FACULTY OF MEDICINE AND HEALTH SCIENCES

Detection of new long-term metabolites for doping analyses

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Gilles Coppieters
Student number: 01309821

Supervisor(s): Prof. Dr. ir. Peter Van Eenoo, Dr. Michaël Polet

A dissertation submitted to Ghent University in partial fulfilment of the requirements for the degree of Master of Science in the Biomedical Sciences

Academic year: 2017 – 2018

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Preface

First, I would like to thank all the awesome people at DoCoLab. If it weren't for you people, this dissertation would not have been feasible. The atmosphere was sociable and there was not a single day that I dreaded doing my experiments at DoCoLab.

I am very grateful to Prof. Dr. ir. Peter Van Eenoo for giving me the opportunity of working on a project at DoCoLab. I would also like to thank Dr. Leen Lootens and Dr. Lore Geldof for giving me a kickstart in the lab last year.

And of course a special thanks to my copromotor Dr. Michaël Polet and mentor Dr. Wim Van Gansbeke. Michaël for his patience with my endless flow of questions, for reading and correcting my dissertation and for his help with my many experiments. Wim also for his immense patience with my questions and for his absolutely amazing guidance of my experiments on GC. If I had a problem with any of the devices, I didn't have to look far for his help. Not a single time did they hesitate to help me out, even if they were busy with their own research.

Last but not least, a warm thank you to all the people who supported me over the years, including my family and friends!

List of abbreviations

AAS	Anabolic androgenic steroids
ACN	Acetonitrile
ALS	Automatic liquid sampler
AR	Androgen receptor
CI	Chemical ionisation
CE	Collision energy
DC	Direct current
DHEA	Dehydroepiandrosterone
DMF	N,N-Dimethylformamide
DoCoLab	Doping control laboratory at Ghent University
E. coli	*Escherichia coli*
EI	Electron ionisation
EtOAc	Ethyl acetate
eV	Elektronvolt
GC-MS	Gas chromatography – mass spectrometry
HPLC	High performance liquid chromatography
H. pomatia	*Helix pomatia*
HRMS	High resolution mass spectrometry
I.d.	Internal diameter
I.m.	Intramuscular
LC-MS	Liquid chromatography – mass spectrometry
LLE	Liquid-liquid extraction
MeOH	Methanol
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSTFA	N-methyl-N-trimethylsilyltrifluoroacetamide
MTBE	Methyl tert-butyl ether
m/z	Mass to charge ratio
OFN	Oxygen free nitrogen
PAPS	3’-phosphoadenosine-5’-phosphosulfate

PICI	Positive ion chemical ionisation
Q	Quadrupole
RF	Radio frequency
RP	Reversed-phase
RPM	Rotations per minute
SdrostM	Sulfated drostanolone metabolite
SmestM	Sulfated mesterolone metabolite
SmetM	Sulfated methenolone metabolite
SIM	Selected ion monitoring
SO_3.py	Sulfur trioxide pyridine complex
SPE	Solid-phase extraction
SRM	Selected reaction monitoring
SULT	Sulfotransferase
T/E	Testosterone to epitestosterone ratio
tMestM	Theoretical sulfated metabolite of mesterolone
tMethM	Theoretical sulfated metabolite of methenolone
TMS	Trimethylsilyl
TMSI	Iodotrimethylsilane
QTOF	Quadrupole Time-of-flight
UDPGA	Uridine 5'-diphospho-glucuronic acid
UGT	Uridine diphosphoglucuronosyl transferases
WADA	World Anti-Doping Agency
WAX	Weak Anion Exchange

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Samenvatting

Achtergrond

Het gebruik van verboden middelen is al eeuwen een storende factor in sportcompetitie. Het doel van deze thesis was om het gebruik van gesulfateerde steroïdmetabolieten als merkers van steroïdgebruik in het kader van dopinganalyses te evalueren.

Methoden

De staalvoorbereiding bestond uit een dubbele vloeistof-vloeistof extractie met ethylacetaat, dit op stalen die bekomen waren aan de hand van excretiestudies. De gebruikte analytische technieken waren gebaseerd op gaschromatografie gekoppeld aan een massaspectrometer. De meeste experimenten zijn uitgevoerd met zowel elektronionisatie als chemische ionisatie. Er is in deze thesis zowel van een drievoudige quadrupool als een time-of-flight massaspectrometer gebruikgemaakt.

Resultaten

Voor **metenolone** waren SmetM1, 2 en 11 detecteerbaar voor 12, 11 en 17 dagen respectievelijk op GC-CI-MS/MS. Dit betekende een verhoging van de detectietijd met een week in vergelijking met het gebruik van geglucuronideerde metabolieten voor metenolone. De gesulfateerde **mesterolone** metabolieten zorgden voor een verdere verlenging van de detectietijd in vergelijking met analyse van de geglucuronideerde fractie. SmestM9, 11, 26 en 36 hadden een detectietijd van 11 tot 21 dagen bij analyse op GC-CI-MS/MS en GC-QTOF-MS. Voor **drostanolone** werd geen verhoging van de detectietijd verkregen door het gebruik van intacte sulfaatmetabolieten al was het verschil met de geglucuronideerde metabolieten slechts een tweetal dagen. Het is uiteraard mogelijk dat er nog onbekende gesulfateerde metabolieten bestaan voor drostanolone die deze detectietijd nog verhogen.

Besluiten

Het gebruik van intacte sulfaten voor het screenen naar steroïden lijkt veelbelovende resultaten op te leveren. Zelfs een kleine verbetering in de detectietijd van steroïden zou een meerwaarde betekenen in de strijd tegen doping.

Summary

Background

The abuse of substances has been a bad influence in competitive sports for centuries. The goal of this thesis was to evaluate the use of sulfated steroid metabolites for detecting steroids in doping control.

Methods

For the sample preparation of the urine samples obtained from excretion studies, a double LLE with ethyl acetate was performed. The techniques for analysis of the sulfated compounds in this thesis were based on gas chromatography coupled with mass spectrometry. Most of the experiments were done with both electron ionisation and chemical ionisation. Mass spectrometry was performed with both a triple quadrupole and a time-of-flight mass spectrometer.

Results

For **methenolone,** SmetM1, 2 and 11 were detectable for 12, 11 and 17 days respectively on GC-CI-MS/MS, which increases the detection time by a week in comparison to the detection of glucuronidated metabolites. The sulfated **mesterolone** metabolites had longer detection times than the glucuronidated fractions. SmestM9, 11, 26 and 36 had detection times of 11-21 days depending on whether GC-CI-MS/MS or GC-QTOF-MS was used. For **drostanolone** however, the sulfated metabolites were two days short of the glucuronidated metabolites in terms of detection time. It is possible of course, that better intact sulfated metabolites for this compound have yet to be identified.

Conclusions

Taking all these results into consideration, the use of intact sulfates to screen for steroids shows a lot of promise. Even a small increase in the detection window of steroids, could prove an asset in the fight against abuse of these substances.

1. Introduction

1.1 Doping in sports

1.1.1 Substance abuse in competitive sports

Drug abuse in sports, usually referred to as the use of doping, has been around for quite some time. However, in the past century the misuse of doping agents and anabolic androgenic steroids (AAS) in particular has skyrocketed. These products are now not only used by competitors at high level, but even by amateurs. The increase of this substance abuse among recreational athletes is likely due to the boost in confidence and well-being sportsmen get as they get closer to the condition which society boasts as "ideal" [1]. Use of doping in competitive sports was first officially banned by sports authorities in the 60's. However, banning the use of performance-enhancing drugs as a deterrent proved insufficient. An extreme example of this would be the systematic drug abuse in competition by the German Democratic Republic. Hundreds of renowned professors, scientists and doctors were involved in unethical experiments using AAS. Many of these experiments even involved administration of these drugs to underage athletes, as they proved to have a significant performance-enhancing effect [2]. The ban did not withhold East Germany from cheating their way to Olympic gold medals and world records. Even nowadays people will still attempt to find new substances to cheat themselves and their fellow competitors. It seems that recognition, praise and fame will continue to be an incentive for the abuse of substances in competitive sports.

1.1.2 World Anti-Doping Agency

This is where the World Anti-Doping Agency (WADA), which was founded on the 10th of November in 1999, comes into play. As its name implies, the WADA is an international anti-doping agency which independently coordinates the global struggle against doping abuse in competitive sports [3]. The prohibited list is an International Standard identifying substances and methods prohibited in-competition, out-of-competition and in particular sports. This list is published and updated annually by WADA. It includes classes of substances such as stimulants, diuretics, anabolic agents, narcotics, peptide hormones and other classes [4-5]. In doping control, the goal is to detect these prohibited substances in matrices such as urine, blood or saliva. These analyses are performed and improved in the so-called WADA accredited laboratories. For a laboratory to achieve accreditation by WADA, several strict criteria and standards must be met and maintained [6]. The experiments for this thesis were performed in the doping control laboratory of the Ghent University (DoCoLab), which is one of these accredited laboratories.

1.2 Anabolic androgenic steroids (AAS)

1.2.1 What are AAS?

As its name implies an anabolic androgenic steroid is a synthetic compound that has both anabolic and androgenic effects, which means it promotes muscle growth while also having a masculinizing effect. It is a group of modified derivatives of the principal male hormone testosterone. Modifications will often slow down the breakdown of the compound in our liver in comparison to testosterone. This means it will be actively binding with androgen receptors (ARs) in our blood for a longer time, and in turn promotes its anabolic and androgenic effects. For sports, the ideal AAS would enhance muscle growth, boost endurance as well as recovery while having minimal androgenic effects such as excessive hair growth, deepening of the voice in females and other unwanted changes of secondary sexual characteristics. It is apparent that these masculinising effects are in most cases not desirable, which is why manufacturers of AAS will keep trying to synthesize new compounds with more anabolic and less androgenic properties. They can however also cause a wide variety of adverse effects, such as acne, gynecomastia, behavioural disorders and cardiovascular risks. As they are one of the most widely abused performance-enhancing substances, it is of great importance that new as well as existing AAS are detectable [7].

1.2.2 Structure of steroids and their properties

Steroids have a common carbon structure consisting of 4 fused rings: 3 cyclohexane rings labelled A, B C and a cyclopentane ring labelled D. The entirety of this carbon core structure is referred to as a sterane ring system. This structure is the basis upon which all the different steroids are built. By introducing a variety of functional groups into the compound, its attributes and behaviour can be altered [8].

In case of testosterone which is the primary male steroid hormone [Figure 1], there is the addition of a carbonyl group at position C3, a double bond in the A-ring in between positions C4 and C5, a hydroxyl group at position C17 and two additional methyl groups numbered C18 and C19. Testosterone is an endogenous steroid which means it is naturally produced in our body. Its synthesis starts from cholesterol and features several enzymatic steps leading to precursors such as dehydroepiandrosterone (DHEA) and androstenedione. An exogenous compound does not naturally occur in our body. It was administered to the body through other means, such as for example oral intake or parenteral injection [8, 9].

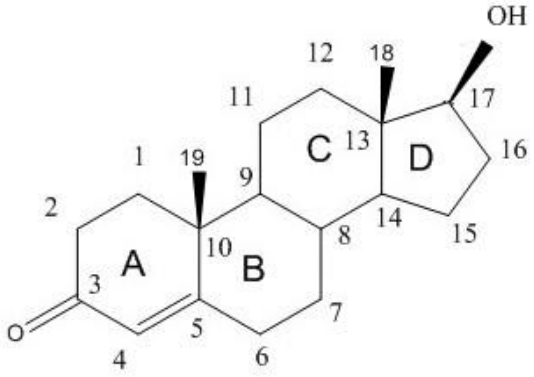


Figure 1: Structure of testosterone.

Other examples of steroids that will be addressed in this thesis are methenolone (17β-hydroxy-1-methyl-5α-androst-1-en-3-one) and mesterolone (17β-hydroxy-1α-methyl-5α-androstan-3-one) [Figure 2]. These are both exogenous steroids.

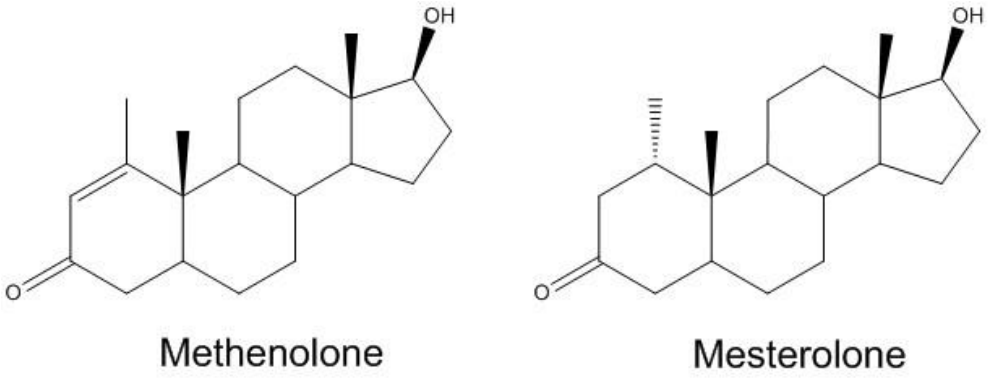


Figure 2: Structures of methenolone and mesterolone.

Different steroids will have varying affinities for the AR due to the properties of certain structural modifications. For example, reducing the carbonyl group at position C3 in the A-ring to a hydroxyl group will lower the steroids binding affinity to the AR [10]. Esterification of the 17β-hydroxyl group using carboxylic acid is often used in production of synthetic AAS that are topically applied or injected intramuscularly (i.m.).

It increases its anabolic properties by prolonging the availability and action of the synthetically derived compound [10]. After oral intake, many substances will be partly metabolized by our liver, after which only a small amount of the active drug reaches the systemic circulation. This is called the first-pass effect. However, some modifications can enhance oral activity of steroids. Substitution of the 17α-H with a methyl or ethyl group will decrease neutralization of a steroid by the first-pass effect. The 17β-hydroxyl group cannot be oxidised as easily due to steric hinder. Introducing a

methyl group at position C1 will also enhance oral activity of a steroid [11]. More modifications can be seen in Figure 3. They serve to enhance anabolic activity while minimizing androgenic activity.

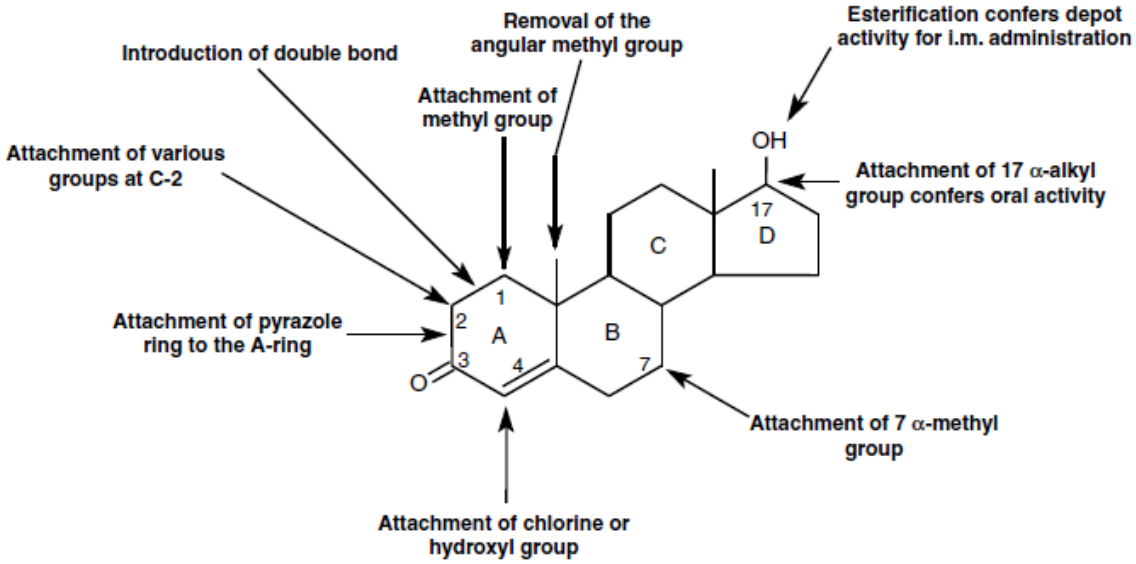


Figure 3: Several structural modifications which serve to enhance anabolic activity of a substance; i.m. = intramuscular [11].

1.2.3 Metabolism of steroids

Prior to excretion in urine, xenobiotics are metabolized in our liver. This metabolic pathway consists of two consecutive steps, referred to as the phase I metabolism and the phase II metabolism. These steps serve to inactivate a compound and make it more polar to facilitate its urinary excretion. In the framework of doping control, a lot of metabolites have a longer detection window in comparison to their parent compounds.

1.2.3.1 Phase I metabolism

As previously mentioned, the compound is enzymatically modified by cytochrome P450 enzymes in order to facilitate its excretion. This can be achieved through oxidation, reduction or hydrolysis of the drug. As an example, the 5α- and 5β-reduction of the A-ring will be discussed, which is the principal step in the metabolism of 3-keto-4-ene steroids such as testosterone. The irreversible reduction of the double bond between positions C4 and C5 by the enzymes 5α- and 5β-reductase, creates an asymmetric centre at position C5, which results in two isomers as can be seen in Figure 4. Later steps include among others the reduction of the carbonyl group at position C3 leading to 3-hydroxy isomers [12]. The B-, C- and D-rings can also be altered during phase I.

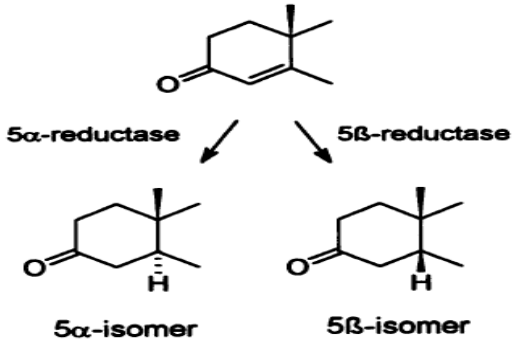


Figure 4: The 5α- and 5β-reduction of the A-ring in 3-keto-4-ene steroids [12].

1.2.3.2 Phase II metabolism

Following the introduction of new functional groups by the phase I reactions, the steroids will now be altered in phase II, also known as the conjugation phase. The vast majority of androgens is conjugated with either glucuronic acid or sulfate, which results in only a very small number of unconjugated metabolites being excreted in urine [12].

