In Vitro Synthesis of Metabolites of three Anabolic Androgenic Steroids, by Human Liver Microsomes

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MASTER THESIS

In Vitro Synthesis of Metabolites of three Anabolic Androgenic Steroids, by Human Liver Microsomes

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Sammendrag

Androgene anabole steroider er i idrettssammenheng et mye brukt ulovlig middel for å øke prestasjonsevnen. For å avdekke misbruk, doping, er en urinprøve velegnet. Identifisering av steroidet og/eller dets metabolitter forutsetter tilgjengelighet av etablerte referanser for sammenligning. Bruk av utskillelsesforsøk for kartleggelse og opparbeidelse av relevante referanse metabolitter, er ressurskrevende og etisk omdiskutert. Bruk av mikrosomer er derfor et aktuelt alternativ. Målet med denne oppgaven var å syntetisere og identifisere metabolitter fra kjente og sjeldne anabole androgene steroider, ved bruk av humane levermikrosomer. Leveren er et viktig organ for steroid metabolismen. Inkubasjon av anabole androgene steroider med humane levermikrosomer, utgjorde en *in vitro* simulering av levermetabolismen. En bekreftelsesmetode for metabolitter bestod i gasskromatografi tandem massespektrometri i fullstendig søk, MRM-modus, eller begge kombinert. 6β-hydroksymetandienon, epimetandienon og 17,17-dimetyl metandienon ble dannet under inkubasjon av metandienon. 17β-hydroksymetyl metandienon ble produsert fra 17,17-dimetyl metabolitten. Henholdsvis tre og én metabolitt(er) ble funnet for "designer steroidene" metylnortestosteron og madol. Metabolittene viste store variasjoner i optimal inkubasjonstid og enzymatiske forutsetninger for dannelse.

Abstract

Anabolic androgenic steroids are substances frequently misused to improve physical performance in sports. To reveal substances misused as doping, athlete urine samples are collected and tested. To identify the steroid and/or its metabolites in urine, reference compounds must exist for comparison. The time-consuming and ethical concerns about *in vivo* excretion studies for the examination of these compounds, make the use of liver fragment microsomes an attractive alternative. The aim of this thesis was to synthesize and identify metabolites from known and rare anabolic androgenic steroids, by the use of human liver microsomes. Liver is an important organ in steroid metabolism. By incubating AAS with human liver microsomes and co factors, an *in vitro* simulation of the liver metabolism was carried out. A confirmation of metabolites was performed by gas chromatography tandem mass spectrometry in full scan, MRM mode, or both. 6β-hydroxymethyl metandienon, epimetandienon and 17,17-dimethylmetandienon were successfully synthesized from metandienon, and the 17β-hydroxymetandienon was produced from the 17,17-dimethyl metabolite. Respectively three and one metabolite(s) were found for the "designer steroids" methylnortestosteron and madol. Metabolite variations were observed regarding the optimal time of incubation, and enzymatic requirements of formation.

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by Tiril SCHJØLBERG

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Chapter 1

Introduction

The practice of enhancing sport performance with supplements, might be as old as sporting itself. In ancient Greece, during the 4th century B.C., Aristotle described how athletes consumed special diets, like testes from strong animals, preferably to improve their performance[9]. Doping in modern time is known from late 19th century, by the use of e.g. caffeine, cocaine or strychnine derived from plants or animals[35]. The International Amateur Athletic Federation tried to prohibit doping in 1928, but made no success as it was both demanding, and in many cases impossible to control or confirm misuse of doping substances at that time[51].

1.1 History of doping

Doping in sports is defined as the use of drugs to enhance performance of an athlete[8]. It tampers with the spirit of fair play, and is considered cheating by sports organizations. Several drugs of various kinds are considered as doping. The most common, are androgenic anabolic steroids[52].

