Etio production.

Regarding, the cysteinyl metabolites, as it can be seen in Figures 8.3b-8.3k, after the administration of dutasteride, an important increase was observed for the concentrations of Δ^6-AED, Δ^6-T and Δ^{15}-AD (between 4 and 7 folds), compared to the increase of less than 1 fold observed for Δ^1-AED. Thus, the increase observed for the cysteinyl steroids is in agreement with that the hypothesis that the inhibition of one of the majors T metabolic pathways would lead to the activation of minor metabolic pathways which increase the biosynthesis of Δ^1-AED, Δ^6-AED, Δ^6-T and Δ^{15}-AD.

Due to the large increase observed for Δ^6-AED and Δ^6-T concentrations (Figures 8.3d and 8.3e), the ratios Δ^1-AED/Δ^6-AED and Δ^1-AED/Δ^6-T exhibited a decrease of 2 and 3 times respectively after the administration (Figures 8.3g and 8.3h). In the other hand, as a consequence of the lower increase observed for Δ^{15}-AD, the ratios involving Δ^{15}-AD showed an increase between 2 to 7 times after the drug intake (Figures 8.3i, 8.3j and 8.3k). The decrease exhibited for these ratios was in disagreement with the increase around 2 folds observed in the T/E values (Figure 8.3b). However, the use of the ratios Δ^1-AED/Δ^6-AED, Δ^1-AED/Δ^6-T and Δ^1-AED/Δ^{15}-AD (Figure 8.3g-8.3i) showed similar increases than the observed for the T/E marker.

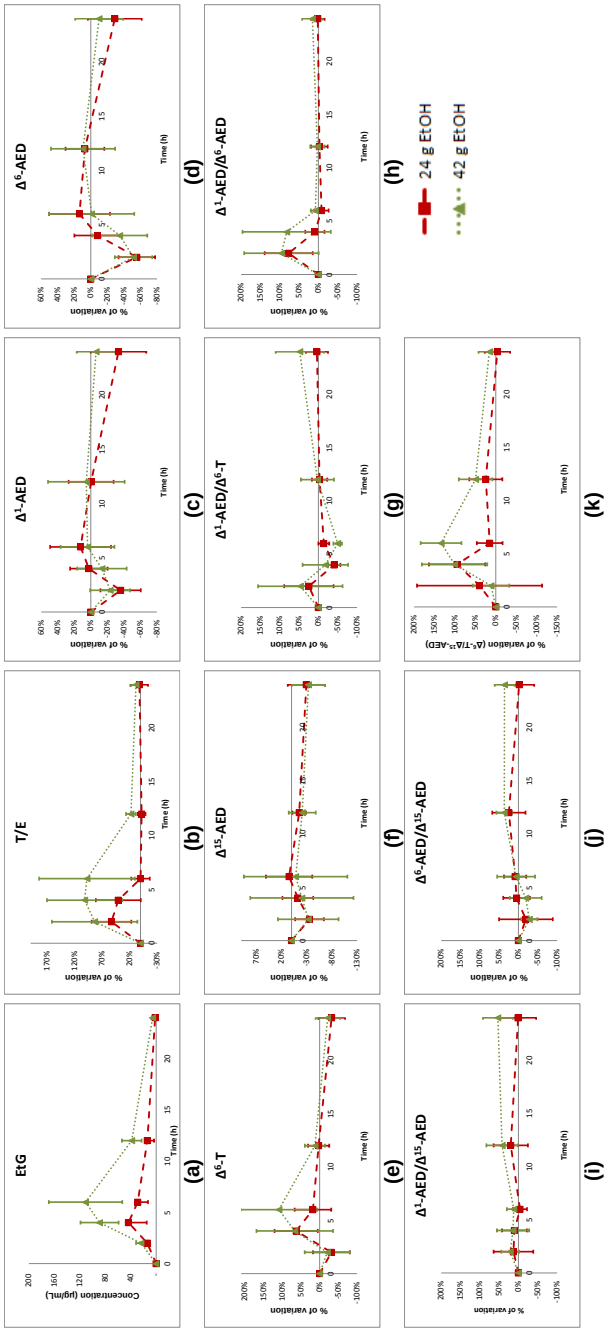

Figure 8.2. Variations observed for the marker for EtOH ingestion **(a)** EtG; and the variations observed for the ratios **(b)** T/E, **(c)** Δ^1-AED, **(d)** Δ^6-AED, **(e)** Δ^6-T, **(f)** Δ^{15}-AED **(g)** Δ^1-AED/Δ^6-AED, **(h)** Δ^1-AED/Δ^6-T, **(i)** Δ^1-AED/Δ^{15}-AD, **(j)** Δ^6-AED/Δ^{15}-AD and **(k)** Δ^6-T/Δ^{15}-AD after the ingestion of 24g or 42g of EtOH.

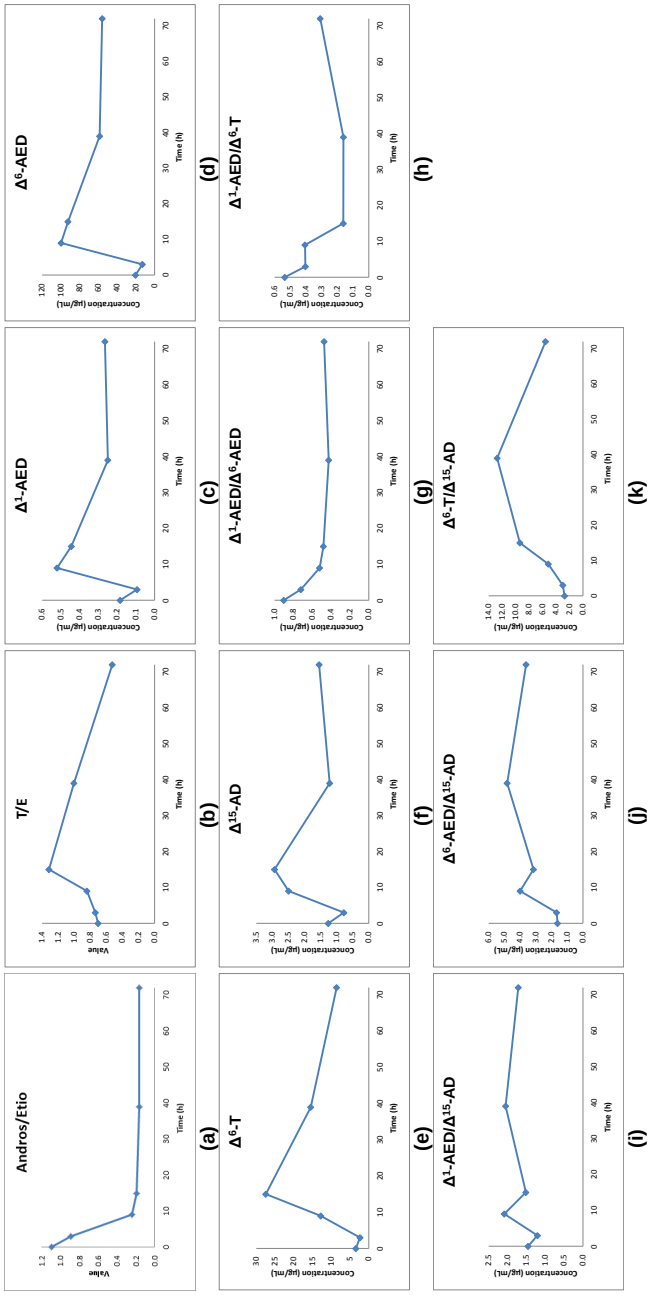

Figure 8.3. Variations observed for the ratios **(a)** Andros/Etio and **(b)** T/E and the variations observed for the concentrations (µg/mL) of the cysteinyl compounds **(c)** Δ^1-AED, (d) Δ^6-AED, **(e)** Δ^6-T, **(f)** Δ^{15}-AED; and the selected ratios between these compounds **(g)** Δ^1-AED/Δ^6-AED, **(h)** Δ^1-AED/Δ^6-T, **(i)** Δ^1-AED/Δ^{15}-AED, **(j)** Δ^6-AED/Δ^{15}-AED and **(k)** Δ^6-T/Δ^{15}-AED after the administration of 5α-reductase inhibitor.

8.6. General conclusions

The influence of the common factors that can alter the steroid profile has been studied for four steroids excreted in urine as conjugated with cysteine; Δ^1-AED, Δ^6-AED, Δ^6-T and Δ^{15}-AD.

These cysteinyl compounds showed to be relatively stables to those factors related to the sample preservation such as microbial degradation and freeze/thaw cycles. The results observed for these compounds were similar than those observed for the classical stable T/E ratio.

Regarding the endogenous factors influencing the excretion of these compounds, similar effects than those observed when studying the T/E ratio were observed. Moderate ultradian and circadian rhythms (with variations less than 30%) were found for the cysteinyl steroids. Gender variations on these metabolites were also revealed. In particular, higher concentrations of Δ^6-AED and Δ^6-T were observed in female urine samples. In addition to that, large alterations on the excretion of the cysteinyl compounds were observed during the earliest stages of pregnancy. As expected, the variations observed in the excretion of T in Asian populations due to the UGT2B17 polymorphism do not have effects in the excretion of the steroids conjugated with cysteine. Thus, the ethnic variations which are one of the major drawbacks when using a general T/E cut-off value can be circumvented by using ratios between cysteinyl compounds.

The uses of substances which are known to influence the steroid profile such as EtOH or 5α-reductase inhibitors lead to variations similar to those observed for the T/E ratio. After the EtOH ingestion, all cysteinyl compounds exhibited a decrease in their concentrations in the first hours after the intake. Only for Δ^6-T an

increase after EtOH administration was observed for several hours. The use of 5α-reductase inhibitors supposes an increase in the excretion of these compounds in urine probably by the activation of minor metabolic pathways.

Altogether, this study showed the potential of these compounds to be implemented in the steroid profile. The inclusion of the cysteinyl compounds would potentially improve the diagnostic specificity allowing the circumvention of some limitations exhibited for the current steroid profile.

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Part III:

New insights for doping control

Abstract

The main objective of this part is to study the usefulness of the cysteinyl compounds (Δ^1-AED, Δ^6-AED, Δ^6-T and Δ^{15}-AD) and ratios involving these compounds for the detection of oral and gel testosterone administration as well as the misuse of other endogenous anabolic androgenic steroids.

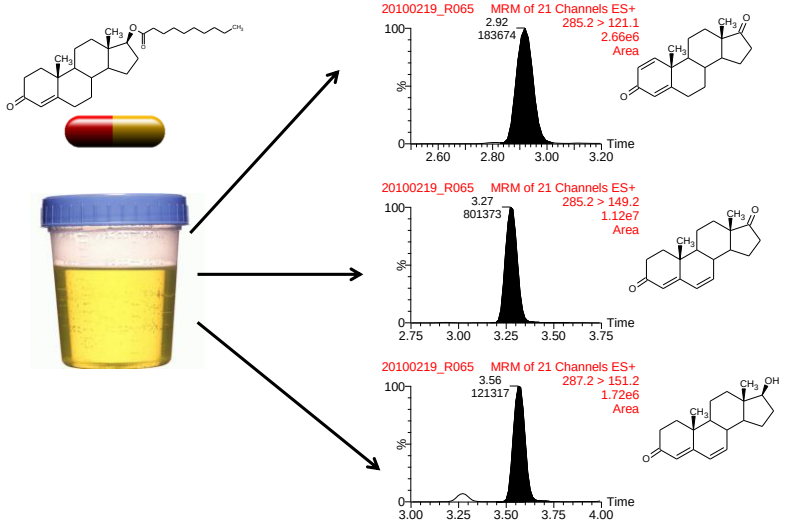
In chapter nine, the usefulness of the cysteine conjugated compounds was evaluated for the detection of testosterone oral administration. The results were compared with those obtained by using the classical marker T/E. Concentrations of Δ^6-AED and Δ^6-T showed similar behavior as the T/E, allowing the detection of the misuse several hours after the administration. More promising results were obtained by quantifying Δ^1-AED and Δ^{15}-AD. The time in which the concentrations of these analytes can be differentiate from the basal levels were between 3 and 6 times longer than the obtained with T/E, as a result, an improvement in the detection of testosterone abuse can be achived. Moreover, several ratios between these compounds were evaluated. Some of them improved the detection of testosterone misuse when comparing with T/E. The best results were obtained with those ratios involving Δ^1-AED.

In chapter ten, the applicability of the cysteinyl compounds was evaluated for the detection of testosterone gel and other different endogenous steroids in a single dose: dihydrotestosterone gel (DHT), oral dehydroepiandrosterone (DHEA), and testosterone gel. After independent administration of these endogenous steroids, a rise in the values of several of the ratios calculated between cysteinyl compounds was noticed. For DHT, a small increase was observed for the ratios Δ^1-AED/Δ^{15}-AD, Δ^6-T/Δ^{15}-AD and Δ^6-

AED/Δ^{15}-AD although only for one volunteer. Better results were obtained for oral DHEA and T gel where an increase was observed in all volunteers for several of the tested ratios. The detection time in which the misuse can be detected was evaluated using two different approaches: (1) comparison with population based reference limits, and (2) comparison with individual threshold levels. The obtained detection times were compared with the results of previously published markers for the misuse of such substances. When using cysteine conjugated compounds, shorter detection times for DHT, similar detection times for DHEA, and the detectability was substantially improved for testosterone gel administration

New insights for the detection of testosterone oral misuse

Chapter 9



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9.1. Introduction

Testosterone (T) is the principal androgenic anabolic steroid in humans. It is mainly produced in the testis and it is involved in the development of several tissues and processes [1]. T is virtually inactive when administered orally because it is rapidly metabolized mostly to inactive compounds. Its administration as an ester derivative can overcome this limitation. Testosterone undecanoate (TU) is an ester of T which can be used for the treatment of male hypogonadism [2].

The main difficulty for the screening of T use is its endogenous nature. Doping control laboratories need to distinguish between T exogenously administered and that endogenously produced. For this purpose, the ratio of urinary testosterone to its isomer epitestosterone (T/E) excreted as glucuronides was proposed in 1983 as the first marker for T misuse [3].