Conjugation with glucuronic acid, also referred to as glucuronidation, is catalysed by uridine diphosphoglucuronosyltransferases (UGTs). The reaction uses uridine-5'-diphosphoglucuronic acid (UDPGA) as a cofactor as is displayed in Figure 5. This conjugation step alters the properties of the molecule which will often lead to inactivation [13].

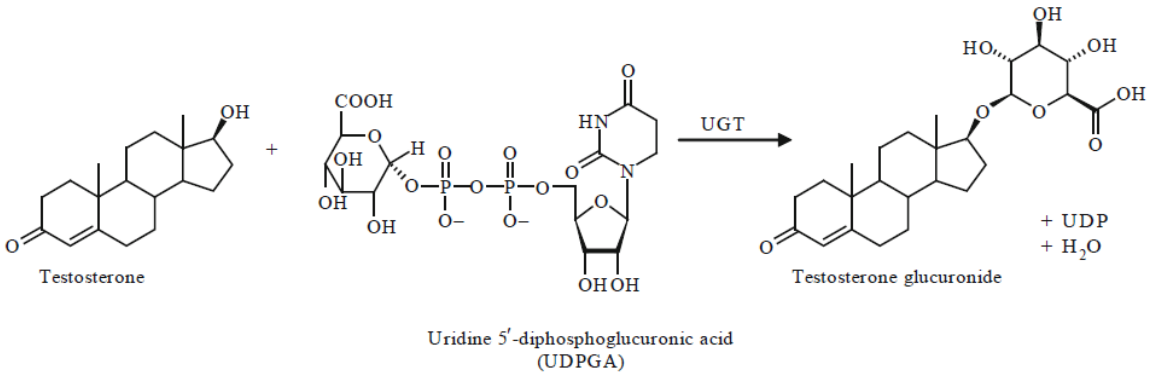


Figure 5: Glucuronidation of testosterone [13].

The second type of conjugation is referred to as sulfate conjugation or sulfation. This reaction employs 3'-phosphoadenosine-5'-phosphosulfate (PAPS) as a co-factor and uses sulfotransferases (SULTs) as catalysing enzymes. During sulfation a sulfate moiety such as sulfur trioxide (SO_3) will be transferred from PAPS to the substrate [Figure 6]. There are membrane-bound SULTs and cytosolic SULTs, only the latter being of any relevance to sulfonation of steroids [13].

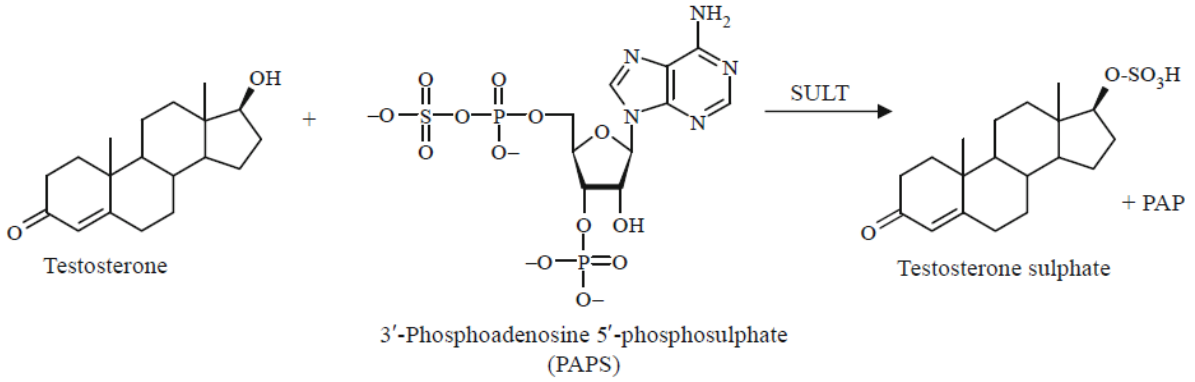


Figure 6: Sulfation of testosterone [13].

While glucuronidation appears to favour steroids with 3α and 17β functional groups, SULTs will mainly sulfate steroids in possession of a 3β-hydroxyl group. This is the case for one of the primary endogenous precursors of testosterone: DHEA [8,13].

1.2.4 Steroid sulfates

Recently, steroid sulfates have been getting a lot more attention in context of doping control analysis. Up until now, analyses usually involved the detection of glucuronidated steroids. Tests such as for example the determination of testosterone/epitestosterone (T/E) ratios, were developed with use of glucuronidated compounds in mind. So, validated methods using sulfated steroids for routine screening purposes have yet to be established. There are a few drawbacks to the use of steroid sulfates. Hydrolysis of steroid sulfates by chemical means such as the use of strong acids, will often lead to degradation of the analyte and increased matrix-derived interference, which in turn impairs sensitivity of analysis [14-16]. However, by limiting analyses to glucuronic acid conjugates, some important steroid sulfate metabolites could potentially be overlooked. These sulfated analytes could enhance sensitivity, prolong detection windows of certain steroids leading to greater retrospectivity and allow easier distinction between exogenous and endogenous androgens [14, 16]. These attributes would make for an incredibly useful asset in the fight against AAS abuse. The fact that reference materials for steroid sulfates are scarce to say the least, constitutes an additional challenge. Having these standards at the ready would facilitate identification of sulfated metabolites, and possibly improve the advancement of appropriate methods to analyse steroid sulfates [16].

1.3 Methods for sample preparation

To ensure the optimal detection of a compound, with minimal background noise, an optimized sample preparation will be employed. Generally, sample preparation will consist of hydrolysis followed by solid-phase extraction (SPE) or liquid-liquid extraction (LLE), evaporation and derivatization as a final step in case of gas chromatography coupled with mass spectrometry (GC-MS) [17]. These steps are illustrated in Figure 7.

Figure 7: General procedure for sample preparation in doping control [17].

Hydrolysis is an important step during sample preparation, especially for GC-MS. Steroids conjugated with glucuronides are often not volatile enough for analysis in GC-MS, which is why they are enzymatically cleaved using a β-glucuronidase. Enzymes for hydrolysis of sulfated steroids are also available, although sulfate conjugates are not as efficiently hydrolysed [17]. However, this was not a problem for the sample preparation in this thesis as hydrolysis was not performed for steroid sulfates. It appears that the sulfate groups are cleaved during injection on GC-MS, which eliminates them from analysis. This cleavage is most likely the result of the high temperatures used during injection of the samples. Detection of these so-called non-hydrolysed steroid sulfates is the main focus of this thesis.

1.3.1 Extraction methods: SPE and LLE

For the extraction and purification of steroid compounds from a urine sample, SPE and LLE methods were applied. The aim of this extraction is to minimise matrix interferences, without significant losses of our analytes.

In LLE, an organic solvent such as for example ethyl acetate is used. The steroid compounds will be partitioned favourably in this solvent [18]. The organic phase containing the steroid compounds is then evaporated using oxygen free nitrogen (OFN) to prevent unwanted oxidation reactions [8].

In order to employ SPE, the sample must pass through some sort of column, for example a cartridge. In case of sulfated steroids, a cartridge device based on weak ion exchange (WAX) is used. Just like in liquid chromatography, the combination of mobile and stationary phases will determine which analytes may pass and which sample compounds are retained. As steroid sulfates

will be negatively charged, they are retained by the positively charged amine groups that are bound to the stationary phase of the WAX cartridge [Figure 8].

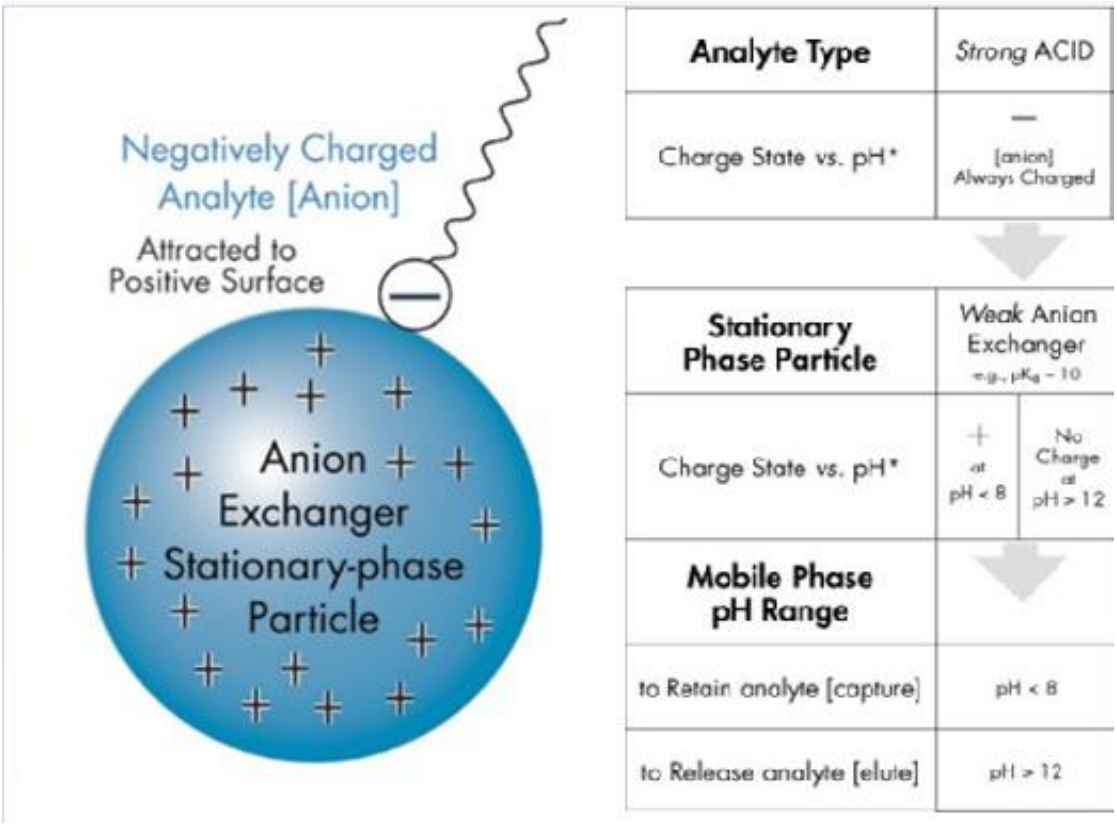


Figure 8: Illustration of WAX principles [19].

As long as the pH is kept below 8 approximately, the compounds will be retained by the stationary phase. When the pH is above 12 however, the weak anion exchanger loses its charge and the analytes will elute [19].

While LLE will usually cause the loss of some analytes, the compounds are efficiently retained using SPE. However, SPE will also retain other interfering compounds. This can result in a lot of background noise during analysis, which additional purification steps will not always be able to reduce.

1.3.2 Derivatisation

GC-MS will often require an additional step of sample preparation, called derivatisation. Derivatisation of substances will enhance their volatility, make them more thermally stable and improve their chromatographic attributes. This is done by converting the hydroxyl and keto functions of the steroid, to trimethylsilyl (TMS) ethers and enol derivatives [18]. To this end, a mixture of N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA), ethanethiol and ammonium iodide (NH_4I) is employed (500:4:2) [Figure 9]. Iodotrimethylsilane (TMSI) will be formed *in-situ* as

a result of MSTFA and NH_4I reacting with each other. TMSI, which serves as a catalyst in this mixture, will fall apart into iodine and react with the steroid compounds. However, ethanethiol will continuously inhibit the formation of this iodine by acting as a reducing agent [17, 18]. By performing this derivatisation at higher temperatures, TMSI will be able to enolise even the more stable keto groups, as TMSI is the most reactive trimethylsilylating reagent [8].

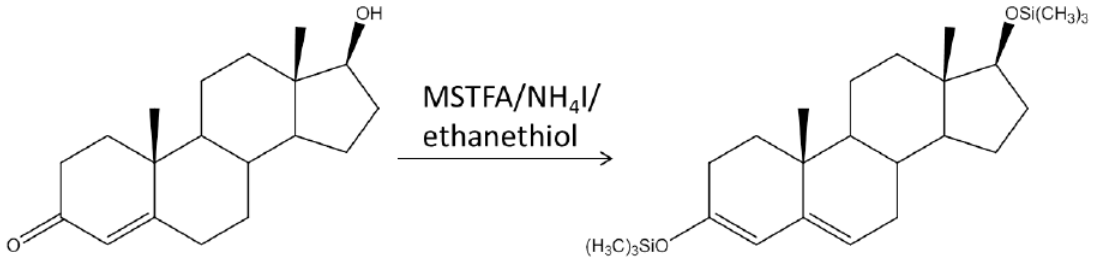


Figure 9: Derivatization scheme of testosterone [17].

1.4 Methods of detection

1.4.1 *Chromatography*

In analytical chemistry, most samples are mixtures of several compounds. In order to isolate the substances of interest, chromatography is often the method of choice in laboratories. This isolation is done by separation of the sample over a column. The column is coated with a stationary phase, along which the sample is led under the guidance of a mobile phase. Compounds that interact with this stationary phase will be held back longer than molecules that do not interact with the stationary phase, the latter will elute first. As the compounds of the mixture elute at different times, they are separated. There are two important subtypes of chromatography that complement each other in doping control analyses: liquid chromatography (LC) and gas chromatography (GC).

1.4.1.1 Liquid chromatography

LC requires analytes to be soluble in the mobile phase. In contrast to GC, compounds do not have to be volatile, thermally stable and non-polar to be compatible with LC. This is the main reason why LC became such a staple in doping analysis. Reversed-phase High Pressure Liquid Chromatography (RP-HPLC) is the most commonly used LC method. In RP-HPLC a non-polar stationary phase is used in combination with a polar/organic mobile phase [20]. Non-polar compounds such as steroids will be retained by hydrophobic interactions with the non-polar stationary phase, while polar compounds will elute faster.

1.4.1.2 Gas chromatography

As mentioned above, compounds in GC need to be volatile. They have to be brought in their gas phases. This is one of the drawbacks of GC, as analytes with a molecular mass above 800 will not have an adequate vapor pressure that allows them to vaporise. The higher temperatures that are required to bring the compounds in the gas phase, may also cause some to decompose [21]. The mobile phase in GC is often referred to as the carrier gas and leads the analyte past the stationary phase. Carrier gasses that are often used in GC are hydrogen, nitrogen and helium. Helium is the ideal carrier gas for doping control as it is less damaging to the ionisation source than hydrogen, generates less background noise and results in much faster analyses than nitrogen. Injection of the sample takes place at temperatures way above the boiling point of the compounds. Usually a temperature above the least volatile compound is chosen. After injection, the compounds will be immediately vaporised and mingle homogeneously with the carrier gas. The mobile phase will lead the compounds to the column which is located in an oven. Here, temperature programming can be employed to obtain separation. Because concentrations of steroids in doping control samples are often very low, the injection methods of choice are splitless injection or on-column injection [22].
During splitless injection the sample is injected while the splitter is closed. The compounds are subsequently vaporised and move to the column [23]. On-column injection is different, as the sample is brought directly onto the column as a liquid using a syringe. Using this technique, larger volumes of diluted samples can be introduced without too many complications related to the solvent [24].
Nowadays, GC and LC are usually employed in combination with mass spectrometry for detection, which enhances analyses both qualitatively and quantitatively.

1.4.2 Mass spectrometry

In mass spectrometry (MS), ions are separated according to their mass-to-charge ratio (*m/z*). The analytes are ionised by the ion source after which they are sent to the mass analyser. Before reaching the mass analyser, the ions are accelerated and transported through a magnetic and/or electric field. Ions with the appropriate *m/z* will be able to pass through and reach the detector. They are then presented in a graph called the mass spectrum [Figure 10].

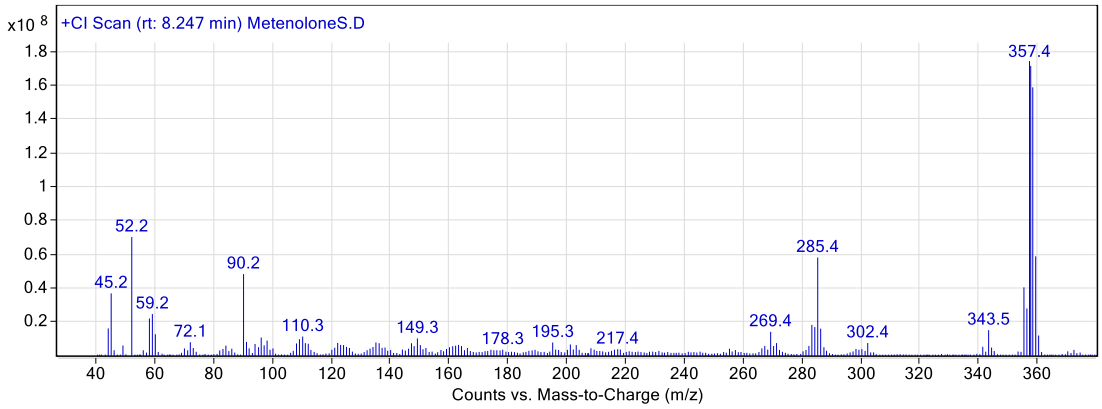


Figure 10: Positive CI full scan mass spectrum of the trimethylsilylated form of metenolone sulfate.

The x-axis illustrates the molecular mass at which an ion peak is generated. On the y-axis, the intensity of the peak is presented. The molecular ion peak arises from the ionisation of the molecule itself and therefore gives us the molecular mass of that compound. The molecular ion peak is however, not necessarily the biggest peak. Some compounds are unstable and easily fall apart into fragment ions during ionisation [26]. This fragmentation of the molecule can be used to gain structural knowledge on the analytes, as the fragments originate from the compound.

1.4.2.1 Ionisation techniques

The amount of fragmentation is highly dependent on the type of ionisation that is used during analysis with MS. These methods of ionisation are generally categorised as hard ionisation methods which generate many fragments, and soft ionisation techniques. The latter will cause less fragmentation. The most commonly employed methods of ionisation in GC-MS, are electron ionisation (EI) and chemical ionisation (CI).

EI is a very popular hard ionisation method and gives a ton of structural information on the analyte. The ion source consists of a filament that emits electrons, these electrons are then sent to the ionisation chamber where they generate ions by collision with the molecules [27]. The positively charged ions are then flung to the exit gap by a repeller plate with positive voltage, after which they are focussed towards the mass analyser under the influence of an electromagnetic field [27].