Misuse of anabolic steroids in sports dates back to the nineteen fifties[24]. Testosterone was the first androgenic anabolic steroid (AAS) suspected to be abused, succeeded by synthetic testosterone analogues. These became available when drug companies met an increasing demand in connection with the discovery of their usefulness, in treating a wide range of endocrinological diseases[35]. As a consequence, the medical committee of the International Olympic Committee (IOC), introduced the first list of prohibited substances ("the code") in 1968[53]. AAS were placed

in "the code" in 1976[24]. Compulsory tests followed in the succeeding winter- and summer- Olympic Games. In 1999, the IOC convened the World Conference on Doping in Sports, as a result of deviating apprehensions and rules in international and national anti-doping organizations. The independent organization, the world anti-doping agency (WADA), was founded, and is today responsible for control and revise of prohibited substances and methods[51]. WADA presents "the code" on a yearly basis, which is the foundation of tests performed by accredited laboratories for control and confirmation of doping.

1.2   Control and confirmation of anabolic steroids

In doping control analysis, body fluids from athletes are tested by WADA accredited laboratories to reveal prohibited substances[4].

Technology has improved the testing techniques[25], focus and communication in the work of doping control analysis. Test intervals and occurrence have improved dramatically[52], and a large number of substances and cover ups are today included in the screening. AAS are the most widespread misused substance in today's doping[52]. Extensive research and control has therefore been put forward to ensure "fair play" in athletic context.

To perform doping control analysis on anabolic steroids, athlete urine samples may be collected at any time, and sent to accredited doping control laboratories for analysis. Since the nineteen sixties, instrumental analytical tools have been used for this purpose[25]. Identification and quantification of AAS can be performed by a combination of chromatography and mass spectrometry[42], a method explained in section 2.7. Accredited laboratories must in most cases identify, but not quantify prohibited substances[26;4]. Hence irrefutable detection of prohibited AAS or their metabolic products need reliable confirmation. This is accomplished by the use of certified reference compounds. Many AAS are completely metabolized in the human body[43]. To identify and/or examine a steroid and its potential metabolites, it is an increasing demand to both understanding and carrying out the steroid metabolism[42;29].

Furnishing of reference compounds has been approached in several ways, among them, through chemical synthesis, and through *in vivo* excretion studies[42]. A chemical synthesis of potential metabolites requires a well understood metabolism,

and a known or suggested metabolite molecular structure. It provides pure com-
pounds, but has limited stereo specificity[30]. An issue possibly solved by isolation
and purification of metabolites from excretion studies, giving healthy volunteers
steroids[40]. The excreted metabolites from urine may prove as references to chemi-
cally synthesized compounds, or in direct comparison to urine samples in a doping
control analysis. Since many steroids produce known or unknown side effects,
both ethical and medical concerns are associated with exploring AAS and their
metabolites in the latter way[29].

In recent years, alternative approaches to obtain reference metabolites have been
proposed. Zöllner *et al.*[58] synthesized the 17β-hydroxymethyl metabolite of the
AAS metandienon by recombinant yeast strains. The strains of *Schizosaccha-
romyces pombe* were modified to express different human hepatic enzymes. This
method proved successful in synthesizing enough reference material for structure
characterization. Each yeast culture expressed different human enzymes, which
made it possible for only one enzyme to be examined at a time.

Kuuranne *et al.*[30;29] examined an alternative approach, to obtain reference metabo-
lites by the use of human liver microsomes. Several metabolites of AAS were ex-
amined for their *in vivo*- *in vitro* correlation with microsomes as an *in vitro* source
of metabolizing enzymes. This technique was recently approved by WADA as a
method to derive reference compounds of steroid metabolites[1], but has never been
performed in Norwegian anti-doping research.

The aim of this thesis was to explore the use of microsomes as a technique to syn-
thesize metabolites from an AAS. Metandienon was chosen as the model AAS, as
its metabolism is well known, while madol and methylnandrolone were substances
with limited information regarding their metabolic pattern. An additional purpose
was to establish an analytical method to determine metabolites resulting from the
synthesis.