Nowadays, the measure of the T/E is still the gold standard for the screening of T administration. The T/E value can be obtained either by gas chromatography coupled to mass spectrometry (GC-MS) analysis after enzymatic hydrolysis of the urine, and silylation of the steroids [4-6], or by the direct detection of the glucuronides by liquid chromatography tandem mass spectrometry (LC-MS/MS) [7-9]. In recent years, a threshold T/E value of 4 was established by WADA [10]. As a result, samples with a T/E value higher than 4 require a confirmation analysis by isotope ratio monitoring gas chromatography mass spectrometry (GC/C/IRMS) [11]. Nevertheless, many of these confirmation analyses turn out negative, due to, either the athlete had a naturally elevated T/E ratio, or the GC/C/IRMS technique was not able to unequivocally

prove that part of the T metabolites present in the urine were of exogenous origin.

In spite of the satisfactory results obtained by this approach, the use of the T/E presents some drawbacks. It has been reported that in some cases, after the administration of T to individuals with low basal T/E (often due to genetic factors), the increased concentration of T is not sufficient to raise the T/E above the cut-off value of 4, thus hampering its detection [12, 13]. Other scenario where the T/E shows limitations has to do with the short-term detection of testosterone after an oral ingestion. In this case, the T/E value can only be distinguished from the basal level for a few hours (less than 12 h after ingestion), and in some samples the T/E is not affected [14-17]. The detection of the unmodified TU in plasma has been compared with the urinary T/E values but similar detection windows have been obtained [14]. Other analytical strategies have been evaluated for the detection of orally administered T, for instance the detection of the intact administered T esters in hair [15-17]. This approach proved its usefulness in detecting the abuse of T in bodybuilders [16] and in patients taking daily TU [16], however, its application after a single dose administration has not been reported yet. The strategy of detecting a comprehensive panel of urinary steroid by GC/MS, followed by an exhaustive study of alternative ratios has also been evaluated, but none of the proposed markers were found to be better than the classical T/E ratio [18-20].

An alternative and emerging approach is the establishment of individual threshold values. By this strategy, an individual threshold value is calculated considering all available samples for the same athlete. A sample is considered as suspicious if values above this individual threshold are obtained. The use of this approach could

overcome the individual and ethnical variations exhibited by the population reference limit [21, 22]. In fact, one year after the publication of these results, WADA adopted individual thresholds for the screening of T misuse by the application of the athlete biological passport [23].

In this thesis, four T metabolites: Δ^1-AED, Δ^6-AED, Δ^6-T and Δ^{15}-AD have been characterized (see chapter 3), and an adequate method for its quantitation in urine samples have been developed and validated in chapter 6. These metabolites were shown to come from a cysteine conjugated as a phase II metabolism (see chapter 4).

The use of these cysteinyl compounds released after basic treatment of the urine can be potentially used for the detection of T misuse but its applicability for doping control analysis has not been proved yet.

Therefore, the goal of this study is to investigate the usefulness of these metabolites conjugated with cysteine for the detection of orally administered T by the application of the reference population limits established in chapter 7. The results are compared with those obtained by the traditional T/E measurements.

9.2. Experimental

9.2.1. Chemicals and reagents

Δ^6-AED and Δ^6-T were obtained from Steraloids Inc. (Newport, USA). Δ^1-AED was purchased from NMI (Pymble, Australia). Methandienone, and methyltestosterone used as internal standard (ISTD) for LC-MS/MS and for GC-MS procedures respectively and testosterone and epitestosterone were obtained from Sigma-Aldrich (St Louis, MO, USA). The β-glucuronidase preparation

(from Escherichia coli type K12) was purchased from Roche Diagnostics (Mannheim, Germany).

Analytical grade potassium carbonate, potassium hydroxide pellets, di-sodium hydrogen phosphate, sodium hydrogen phosphate, tert-butyl-methyl ether, and ammonium iodide were obtained from Merck (Darmstadt, Germany).

Acetonitrile and methanol (LC gradient grade), formic acid, ammonium formate (LC/MS grade) and cyclohexane were purchased from Merck (Darmstadt, Germany). Milli Q water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain). Detectabuse® columns were obtained from Biochemical Diagnostics Inc (Edgewood, New York, USA).

9.2.2. LC-MS/MS instrumentation

Selected reaction monitoring (SRM) method was carried out using a triple quadrupole (Quattro Premier XE) mass spectrometer equipped with an orthogonal Z-spray-electrospray ionization source (ESI) (Waters Associates, Milford, MA, USA) interfaced to an UPLC system, Acquity (Waters Associates) for the chromatographic separation. Drying gas as well as nebulising gas was nitrogen. The desolvation gas flow was set to approximately 1200 L/h and the cone gas flow to 50 L/h. A cone voltage of 25 V, and a capillary voltage of 3.0 kV were used in positive ionization mode. The nitrogen desolvation temperature was set to 450 ºC and the source temperature to 120 ºC.

The LC separation was performed using an Acquity UHPLC BEH C_{18} column (100 mm x 2.1 mm i.d., 1.7 μm particle size) (Waters Associates), at flow rate of 300 μL/min. Water and methanol both with formic acid (0.01%) and ammonium formate (1 mM) were selected as mobile phase solvents. A gradient program was used;

the percentage of organic solvent was linearly changed as follows: at 0 min., 45%; at 1 min., 45%; at 3.5 min., 65%; at 4.5 min., 95%; at 5 min., 95%; at 5.5 min., 45%; at 8 min, 45%.

Analytes were determined by a SRM method including two transitions for each compound (see chapter 6) . The most specific transitions (285>121 for Δ^1-AED, 285>149 for Δ^6-AED, 287>151 for Δ^6-T and 287>95 for Δ^{15}-AD) were selected for quantitative purposes.

9.2.3. GC-MS instrumentation

GC/MS was carried out on a 6890N gas chromatograph coupled with a 5975 MSD (Agilent technologies, Palo Alto, CA, USA). The steroids were separated on a HP-Ultra1 cross-linked methyl-silicone column, 16.5 m x 0.2 mm i.d., film thickness 0.11 μm (J&W Scientific, Folsom, CA, USA). Helium was used as the carrier gas at a constant pressure of 5 psi. A 2 μL aliquot of the final derivatized extract was injected into the system operated in split mode (split ratio 1:15). The GC temperature is ramped as follows: at 0 min, 180 °C; then 3 ºC/ min up to 230 ºC; then 40 ºC/min up to 310 ºC ; then 3 min to 310 ºC.

T and E were determined in SIM mode by monitoring m/z 432 at 13.2 min and 12.3 min respectively. Methyltestosterone used as ISTD was determined by measuring *m/z* 446 at 14.9 min.

9.2.4. Sample preparation

The method used for LC-MS/MS determination was based upon the method described and validated in chapter 6. Briefly, after addition of 50 µL of ISTD (methandienone at 1 µg/mL), 5 mL of urine were basified by addition of 300 µL of KOH (6 M). The mixture was heated at 60 ºC for 15 min, followed by a liquid-liquid

extraction with 6 mL of *tert*-butylmethylether. The sample was centrifuged and the organic layer separated and evaporated. The residue was dissolved into 150 µL of a mixture of water:acetonitrile (50:50, v/v). Finally, 10 µL were directly injected into the LC-MS/MS system.

For GC-MS, after addition of 50 µL of ISTD (methyltestoterone 50 ng/ml), 5 ml of urine were passed through a Detectabuse® column, previously conditioned with 2 mL methanol and 2 mL water. The column was then washed with 2 mL water and finally the analytes were eluted with 2 mL methanol. The methanolic eluate was evaporated under nitrogen stream at 50 °C, reconstituted in 1 mL of sodium phosphate buffer (0.2 M, pH 7), and hydrolysed with 30 µL of β-glucuronidase. The mixture was incubated at 55 °C for 1h. After cooling to room temperature, 250 µL of a 5% potassium carbonate solution were added to the hydrolysate (pH = 9.5). Liquid-liquid extraction was performed by addition of 6 mL tert-butylmethylether. The sample was centrifuged at 3500 rpm, and the organic layer separated, evaporated to dryness under nitrogen stream and dried in a vacuum desiccator over phosphorus pentoxide. The extracts were reconstituted and derivatised with 50 µL of MSTFA:NH_4I:2-Mercaptoethanol (1000:2:6, v/w/v) for 20 min at 60 °C.

9.2.5. Urine samples

For the administration study, a single oral dose of 120 mg of testosterone undecanoate (Androxon™, three 40 mg capsules; Organon) was given to five healthy male volunteers. Ethical approval for the study had been granted by Comité Ètic d'Investigació Clínica of our institute (CEIC-IMAS no.94/467) and

the Spanish Health Ministry (DGFPS no.95/75). All of the subjects participating in the study gave their written informed consent.

Urine samples were collected before TU administration (3 samples) and at 4 h, 8 h, 12 h, 24 h, 36 h, 48 h and 72 h after TU administration. Aliquots of 50 mL of urine were frozen at –20 °C until analysis.

9.2.6. Data analysis

The urinary concentrations of the metabolites were corrected to a specific gravity of 1.020 according to WADAS's technical document on endogenous steroids [10] to reduce variability due to urine dilution.

Urinary concentrations of Δ^1-AED, Δ^6-AED, Δ^6-T and Δ^{15}-AD after alkaline treatment were obtained by comparing with a calibration curve. Due to the fact that authentic Δ^{15}-AD is not commercially available, this steroid was semi-quantified by assuming equal response to Δ^6-T (the analyte with the closest retention time). Ratios between the different metabolites were studied.

Assuming that the administration of T will increase the concentrations of the metabolites, only the upper limit was used in this study, because suspicious samples will be having a concentration higher than the reference limit (RL) established for each compound in the population studies (see chapter 7).

The detection time (DT) was defined as the maximum time after TU administration in which the concentration of each marker is above the upper RL obtained in the population studies. The DT obtained for each metabolite and ratio was compared with the DT obtained for T/E.

9.3. Results

9.3.1. Concentrations

- **Testosterone**

Basal concentrations below the 97.5% RL were obtained in all volunteers from the TU administration study. Relatively low urinary basal concentrations (between 3 and 20 ng/mL) were detected for volunteers 1 and 3, while higher urinary basal concentrations (between 28 and 51 ng/mL) were detected for volunteers 2, 4 and 5.

Marker	Population data	Detection time (h)				
		Volunteer				
	97.5%RL	1	2	3	4	5
T*	89	8	8	-	4	8
Δ^1-AED	0.7	24	36	12	24	24
Δ^6-AED	56	4	8	24	8	12
Δ^6-T	7.9	4	8	4	8	12
Δ^{15}-AD	2.7	36	36	12	24	24
T/E*	3.5	8	8	-	4	8
Δ^1-AED/Δ^6-T (x10)	2.6	24	all	12	24	24
Δ^{15}-AD/ Δ^6-T	1.2	36	24	8	8	8
Δ^1-AED/Δ^6-AED (x100)	3.8	24	48	12	24	24
Δ^{15}-AD/ Δ^6-AED (x10)	1.8	24	24	4	8	12
Δ^1-AED/E (x100)	8.9	12	12	12	12	24
Δ^6-AED /E	4.5	4	-	4	4	4
Δ^6-T/E (x10)	6.6	4	-	4	4	4
Δ^{15}-AD/E (x10)	2.8	8	8	8	8	8

*T and T/E values were obtained after enzymatic hydrolysis with β-glucuronidase (glucuronide+free fraction)

Table 9.1. Detection times for the proposed new markers after TU oral administration

Concentrations of T above the 97.5% RL were reported for all volunteers after TU administration with the main exception of volunteer 3, where no rises in T above de 97.5% RL were detected (Figure 9.1, Table 9.1). The highest concentration of T after TU

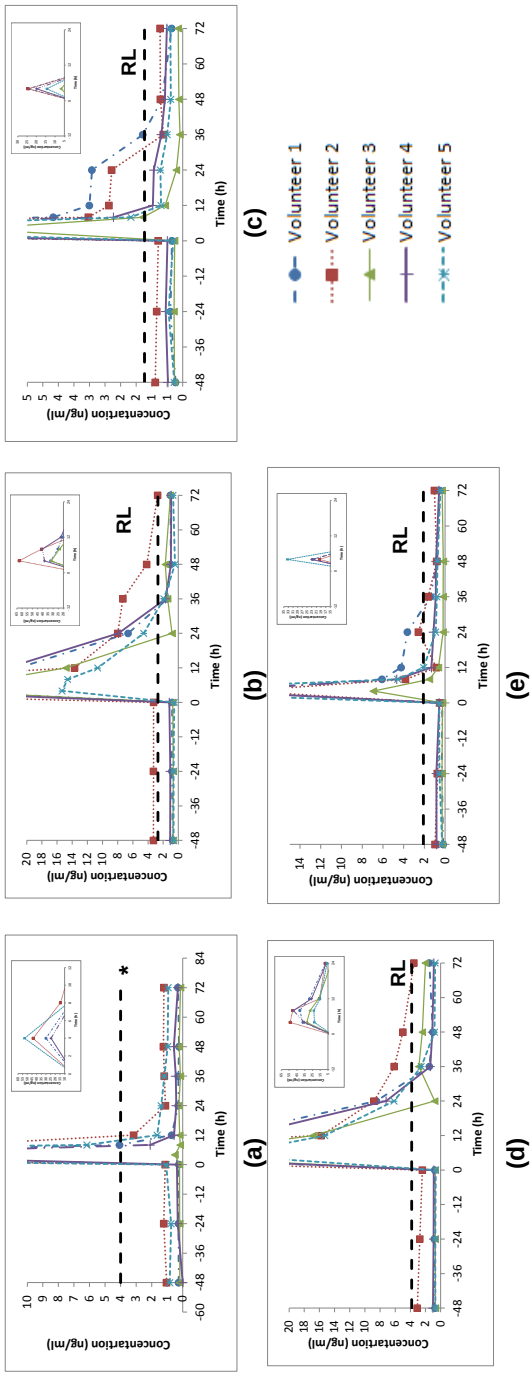
administration was found in the sample collected 4 h after TU administration for volunteer 2 (2456 ng/mL).