As CI is a soft ionization method, it will lead to less fragmentation in comparison to EI. Structural information will be harder to obtain, but the molecular ion mass is easier to distinguish. CI makes use of reagent gasses such as methane or ammonia, which are ionised by electrons originating from a similar filament used in EI [28]. The protonated reagent gas will then in turn protonate the analyte. Ammonia has a higher proton affinity than methane, which leads to less fragmentation and a higher abundance of the molecular ion [25]. Ammonia is the reagent gas of choice for the

analyses performed in this thesis. As we will want to select the desired precursor ions using a full scan, after which we use the product ion scan mode with harsher fragmentation to identify metabolites.

1.4.2.2 Quadrupole as mass analyser

This part of the mass spectrometer will decide which ions can reach the detector. It consists of four poles that are connected to an electrical circuit. Molecules that were ionised by the ion source, will enter a radiofrequency field which is generated by the quadrupole [Figure 11]. The poles that face each other will either get a positive or a negative direct current (DC) voltage, upon which an alternating radio frequency (RF) current is superimposed [29, 30].

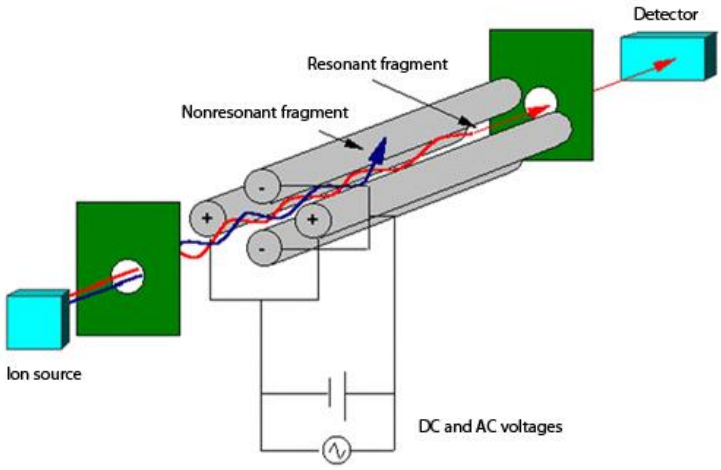


Figure 21: Illustration of a quadrupole [29].

The quadrupole will then serve as a band-pass filter, which means it will only let ions with a certain mass-to-charge ratio or *m/z* pass through towards the detector. By altering the DC and RF voltages, we can scan for ions in the desired mass range [30]. In MS, an electron multiplier will normally be used as detector. This device employs a cascade effect involving electrons to generate the mass spectrum.

1.4.2.3 Tandem MS

Tandem MS (MS/MS) can for example be performed using a triple quadrupole instrument, which consists of three quadrupoles that are connected in series [Figure 12]. MS/MS is a very valuable asset in the search for structural information. In the scan mode, only the first quadrupole Q1 is used to scan a certain mass range. Once we have selected ions with a certain *m/z* that are of interest, we can employ the product ion scan mode. Q1 will select the ions of interest, Q2 will act as a collision cell and fragment these ions. Q3 will then scan for these fragments [32]. By employing this

method, we are certain that the fragments that we see in the mass spectrum generated by Q3 originate from the selected precursor ion in Q1.

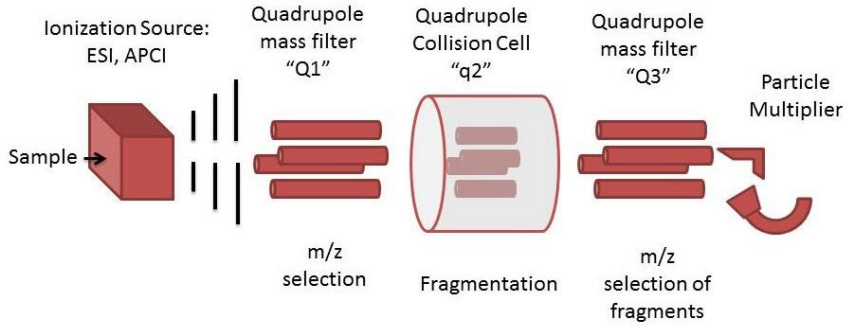


Figure 12: Illustration of a triple quadrupole mass spectrometer [31].

1.4.3 GC-PICI-MS/MS

The experiments in this thesis were mostly performed using a GC-PICI-MS/MS device. This combines GC with a triple quadrupole mass spectrometer. It allows for a short analysis time with high sensitivity and meets the requirements for both quantitative and qualitative screening of substances [33]. Positive ionisation of the analytes is done using CI with ammonia as a reagent gas. The molecular ion is easy to distinguish as fragmentation during a full scan is mild. In comparison with EI however, not a lot of structural information will be gained on the compound using just a scan. The scan is therefore used to select precursor ions. More fragmentation will occur during the subsequent product ion scans using the three-dimensional mass analyser. Structural knowledge on possible metabolites can be obtained by observing their collision-induced dissociation patterns [25]. CI was the most appropriate ionisation technique for the identification of metabolites, as EI leads to harsh fragmentation even during a full scan. Which makes searching for metabolites and elucidating their structures a significant challenge.

1.4.4 GC-Quadrupole Time-Of-Flight (QTOF)-MS

This instrument combines gas chromatography with a quadrupole and time-of-flight analyser, which allows for high-resolution, high mass accuracy analysis of all ions in parallel. It can be described in the simplest way as a triple quadrupole with the last quadrupole section replaced by a TOF analyser. Employing a TOF analyser, the ion's *m/z* will be determined via time measurement. After acceleration, the velocity of the ion will vary based on its *m/z*. This means that the time it takes for this ion to reach the detector, directly correlates with its *m/z*. The high-resolution spectra will

partially or even completely solve the problem of interfering ion peaks that occur at similar nominal mass [34].

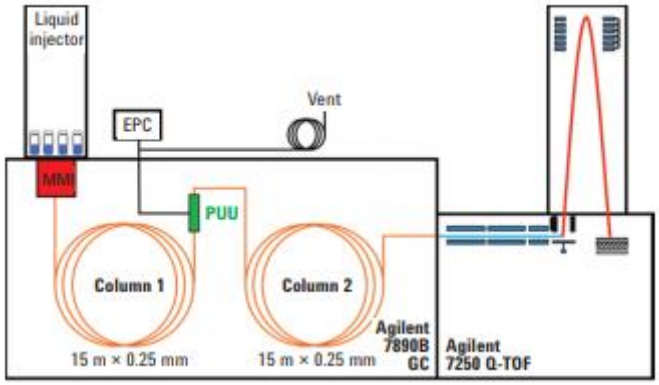


Figure 13: Setup of the Agilent 7250 Quadrupole Time-of-Flight GC/MS system employed in this thesis [35].

2. Materials and methods

2.1 Chemicals and reagents

N,N-Dimethylformamide (DMF), 1,4-dioxane and sulfur trioxide pyridine complex (SO_3.py) 98% were obtained from Sigma (St. Louis, MO, USA). Ethyl acetate (EtOAc) was obtained from Acros Organics (Geel, Belgium). Acetonitrile (ACN) and methyl tert-butyl ether (MTBE) were from Biosolve (Valkenswaard, The Netherlands). Sodium hydrogen carbonate ($NaHCO_3$) was obtained from Fisher Scientific (Loughborough, UK). Sodium chloride (NaCl), sodium sulfate (Na_2SO_4), potassium carbonate (K_2CO_3), disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4) and sodium acetate (NaOAc) were purchased from Merck (Darmstadt, Germany). Isopropanol was from Avantor (Deventer, The Netherlands). The reagents used to make the solution for derivatisation (REA-19) were N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) obtained from Karl Bucher Chemische Fabrik GmbH (Waldstetten, Germany), ethanethiol obtained from Acros (Geel, Belgium) and ammonium iodide (NH_4I) was purchased from Merck (Darmstadt, Germany) employed in a 500:4:2 ratio respectively. The gases helium, ammonia, hydrogen and oxygen-free nitrogen (OFN) were delivered by Air Liquide (Bornem, Belgium). β-Glucuronidase from Escherichia coli K12 and β-glucuronidase/aryl sulfatase from Helix pomatia were obtained from Roche Diagnostics GmbH (Mannheim, Germany).

The carbonate buffer (pH = 9.5) was prepared by dissolving 135g K_2CO_3 and 111g $NaHCO_3$ in 900 mL of double-distilled water. Phosphate buffer (pH = 7) was made by dissolving 7.1 g of $Na_2HPO_4.2H_2O$ and 1.4 g of $NaH_2PO_4.H_2O$ in 100 mL water. The acetate buffer (pH = 5.2) was obtained by dissolving 136 g of NaOAc in 900 mL of water and the addition of acetic acid until a pH of 5.2 was obtained. Formic acid (HCOOH) in H_2O 2% (V/V) was made by dissolving 2 ml of formic acid from Avantor (Deventer, The Netherlands) in 98 ml aqua bidest. Saturated ammonium hydroxide (NH_4OH) in MeOH was made by dissolving 5ml of NH_4OH purchased from Merck (Darmstadt, Germany) in 95ml MeOH obtained from Fisher Scientific (Loughborough, UK).

2.2 Reference standards

Methenolone (17β-hydroxy-1-methyl-5α-androst-1-en-3-one) was a gift from the Drug Control Centre of King's College London (London, UK). Methandienone (17β-hydroxy-17α-methylandrosta-1,4-dien-3-one), epimethendiol (17β-methyl-5β-androst-1-ene-3α,17α-diol), mesterolone metabolite (3α-hydroxy-1α-methyl-5α-androstan-17-one), methenolone metabolite (3α-hydroxy-1-methylen-5α-androstan-17-one) and drostanolone (17β-hydroxy-2α-methyl-5α-androstan-3-one) were obtained from NMI (Pymble, Australia). Mesterolone (17β-hydroxy-1α-methyl-5α-androstan-3-one) was from Schering (Berlin, Germany).

2.3 Synthesis and SPE of steroid sulfates

2.3.1 Synthesis

The protocol used to synthesize the steroid sulfates had already been published [16]. Two mL of the steroid compound (100 µg/mL) is transferred to a test tube, which is then evaporated to dryness using OFN. Then 100 µL of 1,4-dioxane is added to the test tube containing the dried steroid. In the next step, 10 mg of SO_3.py is dissolved in 100 µL of DMF and added to the same tube. The mixture is transferred to a vial, which is then stirred (1000 rpm) at room temperature for 4 hours. After 4 hours 1.5 mL of water is added to stop the reaction.

2.3.2 Solid-phase extraction

The next step is SPE using an Oasis WAX 3cc cartridge (60mg, 30 µm) to purify the sulfated compound. After pre-conditioning with 5 mL of methanol followed by 5 ml of water, 1.7 mL of the reaction mixture is loaded onto the WAX cartridge. The washing step involved 5 mL of HCOOH in

H_2O 2% (V/V) followed by 5 mL of water. Any remaining free steroids were then eluted in vacuum using 5 mL of methanol. The sulfated steroids were eluted with 5 mL of a saturated ammonia solution in methanol 5% (V/V). After evaporation to dryness with OFN the compound is ready for derivatisation followed by analysis. If the sulfated compounds are destined for storage and later use, they are dissolved in acetonitrile after evaporation to dryness.

2.4 Sample preparation

2.4.1 Non-hydrolysed steroid sulfates

The LLE protocol for sulfated compounds without an additional hydrolysis step required 2 mL of a urine sample in combination with 1 mL of carbonate buffer (pH = 9.5). Then 1 scoop of NaCl was added, followed by 3 mL of ethyl acetate. Extraction was performed by rolling the test tubes for 15 minutes, after which they were centrifuged at 2000 rpm for 5 minutes. Then the organic layer was transferred to a second tube. Another 3 mL of EtOAc is added to the original test tube, it is rolled for 15 minutes and centrifuged for 5 more minutes at 2000 rpm. After transferring the organic layer a second time, we have performed a double extraction. After evaporation to dryness using OFN, the compound is now ready for the derivatisation step.

2.4.2 Hydrolysis of steroid sulfates with Helix pomatia

Urine samples (2 mL) were hydrolysed by the addition of 1 mL acetic buffer (pH = 5.2) and 50 µL of β-glucuronidase/aryl sulfatase from *Helix pomatia.* The samples were then incubated for 1.5 hours at 56 °C. After the incubation, 1 mL of carbonate buffer (pH = 9.5) was added in combination with 5 mL of MTBE. The test tubes were then rolled for 20 minutes, after which the organic layer was transferred to a new test tube. It was then evaporated to dryness by employing OFN.

2.4.3 Hydrolysis of glucuronidated steroids

For experiments that involved glucuronidated compounds, 2 mL of the urine sample was hydrolysed by adding 1 mL phosphate buffer (pH = 7) in combination with 50 µL of β-glucuronidase from *E. coli*. The samples were then incubated for 1 hour at 56 °C. Subsequent steps of this protocol were identical to those described in *2.4.2*.

2.4.4 Derivatisation

Here, the steroid will be converted into its trimethylsilylmethyl derivative. After adding 60 µL of MSTFA/ethanethiol/NH_4I (500:4:2) to the dried residues, the residue is transferred to an appropriate vial and placed in an oven (80 °C) for 30 minutes. The derivatised compound is now ready for analysis.

2.5 Instrumentation

2.5.1 GC-CI-MS/MS and GC-EI-MS/MS

An Agilent GC 7890 gas chromatograph coupled to an Agilent 7010B triple quadrupole mass spectrometer (QQQMS, Agilent Technologies, Palo Alto, USA) was employed. The sample was prepared and introduced using the high-end PAL3 sample injector (CTC analytics, Switzerland). In CI experiments ammonia was utilised as the reagent gas at a gas flow of 20% (standard configuration of the Agilent 7000B QQQMS), while EI was employed at 70 eV. The mass spectrometer utilised nitrogen as collision gas (flow rate = 1.5 mL/min), and helium was purged into the collision cell (flow rate = 2.25 mL/min). The GC was equipped with a 12 m × 250 µm I.D., 0.25 µm film thickness HP-1MS column (Agilent Technologies, Palo Alto, USA). The oven temperature program is described in [Table 1].

Table 1: Oven temperature program for GC-CI/EI-MS/MS.

	Rate (°C/min)	Value (°C)	Hold time (min)	Run time (min)
Initial		110	0.25	0.25
Ramp 1	70	125	0.2	0.66429
Ramp 2	35	186	0.2	2.6071
Ramp 3	2.2	204	0	10.789
Ramp 4	22	245	0	12.653
Ramp 5	45	270	0	13.208
Ramp 6	75	320	0.9	14.775

Helium was used as carrier gas at a constant flow rate of 0.9 ml/min for 9.2 min and then increased with 1.5 mL/min to 4 mL/min in order to flush out the column; 1.5 µl was injected pulsed splitless at 275 °C.

The GC-QQQMS was controlled with GCMS MassHunter software (version B.08, Agilent Technologies, Waldbronn, Germany), while the Gerstel equipment was controlled by Maestro software (version 1.4, Gerstel).

2.5.2 GC-QTOF-MS

An Agilent 7250 Quadrupole Time-of-Flight GC/MS system with an EI ion source was employed in these experiments. The sample was prepared and introduced pulsed splitless at 250 °C using the Agilent 7693A ALS (Automatic Liquid Sampler). The GC was equipped with a 5 m + 10 m × 200 µm I.D., 0.11 µm film thickness HP-1MS column (Agilent Technologies, Palo Alto, USA). Oven temperature program can be found in [Table 2]. The mass spectrometer utilised nitrogen as collision gas (flow rate = 1 mL/min), and helium was purged into the collision cell (flow rate = 4 mL/min).

Table 2: Oven temperature program for GC-QTOF-MS.

	Rate (°C/min)	Value (°C)	Hold time (min)	Run time (min)
Initial		110	0.1	0.1
Ramp 1	75	125	0.1	0.4
Ramp 2	50	185	0	1.6
Ramp 3	3	209	0	9.6
Ramp 4	15	245	0	12
Ramp 5	75	320	1	14

2.6 Excretion studies

For the first long-term methenolone excretion study, a single dose of 50 mg of methenolone (Primobolan, Schering) was administered orally by a healthy male volunteer (36 years, 65 kg). A pre-administration (blank) urine sample was collected at 0 h and subsequent urine samples were collected at 3 h, 5 h, 8 h and every 12 hours from day 1 until day 23. The second methenolone excretion study was obtained from Kazakhstan. A single dose of 50 mg of methenolone was administered orally to a healthy male volunteer. Four blank urine samples were collected before administration. Subsequent urine samples were collected every 24 hours from day 1 post-administration until day 51.

For the long-term mesterolone excretion studies, a single dose of 25 mg of mesterolone (Proviron, Bayer) was administered orally to three healthy male volunteers (36 years, 65 kg; 30 years, 80 kg;

45 years, 96 kg). A pre-administration (blank) urine sample was collected at 0 h and subsequent urine samples were collected at 2 hours (h), every 2 h until 12 h, and every 24 h from day 2 until either day 15 (in case of excretion study 1), day 22 (for excretion study 2) or day 29 (excretion study 3).

The long-term drostanolone excretion study was done by oral intake of a single dose of 25 mg of drostanolone by a healthy male volunteer (36 years, 65 kg). A pre-administration (blank) urine sample was collected at 0 h and subsequent urine samples were collected at 1 h, 3 h, 5 h, 8 h, 12 h and every 12 h until day 5. Urines samples after day 5 were taken every 24 hours up to day 29.

3. Results and discussion

3.1 Sample preparation techniques

To compare the efficiency of different techniques for the extraction of non-hydrolysed sulfated steroids, five reference standards were sulfated by employing the protocol described in *2.3*. These compounds were epimetendiol, mesterolone, methandienone and methenolone along with its metabolite (3α-hydroxy-1-methylen-5α-androstan-17-one). These compounds were then used to evaluate possible techniques for their extraction from urine. The addition of a sulfate group will make a steroid compound more polar. To extract this rather polar sulfated steroid from urine, a polar solvent will be required.