First, a brief review of some basic theory is given. Section 2.2 provides a frame-
work to understand anabolic steroids and its metabolites in general. Section 2.4
is devoted to the general concepts behind the liver metabolism, with an addi-
tional subsection on microsomes. Sample processing in doping control analysis is
reviewed in section 2.6. The concepts behind gas chromatography tandem mass
spectrometry is outlined in section 2.7, with emphasis on method optimization by
multiple reaction monitoring transition and general steroid detection. Next, in

chapter 3, establishing the method for detection, and assay of *in vitro* synthesis of metabolites are presented. Results of establishing the detection-method, and synthesized metabolites are presented and discussed in chapter 4. The conclusion is given at the end, together with suggestions for future work.

Chapter 2

Theory

The theory given here, explains the chemical basis and therapeutic application of use of anabolic steroids. This serve as a background for the understanding of its usefulness, burden and complexity. All are important aspects to consider in the fight against doping in sports. Both general characteristics of anabolic androgenic steroids and some specific prohibited compounds are covered in this chapter. As a framework for the experimental design, sample preparation in doping control analysis and analysis by gas chromatography tandem mass spectrometry is outlined too.

2.1 Steroids

Steroids are a large group of chemical components classified collectively by their structural likeness. The structural core of a steroid consists of four rings of carbon[8], as shown in figure 2.1. Three rings (A, B and C) with six carbon atoms, and one (D) with five.

Double bonds and ring substituents are crucial for the steroid interaction with receptors associated with the cellular membrane, where it may function as a ligand[16]. Steroids are important "biological regulators", often with powerful physiologic effect when administered to living organisms[46]. Among these are the male and female sex hormones, adrenocortical hormones, and D-vitamins. The sex hormones are further classified in three major groups: estrogens (female sex hormones), androgens (the male sex hormones) and progestines (pregnancy hormones)[46].
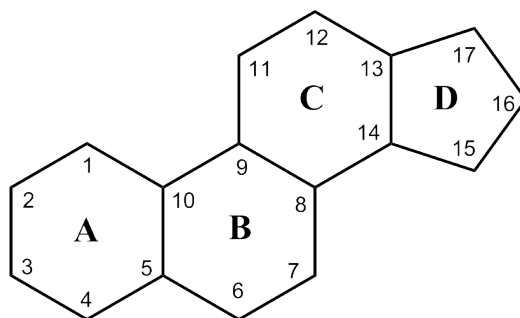
FIGURE 2.1: The steroid core molecular structure. The characteristic carbon rings: A, B, C and D are labeled.

2.2 Anabolic androgenic steroids

Anabolic and androgenic are terms describing effects of some steroid hormones. Anabolic actions refer to affected protein metabolism through increased protein synthesis and/or inhibited protein breakdown[23]. Androgenic, refer to development of male characteristics, such as hair growth and deepened voice[46]. In the WADA prohibited list, the steroid hormones are divided into two classes: endogenous and exogenous steroids[27].

Some androgenic anabolic steroids (AAS) are natural internally produced, and function as hormone signals in the human body[17]. These are termed endogenous AAS (endogens), as the word endogenous stands for internal origin[8]. Endogens are needed for normal cell function. Two endogens are testosterone, shown in figure 2.2, and dihydrotestosterone[4]. Endogens are usually well regulated by the human signal system, unless pathologically influenced, or subjected to compounds that compete for the same receptors.
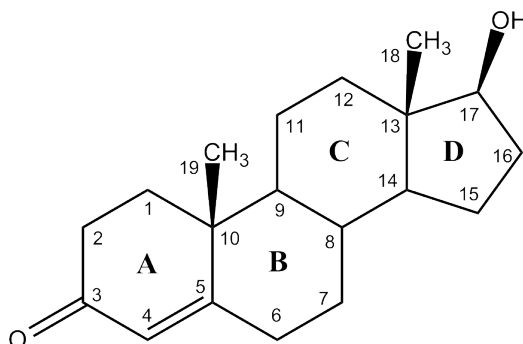

FIGURE 2.2: The molecular structure of testosterone, labeled with carbon- ring names and numbers.