The DTs obtained for all volunteers using T concentration as a marker were between 4 and 8 h for all volunteers except for volunteer 3 (Table 9.1).

- **Cysteinyl metabolites**

Basal concentrations in the five volunteers participating in the TU administration study were below the 97.5% RLs for the Δ^1-AED (concentrations between 0.18 to 0.5 ng/mL) and Δ^6-T (concentrations between 0.7 to 4.2 ng/mL). In the case of Δ^{15}-AD and Δ^6-AED, most of the volunteers also presented basal concentration values lower the 97.5% RL. However, volunteer 4 had basal concentrations close to the 97.5% RL and in one of the three collected basal samples exceeded this value (Figure 9.1).

All four metabolites released after alkaline treatment raised their concentrations above the 97.5% RL after TU administration in all volunteers. The major increases were observed for Δ^1-AED and Δ^{15}-AD where concentrations up to 100 times higher than the 97.5% RL were found in the samples collected 4 h after TU administration. This increase was more moderate for Δ^6-AED and Δ^6-T where concentrations 3-4 times higher than the 97.5% RL were obtained in the samples collected 4 h after TU administration (Figure 9.1).



*WADA threshold value

Figure 9.1. Excretion profiles for urinary concentrations of **(a)** T glucuronide hydrolysis and **(b)** Δ^1-AED, **(c)** Δ^6-AED, **(d)** Δ^6-T, **(e)** Δ^{15}-AD with its own 97.5% RL.

All four metabolites released after alkaline treatment raised their concentrations above the 97.5% RL after TU administration in all volunteers. The major increases were observed for Δ^1-AED and Δ^{15}-AD where concentrations up to 100 times higher than the 97.5% RL were found in the samples collected 4 h after TU administration. This increase was more moderate for Δ^6-AED and Δ^6-T where concentrations 3-4 times higher than the 97.5% RL were obtained in the samples collected 4 h after TU administration (Figure. 9.1).

The use of Δ^6-AED and Δ^6-T concentrations as markers for TU misuse allows for DTs between 4 and 12 h. However, using the concentrations of Δ^1-AED and Δ^{15}-AD, DTs greater than 24 h were in general obtained. Shorter DTs were always observed for volunteer 3 (Table 9.1).

9.3.2. Ratios

- **T/E**

All the volunteers in the TU administration study showed basal T/E values below the 97.5% RL and the threshold established by WADA [24]. Three out of the five volunteers had basal T/E values close to the first maximum of the bimodal distribution. Thus, volunteers 1, 3 and 4 had basal T/E values of 0.2, 0.15 and 0.4 respectively. The basal T/E values for the other two volunteers were close to the second maximum of the population distribution (0.9 for volunteer 5 and 1.1 for volunteer 2).

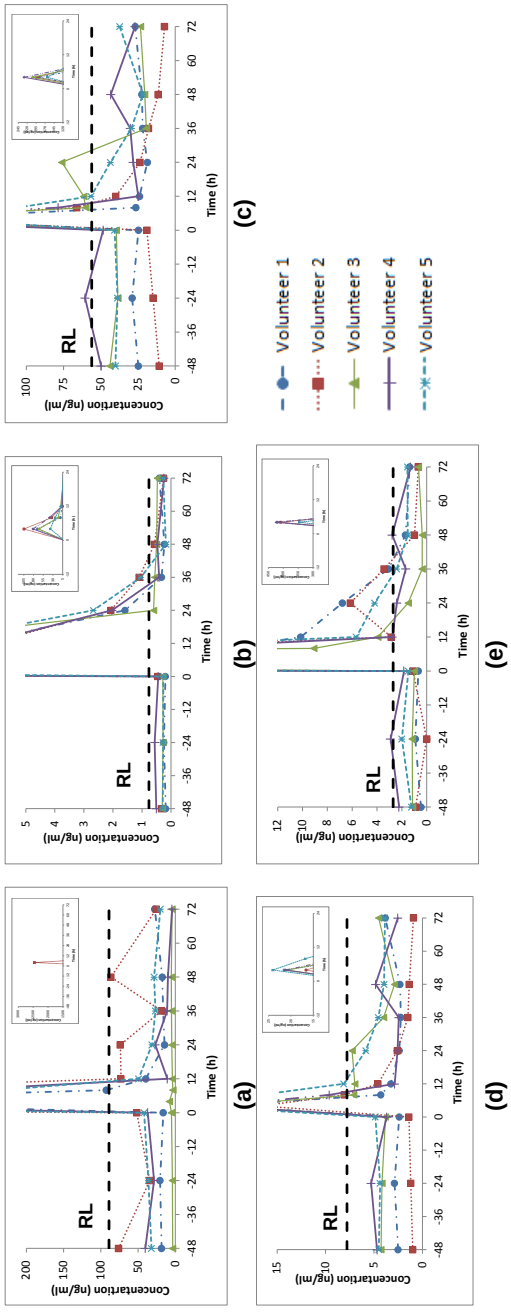


Figure 9.2. Excretion profile for **a)** T/E glucuronide hydrolysis and **(b)** Δ^1-AED/Δ^6-T, **(c)** Δ^{15}-AD/Δ^6-T, **(d)** Δ^1-AED/Δ^6-AED and **(e)** Δ^{15}-AD/Δ^6-AED compared with its own 97.5% RL

After TU administration, T/E values were more than 50 times higher than the basal ones. The values were normally above the 97.5% RL and the threshold established by WADA (Figure 9.2). The main exception was volunteer 3 in which no variation in T/E was observed. The major value in T/E levels after TU administration was observed for volunteer 5 (T/E=52.9).

Using T/E as marker for T misuse, DTs between 4 to 8h were obtained for all volunteers with the exception of volunteer 3.

- **Cysteinyl metabolites**

Basal values normally below the 97.5%RLs were obtained for all ratios for all the volunteers from the TU administration study. The main exception for this behaviour was volunteer 2 where the basal values for some ratios like Δ^1-AED/ Δ^6-T were above the 97.5% RL. After TU administration, the 97.5% RL was exceeded in only four of the twelve tested ratios (Δ^1-AED/Δ^6-T, Δ^1-AED/Δ^6-AED, Δ^{15}-AD/ Δ^6-T, Δ^{15}-AD/Δ^6-AED). Therefore, these were the only ratios studied and discussed in this study (Figure 9.2). Increases of more than 20 times in the value of these ratios were normally observed after TU administration (Figure 9.2). This rise was observed for all volunteers in all ratios.

The use of Δ^{15}-AD/Δ^6-AED and Δ^{15}-AD/Δ^6-T as markers for TU misuse allowed for DTs between 4 and 24 h while longer DTs (between 12 and 48 h) were obtained by the use of Δ^1-AED/ Δ^6-T and Δ^1-AED/Δ^6-AED (Table 9.1).

- **Cysteinyl metabolites and E**

Basal values below the 97,5% RL were reported for all volunteers from the TU administration study (see supplementary material S-9.12). After oral TU administration, all of the four ratios within the

cysteine conjugated metabolites and E were normally above the 97.5% RL between 4 to 12h (Table 9.1). However, using Δ^6-AED/E and Δ^6-T/E no values above the 97,5% RL were reported for volunteer 3 (Table 9.1).

9.4. Discussion

The study of TU administration samples revealed several of the limitations of using either T concentrations or T/E as markers for oral T administration. On one hand, short DTs (below 8 h) were obtained, and, on the other hand, in the case of volunteer 3, even after administration of 120 mg of TU, neither the concentration nor the T/E ratio increased above the established thresholds (Table 9.1).

The use of Δ^6-AED and Δ^6-T concentrations as markers for TU misuse did not improve significantly the retrospectivity of T and T/E, meaning that similar DTs were obtained (Figure·9.1). More promising results were obtained when using Δ^1-AED and Δ^{15}-AD concentrations as markers. In these cases, an important improvement in the retrospectivity (between 3 to 6 times compared to T/E) was obtained (Table 9.1).

The use of concentration ratios can minimize the variations in the sample caused by urinary flow rate. For this reason, several ratios have been proposed specifically as markers for detecting T misuse, although, none of them have improved the discriminatory capability of the T/E ratio for the oral administration of T [14, 25]. One of the most critical steps before performing ratios is the selection of the reference compound. Ideally, the reference compound should (i) show remain unaltered after the administration, (ii) be excreted as phase II metabolite in the same way of the analyte and (iii) be

detected in the same method of the analyte. In the particular case of T, E satisfies all these conditions and therefore it can be considered as the ideal reference compound for ratios involving T [4].

Contrarily to T and E, the formations of the four studied T metabolites (Δ^1-AED, Δ^6-AED, Δ^6-T and Δ^{15}-AD) were showed to arise from the release of cysteine conjugates (see chapter 4). Besides, these metabolites and E are determined using different methodologies. Therefore, and taking into account the previously stated considerations, the use of E as reference compound for basic released metabolites is theoretically less suitable than in the case of T.

On the other hand, the four studied T metabolites were quantified using the same analytical method and theoretically, they are formed after realising the cysteine conjugate. However, all the metabolites released after basic treatment showed an increase after T administration. Therefore, none of them was the ideal endogenous reference compound to perform ratios. Among them, Δ^6-T and Δ^6-AED exhibited only moderate increases after T administration and, additionally, they showed the lower DTs (Figure 9.2). For these reasons, Δ^6-T and Δ^6-AED would be considered as potential endogenous reference compounds when performing ratios. Since no ideal reference compound for performing ratios could be selected, two different approaches were evaluated in this study: (i) performing ratios with E and (ii) performing ratios with Δ^6-AED and Δ^6-T.

The eight ratios studied (using E, Δ^6-T and Δ^6-AED as reference compound) followed a monomodal distribution (see chapter 7). This fact theoretically represents a valuable advantage compared with the T/E where some of the population has low basal T/E values.

Individuals with low basal T/E values, will need a substantial increase in T/E ratio before raising the population threshold, so the suspicion of T administration on these samples will be difficult. The use of markers with monomodal distribution can minimize this drawback.

The first tested approach was the use of the ratios within cysteinyl metabolites and E. A slightly improvement in DTs compared to T/E were observed Δ^1-AED/E and Δ^{15}-AD/E, whereas worse DTs for Δ^6-AED/E and Δ^6-T/E were calculated. The restrospectivity obtained for all these ratios was always smaller than the observed directly by the use of Δ^1-AED or Δ^{15}-AD concentrations. The high RSD showed for these ratios (higher than 130%) indicates a high variability among the population values which entails high RLs and decreases the retrospectivity. This fact hampers the use of ratios between the studied metabolites and E for the detection of oral T misuse. More promising results were found by performing ratios with Δ^6-AED and Δ^6-T. Using Δ^1-AED/Δ^6-T and Δ^1-AED/Δ^6-AED a significant improvement in retrospectivity (between 3 to 6 times compared to T/E) were found (Table 9.1 and Figure 9.2). These results are similar to those obtained with the concentration of Δ^1-AED (Table 9.1). However, as stated above the application of ratios is always preferred because it can minimize the variations in the sample produced by urinary dilution. Therefore, the establishment of Δ^1-AED/Δ^6-T and Δ^1-AED/Δ^6-AED can be valuable alternatives for the detection of oral T misuse. This fact is illustrated in Figure 9.3 where the chromatograms obtained for volunteer 4 using T/E and Δ^1-AED/Δ^6-T before and after oral T administration are shown. No significant differences were observed for T/E between the chromatograms obtained before and 24 h after TU administration.

However, using Δ^1-AED/Δ^6-T even 24 h after TU administration clear differences between both chromatograms were found.

The use of ratios involving Δ^{15}-AD (Δ^{15}-AD/Δ^6-AED and Δ^{15}-AD/Δ^6-T) also allowed for a two fold increase in the DT when compared to those obtained with T/E. Nevertheless, their DTs were shorter than those obtained with Δ^1-AED/Δ^6-T and Δ^1-AED/Δ^6-AED.

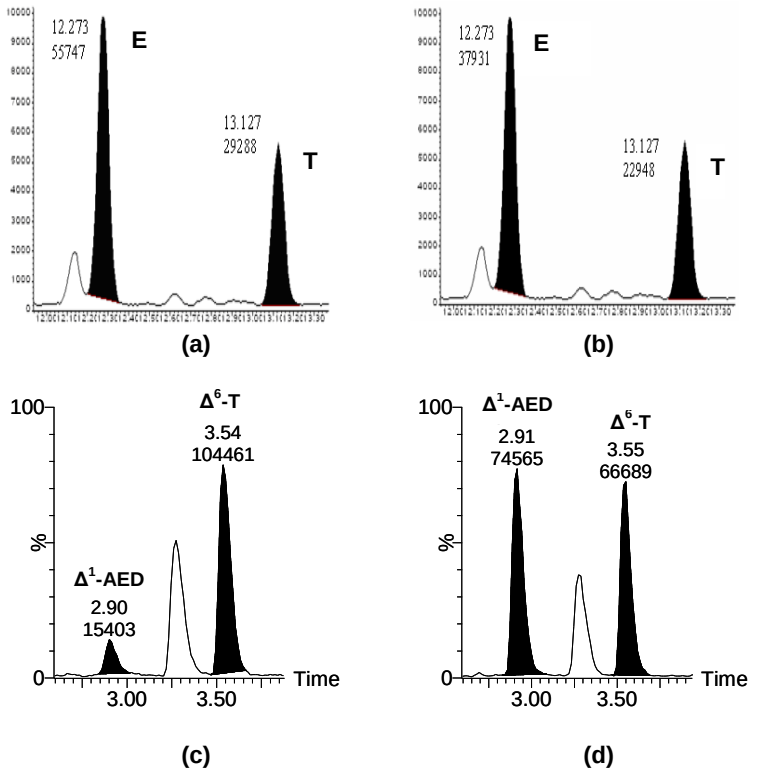


Figure 9.3. Chromatograms of T and E **(a)** before TU administration and **(b)** 24h post-administration and Δ^1-AED and Δ^6-T **(c)** before TU administration and **(d)** 24h post-administration for volunteer 4

It is interesting to comment the case of volunteer 3. T/E ratio was no useful in this volunteer to detect TU misuse since no rises above the RL were obtained. This behaviour can be explained by with the low basal T/E for this volunteer, related to the deletion polymorphism in the gene coding for UGT2B17 [26]. The gene UGT2B17 is associated with T glucuronide levels in urine. Subjects with such deletion in this gene could have a T/E lower than 0.4 [27, 28]. Although this deletion can contribute to explain the absence of variation in T/E values after TU administration, it has to be taken into account that volunteers 1 and 4 also have low basal T/E values and the detection of the misuse was still possible. Since these compounds are coming from a conjugated with cysteine, the use of these metabolites can minimize those limitations associated with the glucuronidation step. This fact is demonstrated with volunteer 3 where the use of the metabolites released after alkaline treatment and ratios between these compounds, allowed for the detection of T misuse up to 12 h in the best cases.