3.1.1 LLE with EtOAc: single extraction

First liquid-liquid extraction using EtOAc was tested. EtOAc is a polar solvent that does not fully mix with water, which should make it the ideal candidate for the extraction of steroid sulfates. Two different negative urines were spiked with 2.5 µg (25 µL of 100 µg/mL) of the 5 synthesized sulfated compounds. Both negative urines (i.e., control samples) will additionally be spiked after the extraction step to evaluate the efficiency of the extraction. The protocol for extraction is identical to the one described in *2.4.1*, that a single extraction with 5 mL of EtOAc was used instead of a double extraction employing 3 mL of EtOAc. The control samples which are spiked after the evaporation step with OFN, should in theory yield 100% of the sulfated steroid. For the single extraction, the yield ranged from 28% to 96% depending on the compound. With an average yield of 62% this is

an efficient method for extraction, especially considering the small amount of time required to carry out this protocol.

3.1.2 LLE with EtOAc: double extraction

The protocol for this extraction method has already been described in *2.4.1*. The yield of sulfated steroids using this method is comparable to the yield found for the single extraction. It also averaged around 62%. However, for the compounds that are of most interest for this thesis (methenolone, mesterolone and their metabolites) the yield obtained with a double extraction was clearly superior when compared to the single extraction with EtOAc. Extraction using plastic test tubes was also explored, but was inferior to the use of glass test tubes.
A question that remained was whether SPE could be more efficient than LLE. The next step was testing possible SPE methods.

3.1.3 Solid-phase extraction

Analogous with LLE, two different negative urines were spiked before and after SPE to evaluate the yield of the sulfated compounds. A detailed description of the protocol employed can be found under *2.3.2*. After analysis the samples prepared with SPE were found to have a lot of interfering compounds which results in background noise. To reduce these interferences, some additional purification steps were added. One mL TBME was added to the test tube after evaporation with OFN, which was then transferred to a new tube and dried again. Then the same step was repeated but using EtOAc. Employing these additional washing steps, the yield of compounds was only 10% which is vastly inferior to LLE. Introduction of a washing step with more polar solvents such as acetonitrile and 2-propanol was also evaluated, but did not lead to better results than the double LLE.

Taking both the amount of labour and the yield in consideration, double LLE was the method of choice. It was therefore applied for sample preparation of non-hydrolysed sulfated compounds in the remainder of this thesis.

3.2 Methenolone

Methenolone (17β-hydroxy-1-methyl-5α-androst-1-en-3-one) has a molecular weight of 302.458 g/mol. It is an anabolic steroid with weak masculinising properties, which makes it a tempting drug for abuse by athletes. Its structure was illustrated earlier [Figure 2]. Note that the non-hydrolysed sulfated form of methenolone was employed in these experiments, unless explicitly stated otherwise. Secondly, when sulfated steroids are analysed on GC, the sulfate group is cleaved of during injection resulting in a loss of 90 *m/z*.

3.2.1 Searching for (long-term) metabolites with GC-CI-MS/MS

The approach for finding new long-term metabolites by employing GC-CI-MS/MS in this thesis was mostly analogous to the detection protocol described earlier [36, 37].
Initially a theoretical list of potential metabolites was compiled [Figure 14]. This list is generated by taking the most common metabolic pathways of steroids in humans into account. These metabolites are generated by introduction of hydroxyl functions, 17-epimerisation, reducing oxo groups and double bonds. Their expected precursor ion and product ions were selected. This was done based on their assumed fragmentation behaviour which can be theoretically derived from their structures [25]. These transitions were then used to set up a multiple reaction monitoring (MRM) GC-CI-MS/MS method. As an example, for the theoretical sulfated metabolite 1 of methenolone (tmethM 1), the precursor ion selected was 357 *m/z* in combination with the product ions 187, 147, 209, 181, 241 and 267 *m/z* at a collision energy of 30 eV.
Next, urine samples from the first long-term excretion study for methenolone were prepared (using the sample preparation described in *2.4.1)* for analysis.
Using the MRM method, a pre-administration sample (taken at 0h) and an excretion sample (8h) were analysed with GC-CI-MS/MS. Possible metabolites were then identified by looking for peaks that are present in the 8h excretion sample, but absent in the pre-administration or blank sample. Transitions originating from peaks that are absent in the blank sample are registered along with their corresponding retention times. The precursor ions of these peaks are selected and a product ion scan (at collision energy 10 and 20 eV) is performed on each of these precursors.

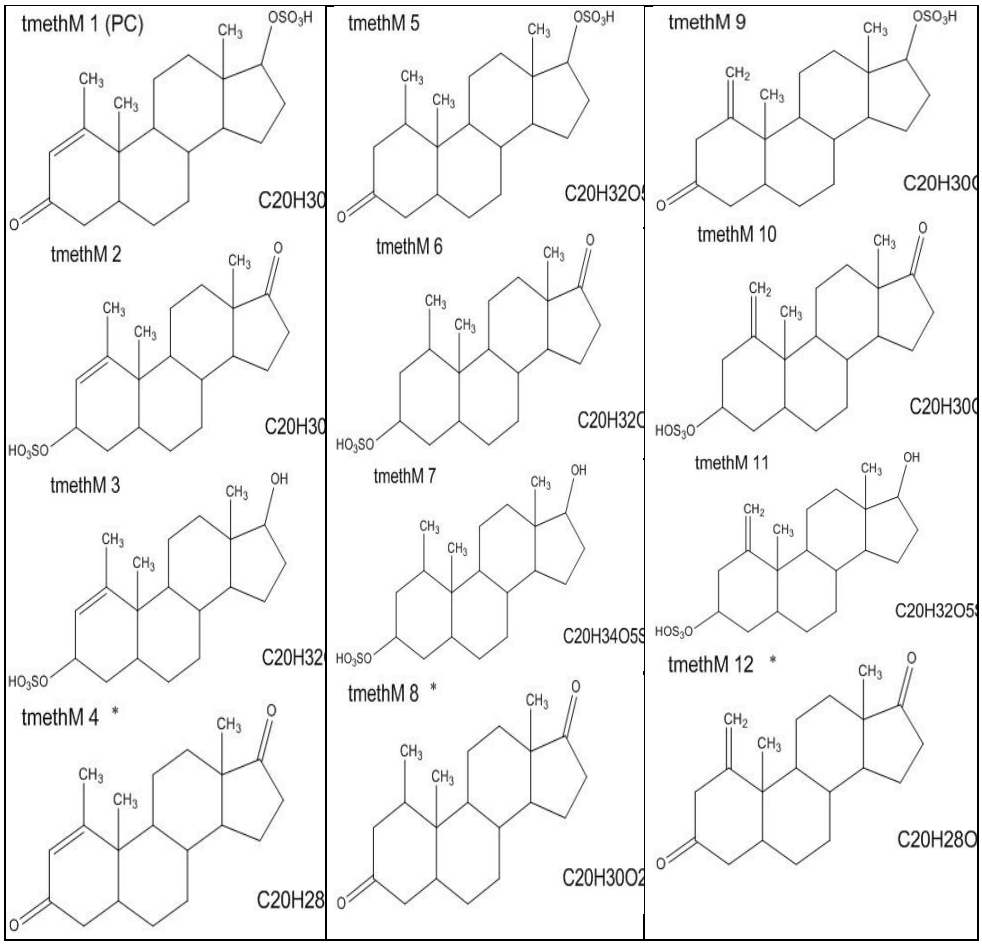


*Figure 14: Structures of theoretically possible non-hydrolysed sulfated methenolone metabolites. For each of these metabolites 1 or 2 hydroxyl groups can additionally be introduced, which makes a total of 33 theoretically possible sulfated metabolites. *tMethM 4, 8 and 12 will not be sulfated because there is no hydroxyl group present. These metabolites can however be sulfated if 1 or 2 additional hydroxyl groups are present.*

The transitions found with these product ion scans are used to set up a dynamic MRM (dMRM) method, which is tested using the blank and 8h excretion sample. After removal of endogenous compounds and interfering transitions, the method is ready to be used for analysis of the first long-term excretion experiment. Using that data, the metabolites that were found and processed using the dMRM method will be evaluated on their respective detection times. The blank sample and excretion urines ranging from 3 hours post-administration to 552 hours (23 days) were analysed on the GC-CI-MS/MS.

The detection window of each metabolite was checked to see if it has potential for screening. A long-term metabolite would be a metabolite that is clearly distinguishable during several days or even weeks after intake of the drug. All detected methenolone metabolites and their detection times can be found in Figure 15. Sulfated methenolone metabolite 11 (SmetM11) seems very promising with a detection time of over 2 weeks when employing GC-CI-MS/MS.

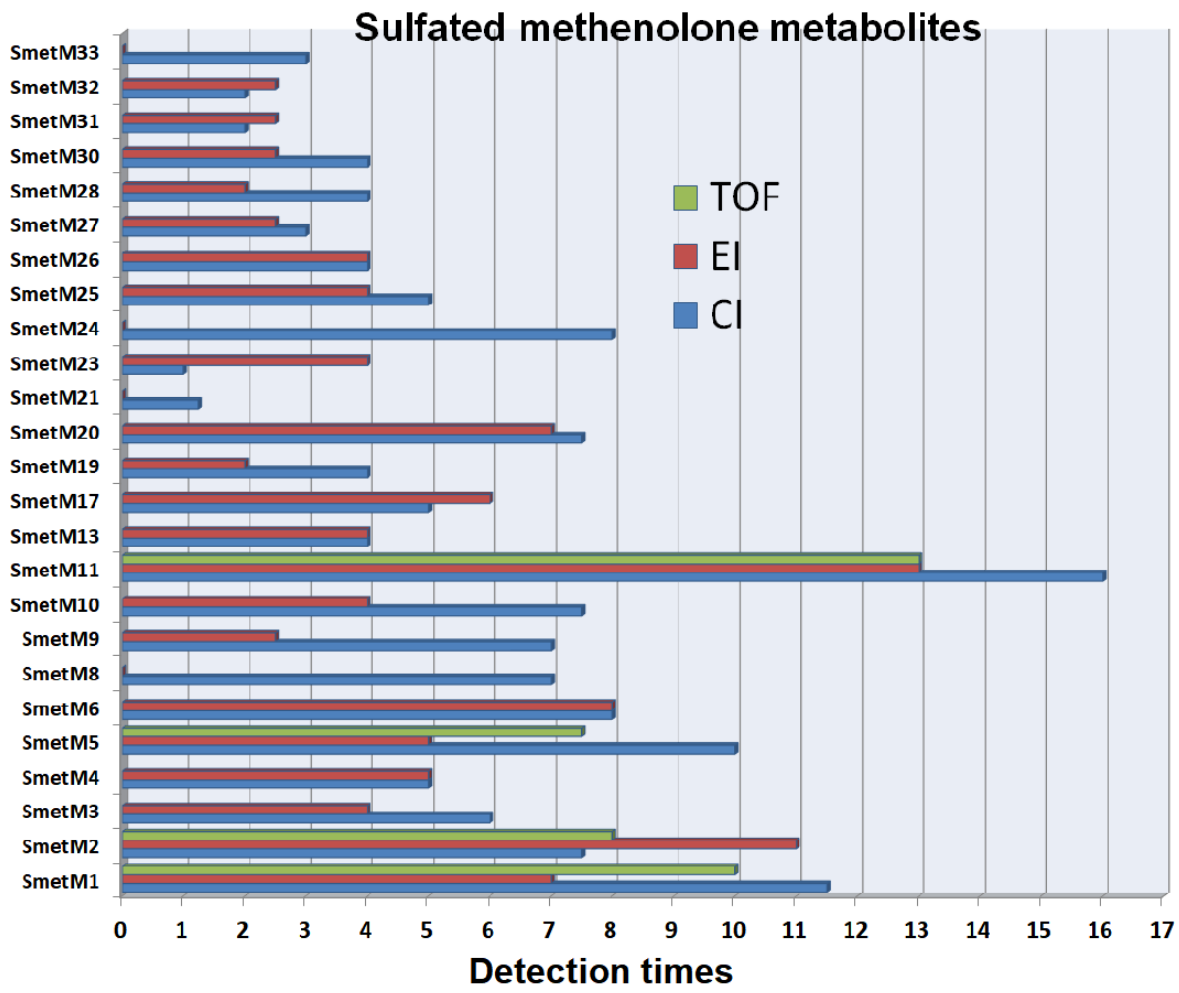


Figure 15: Data from excretion study 1. All found non-hydrolysed sulfated methenolone metabolites and their respective detection times. Different techniques were also compared by their detection times for each metabolite. In case of GC-QTOF-MS only the 4 most promising metabolites monitored.

3.2.2 Final optimisation and analysis of the second excretion study

Metabolites that seem promising are selected to see if their detection window can be prolonged by further optimising their transitions via an additional product ion scan. Compounds that were visible

for more than 120 hours (5 days) were selected, i.e. SmetM 1, 2, 5, 8, 11, 20 and 24. The sample taken at 120 hours (5 days) was then injected 7 times using the dMRM acquisition method. For each injection, the collision energy in the method was changed (i.e., 2, 5, 10, 15, 20, 25 and 30 eV). Of the selected metabolites, each transition was then evaluated at these different collision energies in order to optimise the transitions individually. A certain transition may result in a higher peak at a collision energy of 25 eV, while another transition of the same metabolite may have a higher peak with less background interferences at a collision energy of 2 eV. After injecting the long-term excretion samples (120h - 552h), the optimised data can be used to fully characterise the metabolites of choice. Chromatograms of SmetM 1, 2, 5, 11 and their respective detection times are illustrated in Figure 16. Especially SmetM11 seems very promising with a very long detection time of 384 hours (16 days).

In order to make sure that the detected metabolites are not produced solely in one person, a second long-term excretion experiment was analysed (non-hydrolysed) using the very same method. The optimised metabolites were also detected in these samples and had a detection window of 7 to 10 days. SmetM5 however, was not detected in the second excretion urine. Yet again, SmetM11 had the longest detection time with 240 hours (10 days) [Figure 17].

As an extra measure of control, 20 blank samples from different individuals were then run on GC-CI-MS/MS to see if none of the long-term metabolites show up in their chromatograms. This was checked for SmetM 1, 2, 5 and 11. None of these promising metabolites showed up in the blank samples, which shows that especially SmetM11 has a lot of potential in screening for methenolone. Afterwards, the excretion experiments were also repeated on GC-EI-MS/MS and on GC-QTOF-MS. This allows for an elaborate comparison of these analytical techniques in context of analysing metabolites that originate from non-hydrolysed sulfated steroid compounds.

Additionally, the results from the experiments with non-hydrolysed steroid sulfates were compared to those of hydrolysed compounds. Hydrolysation was performed with either β-glucuronidase/aryl sulfatase from *Helix pomatia* (to hydrolyse both the sulfated steroids and the glucuronidated fractions), or with β-glucuronidase from *E. coli* (to hydrolyse only the glucuronidated compounds). These results will help evaluate which sample preparation method can be combined with the most effective analytical approach.

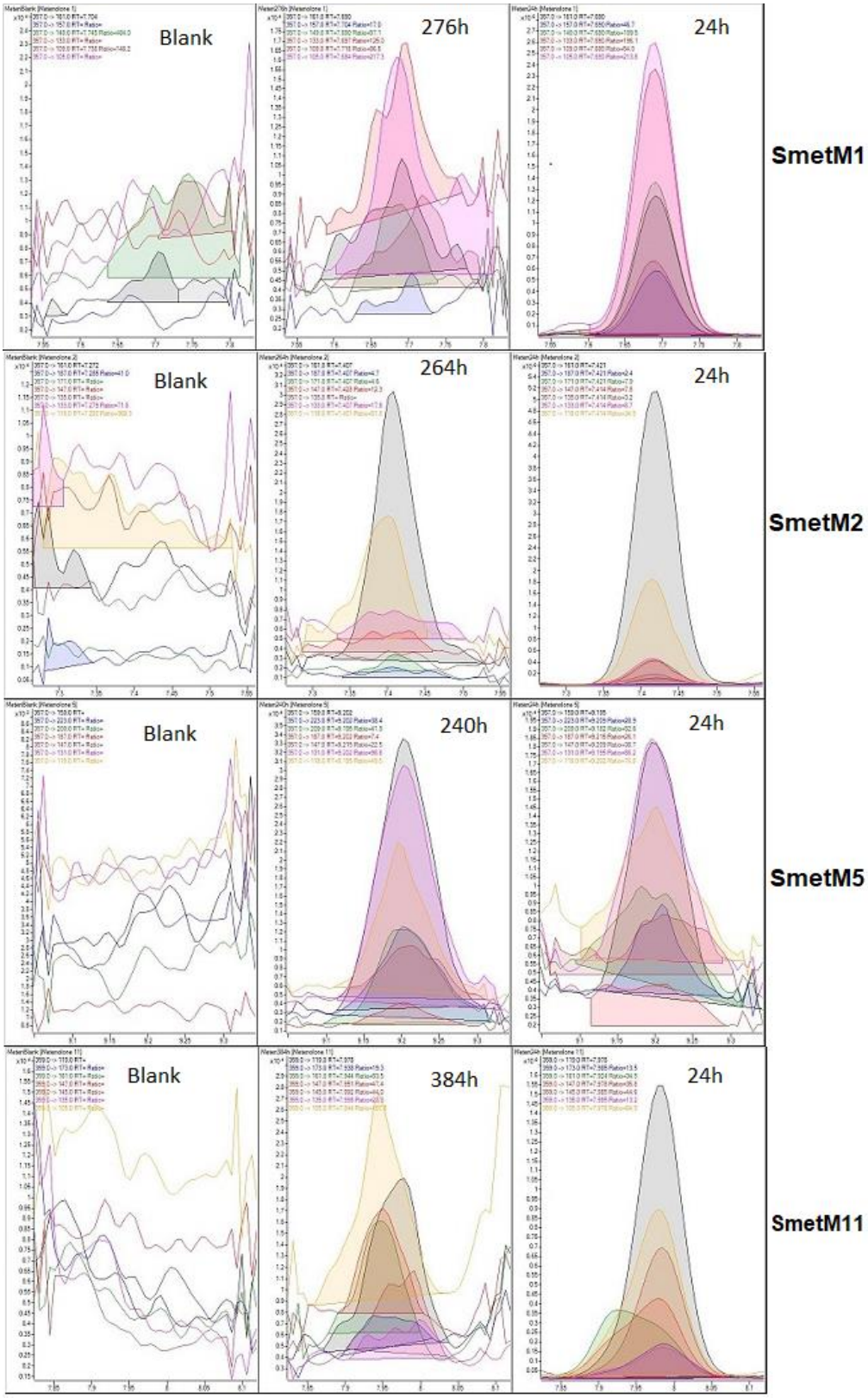


Figure 16: GC-CI-MS/MS chromatograms of SmetM 1, 2, 5 and 11 (data acquired from analysis of the first excretion study).