The history of anabolic steroids began with the discovery and synthesis of testosterone, the male sex hormone. In the mid 19th century, Arnold Berthold were one of the first scientists that acknowledged the testes influence on sexual and behavioral characteristics. He suggested that the characteristics resulted from the testes secreting a substance into the bloodstream[17]. Later, in 1889, Charles Edouard Brown-Séquard reported an increase in strength and mental abilities (!) as a result of self-injections of extracts derived from animal testicles. In the beginning of the 20th century, two British scientists concluded that this substance (among others) function as blood borne messengers, which they named hormones. They argued that the hormones regulated many body functions far from the tissue of origin[17]. The synthesis of testosterone was achieved and published in 1935, almost simultaneously by Butenandt and Hanish, and Ruzicka and Wettstein.

2.2.1   Testosterone analogues

Exogenic AAS (exogens) are synthetic analogues of testosterone[23]. The discovery of 17α-alkylated testosterone revolutionized the prospective of anabolic steroids. 17α-alkylation gave the potential of activity upon oral administration of steroids[5].

Many exogenic compounds do exist for therapeutic purposes. A goal for the development of therapeutic AAS has been to minimize the androgenic and maximize the anabolic action[28]. Methylandrostenediol was the first steroid that showed a differentiation in anabolic and androgenic action. Research on which modifications that gave anabolic properties without the androgenic characteristics has succeeded[49], but also revealed that these effects are closely resembled. Testosterone and synthetic testosterone derivatives are often termed as androgens. Androgens mediate their action through biding to the androgen receptor (AR). The different structural features of steroids, such as β-connected hydroxy-group in the 17-position, presence of C-4, -5 double bond, and 3-keto group in the A-ring, all influence the androgenic and/or anabolic activity[14]. Today, still no steroid is solely anabolic.

When orally administered, testosterone is rapidly absorbed in the portal vein, and is subjected to extensive metabolism (often with loss of effect) in the liver (first-pass effect)[41]. Chemically modified testosterone can circumvent the first-pass effect, providing an increased oral availability[28]. Even though exogens mainly were develop for medical purposes, they are unfortunately also widely misused as doping. The metabolic profile of the exogenous steroids show similarities with

that of testosterone[39], and is to a large extent altered at positions of functional groups. A wide variety of metabolites can be found in human urine subsequent to the administration of synthetic AAS.

2.2.2 Metandienon

Metandienon (MD, 17β-hydroxy-17α-methylandrosta-1,4-dien-3-one) is an exogenic AAS. MD possesses a range of different names, among them dianabol. The chemical structure in figure 2.3 manifests that MD is a 17α-methylated testosterone (methyltestosterone) with an introduced double bond between the first and second C-atom[39].
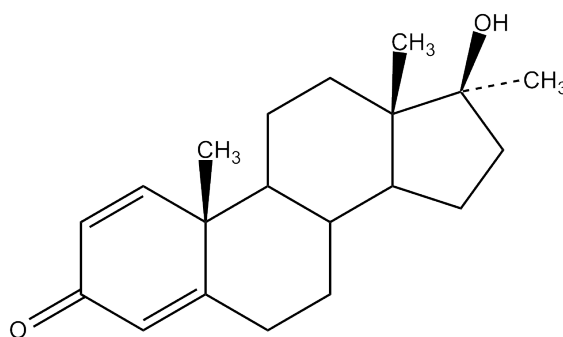

FIGURE 2.3: The molecular structure of metandienon.

MD has anabolic effects, but exhibit less androgenic effects than its endogenic precursor[5]. This might be due to the change in structure, or the potentially reduced testosterone level when administered in high dosages[23].

MD was for the first time synthesized in 1958 and further permitted for use in pediatrics, due to its low androgenic activity[5]. Unfortunately it was misused as doping already in the world championship for weightlifters in 1962[35], and became popular as a doping preparation. Today, MD is the most misused AAS reported by WADA accredited laboratories[52]. MD and other 17α-alkylated steroids do often affect the liver function, the heart, and can result in changes in human behavior[5].