9.5. Conclusions

The usefulness of the cysteine conjugated markers has been demonstrated for oral T misuse. It will be of interest to evaluate the usefulness of these markers for the detection of the administration of other forms of T i.e. topic and intramuscular administration. For this purpose, the RLs established in the present study could be used. Samples collected after the administration of testosterone through different routes should be analysed using the same approach.

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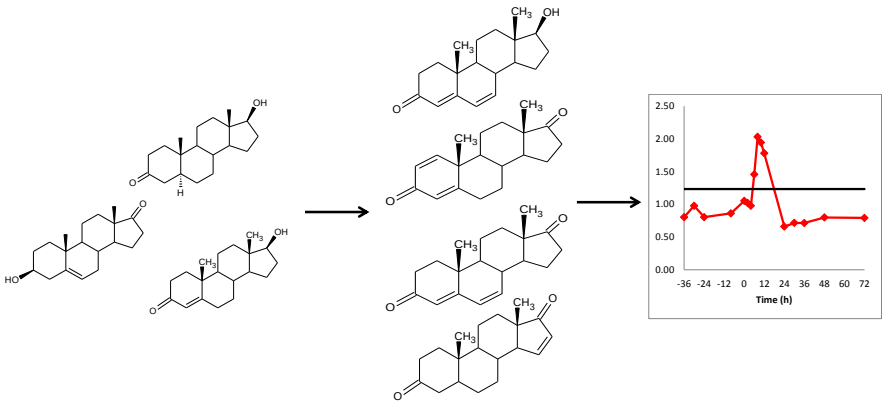
New insights for the detection of testosterone gel and other EAAS

Chapter 10



This chapter has been adapted from:

Fabregat A, Pozo OJ, Van Renterghem P, Van Eenoo P, Marcos J, Segura J, Ventura, R. Detection of dihydrotestosterone gel, dehydroepiandrosterone oral and testosterone gel misuse through the quantification of testosterone metabolites released after alkaline treatment. Drug Test Anal. 2011. 3(11-12):828-835. DOI: 10.1002/dta.351

10.1. Introduction

Testosterone (T) is an endogenous anabolic androgenic steroid (AAS) which was purified and synthesised in 1935. Since then, analogous compounds either naturally present in the body or new synthetic steroids have been marketed and introduced in sports as anabolic agents[1]. AAS are misused by athletes in order to increase lean body mass. For this reason, AAS are included in the list of prohibited substances published by the World Anti-Doping Agency (WADA) [2].

The detection of endogenous AAS misuse is a great challenge for doping control laboratories due to their natural presence in the body and availability as different substances and administration forms (dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), testosterone (T) or androstendione administered orally, intramuscularly or topically). Natural occurrence of these substances make that anti-doping control laboratories have to differentiate between endogenous AAS exogenously administered.

The gold-standard for this differentiation is the analysis by gas chromatography coupled to isotope ratio mass spectrometry (GC/C/IRMS) [3, 4]. However, this analysis is time-consuming and from a practical point of view cannot be applied, as screening method, to the large number of samples managed in doping control field. Therefore, GC/C/IRMS is only used only as a confirmatory tool, falling under the screening methods the task to discern between negative and suspicious samples.

The screening for endogenous AAS is normally based on the determination of the steroid profile and the comparison with established reference limits (RLs). Only when the RLs are

exceeded, these samples are considered as suspicious. The main problem of this approach is that the RLs are based upon population statistics which encompasses big inter-individual variations. Nevertheless, the urinary concentrations of some steroids can show individual and ethnical variations making the establishment of a unique RL difficult. That is the case of T which detection was proposed in 1983 by the ratio between the urinary testosterone and to its isomer epitestosterone (T/E) excreted as glucuronide [5]. In recent years, a T/E threshold of 4 was established by WADA [6]. Samples with a T/E value above this limit are considered suspicious for T misuse. Due to a genetic polymorphism a part of the population have T/E basal values far below the threshold limit, whereas other subjects are always above this threshold [7, 8]. In the last years, in order to overcome the limitations exhibited for the RLs, the establishment of subject-based or genotype based reference ranges has been proposed as a suitable tool [9-11]. In the last version of the technical (2014) WADA's established the application of the athlete biological passport for the screening of the endogenous AAS misuse [6]. This approach is based in the establishment of an individual threshold for each athlete as explained in chapter 1.

T is the most reported endogenous AAS [12]. It is available in different forms like pills, injectable solutions or gels. The application of the gel is approved in testosterone replacement therapy in men with low testosterone conditions. The principal problem is the poor absorption through the skin. Only 9-14% of the total administered T is absorbed making it an ideal preparation to administer micro-doses for cheating athletes [13]. As a consequence, changes in the steroid profile are low which leads to

additional problems to detect T gel misuse with the actual available methods [13-15]. Besides the T/E, other ratios like 5α-androstane-3α,17β-diol (5ααβ-Adiol)/E and androsterone/E have been reported for long-term T gel administration detection [15] .

DHEA is available as a supplement in tablet, capsule, liquid, and sublingual forms. It is a weak endogenous androgen that may be used by athletes with the aim of increasing levels of more active androgens such as T and DHT [16]. According to WADA's statistics, DHEA is the second most reported endogenous AAS [12]. The detection of DHEA administration has been difficult due to incomplete understanding of DHEA metabolism [17-19]. The administration of DHEA has shown to have minimal effects on urine T/E ratio [17]. Therefore several markers have been proposed for the detection of DHEA administration like the concentrations of DHEA or its 3α,5-cyclo metabolites, or the measurement of several ratios like 16α-hydroxy-DHEA/E, 7β-hydroxy-DHEA/E or DHEA /E [18, 20].

DHT is considered more a potent than its precursor T. DHT binds with greater affinity to the androgen receptor and dissociates from the complex more slowly [21]. The enzymatic 5α- reductase promotes the conversion of T to DHT [21]. Several ratios like those between DHT and E or between 5ααβ-Adiol and 5β-androstane-3α,17β-diol (5βαβ-Adiol) were postulated in order to detect its misuse [20, 22, 23].

In this thesis, four cysteinyl T metabolites released after alkaline treatment of the urine, Δ^1-AED, Δ^6-AED, Δ^6-T and Δ^{15}-AD have been characterized and a quantitative method has been developed (see chapters 3, 4 and 6). The application of these metabolites for

the detection of oral testosterone undecanoate administration has been demonstrated in chapter 9.

The goal of this study is to evaluate the variations observed for markers based on these four cysteine conjugated metabolites released after basic treatment after a single dose administration of DHT gel and oral DHEA. Two statistical approaches are evaluated, based either on, population reference limits established in chapter 7 or the individual thresholds, and the results compared with previously published markers.

10.2. Experimental

10.2.1. Chemicals and reagents

Δ^6-AED and Δ^6-T were obtained from Steraloids Inc. (Newport, USA). Δ^1-AED was purchased from NMI (Pymble, Australia), testosterone, epitestosterone, methandienone (used as internal standard for LC-MS/MS) and methyltestosterone (used as internal standard for GC-MS) were obtained from Sigma-Aldrich (St Louis, MO, USA). The β-glucuronidase preparation (from *Escherichia coli* type K12) was purchased from Roche Diagnostics (Mannheim, Germany).

Analytical grade potassium carbonate, potassium hydroxide pellets, di-sodium hydrogen phosphate, sodium hydrogen phosphate and *tert*-butyl-methyl ether, and ammonium iodide were obtained from Merck (Darmstadt, Germany). The derivatization reagent *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) was from Karl Bucher Chemische Fabrik GmbH (Waldstetten, Germany) and 2-mercaptoethanol was from Sigma-Aldrich (St Louis, MO, USA).

Diethyl ether was purchased from Fisher scientific (Leicestershire, UK).

Acetonitrile and methanol (LC gradient grade), formic acid, ammonium formate (LC/MS grade) and cyclohexane were purchased from Merck (Darmstadt, Germany). Milli Q water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).

10.2.2. LC-MS/MS instrumentation

Selected reaction monitoring (SRM) method was carried out using a triple quadrupole (Quattro Premier XE) mass spectrometer equipped with an orthogonal Z-spray-electrospray ionization source (ESI) (Waters Associates, Milford, MA, USA) interfaced to an UPLC system, Acquity (Waters Associates) for the chromatographic separation. Drying gas as well as nebulising gas was nitrogen. The desolvation gas flow was set to approximately 1200 L/h and the cone gas flow to 50 L/h. A cone voltage of 25 V, and a capillary voltage of 3.0 kV were used in positive ionization mode. The nitrogen desolvation temperature was set to 450 ºC and the source temperature to 120 ºC.

The LC separation was performed using an Acquity UHPLC BEH C_{18} column (100mm x 2.1 mm i.d., 1.7 μm particle size) (Waters Associates), at flow rate of 300 μL/min. Water and methanol both with formic acid (0.01%) and ammonium formate (1 mM) were selected as mobile phase solvents. A gradient program was used; the percentage of organic solvent was linearly changed as follows: at 0 min., 45%; at 1 min., 45%; at 3 min., 50%; at 10 min., 50%; at 11 min., 95%; at 11.5 min., 95%; at 12 min, 45%; at 15 min., 45%.

Analytes were determined by a SRM method including two transitions for each compound (see chapter 6). The most abundant and specific transitions (285>121 for Δ^1-AED, 285>149 for Δ^6-AED, 287>151 for Δ^6-T and 287>95 for Δ^{15}-AD) were selected for quantitative purposes. For Δ^{15}-AD a semi-quantification was made using Δ^6-T as a reference compound due to the impossibility of a commercial reference standard.

10.2.3. GC-MS instrumentation

The GC–MS analysis was performed on an Agilent 6890 GC system coupled to a 5975B Vl MSD mass spectrometer from Agilent Technologies (Palo Alto, USA). The instrument was equipped with a 17m J&W Ultra1 column (internal diameter 0.2mm, film thickness 0.11 µm) (Palo Alto, USA). The GC temperature program was: at 0 min, 120 ºC; then 70 ºC/min up to 177 ºC, then 4 ºC/min up to 231 ºC, then 30 ºC/min up to 300 ºC, then 2 min to 300 ºC. The temperatures of other instrument parts were 270 ºC for the injector, 250 ºC for the transfer line, 230 ºC for the ion source and 150 ºC for the quadrupole. 0.5 µL were injected in splitless mode.

Helium was used as carrier gas which was under constant pressure of 84.9 kPa. The instrument was operated in full scan mode for qualitative purposes between the *m/z* 50 and 650. For steroid quantification Selective Ion Monitoring (SIM) was used with a dwelltime of 20 ms for all monitored ions [24]. Additionally, *m/z* 272 was monitored to screen for possible presence of the mono-trimethylsilyl derivatives of androsterone and etiocholanolone which

is an indication of incomplete derivatisation by the MSTFA/NH_4I/ethanethiol (1150/3/6, v/w/v) mixture.

10.2.4. Sample preparation

The method used for LC-MS/MS determination was based on the method described and validated in chapter 6. Briefly, after addition of 50 µL of ISTD solution (methandienone, 1 µg/mL), 2.5 mL of urine were basified by addition of 300 µL of 6 M KOH. The mixture was heated at 60 ºC for 15 min, followed by a liquid-liquid extraction with 6 mL of *tert*-butylmethylether. The sample was centrifuged and the organic layer separated and evaporated. The residue was dissolved into 150 µL of a mixture of water:acetonitrile (1:1, v/v). Finally, 10 µL were directly injected into the LC-MS/MS system.

For GC-MS, the procedure is based upon the methods developed elsewhere [24]. After addition of 50 µL of ISTD solution (methyltestoterone at 2 µg/mL), 1 mL of phosphate buffer (0.1 M $Na_2HPO_4 \cdot {}_2H_2O$/$NaH_2PO_4 \cdot$H2O, pH 7) was added, and hydrolyzed with 50 µL of β-glucuronidase during 2.5 h at 56 ºC. After cooling, 200 mg of NaHCO3/K_2CO_3 (2/1, w/w) buffer were added. Liquid-liquid extraction was performed by addition of 5 mL of freshly distilled diethyl ether. The tubes were centrifuged at 2700 rpm for 5 min and the organic layer was separated, dried over anhydrous Na_2SO_4 and evaporated under oxygen free nitrogen (OFN). The dry residue was derivatised with 100 µL of MSTFA/NH_4I/ethanethiol (1150/3/6, v/w/v) for 1 h at 80 ºC.

10.2.5. Urine samples

For the excretion studies, the following single doses (one per week) were administered to 3 male volunteers (age 23-26 years): A therapeutic preparation of 50 mg DHEA ingested orally; a topical testosterone application (Androgel, Laboratories Besins International, France) and a topical application of DHT (Andractim, Besins International, Belgium) applied on upper arm, chest, thorax and abdomen in doses containing 100 mg and 250 mg active compound respectively. All participants gave their written informed consent and the study was approved by the Ethical Committee of the Ghent University Hospital (B67020064707).

Urine samples collected before administration (5 samples) and at 2, 4, 6, 8, 10, 12, 24, 30, 36, 48 and 72 h after DHT gel and DHEA or T gel administration were analyzed. The urine was stored at -20 ºC until analysis.

10.2.6. Data analysis

Two different approaches were applied in order to evaluate the usefulness of these markers for the detection of DHT gel and DHEA oral administration and other previously published markers.

Firstly, a comparison was performed between the results obtained after administration and the population based RLs previously published (see chapter 7 and [14]). In this approach a sample is considered suspicious if the value of the marker is higher than the RL.