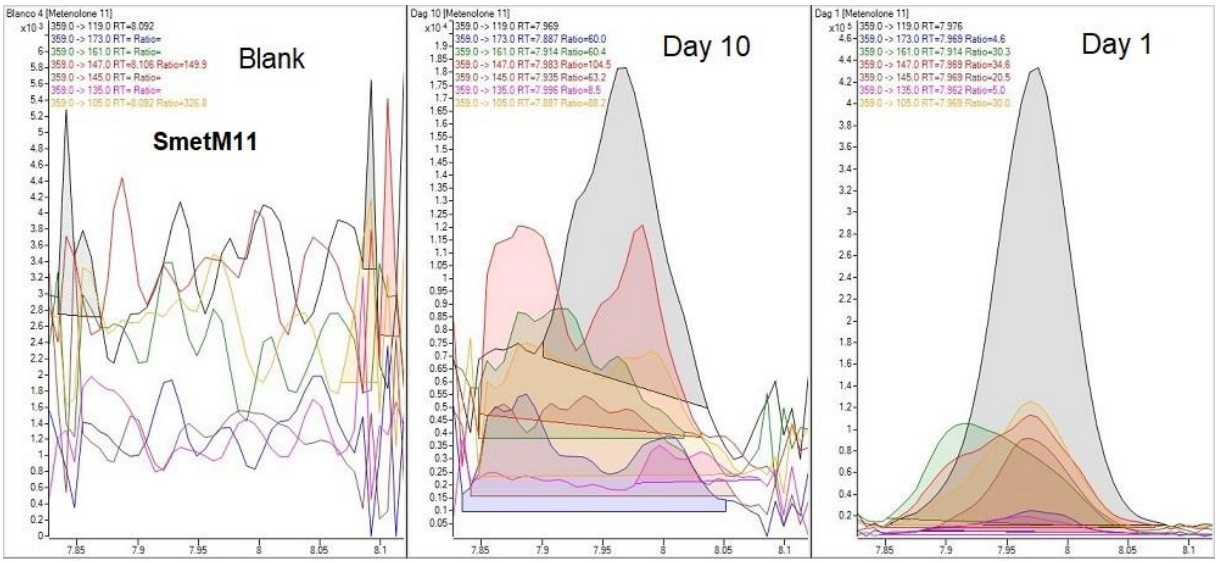


Figure 17: GC-CI-MS/MS chromatogram of SmetM11 (second excretion study).

3.2.3 Characterisation of optimised metabolites

Characterisation can be done by injecting a reference standard (if available) and comparing its retention time and fragmentation pattern with the found metabolites. This was done for SmetM11. The synthesized mesterolone metabolite (3α-hydroxy-1α-methyl-5α-androstan-17-one) sulfate was derivatised and analysed for comparison with SmetM11. Although it appeared not to be the same compound, there is still the possibility that SmetM11 is the 1β-epimer of the synthesized mesterolone metabolite sulfate [Figure 18]. This metabolite was also found by Fragkaki et al [38]. The metabolite S3 that they found is most likely the exact same methenolone metabolite sulfate as SmetM11. S3 has its molecular ion peak at 448 *m/z*, which corresponds to the non-hydrolysed 359 *m/z* (358 with EI) of SmetM11. After hydrolysis, metabolite S3 lost its sulfate group which was then replaced by an O-TMS group. The sulfate group of SmetM11 was lost during injection which leads to the loss of 90 *m/z*.

Metabolites S1-a and S-1b (446 *m/z*;) from the same publication of Fragkaki et al [38] correspond similarly with SmetM1 and 2 (357 *m/z*). This means SmetM1 is the sulfated form of methenolone itself (17β-hydroxy-1-methyl-5α-androst-1-en-3-one sulfate), while SmetM2 tallies with 3α-hydroxy-1-methylen-5α-androstan-17-one sulfate [38]. The proposed structures are illustrated in Figure 18.

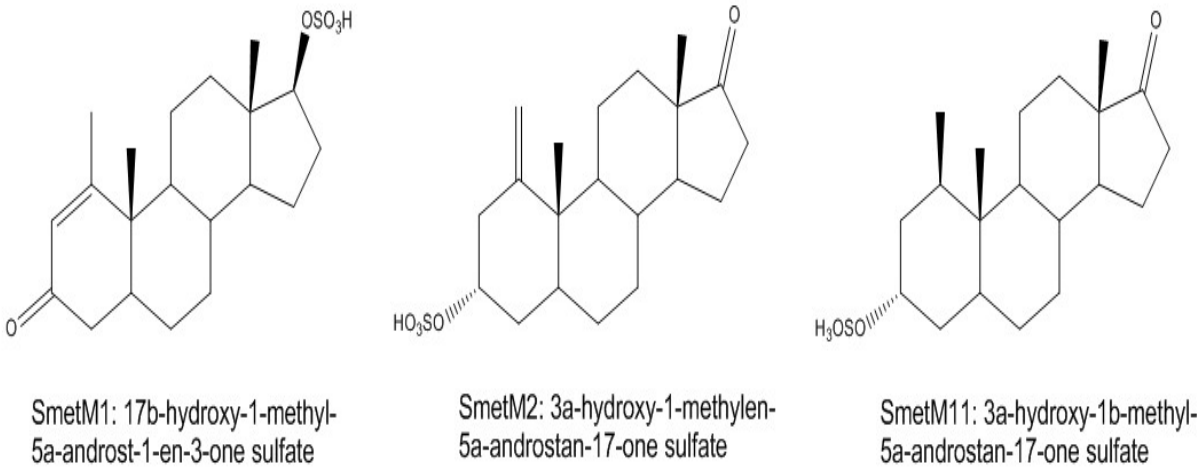


Figure 18: Proposed structures for SmetM 1, 2 and 11.

3.2.4 Analysis on GC-EI-MS/MS

As previously mentioned, comparison of detection times for the metabolites between CI and EI was also done, to evaluate whether there is a large difference. The ion source was changed to EI and production ion scans were taken of the same precursor ions. Note that their *m/z* is 1 lower compared to the precursor ions generated with positive CI. Almost all of the sulfated metabolites were identified by comparing the retention times and fragmentation patterns. Using this data, a new dMRM method was set up. Both excretion studies were then analysed (non-hydrolysed) using this acquisition method. Retention times were adjusted and interfering transitions were removed. After optimisation, detection windows of the metabolites were compared to the CI detection windows. CI seems to be the superior method of ionisation, as the non-hydrolysed metabolites were visible for a much longer time in most cases compared to EI [Figure 15]. The chromatogram for both excretion urines of the longest detectable metabolite (SmetM11) can be seen in Figure 19.

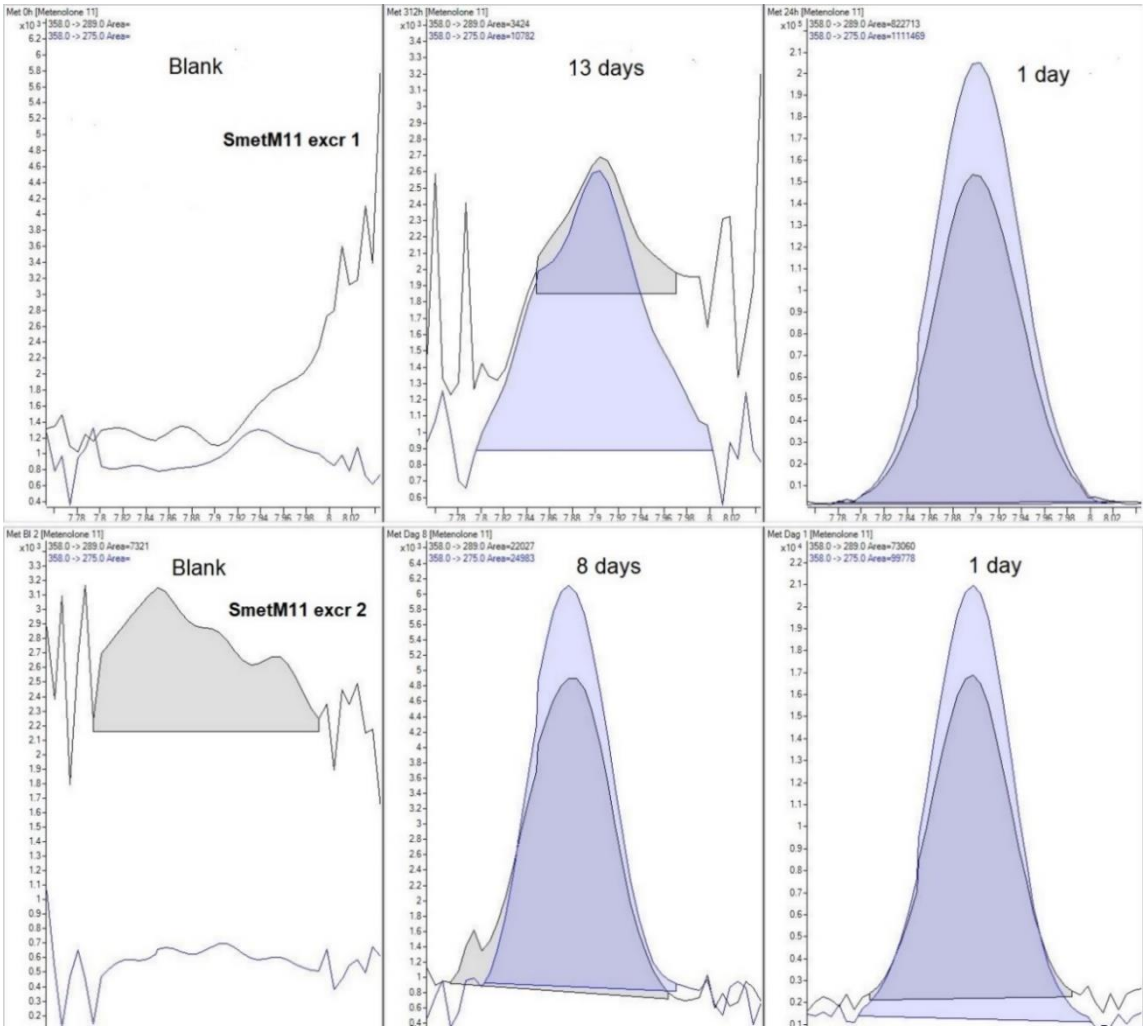


Figure 19: GC-EI-MS/MS chromatograms of SmetM11 (both excretion studies).

3.2.5 Analysis using GC-QTOF-MS

To find the non-hydrolysed sulfated long-term metabolites on this device, the first excretion study was analysed using TOF. Identifying the correct metabolites can prove challenging but by evaluating the retention times and fragmentation patterns, SmetM 1, 2, 5 and 11 were found. Surprisingly, the detection windows of these metabolites were superior to those found with GC-EI-MS/MS. It was however still inferior to GC-CI-MS/MS in terms of how long the metabolites were visible. SmetM 11 was yet again the longest detectable metabolite with 13 days, its chromatogram is displayed in Figure 20.

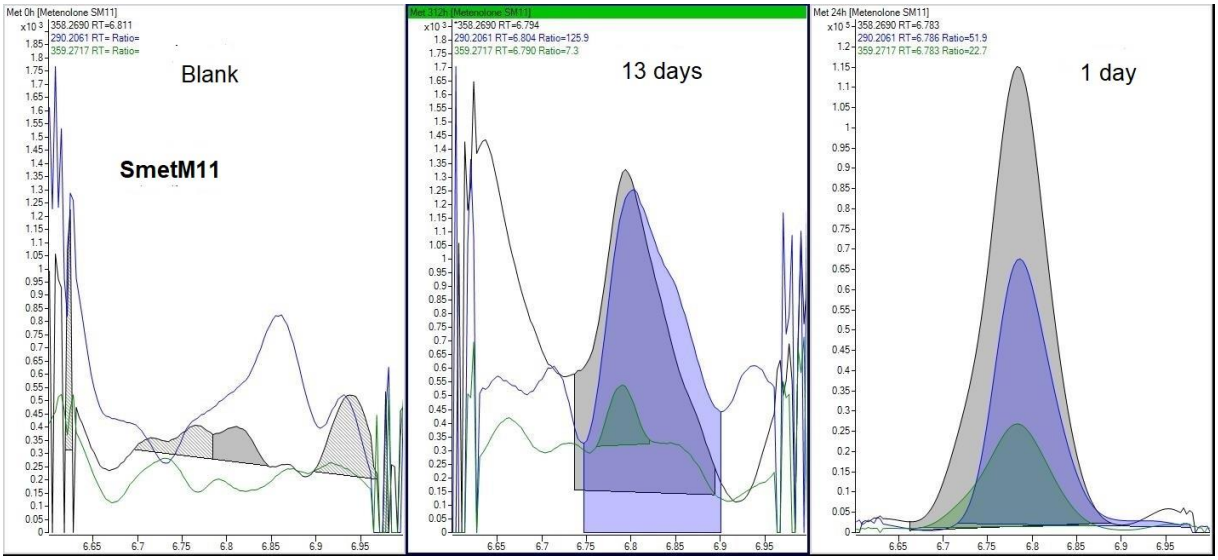


Figure 20: GC-QTOF-MS chromatogram of methenolone metabolite 11 (excretion study 1).

3.2.6 Non-hydrolysed versus hydrolysed metabolites on GC-CI-MS/MS

Excretion study 1 had been analysed on GC-CI-MS/MS in the past after both hydrolysis with glucuronidase from *E. coli* and β-glucuronidase/aryl sulfatase from *Helix pomatia.* The longest detectable metabolites that were found at that time were M15 and M19 (533 *m/z*). The non-hydrolysed forms of these metabolites were most likely not detected for long because the introduction of an extra hydroxyl group makes them very polar and harder to extract with our method of sample preparation.

In Figure 21 detection times of these hydrolysed metabolites M15 and M19 were compared to GC-CI-MS/MS data on the non-hydrolysed metabolites SmetM 1, 2 (both 357 *m/z*) and 11 (359 *m/z*). Even though the non-hydrolysed methenolone metabolites are different from the hydrolysed metabolites M15 and M19, it is clear that the non-hydrolysed metabolites allow for superior detection times on GC-CI-MS/MS.

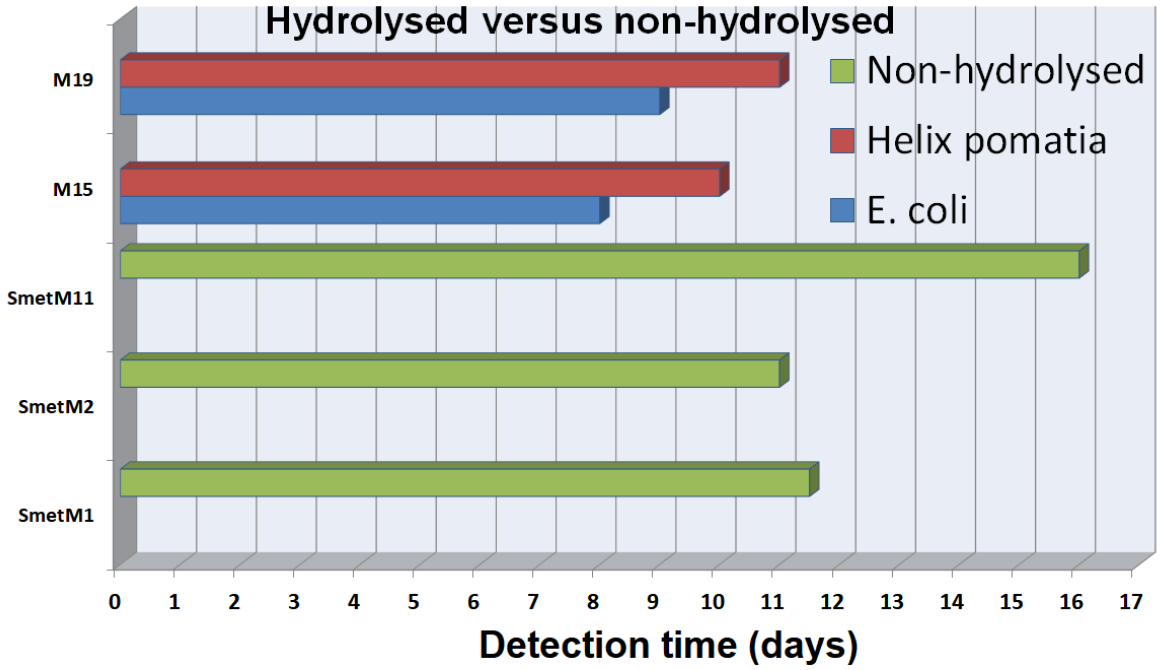


Figure 21: Long-term methenolone metabolites: M15 and M19 on GC-CI-MS/MS (after hydrolysis with glucuronidase from E. coli or with β-glucuronidase/aryl sulfatase from Helix pomatia); SmetM 1, 2 and 11 on GC-CI-MS/MS (non-hydrolysed).

3.3 Mesterolone

Mesterolone (1α-methyl-5α-androstan-17β-ol-3-one) is a synthetic AAS that has seen abuse in both human and equine sports. It has a molecular weight of 304.474 g/mol [39]. Mesterolone is also still frequently used to treat health problems in males that are associated with hypogonadism, low androgen levels and infertility [36]. Its structure can be seen in Figure 22 as tmestM1.

3.3.1 Searching for (long-term) metabolites with GC-CI-MS/MS

The approach for finding long-term metabolites of mesterolone was analogous to the method used for methenolone. Theoretically possible metabolites for mesterolone have already been described [36]. The non-hydrolysed sulfated forms of these theoretical metabolites are illustrated in [Figure 22].

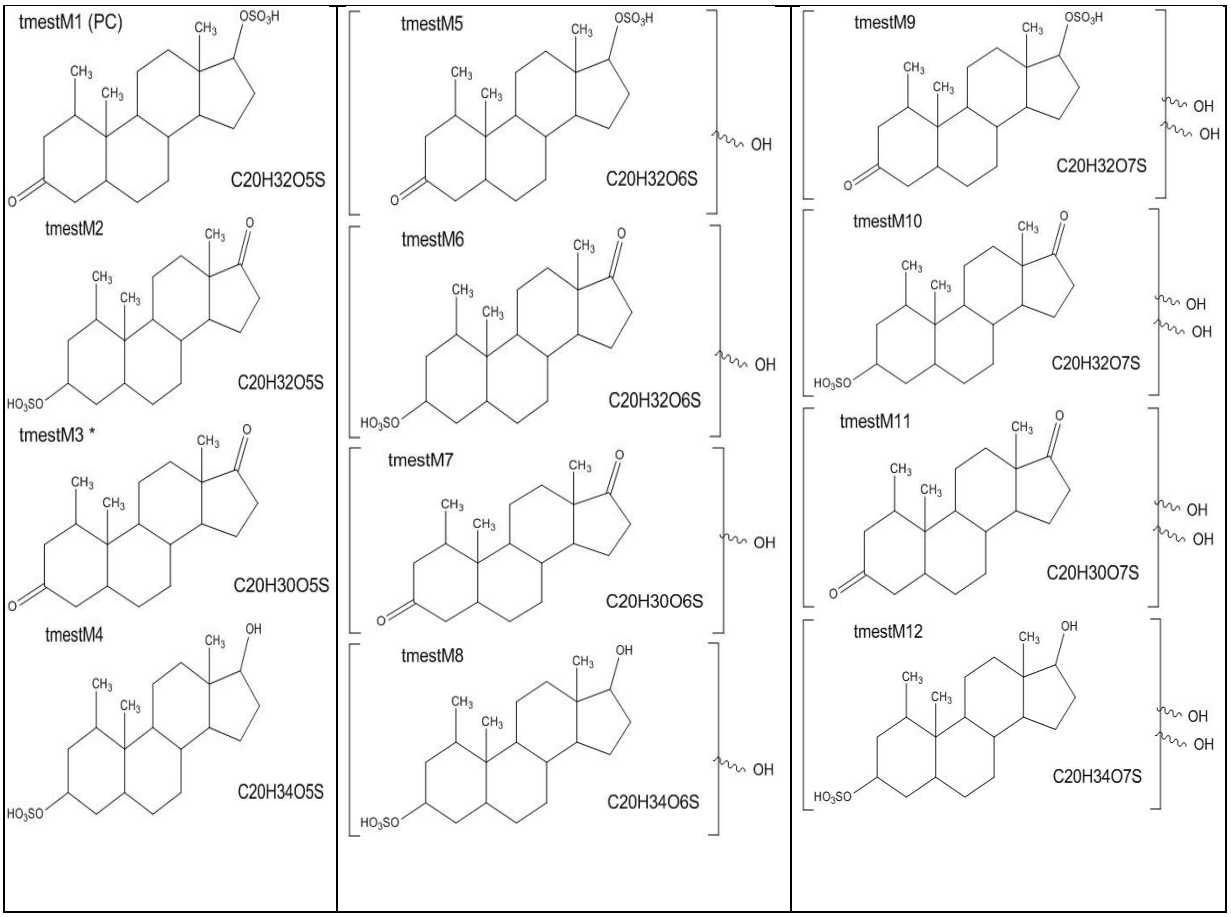


*Figure 22: Structures of theoretically possible non-hydrolysed sulfated mesterolone metabolites. *Note that tmestM3 does not count as a sulfated mesterolone metabolite. It was included in the figure because additional hydroxyl groups can be introduced and sulfated.*

To set up the GC-CI-MS/MS MRM method, the theoretical transitions from [36] were used. Although the product ions will be identical, the precursor ions will have a *m/z* that is 90 units lower compared to those in [36]. During hydrolysis the sulfate or glucuronic acid group will be cleaved, which means the hydroxyl is now available for derivatisation with formation of an O-TMS group. The sulfate group on the compounds used here was not cleaved before derivatisation, but is lost during injection which results in a loss of 90 *m/z*.

The pre-administration sample (0h) and a urine sample taken at 8 hours of the first excretion experiment were analysed using the MRM method containing the theoretical transitions. This GC-CI-MS/MS data was then searched for metabolites that are unique to the 8-hour sample. Product ion scans (at collision energy 10 and 20 eV) were taken for the precursor ions of the metabolites

that were found. The transitions found with these scans were then used to compile a dMRM method for mesterolone. After a test run with the 0 and 8-hour samples, the MRM method was optimised by removing transitions that deteriorate the detection of the corresponding metabolites. It was then used to analyse the first excretion study, these samples ranged from the blank to 360 hours (15 days). After another round of optimisation, the most promising metabolites were selected: SmestM 9, 11, 20, 26, 27, 36 and 55.

3.3.2 Final optimisation and analysis of two long-term excretion studies on GC-CI-MS/MS

Final optimisation was achieved by finding additional transitions that improved detection time and by testing which collision energy (i.e. 5, 10, 15, 20, 25 or 30 eV) was optimal for each transition. For this purpose, the 144-hour (6 days) sample was analysed.

Using the optimised dMRM method, samples 144h to 360h and the blank of excretion study 1 were analysed on GC-CI-MS/MS. The results were very promising as detection times for the selected metabolites ranged from 7.5 days to 15 days. However, the transitions of SmestM9 and 11 are also visible in the blank samples albeit it at lower abundancies. It is probable that an endogenous compound is causing interference. Therefore, a series of 20 blank samples of different individuals was analysed on GC-CI-MS/MS using the dMRM method. Background noise made SmestM9 hard to distinguish in about 50% of these samples. But after removing some transitions, this background noise was reduced and Smest9 was optimised for detection. For SmestM11, the interferences were not eliminated after optimisation. SmestM26 and 36 did not appear in the series of blank samples. Chromatograms of SmestM 9, 11, 26 and 36 and their respective detection times for excretion study 1 are illustrated in Figure 23.

Excretion study 2 which covered up to day 22 was then analysed using the same GC-CI-MS/MS dMRM method. Detection times ranged from 8 days to 21 days, SmestM9 and 11 could be detected until day 21. Note that SmestM11 is not a reliable long-term metabolite. It was still included because it might be of interest when used in combination with SmestM9. SmestM36 could be seen until day 16 as opposed to excretion study 1, where it was detectable up to 13 days. SmestM26 was visible for 10 days, which is less than the 14 days observed in excretion study 1. Their chromatograms can be seen in Figure 24.

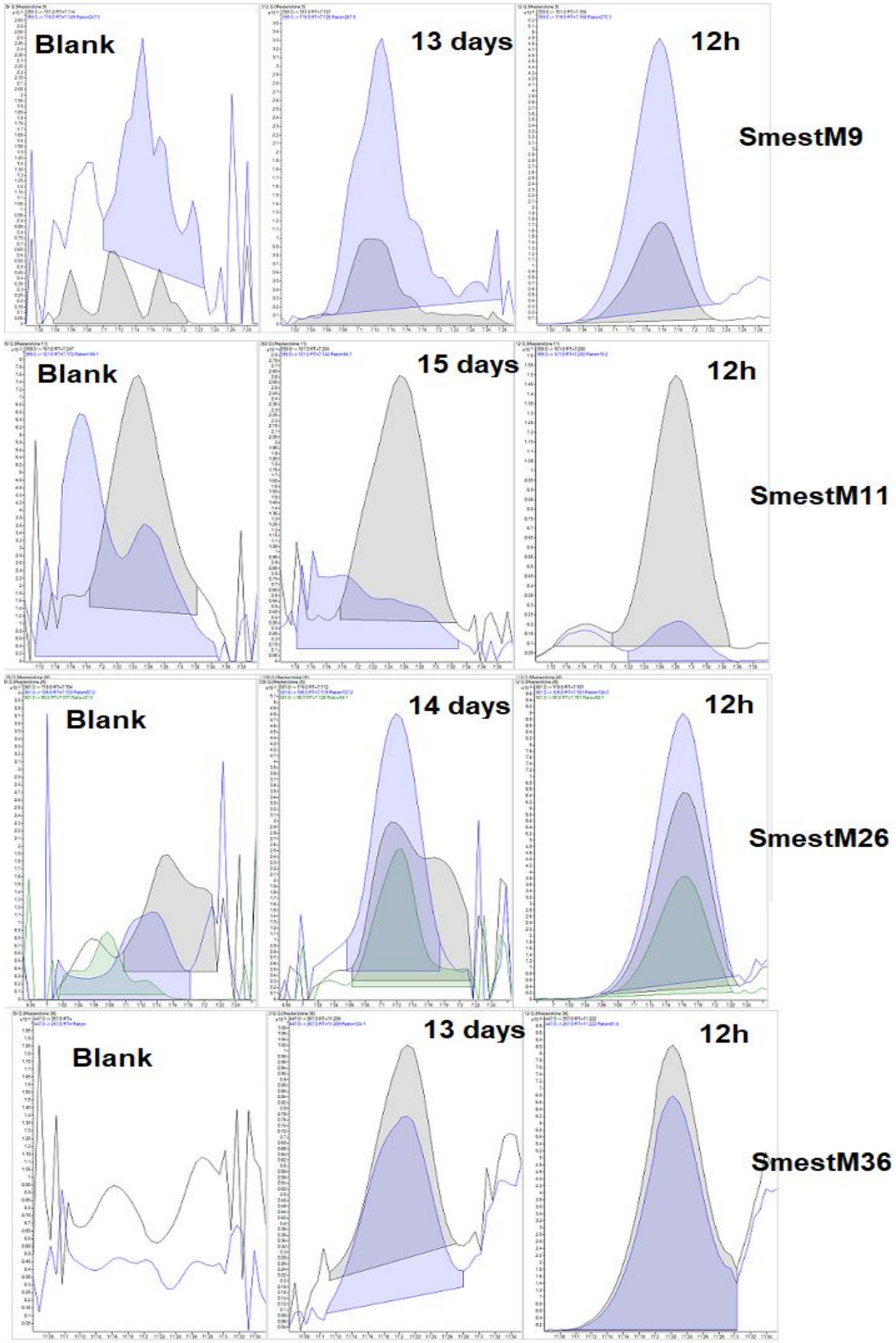


Figure 23: GC-CI-MS/MS chromatograms of SmestM 9, 11, 26 and 36 after optimisation (excretion study 1).

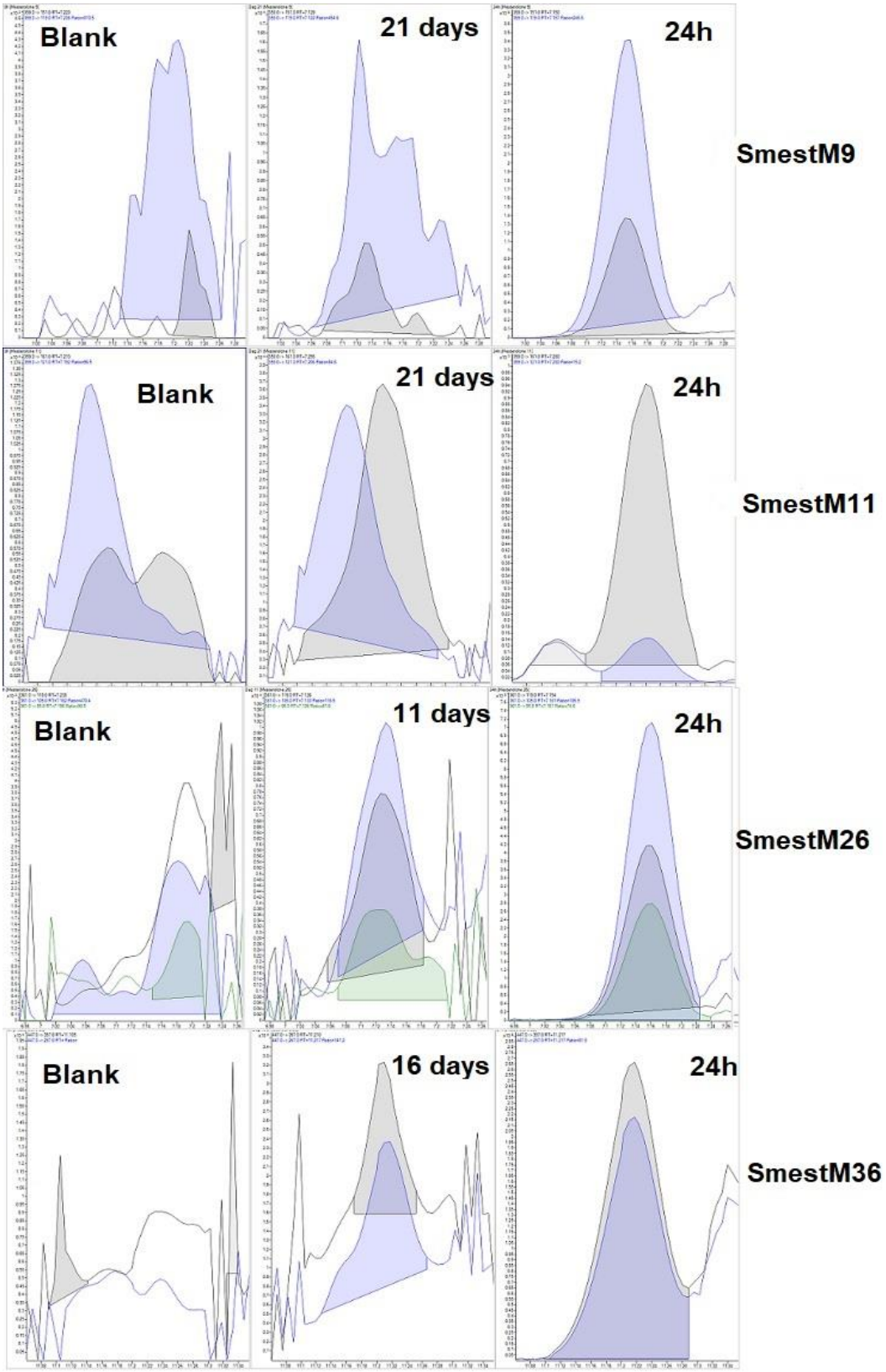


Figure 24: GC-CI-MS/MS chromatograms of SmestM 9, 11, 26 and 36 (excretion study 2).

3.3.3 Characterisation of the long-term metabolites of mesterolone

The long-term metabolites 9 and 11 were thought to be epimers of the same sulfated compound after cleavage, namely mesterolone metabolite (3α-hydroxy-1α-methyl-5α-androstan-17-one sulfate). This theory was confirmed by derivatisation of 15 µL of this sulfated compound (which was synthesized earlier in this thesis) and analysis with the GC-CI-MS/MS dMRM method. Retention times and ratios of the sulfated mesterolone metabolite were identical to those of SmestM9 and 11. The structure of both epimers can be seen in Figure 25.

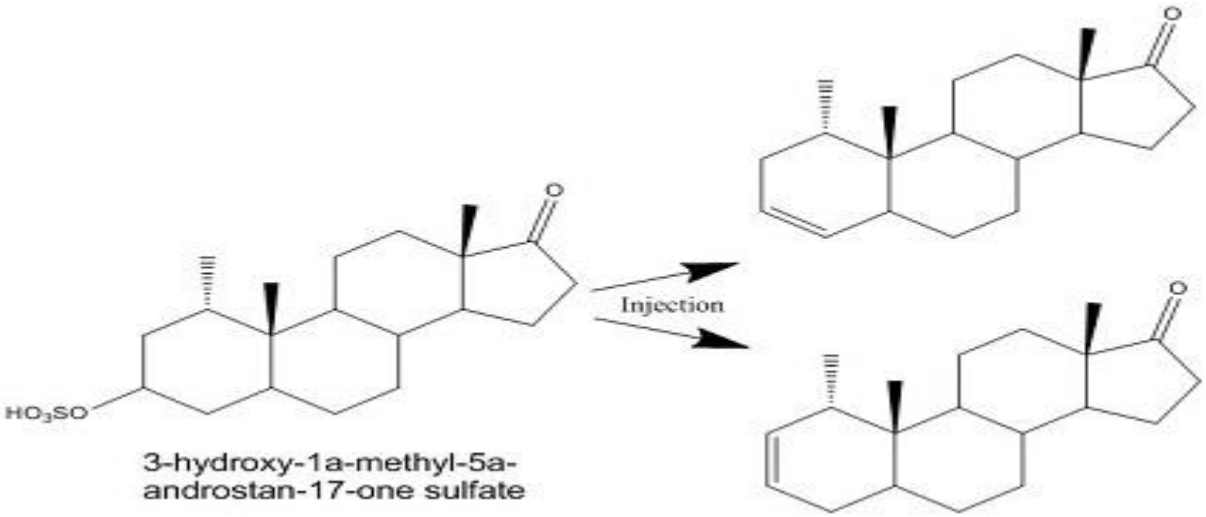


Figure 25: Both epimers created after cleavage of the mesterolone metabolite sulfate during injection; one of these epimers is supposed to be SmestM9 while the other one is SmestM11.

3.3.4 Analysis on GC-EI-MS/MS

For the EI experiments, product ion scans were taken from the same precursor ions used in CI (minus 1 *m/z*). After registering the most important transitions and their retention times, a new dMRM method was created. This method was used to run a blank and 24-hour sample from excretion study 2. SmestM 9 and 11 were identified and monitored by analysing both excretion studies using the dMRM method. Yet again something was causing a signal in the blank. After running a series of blanks, no way of optimisation was found to eliminate these disturbances. This makes the detection time of SmestM9 and 11 very hard to interpret in GC-EI-MS/MS. This interference could possibly be avoided by employing GC-QTOF-MS which also employs an EI

source. Using this technique, mestM9 and 11 should be distinguishable from the interfering ion peaks that occur at similar nominal mass using the high-resolution spectra.

3.3.5 Analysis on GC-QTOF-MS

First the synthesized sulfated mesterolone metabolite (3α-hydroxy-1α-methyl-5α-androstan-17-one) was injected on GC-QTOF-MS. SmestM9 and 11, which were found to be the epimers after cleavage of the sulfate group during injection of this metabolite and could easily be identified in the chromatogram. Their high-resolution transitions were registered. Excretion study 1 was then analysed. Using the 84-hour sample, SmestM9, 11 and 36 were found and afterwards their respective detection times were evaluated [Figure 26].