Secondly, individual thresholds (IT) for the tested markers for each volunteer after the analysis of 5 samples before the administration

were established, followed by the application of the equation: Mean±3*Standard deviation (SD) [9]. In this approach a sample is considered suspicious if the value of the marker is higher than the IT.

The detection time (DT) was defined as the maximum time after the application of the AAS in which the sample is considered suspicious i.e. the values of the marker is above the upper RL in the first approach or above the upper IT in the second one. In order to calculate the DTs, the excretion studies obtained after the administration of a single dose have been used.

10.3. Results

10.3.1. Population based reference limits (RLs)

- **Basal values**

Twelve ratios can be calculated between the four metabolites released in alkaline conditions. Some of them were multiplied by a factor in order to obtain data easier to interpret. Basal values normally below the RL previously established (see chapter 7) were obtained. The main exceptions were the ratios Δ^6-AED/Δ^1-AED and Δ^6-T/Δ^1-AED for volunteer 1. For this volunteer, the mean of the basal values were 1.7 (Δ^6-AED/Δ^1-AED) and 3.3 (Δ^1-T/Δ^1-AED) which are close to the RLs (1.85 and 2.9, respectively). These values together with the standard deviation (0.13 and 0.28, respectively) made that some basal values exceeded the RL.

Similar situation was observed for some previously reported markers. Regarding T/E, basal values for volunteer 1 were below the limit established by WADA of 4 (mean 1.7, standard deviation

0.4), however higher T/E basal values were observed for the other volunteers. For volunteer 2, one basal value was above this limit (mean 3.8, standard deviation 0.8) and for volunteer 2, basal values always above the WADA's threshold were found (mean 5.1, standard deviation 1.4). Similarly, basal values for 5βαβ-Adiol/5ααβ-Adiol and 16α-hidroxy-DHEA/E were close to or even higher than the RL for volunteers 2 and 3. Basal values below the RL were found for the rest of markers.

DHT							
Marker		**DT (h) (with RL)**			**DT (h) (with IT)**		
	97.5% RL	**Volunteer**			**Volunteer**		
		1	**2**	**3**	**1**	**2**	**3**
5ααβ-Adiol/5βαβ-Adiol	1.6	-	24	-	-	72	72
DHT/5βαβ-Adiol	0.4	-	-	-	8	24	72
DHT/E	1.0	-	24	72	12	24	72
Δ^6-AED/Δ^1-AED (x1/100)	1.8	10	-	-	10	-	-
Δ^1-AED/Δ^{15}-AD (x10)	14.1	-	-	-	12	-	-
Δ^6-T/Δ^{15}-AD	15.6	-	-	-	12	-	-
Δ^6-AED/Δ^{15}-AD (x10)	7.7	-	-	-	12	-	-

Table 10.1. DTs using both the 97.5% RL and ITs for the ratios within cysteinyl compounds and ratios previously reported for DHT administration.

- **DHT gel**

From the twelve potential ratios between the basic released metabolites, four of them (Δ^6-AED/Δ^1-AED, Δ^1-AED/Δ^{15}-AD, Δ^1-T/Δ^{15}-AD and Δ^6-AED/Δ^{15}-AD) increased after DHT gel administration only in one of the volunteer (volunteer 1). Nevertheless, none of these ratios increased above the RL.

Regarding previously reported markers, values above the RL were obtained for DHT/E for volunteers 2 and 3 with DTs higher than 24 h for all of them (Table 10.1). Interestingly, this marker did not provide successful results for volunteer 1.

- **DHEA**

Five out of the twelve potential ratios (Δ^6-AED/Δ^1-AED, Δ^6-AED/Δ^1-AED, Δ^6-T/Δ^{15}-AED, Δ^6-AED/Δ^{15}-AED and Δ^1-AED/Δ^{15}-AED) increased after DHEA administration. The use of RLs for some of these markers like Δ^6-AED/Δ^1-AED and Δ^6-T/Δ^1-AED resulted in DTs of 4 h for volunteers 2 and 3 (Table 10.2), whereas most of the samples were above the RL for volunteer 1 due the high basal value.

Among previously described markers, the use of 7β-hydroxy-DHEA/E allowed for the detection of DHEA misuse in the three volunteers with DTs between 2 h and 12 h. Similar DTs were observed with DHEA/E although it was not able to detect DHEA misuse for volunteer 1. The longest DTs (72 h) were observed with 5βαβ-Adiol/5ααβ-Adiol although these satisfactory results could not be obtained in volunteer 1. For 16α-hydroxy-DHEA/E relatively high DTs (between 4 h and 48 h) were observed. However, as

explained before, it has to be taken into account that several samples were above the RL for volunteer 2 and 3 due to the high basal values for these markers (see section 10.3.1).

DHEA							
Marker		**DT (h) (with RL)**			**DT (h) (with IT)**		
	97.5% RL	**Volunteer**			**Volunteer**		
		1	**2**	**3**	**1**	**2**	**3**
5βαβ-Adiol/5ααβ-Adiol	8.3	-	72	72	30	72	72
16α-OH-DHEA/E	2.3	4	24	48	8	30	30
7β-OH-DHEA/E	8.4	2	6	12	8	30	12
DHEA/E	6.7	-	10	6	8	30	6
Δ^6-T/Δ^1-AED (x1/10)	2.9	All	4	4	12	12	4
Δ^6-AED/Δ^1-AED (x1/100)	1.8	72	4	4	12	4	-
Δ^6-T/Δ^{15}-AD	15.6	-	-	-	12	24	12
Δ^6-AED/Δ^{15}-AD (x1/10)	7.7	-	-	-	12	12	8
Δ^1-AED/Δ^{15}-AD (x10)	14.1	-	-	-	-	10	12

Table 10.2. DTs obtained using both the 97.5% RL and ITs for the ratios within cysteinyl compounds and ratios previously reported for DHEA administration

- **T gel**

Four ratios between metabolites released after alkaline treatment increased after T gel administration. However, none of these markers (Δ^1-AED/Δ^{15}-AD, Δ^6-T/Δ^{15}-AD, Δ^6-AED/Δ^{15}-AD and Δ^6-AED/Δ^1-AED) showed values above their RL (Table 10.3). In volunteer 1, some samples collected after T gel were above the RL

established for Δ^6-AED/Δ^1-AED (97.5% RL=1.8) probably due to the high basal values for this volunteer.

T gel							
Marker		**DT (h) (with RL)**			**DT (h) (with IT)**		
	97.5% RL	**Volunteer**			**Volunteer**		
		1	**2**	**3**	**1**	**2**	**3**
T/E	**4***	**-**	**All**	**All**	**-**	**-**	**-**
1,4-AD/15-AD (x10)	**14.1**	**-**	**-**	**-**	**12**	**10**	**10**
6-T/15-AD	**15.6**	**-**	**-**	**-**	**10**	**10**	**-**
4,6-AD/15-AD (x1/10)	**7.7**	**-**	**-**	**-**	**12**	**10**	**-**

Table 10.3. DTs obtained using both the 97.5% RL and ITs for the ratios within cysteinyl compounds and ratios previously reported for T gel administration

Similar effect was observed for T/E. After T gel administration, several samples were above the WADA's threshold for T/E in volunteer 2 and all of them were above this limit in volunteer 3 due to the elevated basal values for these volunteers.

10.3.2. Individual threshold (IT)

- **DHT gel**

As explained before, variations in the values of ratios between basic released metabolites were only observed after DHT gel administration for volunteer 1. In this case, DTs between 10 h and 12 h were obtained by the use of IT of the four selected markers

(Δ^1-AED/Δ^{15}-AD, Δ^6-T/Δ^{15}-AD, Δ^6-AED/Δ^{15}-AD and Δ^6-AED/Δ^1-AED) (Table 10.1 and Figures 10.1d- 10.1g). Remarkably, none of these markers allowed for the detection of DHT gel misuse in volunteer 2 and 3.

Regarding the previously published markers for the DHT detection, DTs between 8 h and 72 h were reported using DHT/5βαβ-Adiol and DHT/E for all volunteers (Figures 10.1b and 10.1c). DTs of 72 h were reported using 5ααβ-Adiol/5βαβ-Adiol for volunteer 2 and 3 although this marker did not provide satisfactory results for volunteer 1.

- **DHEA**

After DHEA administration, five of the tested markers exhibited an increase in their values. Markers giving longer DTs were found to be Δ^6-T/Δ^{15}-AD, Δ^6-AED/Δ^{15}-AD, Δ^6-T/Δ^1-AED and Δ^6-AED/Δ^1-AED (Figures 10.2e-10.2h). Two of the markers (Δ^6-T/Δ^{15}-AD and Δ^6-AED/Δ^{15}-AD) allowed for the detection of DHEA administration in all three volunteers whereas the other three gave satisfactory results for two of the volunteers (Table 10.2). DTs between 8 h and 24 h were obtained with these markers.

Similar DTs (between 6 h and 30 h) were found when using some ratios proposed in the literature for DHEA misuse: 5βαβ-Adiol/5ααβ-Adiol, 16αOH-DHEA/E, 7βOH-DHEA/E and DHEA /E (Figures 10.2a-10.2d). The longest DTs were obtained using the ratio 5βαβ-Adiol/5ααβ-Adiol (Table 10.2).

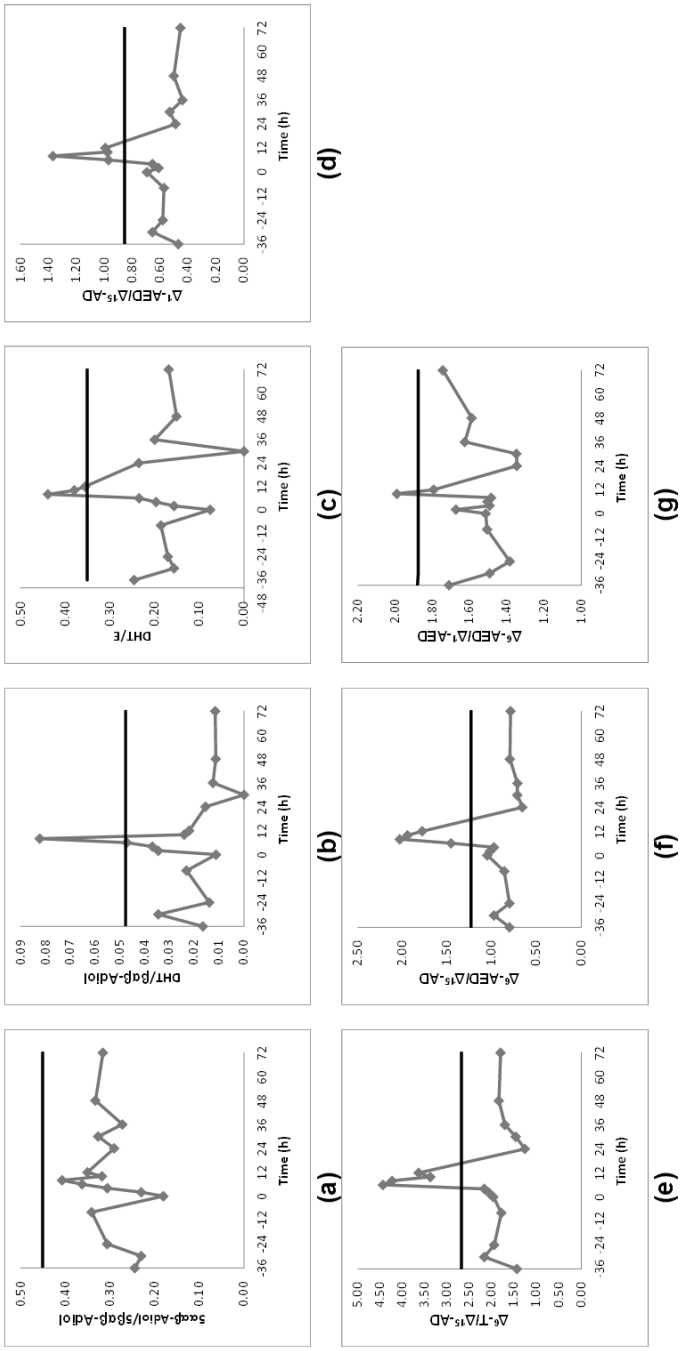


Figure 10.1. Results of DHT gel administration in volunteer 1: ratios observed for cysteinyl T metabolites **(d)** Δ^1-AED/Δ^{15}-AD; **(e)** Δ^6-T/Δ^{15}-AD; **(f)** Δ^6-AED/Δ^{15}-AD and **(g)** Δ^6-AED/Δ^1-AED and ratios previously published **(a)** 5αaβ-Adiol/5ααβ-Adiol; **(b)** DHT/5βαβ-Adiol; **(c)** DHT/E. Bold lines indicate individual thresholds.