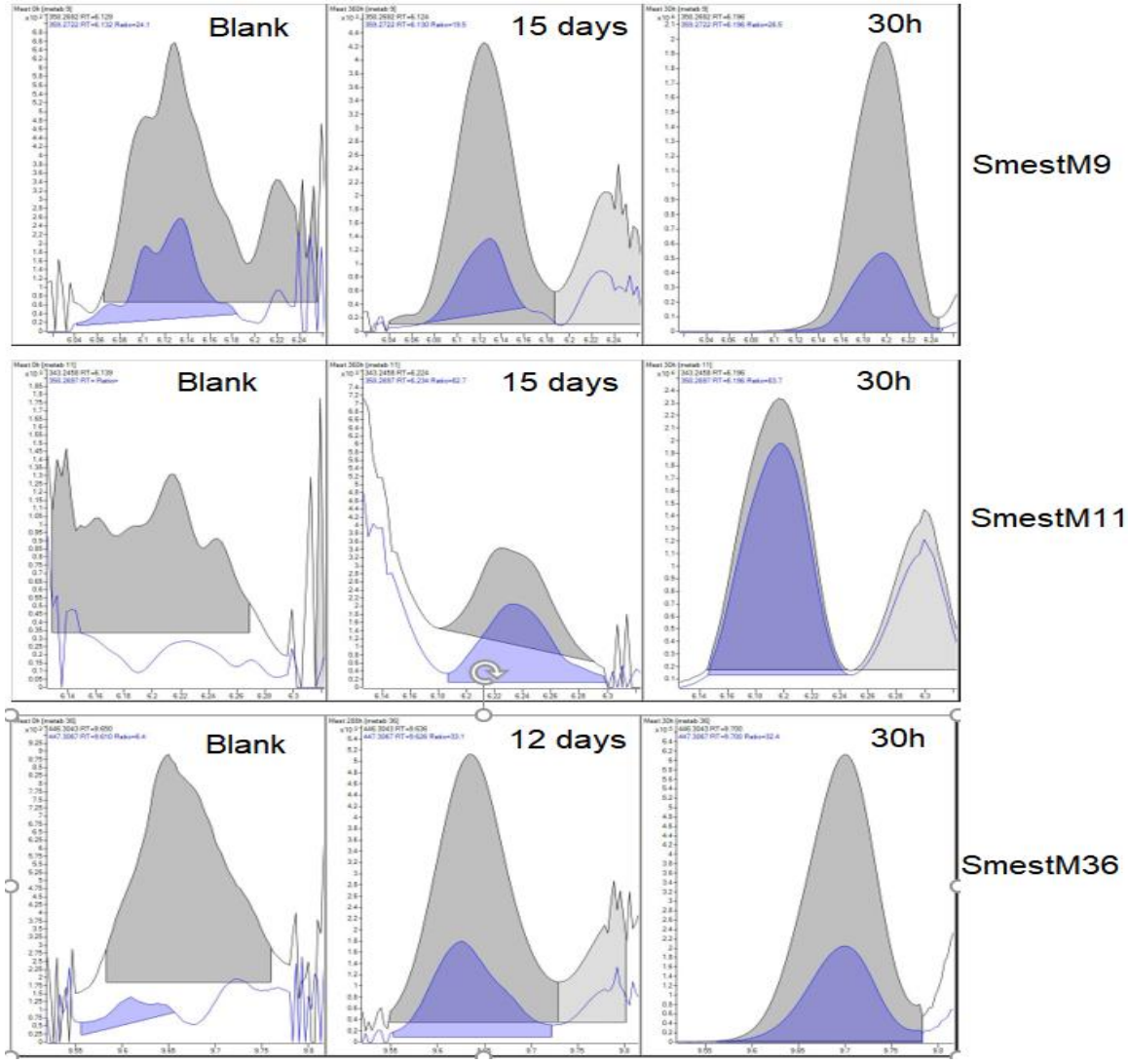


Figure 26: GC-QTOF-MS chromatograms of SmestM9, 11 and 36 (excretion study 1).

SmestM9 and 11 had detection times of 15 days (last collected sample of excretion study 1), SmestM36 was visible until day 12. These results are comparable with the ones found for GC-CI-MS/MS. GC-QTOF-MS appears to have a good sensitivity for these sulfated mesterolone metabolites. It should also be mentioned that the interferences for SmestM9 and 11 are barely noticeable (in order of magnitude 10^2 for the SmestM9 blank sample) here. To see if these metabolites may have even longer detection times than 15 days, excretion study 2 was also analysed [Figure 27].

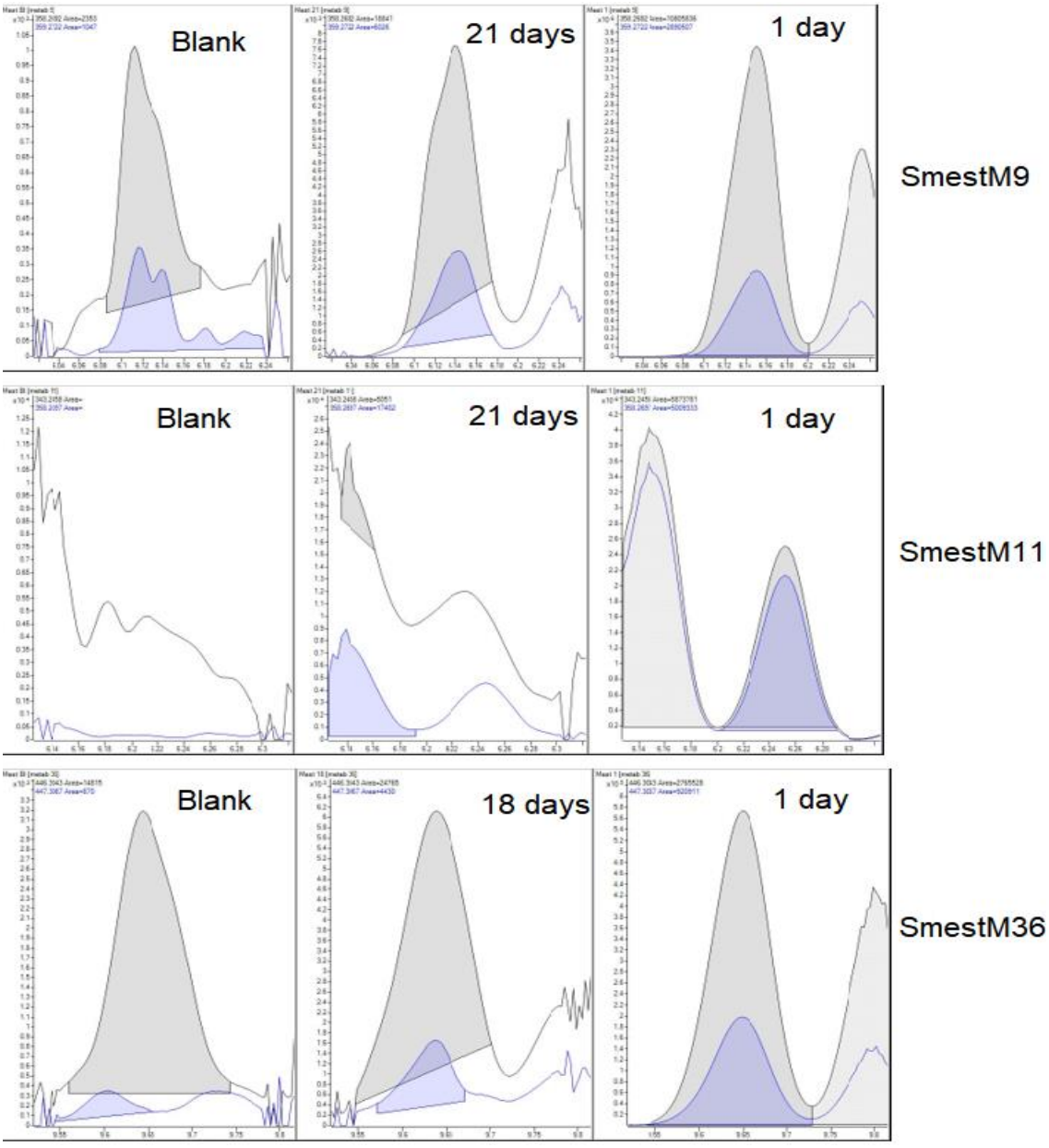


Figure 27: GC-QTOF-MS chromatograms of SmestM9, 11 and 36 (excretion study 2).

Yet again the detection times were about the same as those registered with GC-CI-MS/MS, SmestM36 was detectable for an additional 2 days. The chromatograms of SmestM9 and 36 for the blank samples are not completely unambiguous, but the background noise is nowhere near as bad in comparison to GC-EI-MS/MS. This is most likely due to the employment of high-resolution, high mass accuracy spectra by GC-QTOF-MS. Even though the device was limited to full-scan spectra, it is fair to say that these detection times show that this method is well capable of detecting certain sulfated long-term metabolites. A comparison between the detection times of GC-QTOF-MS and GC-CI-MS/MS for the sulfated mesterolone metabolites can be found in [Table 3].

*Table 3: Most promising sulfated mesterolone metabolites and their respective detection times for both GC-CI-MS/MS and GC-QTOF-MS. EI was not included as the results were prone to interference. *Excretion study 1 ended at 15 days. **m/z used as precursor for the product ion scans in GC-CI-MS/MS*

Mesterolone	*m/z***	CI		Q-TOF	
Excretion st.		1	2	1	2
SmestM9	359	13 days	21 days	/	/
SmestM11	359	15 days*	21 days	15 days*	21 days
SmestM26	361	14 days	11 days	15 days*	21 days
SmestM36	447	13 days	16 days	12 days	18 days

3.3.6 Hydrolysed mesterolone metabolites

In earlier studies [36], long-term mesterolone metabolites were described after hydrolysis with both *H. pomatia* and *E. coli.* The most promising metabolites were mestM20 and mestM22 both with a mass-to-charge ratio of 625 on CI. These metabolites were identified as 3,6,18-trihydroxy-1α-methyl-5α-androstan-17-one and 3,6,16-trihydroxy-1α-methyl-5α-androstan-17-one respectively. Because the exact same excretion studies were used and analysed on GC-CI-MS/MS, these metabolites can be directly compared with the non-hydrolysed metabolites found in this thesis. This was done for both excretion studies, detection times for excretion study 1 and 2 are illustrated in Figure 28 and Figure 29 respectively.

Note that mestM20 and mestM22 are not the hydrolysed counterparts of the found non-hydrolysed mesterolone metabolites. Because mestM20 and 22 have 2 additional hydroxyl groups, the non-hydrolysed counterparts were most likely too polar to extract with EtOAc.

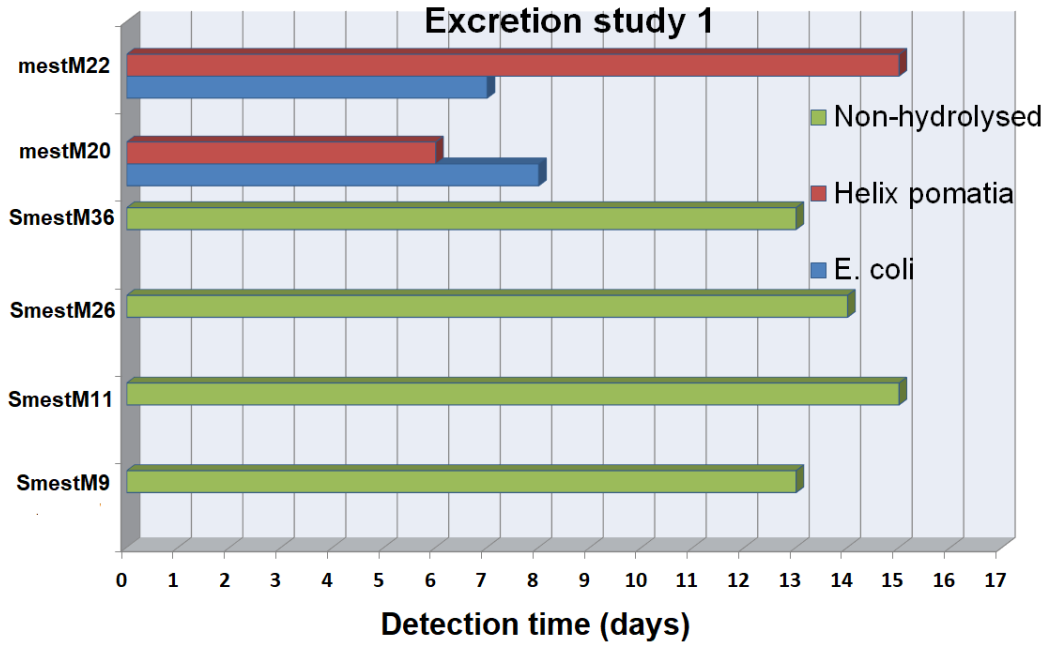


Figure 28: Long-term mesterolone metabolites: M20 and M22 [36] (after hydrolysis with glucuronidase from E. coli or with β-glucuronidase/aryl sulfatase from Helix pomatia); SmetM9, 11, 26 and 36 (non-hydrolysed). Excretion study 1 analysed on GC-CI-MS/MS.

Figure 29: Long-term mesterolone metabolites: M20 and M22 [36] (after hydrolysis with glucuronidase from E. coli or with β-glucuronidase/aryl sulfatase from Helix pomatia); SmetM9, 11, 26 and 36 (non-hydrolysed). Excretion study 2 analysed on GC-CI-MS/MS.

Figure 28 clearly shows that only mestM22 after hydrolysis with *H. pomatia* can rival the detection times of the intact sulfated mesterolone metabolites with a detection time of 15 days. With a detection time of 6-8 days, the glucuronidated form of mestM22 and both forms of mestM20 fall short of those found for the non-hydrolysed metabolites in excretion study 1.

From Figure 29 on the second excretion study, it is fair to say that the comparison is not even close. The non-hydrolysed sulfated metabolites were visible for a much longer time with detection times of 11-21 days, compared to 4-7 days for the hydrolysed metabolites.

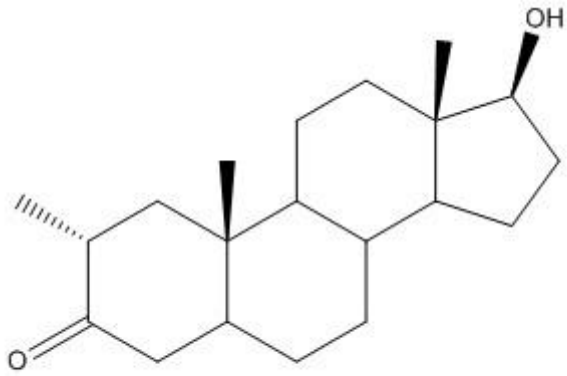
These findings suggest that analysis of the non-hydrolysed steroid sulfates on GC-CI-MS/MS would be a massive improvement for the detection of mesterolone in urine.

3.3.7 Third excretion study on GC-CI-MS/MS

As an extra control a third excretion experiment was performed with mesterolone. Due to time restrictions however, the samples were only analysed with GC-CI-MS/MS.
SmestM26 had a detection time of 16 days, while SmestM36 was visible up to 15 days. SmestM9 and 11 were detectable for up to 18 and 19 days respectively, which is more or less in line with the other excretion studies.

3.4 Drostanolone

The third compound addressed in this thesis is drostanolone (17β-hydroxy-2α-methyl-5α-androstan-3-one) [Figure 30]. The only difference from mesterolone is a 2α-methyl group instead of a 1α-methyl group. Obviously its molecular formula ($C_{20}H_{32}O_2$) and molecular weight (304.474 g/mol) are identical to those of mesterolone.



17b-hydroxy-2a-methyl-5a-androstan-3-one

Figure 30: structure of drostanolone.

3.4.1 Searching for (long-term) metabolites with GC-CI-MS/MS and their optimalisation

Because the structure of drostanolone is identical to that of mesterolone except for the methyl group being at the 2α instead of 1α position, the theoretically possible non-hydrolysed sulfated drostanolone metabolites were not listed in a new figure [Figure 22]. The GC-CI-MS/MS MRM method containing the theoretical transitions from mesterolone was used to search for metabolites of drostanolone, as the theoretical metabolites of these compound have the same mass and differ only in the position of an extra methyl group.

A pre-administration sample (0h) and a urine sample taken at 12 hours of the excretion experiment were analysed using this MRM method. After selecting the metabolites that were solely seen in the 12-hour sample, product ion scans (at collision energy 10 and 20 eV) were taken of their precursor ions. A dMRM method for drostanolone was set up using the data from these product ion scans and it was tested using the aforementioned blank and 12-hour samples. The method was cleaned up by removing unnecessary transitions, and the excretion study (12h to day 29) was analysed. The most promising metabolites were selected: SdrostM 1, 2, 4, 11 and 13. They were optimised by analysing the urine sample at day 9 at different collision energies (i.e. 5, 10, 15, 20, 25 or 30 eV) and selecting the optimal collision energy for each transition. The excretion samples (12h to day 29) were analysed again, and the detection time for each of the non-hydrolysed sulfated metabolites was registered. SdrostM 2, 4 and 11 were the longest detectable metabolites, their chromatograms and respective detection times are illustrated in Figure 31.

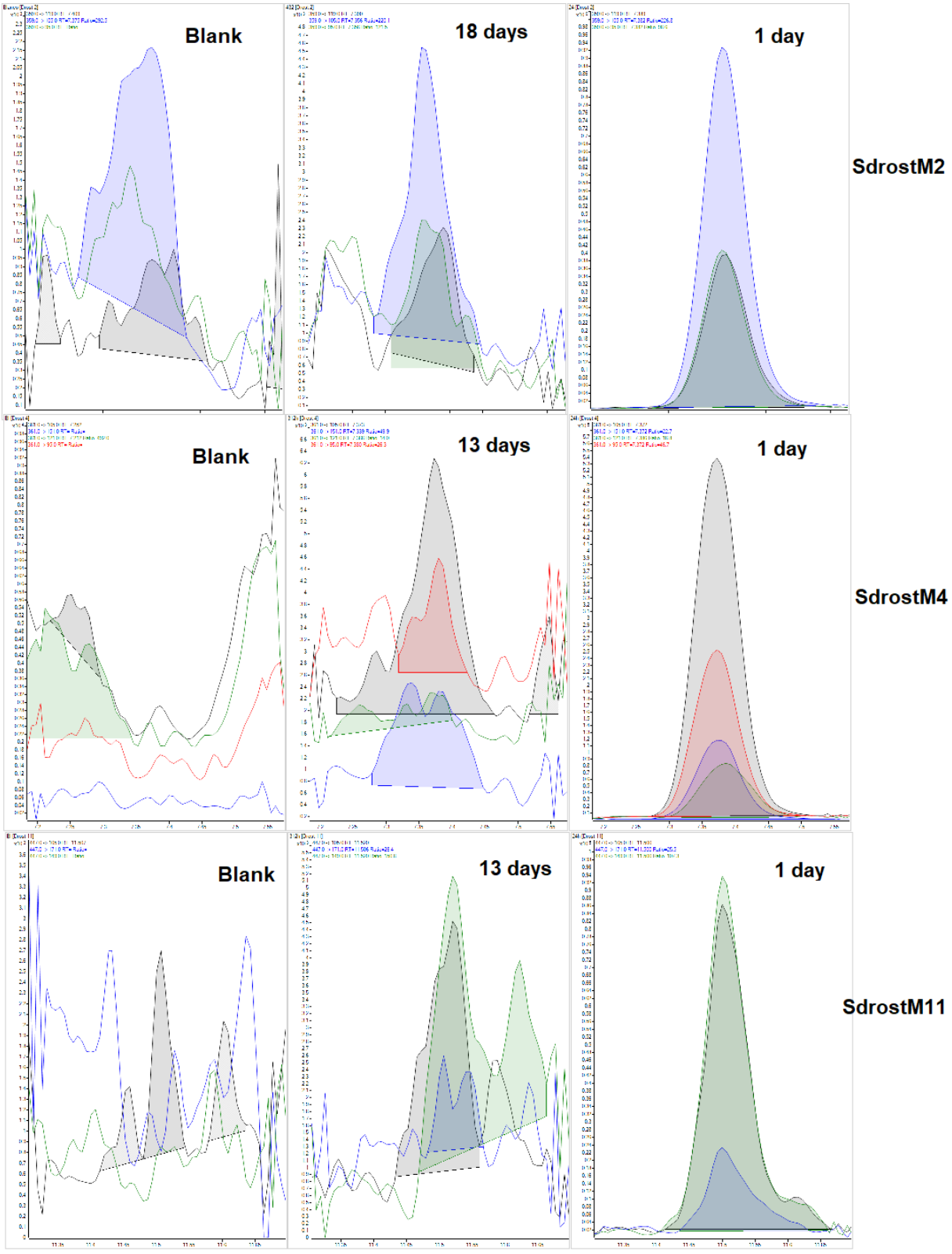


Figure 31: GC-CI-MS/MS chromatograms of SdrostM 2, 4, 11 and their respective detection times.

3.4.2 Characterisation of the selected metabolites

Especially SdrostM2 has a very long detection time of 18 days, this metabolite is most likely the non-hydrolysed sulfated form of a metabolite that is already being used for detection of drostanolone. This can be verified by sulfation of this known metabolite (3α-hydroxy-2α-methyl-5α-androstane-17-one) and analysis on GC-CI-MS/MS using the dMRM method [Figure 32]. The sulfated known drostanolone metabolite gave a signal in order of magnitude 10^7 for SdrostM2 which confirms that this is indeed the same compound.

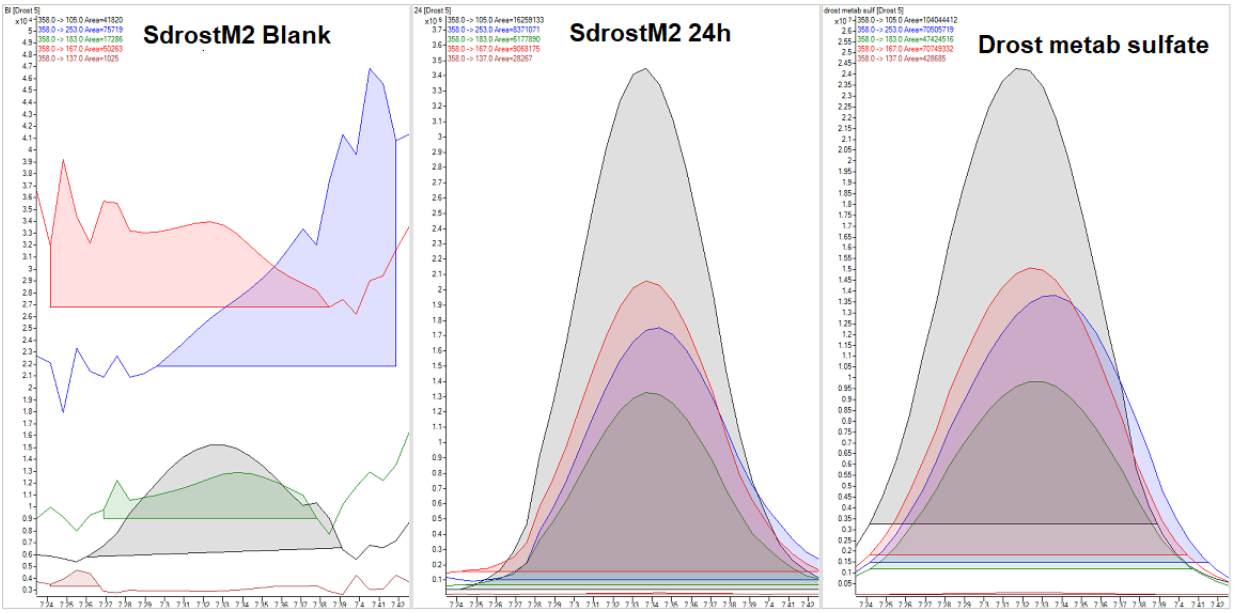


Figure 32: Chromatograms of the sulfated drostanolone metabolite (3α-hydroxy-2α-methyl-5α-androstane-17-one), a blank and the 24-hour sample from the excretion study processed as SdrostM2.

3.4.3 Analysis on GC-EI-MS/MS

The ion source was changed from CI to EI and product ion scans were taken from the same precursors used in CI (minus 1 *m/z).* This data was used to set up a dMRM method for EI, then both the blank sample and the 24-hour sample from the excretion study were analysed. SdrostM2 and SdrostM11 were both identified. Their detection times were inferior to both GC-CI-MS/MS and GC-QTOF-MS however. SdrostM2 was detectable only up to 11 days [Figure 33], while SdrostM11 appeared to be prone to a lot of interferences which made it hard to evaluate its detection time.

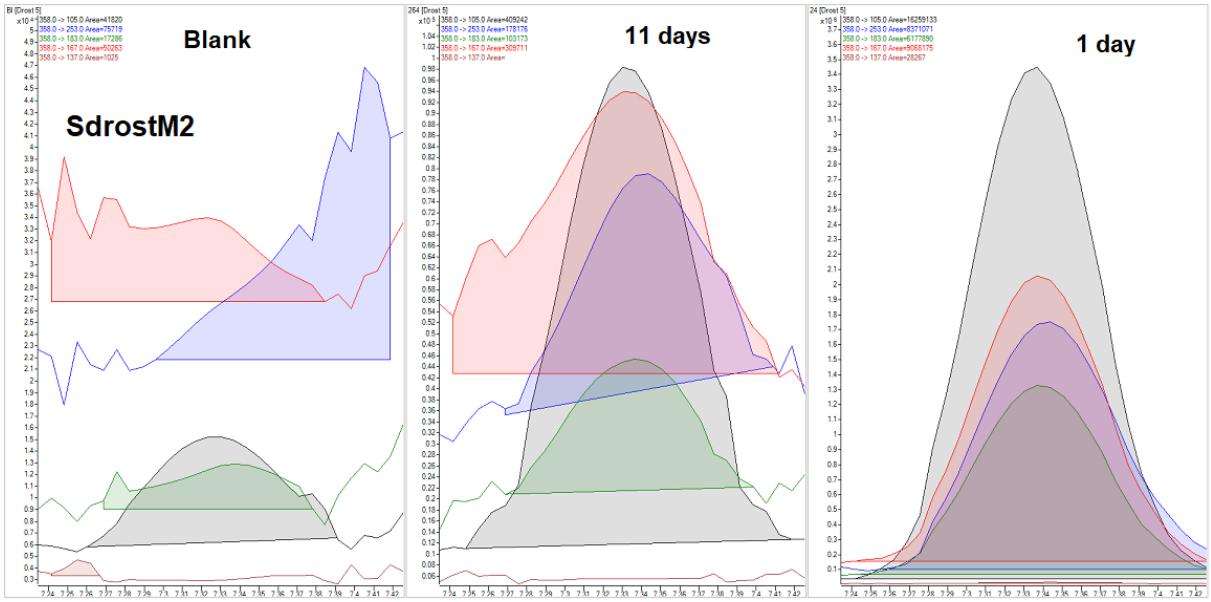


Figure 33: Chromatogram and the detection time of SdrostM2 on GC-EI-MS/MS.

3.4.4 Analysis on GC-QTOF-MS

The excretion study was also analysed on the QTOF device. Using the 24-hour sample, SdrostM2 and 11 were identified in the full-scan spectrum because their approximate masses and retention times were known from the CI experiments. Their detection times were 19 and 16 days respectively [Figure 34], these results are superior to the detection times found with GC-CI-MS/MS.

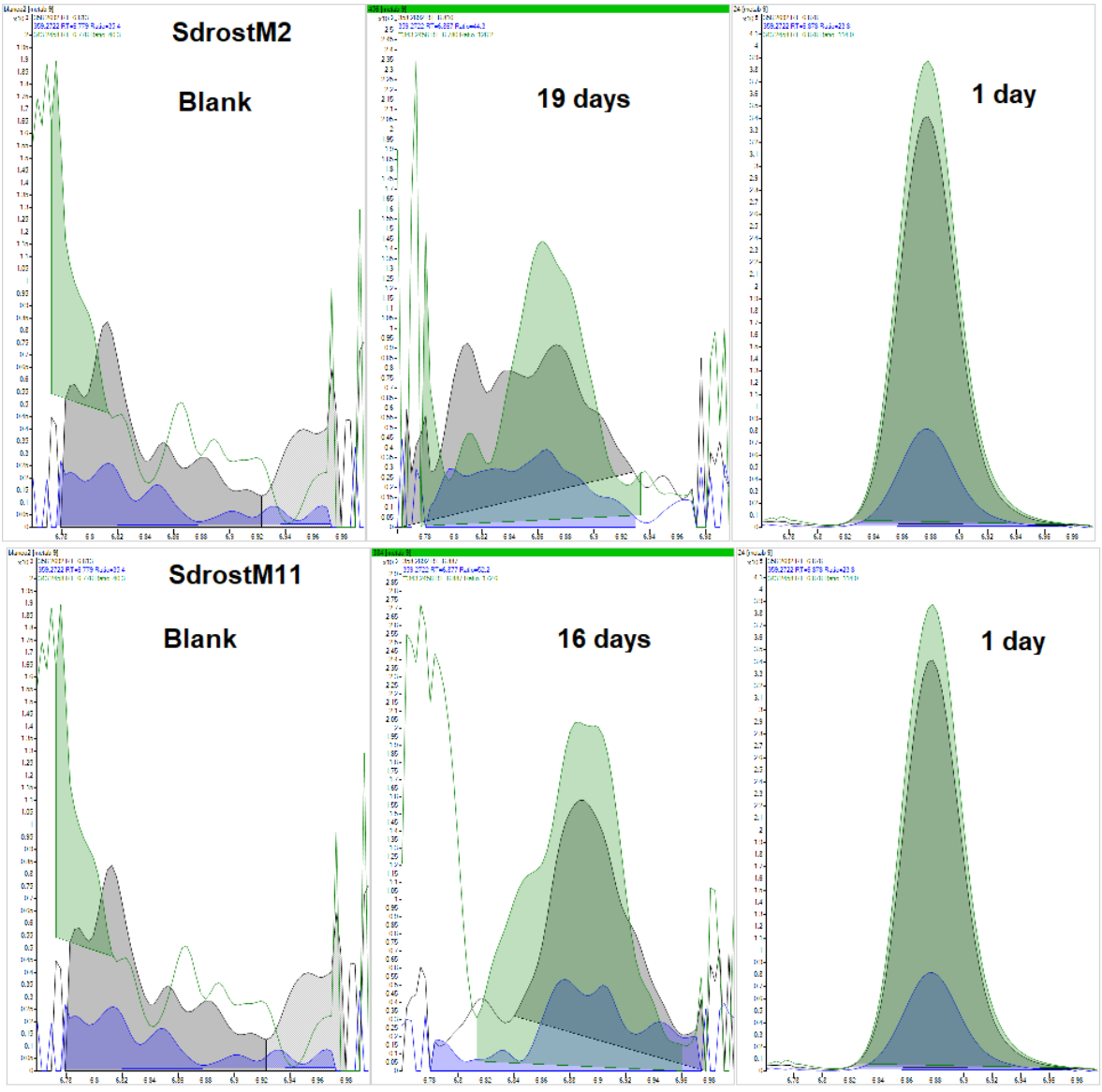


Figure 34: GC-QTOF-MS chromatograms of SdrostM 2 and 11 and their respective detection times.

3.4.5 Hydrolysed drostanolone metabolites on GC-CI-MS-MS

The drostanolone excretion study was also used for analysis after hydrolysis with both *H. pomatia* and *E. coli*. The most promising metabolite SdrostM2 (which we were able to verify as the drostanonlone metabolite 3α-hydroxy-2α-methyl-5α-androstane-17-one) was identified at 449 *m/z* due to an additional TMS group. In the samples that were hydrolysed with *H. pomatia*, drostanolone metabolite was detectable up to 19 days after intake. For the *E. coli* samples, the detection time of this metabolite was even longer at 20 days [Figure 35]. It appears that for the detection of this metabolite, hydrolysis with *E. coli* and analysis on GC-CI-MS/MS is still the best option. The detection of drostanolone metabolite as an intact sulfate does not allow for superior detection times.

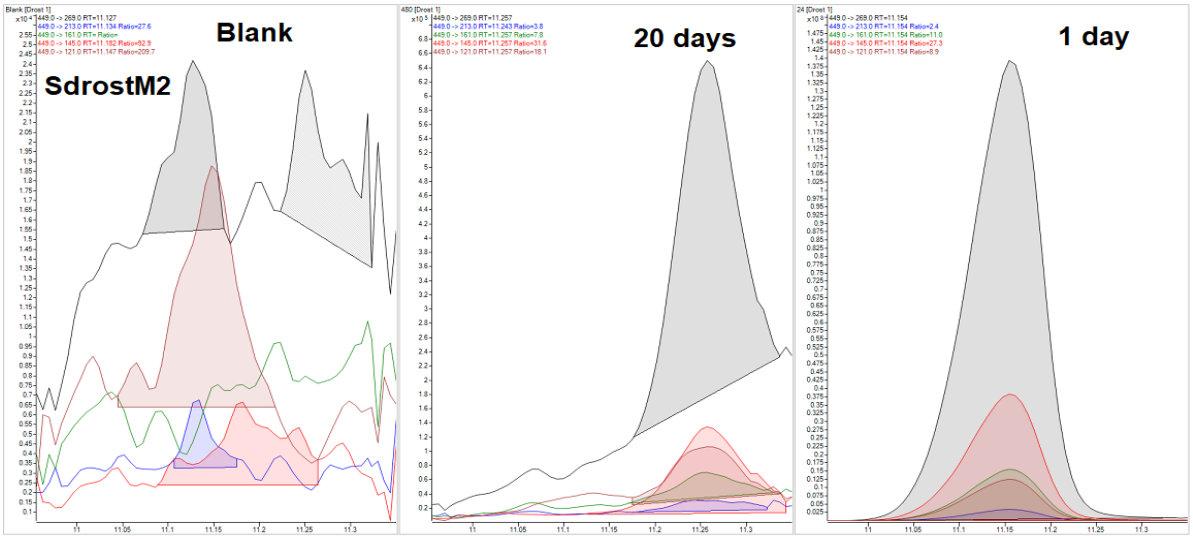


Figure 35: Chromatogram and detection time of drostanolone metabolite (3α-hydroxy-2α-methyl-5α-androstane-17-one) after hydrolysis with β-glucuronidase from E. coli.

An overview of the different techniques and their corresponding detection times for drostanolone metabolite after hydrolysis or SdrostM2 in its sulfated form, can be seen in Table 4.

*Table 4: Detection time of drostanolone metabolite (3α-hydroxy-2α-methyl-5α-androstane-17-one) as an intact sulfate using GC-CI-MS/MS, GC-EI-MS/MS and GC-QTOF-MS. Its hydrolysed forms were also analysed on GC-CI-MS/MS. *m/z used for the product ion scans in GC-CI-MS/MS*

Drostanolone metabolite	*m/z**	CI	EI	Q-TOF
Intact sulfate	359	18 days	11 days	19 days
E. coli	449	20 days	/	/
H. pomatia	449	19 days	/	/

4. Conclusion

The findings in this thesis suggest that there is much about sulfated steroid metabolites that has yet to be explored. Many of the metabolites that were identified are an asset in the long-term detection of the respective steroids. For **methenolone** SmetM1, 2 and 11 were detectable for 12, 11 and 17 days respectively on GC-CI-MS/MS. This is an increase in detection time of a few days up to over a week in comparison to the detection of glucuronidated methenolone metabolites.

For **mesterolone** the difference between detection times of glucuronidated fractions and intact sulfates was even bigger. SmestM9, 11, 26 and 36 had incredible detection times of 11-21 days depending on whether GC-CI-MS/MS or GC-QTOF-MS was used. Generally, these techniques were comparable in terms of detection time throughout this thesis. Even though the GC-QTOF-MS showed some very surprising results, the focus was kept on GC-CI-MS/MS as this is the technique used for routine screening in DoCoLab. Analysis of intact sulfates on GC-EI-MS/MS did not yield very good results in comparison to the other two techniques, which is why it was not explored as elaborately in this thesis.

Detection time of SdrostM2 (18 days), which is the intact sulfate of the classic **drostanolone** metabolite (3α-hydroxy-2α-methyl-5α-androstane-17-one), was inferior to the glucuronidated form (20 days) on GC-CI-MS/MS.

This implies that detection of intact sulfates may not always be superior to glucuronidated metabolites, some compounds may benefit more from the detection of classic glucoronidated long-term metabolites. It is possible of course, that better intact sulfated metabolites of these compounds have yet to be identified. Taking all these results into consideration, the use of intact sulfates to screen for steroids shows a lot of promise. Even a small increase in the detection window of steroids, could prove an incredible asset in the fight against abuse of these substances.

5. References

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