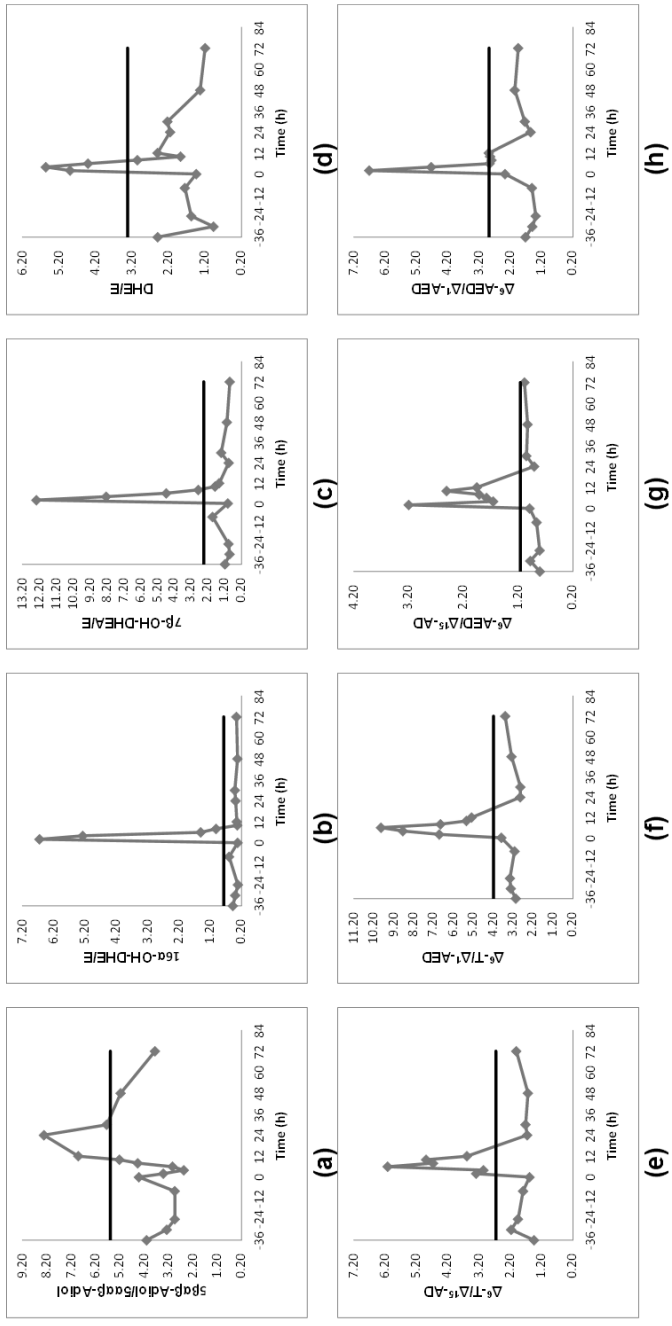


Figure 10.2. Results of DHEA administration in volunteer 1: ratios observed for cysteinyl T metabolites **(e)** Δ^6-T/Δ^{15}-AD; **(f)** Δ^6-T/Δ^1-AED; **(g)** Δ^6-AED/Δ^{15}-AD; **(h)** Δ^6-AED/Δ^1-AED and ratios previously published **(a)** 5αα**β**-Adiol/5αα**β**-Adiol; **(b)** 16α-OH-DHEA/E; **(c)** 7**β**-OH-DHEA/E and **(d)** DHE/E. Bold lines indicate individual thresholds.

- **T gel**

Three of the potential markers allowed for the detection of T gel administration. Whereas the use of Δ^1-AED/Δ^{15}-AD allowed the detection of T gel misuse in all volunteers (Table 10.3), the use of Δ^6-T/Δ^{15}-AD and Δ^6-AED/Δ^{15}-AD made possible the detection of T gel administration in volunteer 1 and 2 (Figures 10.3c and 10.3d). DTs between 10-12 h were obtained by the use of these markers. The marker Δ^1-AED/Δ^6-AED which was found to be the most useful for the detection of oral administration of testosterone [26] also increased for all volunteers but it only exceeded the threshold value in one of them.

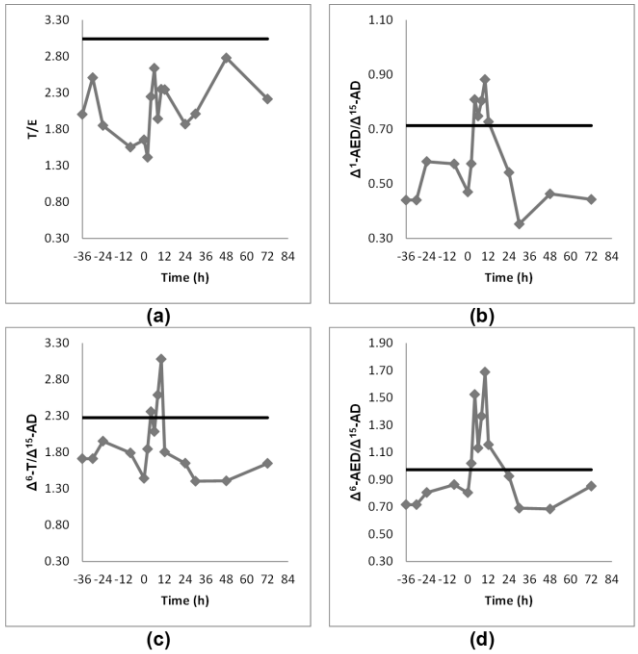


Figure 10.3. Results of T gel administration in volunteer 1: **(a)** T/E and ratios observed for cysteinyl T metabolites; **(b)** Δ^1-AED/Δ^{15}-AD; **(c)** Δ^6-T/Δ^{15}-AD; **(d)** Δ^6-AED/Δ^{15}-AD. Bold lines indicate

individual thresholds (IT)

No significant changes in the common marker for T misuse the T/E were observed after T gel administration (Figure 10.3a), all the values being below the established IT.

10.4. Discussion

The usefulness of four T metabolites released after alkaline treatment have been evaluated for the detection of a single dose administration of DHT gel, oral DHEA and T gel. Two alternative approaches for the detection of a suspicious sample have been tested: (i) the use of population based reference limits and (ii) the use of individual threshold limits.

The application of population based RLs for cysteinyl T metabolites showed most of the limitations of this approach already reported for conventional enzymatic released metabolites. On the one hand, subjects having a basal value far below the threshold and exhibiting a significant increase cannot be detected by the RLs approach. On the other hand, subjects show a basal value higher than the established RL and therefore the RL is inadequate to distinguish between samples collected before and after administration. This fact is illustrated by the detection of DHEA administration by means of the Δ^6-T/Δ^1-AED ratio (Table 10.2). Since the basal value for volunteer 1 (mean 3.3, standard deviation 0.28) is higher than the RL (2.9) all the samples from this subject would be considered as suspicious by this approach causing a risk of false positive findings. Aditionally, the low basal value for

volunteer 2 (mean 1.3, standard deviation 0.30) made that the administration could be detected only in samples collected 4h after DHEA intake. Similar results have been reported for the steroid profile [25] making the use of individual threshold limits necessary to improve detection sensitivity in the doping control [9, 10, 15, 23, 26].

Similarly to what was observed with conventional metabolites, the efficacy of cysteinyl T metabolites was improved by the use of IT limits. Using this strategy valuable piece of information can be extracted for the three AAS tested.

The formation of Δ^1-AED, Δ^6-AED and Δ^6-T from DHT would imply the addition of two double bonds and the inherent loss of the 5α-hydrogen. Therefore, it is not expected that the amount of these metabolites would increase after DHT administration. In contrast, it seems more rational the use of ratios between 5α-containing metabolites and 5α-non-containing metabolites (5β or 4-ene metabolites) as DHT marker.This factwas confirmed after sublingual DHT administration, where the marker 5αaβ-Adiol/5βaβ-Adiol increased substantially [23]. Similar results were also observed after DHT gel administration in volunteers 2 and 3 where the use of 5αaβ-Adiol/5βaβ-Adiol, DHT/5βaβ-Adiol and DHT/E provide DTs higher than 24 h, whereas the markers based on basic released metabolites did not exhibit any increase (Table 10.1). An exception for this behavior was volunteer 1, in which 5αaβ-Adiol/5βaβ-Adiol did not increase, thus showing an unexpected behavior for DHT. For this volunteer, DTs lower than 12h were obtained using DHT/5βaβ-Adiol and DHT/E as markers. Similar DTs for volunteer 1 were observed when using markers based on basic released metabolites. The most satisfactory markers were

those related with Δ^{15}-AD, which might indicate a drop in Δ^{15}-AD concentration after topical DHT administration. In any case, the use of basic released metabolites did not provide any substantial improvement in the detection of DHT administration.

Several ratios based on cysteinyl metabolites were found to be useful for the detection of DHEA administration. Metabolites Δ^{6}-AED and Δ^{6}-T were involved in most of these ratios suggesting an increase in the concentration of these metabolites after oral DHEA administration. The formation of these metabolites from DHEA would imply either oxidative transformations from 5-ene to 4,6-diene and from 3-hydroxy to 3-oxo or conversion from DHEA to T and subsequent transformation from T to Δ^{6}-AED and Δ^{6}-T. Since Δ^{1}-AED is also a direct T metabolite (see chapter 3) and no increase of this metabolite is observed, the first hypothesis is more likely to occur. Using these metabolites, Δ^{6}-T/Δ^{15}-AD was the best marker found for DHEA administration. DTs between 12 and 24 h were obtained (Table 10.2). These results were comparable to those obtained with previously reported markers. The longest DTs were obtained using 5βαβ-Adiol/5ααβ-Adiol although the increase of this ratio was less significant than the observed with the other ratios (Figure 10.2).

The four compounds evaluated in this study were reported as T metabolites and all of them increased after oral T intake being the highest increase for Δ^{1}-AED and Δ^{15}-AD [24]. After topic T administration, several markers based on these metabolites were found. Most of them were based on Δ^{15}-AD suggesting either a decrease on Δ^{15}-AD concentration or a simultaneous increase in the concentration of the other three compounds. These results differed drastically from those generated after an oral T

administration where the highest increase was observed for Δ^{15}-AD. A possible explanation could be the lack in the skin of the enzymes needed for Δ^{15}-AD formation. The best marker was found to be Δ^{1}-AED/Δ^{15}-AD which allows for the detection of T gel administration in all the volunteers with DTs higher than 10 h (Table 10.3, Figure 10.3). These results represent a significant advance in T gel detection since it could not be detected using previously reported markers like T/E. Currently, the main disadvantage of the use of these ratios is the absence of commercially available Δ^{15}-AD. Once it will be available the usefulness of these results should be confirmed by using a quantitative method also for Δ^{15}-AD.

10.5. Conclusion

The use of cysteinyl metabolites can be useful for doping control analysis mainly in the detection of oral DHEA and T gel administration. However, most of the useful markers involve the quantification of Δ^{15}-AD. A semi-quantitative method was used for the quantification of this compound due to the lack of pure standard. It is expected to obtain more reliable results with a method including the Δ^{15}-AD standard.

In the present study, excretion studies obtained after a single administration of drugs were evaluated. It has to be taken into account that this is not the most likely scenario when dealing with AAS users. Prolonged administrations of AAS are needed in order to obtain the expected benefits. The analysis of samples obtained after multiple doses have shown that some markers like T/E, 5αβ-Adiol/E and androsterone/E which are not useful for single dose

administration of T gel can be applicable for the detection of T gel after administration for longer periods [13, 15]. In this sense, it will be valuable to test the applicability of the markers presented in this study for the evaluation of multiple administrations of endogenous AAS. The evaluation of these results would help in the consideration of these metabolites as complement of the analytes currently included in the steroid profile.

10.6. References

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Discussion and suggestion for future works

Chapter 11

11.1. Discussion

Common approaches for metabolic studies in doping control are normally focused in either the glucuronide or the sulphate metabolic fraction of the urine. This thesis has pointed out the usefulness of focusing the metabolic studies into unexplored fractions of the urine. Concretely, by using an extraction method including an alkaline treatment of the urine, four unreported testosterone metabolites (1,4-androstadien-3,17-dione, 4,6-androstadien-3,17-dione, 4,6-androstadien-17β-ol3-one and 15-adrostene-3,17-dione (Δ^{15}-AD)) arising form conjugates with cysteine have been detected by LC-MS/MS approaches. In the same way, other compounds might be elucidated by using new extraction approaches of the urine.

These metabolites might be useful for the detection of endogenous anabolic androgenic steroid (EAAS) misuse and, therefore, their implementation in the steroid profile of the athlete biological passport (ABP) might improve the screening capabilities of the ABP. In that sense, the cysteinyl conjugated metabolites have shown their usefulness for the detection of testosterone and other EAAS misuse circumventing in some cases the limitations exhibited for the markers of the current steroid profile. The fact, that some of the useful markers were based on ratios involving Δ^{15}-AD showed the need to synthesize authentic reference material of this compound for a proper evaluation of the markers. Additionally, none of the four cysteinyl metabolites is an ideal endogenous reference compound for performing ratios. This fact makes that the results observed when using ratios between the cysteinyl metabolites might be underestimated. Therefore, the

elucidation of an endogenous cysteinyl compound which did not increase after the administration might improve the detection capabilities exhibited for cysteinyl markers.

Ideally, the elucidated markers have to be detected together with the current markers of the steroid profile. The fact that cysteinyl compounds are detected after an alkaline treatment of the urine (which implies an additional extraction of the urine) is a serious limitation for its routine implementation in the steroid profile. The development of LC-MS/MS method based on the direct detection of metabolites conjugated with glucuronide and cysteine might circumvent this limitation.

In this thesis an in deep study of the factors that can influence the excretion of cysteinyl metabolites has been performed. Factors reported to affect the excretion of metabolites conjugated with glucuronic acid have been studied. Since these compounds are excreted conjugated with cysteine moiety it cannot be discarded that other factors not monitored are affecting the excretion of these new metabolites. For instance, the production of sporadic artefacts in urine due to the harsh conditions used (6 M of KOH) for the hydrolysis of the cysteine moiety can not be discarded.

The detection of similar cysteinyl compounds for progesterone and cortisol confirmed this alternative metabolic pathway unreported until now common to all steroid compounds. Thus, this new pathway can open new perspectives not only in doping field but for other clinical purposes.

Finally, it can be concluded that a comprehensive study of the whole amount of fractions of the urine might be useful to elucidate and characterize steroid metabolites unnoticed until now. The implementation of these new metabolites in the

current steroid profile might either minimize the limitations exhibited or increase the diagnostic specificity of this approach.

11.2. Suggestion for future works

From the main conclusions extracted of this work, several research lines can be suggested:

- Similarly to the approach used for the discovery of cysteinyl compounds, the study of other fractions of the urine like the fraction remained after the hydrolysis with β-glucuronidase.
- Study the detection capabilities of the cysteinyl markers by the application of the bayesian model.
- Testing metabolomics based approaches as new alternatives for metabolic studies.
- Evaluation of the cysteinyl metabolites as novel opportunities for GC/C/IRMS analysis.
- Study the presence of similar cysteinyl metabolites for exogenous AAS.
- Evaluate the usefulness of the cysteinyl metabolites as a biomarker for clinical purposes.

Conclusions

Chapter 12

The most important conclusions of this thesis are summarized below.

1. Four previously unreported testosterone metabolites were detected after alkaline treatment of urine samples. The metabolites were identified as 1,4-androstadien-3,17-dione (Δ^1-AED), 4,6-androstadien-3,17-dione (Δ^6-AED), 4,6-androstadien-17β-ol-3-one (Δ^6-T) and 15-androsten-3,17-dione (Δ^{15}-AD). Additionally, the occurrences of boldenone (1,4-androstadien-17β-ol-3-one (Δ^1-T)) and boldenone metabolite (5β-androsta-1-en-17β-ol-3-one) were also detected in testosterone post-administration samples after alkaline treatment of the urine.

2. The new metabolites are produced from degradation of cysteine conjugates. The presence of Δ^6-AED and Δ^6-T in urine after basic treatment arise from 7α-cystein-S-yl-4-androsten-3,17-dione and 7α-cystein-S-yl-4-androsten-17β-ol-3-one, respectively. Additionally, the presence of analogous conjugates for progesterone and cortisol was confirmed, showing that the conjugation with cysteine is not restricted to testosterone but common to other related steroid compounds.

3. The incubation of testosterone in an hepatocyte cells system resulted in the formation of Δ^1-T, Δ^6-T, Δ^1-AED and Δ^6-AED, confirming that 1,2- and 6,7- dehydrogenations are phase I metabolic reactions of testosterone.

4. Due to the low sensitivity exhibited for the cysteine conjugates, an indirect method based on the release of the cysteine moiety was developed to quantify these metabolites in urine. The indirect method was able to quantify either low endogenous concentrations or high concentrations observed after testosterone administration.

5. The analyses of the 173 urine allowed to determine the 97.5 and 99% reference population limits with a confidence interval of 95% for these compounds and the ratios between them, to be used for doping control purposes. The conjugates with cysteine showed a monomodal distribution in contrast with T/E ratio that exhibited a bimodal distribution.

6. Similar variations of cysteine conjugates than those observed for T/E were in general observed for the factors that can influence the steroid profile. Cysteinyl metabolites were relatively stables to factors affecting sample preservation. Regarding endogenous factors, moderate ultradian and circadian rhythms, differences between genders and high differences in the earliest stages of pregnancy, were observed for conjugates with cysteine. As expected, UGT2B17 polymorphism did not affect the excretion of the cysteinyl compounds. The use of exogenous substances (ethanol and aromatase inhibitors) showed similar effect on conjugates with cysteine than those observed for T/E

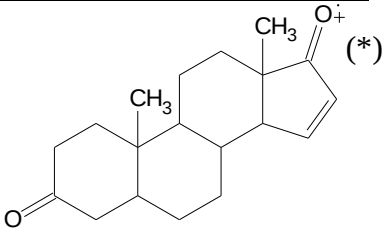
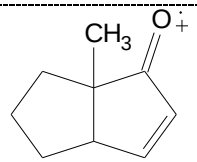
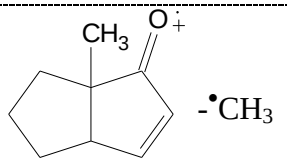
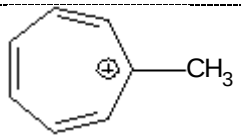
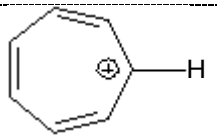
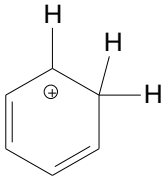
7. The use of markers between cysteinyl metabolites and the application of reference population limits allowed to increase the detection time of a single oral dose of testosterone between 3 to 6 times when comparing with T/E. Furthermore, the use of ratios between cysteinyl compounds allowed the detection of testosterone oral administration in individuals where no increase of T/E was observed after the oral administration. The use of individual thresholds and the application of ratios involving Δ^{15}-AD allowed the detection of testosterone gel whereas using the common marker T/E the detection remained impossible

8. The use of cysteine metabolites and individual threshold limits allowed the detection of the administration of other endogenous steroids administration forms, such as DHT gel and oral DHEA. However, cysteine conjugates did not improve the detection times compared with the common markers of the steroid profile.

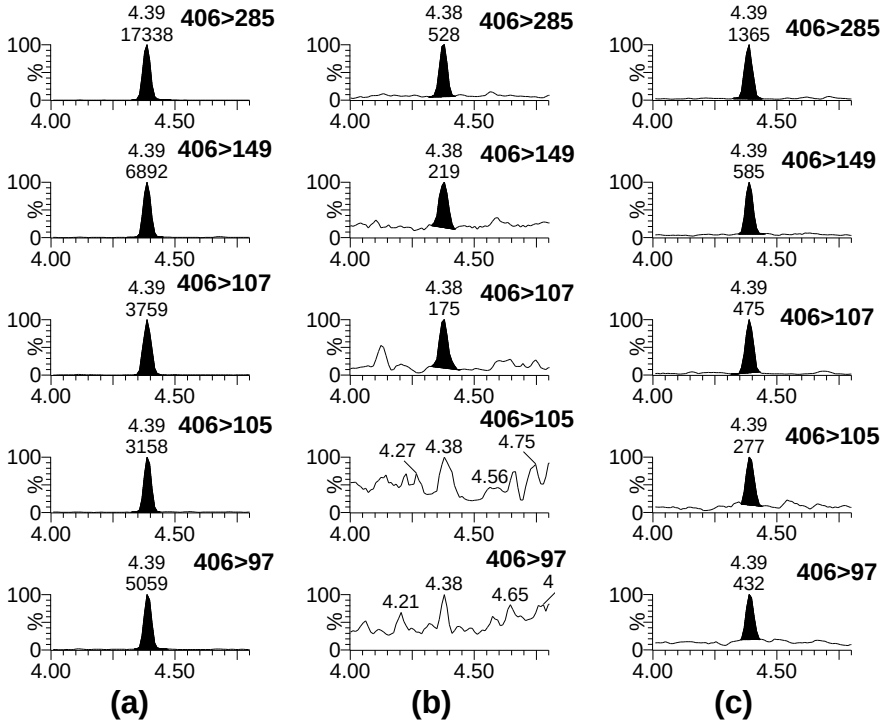
9. The use of liquid chromatography coupled to tandem mass spectrometry has allowed the identification of previously unreported phase I and phase II metabolic reactions for anabolic androgenic steroids that have demonstrated to be useful for doping control purposes. The usefulness of these metabolic pathways in other fields (e.g., clinical field) should be evaluated. The results also demonstrated the need of a re-evaluation of steroid metabolism in order to look for other new metabolic pathways.

Annexes I

Supplementary material

Ion (m/z)	Formula	Error (mDa)	Proposed pathway
286.1943	$C_{19}H_{26}O_2$	1.0	 (*)
271.1718	$C_{18}H_{23}O_2$	2.0	$-^{\bullet}CH_3$
268.1818	$C_{19}H_{24}O$	0.9	$-H_2O$
258.1620	$C_{17}H_{22}O_2$	0.2	$-H_2C{=}CH_2$
257.1554	$C_{17}H_{21}O_2$	1.2	$-^{\bullet}CH_2CH_3$
253.1595	$C_{18}H_{21}O$	0.3	$-H_2O$ $-^{\bullet}CH_3$
136.0896	$C_9H_{12}O$	0.8	
121.0675	C_8H_9O	2.2	
105.0702	C_8H_9	0.2	
91.0541	C_7H_7	0.7	
79.0537	C_6H_7	1.1	

S-3.1. Abundant ions obtained in GC-TOF for underivatized M4



S-4.2. Indirect SRM confirmation of the presence of Δ^6-AED-Cys in plasma. **(a)** Standard at 500 pg/mL, **(b)** free fraction of the plasma, and **(c)** plasma remaining after removing the free fraction treated in alkaline conditions.

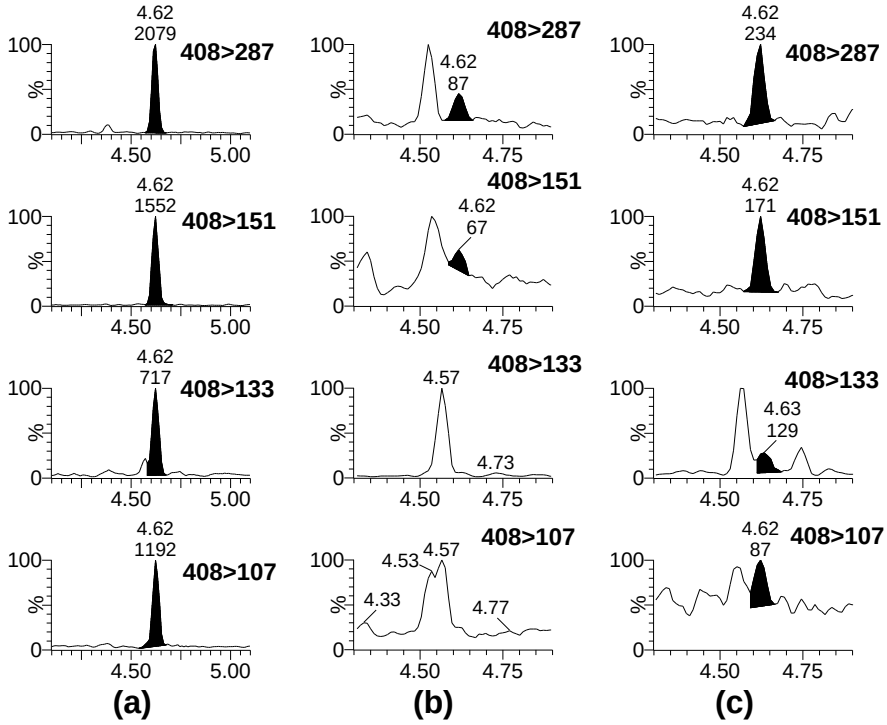


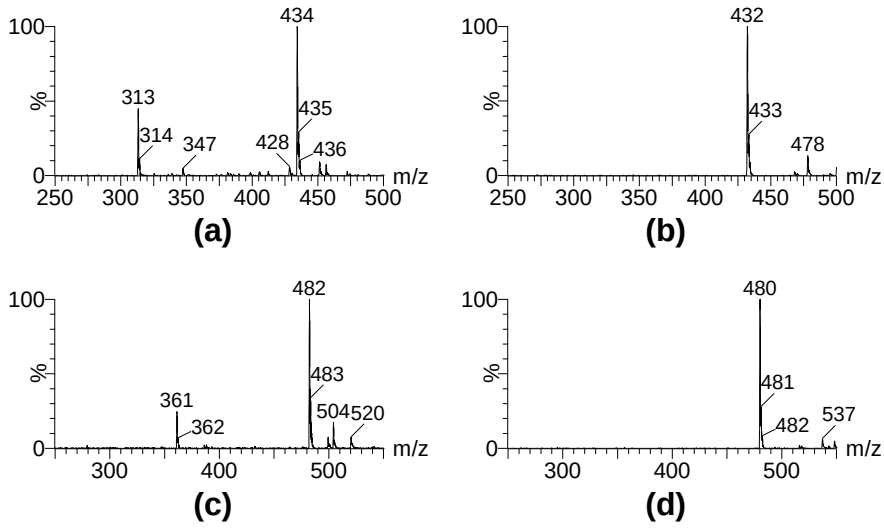
Figure S-4.3. Indirect SRM confirmation of the presence of Δ^6-T-Cys in plasma. **(a)** Standard at 50 pg/mL, **(b)** free fraction of the plasma, and **(c)** plasma remaining after removing the free fraction treated in alkaline conditions.

Transition	Standard	Free fraction	Fraction treated in alkaline conditions
285 → 149	100 %	100 %	100 %
285 → 107	40 %	41 %.	43 %
285 → 97	27 %	33 %	35 %
285 → 83	18 %	*n.d.*	20 %
285 → 79	31 %	*n.d.*	32 %

S-4.4. Relative abundances of the transitions acquired for Δ^6-AED in the standard solution, in the free fraction of the plasma and in the plasma remaining after removing the free fraction treated in alkaline conditions.

Transition	Standard	Free fraction	Fraction treated in alkaline conditions
287 → 151	100 %	100 %	100 %
287 → 133	75 %	77 %.	73 %
287 → 97	34 %	*n.d.*	55 %
406 → 79	57 %	*n.d.*	37 %

S-4.5. Relative abundances of the transitions acquired for Δ^6-T in the standard solution, in the free fraction of the plasma and in the plasma remaining after removing the free fraction treated in alkaline conditions.



S-4.6. Full scan spectra for **(a)** Δ^6-Prog-Cys in positive ionization mode, **(b)** Δ^6-Prog-Cys in negative ionization mode, **(c)** Δ^6-Cort-Cys in positive ionization mode, and **(d)** Δ^6-Cort-Cys in negative ionization mode.

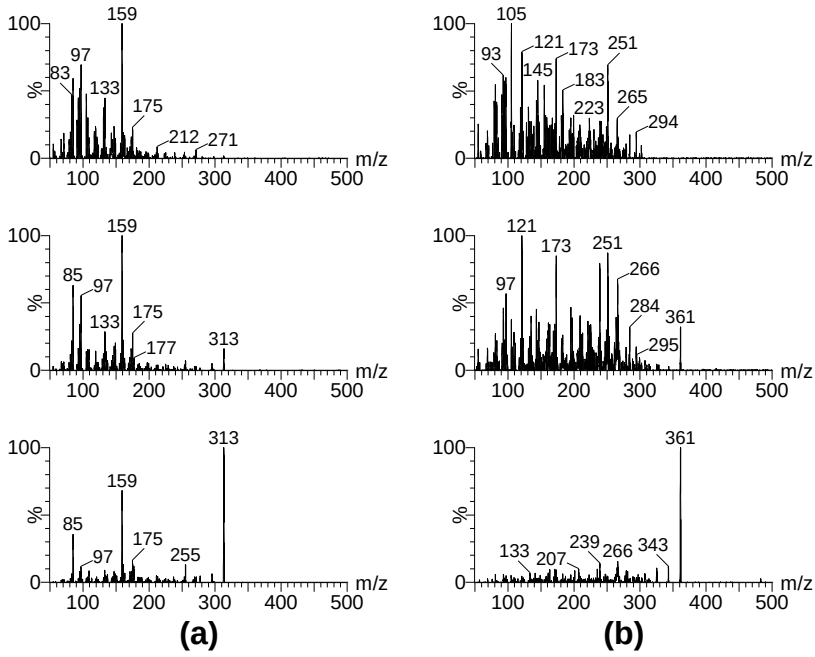


Figure S-4.7. Product ion spectra in ESI+ for **(a)** Δ^6-Prog-Cys (precursor ion *m/z* 434), **(b)** Δ^6-Cort-Cys (precursor ion *m/z* 482) acquired at 20 eV (bottom), 30 eV (medium), and 40 eV (top)

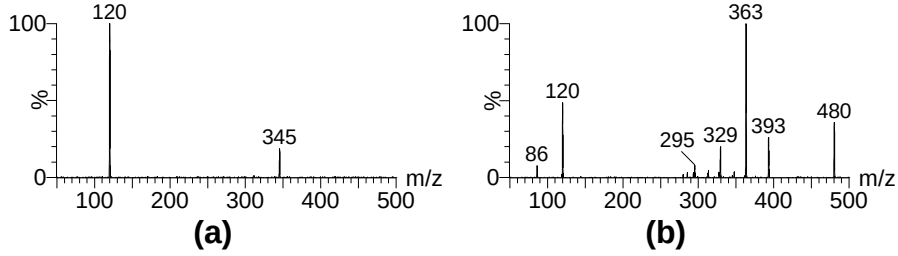
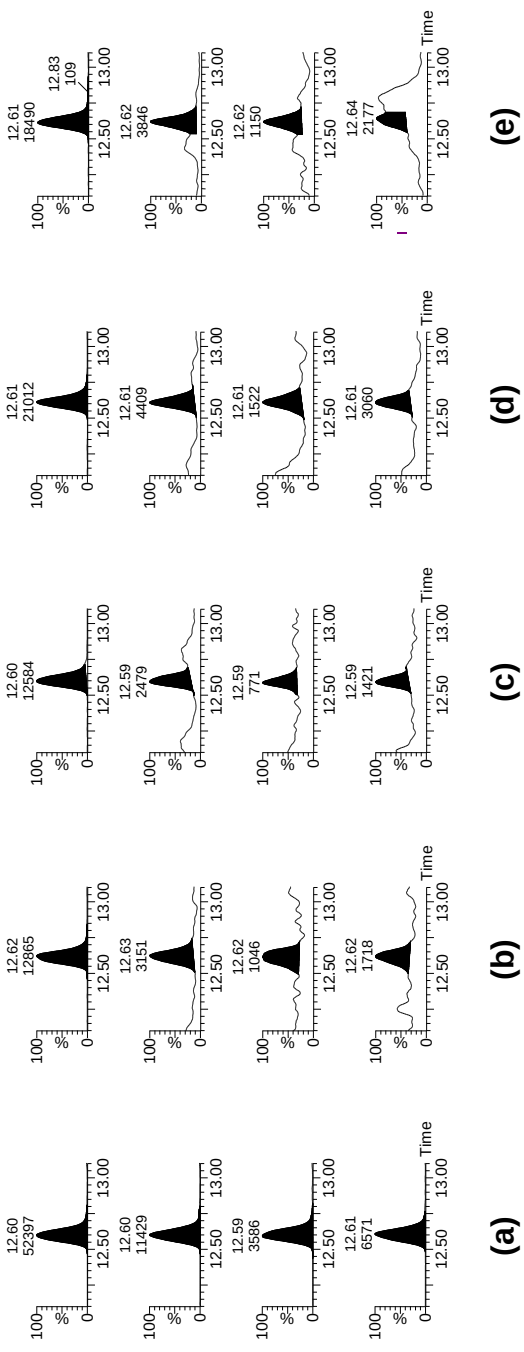
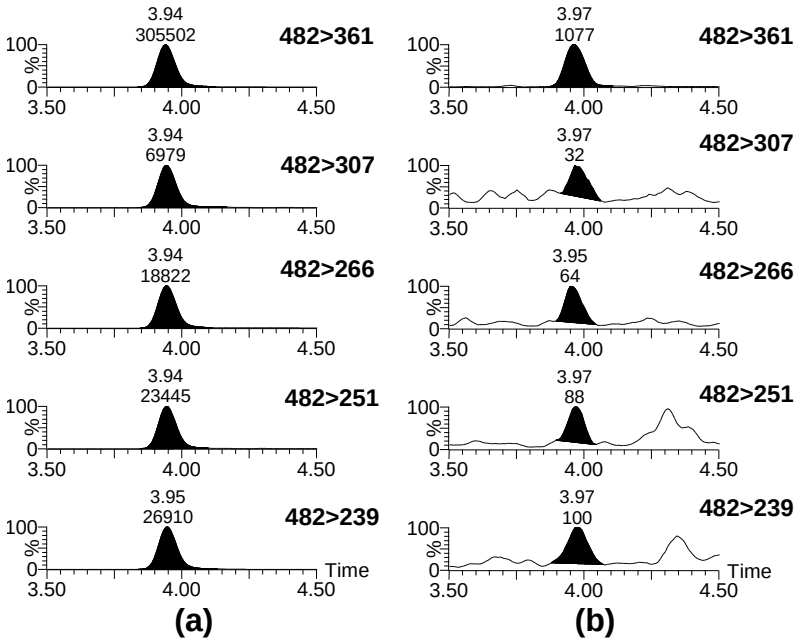


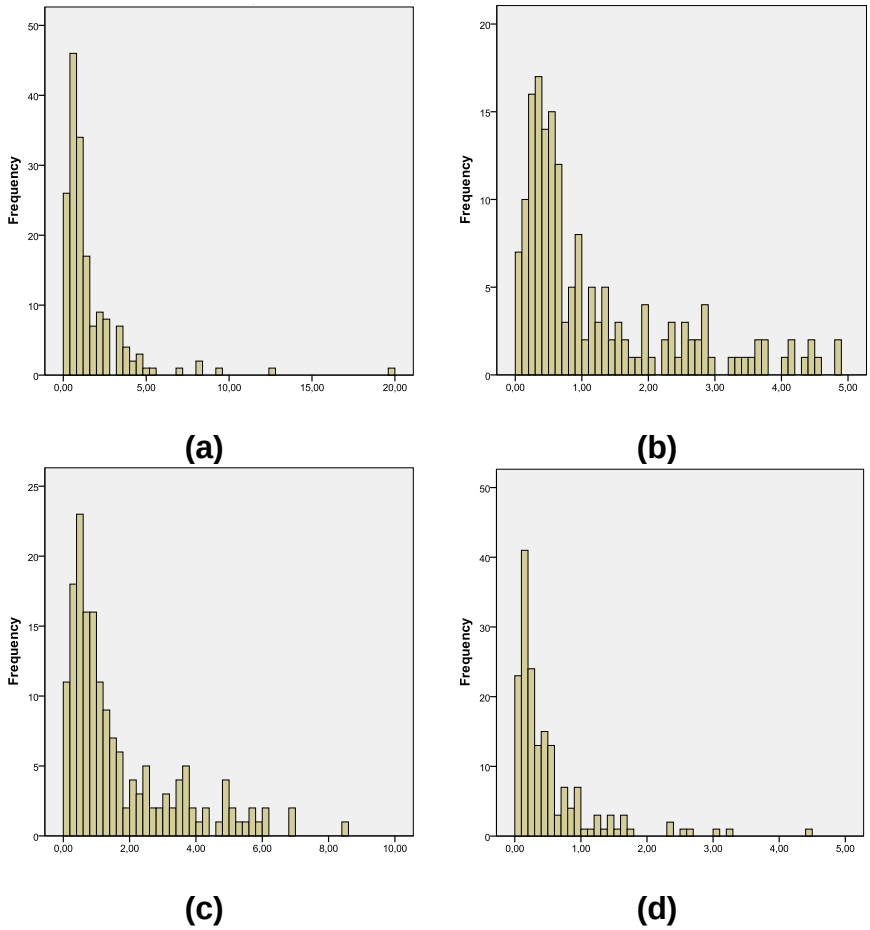
Figure S-4.8. Product ion spectra in ESI- acquired at 20 eV for **(a)** Δ^6-Prog-Cys (precursor ion *m/z* 432), **(b)** Δ^6-Cort-Cys (precursor ion *m/z* 480)



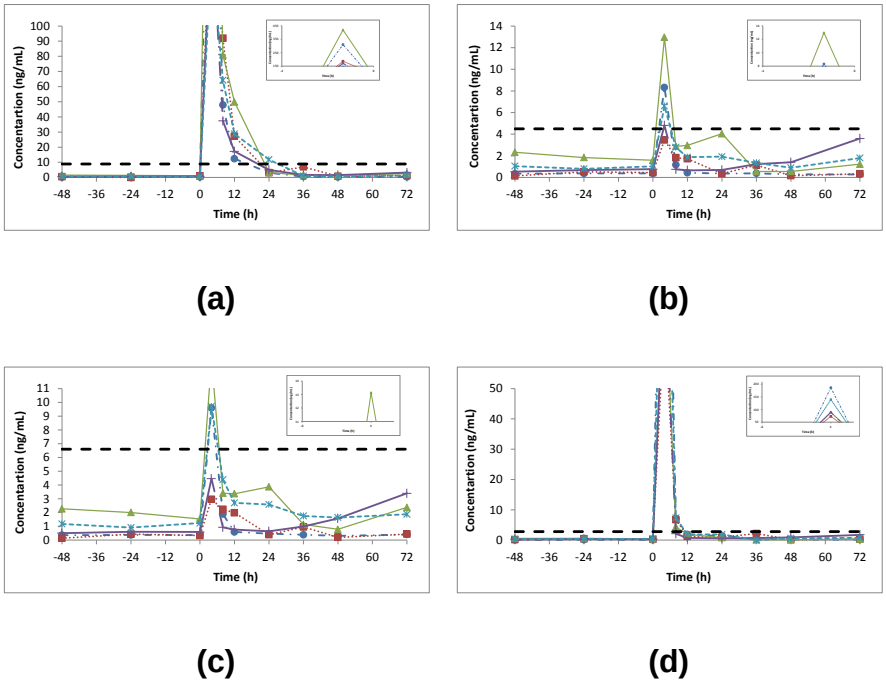
S-4.9. SRM confirmation of the presence of Δ^6-Prog-Cys in urine collected from pregnant woman. **(a)** Synthesized material, **(b) – (e)** extracted urine.



S-4.10. SRM confirmation of the presence of Δ^6-Cort-Cys in urine collected from Cushing's syndrome patient. **(a)** Synthesized material, **(b)** extracted urine.



S-7.11. Histograms obtained for the four ratios between the cysteinyl metabolites and E **(a)** Δ^1-AED/E, **(b)** Δ^6-AED/E, **(c)** Δ^6-T/E and (d) Δ^{15}-AD/E.



S-9.12. Excretion profile for ratios between cysteinyl metabolites and E compared with its own 97.5% RL **(a)** Δ^1-AED/E, **(b)** Δ^6-AED/E, **(c)** Δ^6-T/E and **(d)** Δ^{15}-AD/E.

Annexes II

Scientific papers presented in this thesis

- **A. Fabregat,** O.J. Pozo, J. Marcos, J. Segura, R. Ventura. Factors affecting urinary excretion of testosterone metabolites conjugated with cysteine. Drug Test Anal (2014). *Submitted*
- **A. Fabregat**, J. Marcos, R. Ventura, G. Casals, W. Jimenez, Reichenbach, V, J. Segura, O.J. Pozo. Formation of Δ^1 and Δ^6 testosterone metabolites by human hepatocytes. Steroid (2014). *Submitted.*

- **A. Fabregat** , A. Kotronoulas, J. Marcos, J. Joglar, I. Alfonso, J. Segura, O.J. Pozo. Detection, synthesis and characterization of metabolites of steroid hormones conjugated with cysteine. Steroids (2012). 78 (3): 327-336.

- **Fabregat**, O.J. Pozo, P. Van Renterghem , P. Van Eenoo, J. Marcos, J. Segura, R. Ventura. Detection of dihydrotestosterone gel, oral dehydroepiandrosterone, and testosterone gel misuse through the quantification of testosterone metabolites released after alkaline treatment. Drug Test Anal (2011). 3 (11-12):828

- **A. Fabregat**, O.J. Pozo, J. Marcos, J. Segura, R. Ventura, Alternative Markers for the long-term detection of oral testosterone misuse. Steroids (2011). 76 (12): 1367-1376.

- **A. Fabregat**, O. J. Pozo, J. Marcos, J. Segura, R. Ventura, Quantification of testosterone and metabolites released after alkaline treatment in human urine. Drug Testing and Analysis (2010). 2:630-636.

- O. J. Pozo, J. Marcos, R. Ventura, **A. Fabregat**, J. Segura. Testosterone metabolism revisited: discovery of new metabolites. Analytical Bioanalytical Chemistry (2010). 389:1759-1770.

Annexes III

Other scientific papers published by the author

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

- **A. Fabregat**, J. Marcos, L. Garrostas, J. Segura, O.J. Pozo, R. Ventura. Evaluation of urinary excretion of androgens conjugated to cysteine in human pregnancy by mass spectrometry. J Steroid Biochem & Mol Biol (2014). 139:192-200.

- O. J. Pozo, J. Marcos, **A. Fabregat**, R. Ventura, G. Casals, P. Aguilera, J. Segura , J. To-Figueras. Adrenal hormonal imbalance in acute intermittent porphyria patients: Results from a case control study. Orphanet Journal of Rare Diseases (2014). Orphanet J Rare Dis (2014). 9(1): 54.

- O.J. Pozo, N. Brabanter, **A. Fabregat**, J. Segura, R. Ventura, P. Van Eenoo, K. Deventer. 10 years after THG discovery. Current status and bioanalytical challenges in the detection of unknown anabolic androgenic steroids in doping control analysis. Bioanalysis (2013). 5(21):2661-2667

- **A. Fabregat**, O.J. Pozo, J. Marcos, J. Segura, R. Ventura. The use of LC-MS/MS for the open detection of steroid metabolites conjugated with glucuronic acid. Anal Chem (2013). 85(10): 5005-5014.

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