2.3   "Designer steroids"

"Designer steroids" are steroids produced to closely resemble existing AAS, but with sufficient structural differences to avoid detection by WADA accredited laboratories[27]. Some "designer steroids" were partly examined in trials decades ago, but never commercially marketed, nor used in pediatrics. Only limited or no available data exists regarding their toxicological and pharmacological effects in humans[4;27]. The first revealed "designer steroid" was norbolethone, a steroid tested decades ago for medical purposes, but never launched as a commercial product[6]. After discovering a second "designer steroid", the WADA science director expressed a concern regarding the progress of steroid abuse, with the sole purpose of "designer steroids" being used as doping agents[50]. "New" "designer steroids" are not registered on WADA list of prohibited substances, but are covered through the implemented phrase: "and related compounds"[54]. Since their structure differs from known steroids, "designer steroids" are often not embedded in the screening in regular doping analysis[44], and are therefore more difficult and often impossible to detect.

2.3.1   Methylnortestosterone

The exogenous AAS, methylnandrolone or methylnortestosterone (MeNT, 17β-hydroxy-17α-methyl-5α-estr-4-ene-3-one), is a 3-keto-4-ene steroid, shown in figure 2.4. The distribution of MeNT as a potential pharmaceutical was closed decades ago. It recently reappeared as a drug available through the Internet market[7], and is considered as a "designer steroid".
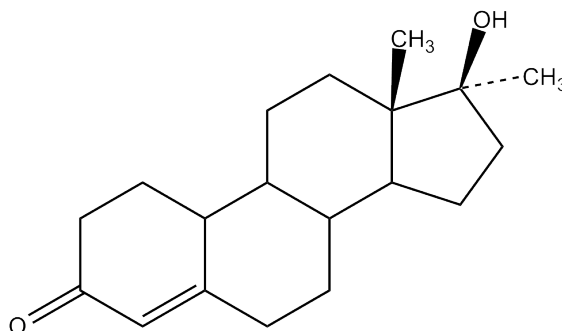

FIGURE 2.4: The molecular structure of methylnortestosterone

MeNT does most likely exert its action on AR through providing androgenic and anabolic action. Speculations on the steroid activity follows the reasoning by

Fragkaki *et al.*[14]. The structure of MeNT has similarities with that of MD and nandrolone. Compared to testosterone, 17α-alkylation and 19-methyl removal (19-nor steroids) favours anabolic activity. A 19-nor structure also reduces androgenic activity. In that way, MeNT may very well be a potent anabolic inducer.

2.3.2 Madol

The exogenic AAS Madol (17α-methyl-5α-androst-2-en-17β-ol) (figure 2.5), also known as desoxymethyltestosterone, is one of the first known designer steroids[44]. The patent on Madol was awarded decades ago, but for unknown reasons, never launched as a pharmaceutical[10]. In early 2005, the WADA accredited UCLA Olympic Analytical Laboratory (USA) and Montreal laboratories (Canada), simultaneously, and independently revealed their discovery of madol from an oily substance[44;50]. They discovered this substance as a result of intensive and collaborative investigation nationally. The madol mixture showed mainly madol, but also traces of its 3-ene isomer. Impurity in a drug preparative is indicative of a manufacture outside the professional pharmaceutical industry[50].
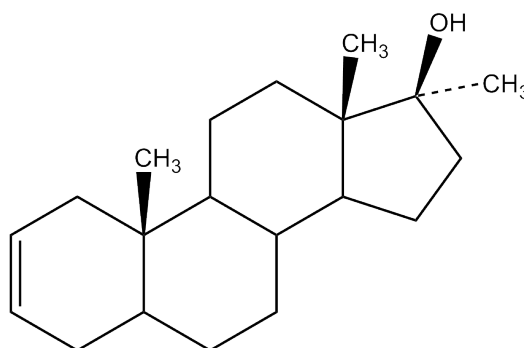

FIGURE 2.5: The molecular structure of madol

Diel *et al.*[10] used tests for biological activity in orchiectomised rats (i.e. had their testes removed), to characterize the pharmacological profile of madol. They concluded that madol is a powerful anabolic steroid, but they also addressed toxic side effects, such as a hypertrophic heart. Madol is placed on WADA prohibited list[54], but has limited detectability in today's screening. Several websites offer madol as a performance enhancing "supplement".

It should be mentioned that additional side effects may result from the use of madol. Even though Diel *et al.*[10] characterized the pharmacological profile of madol, they used pure madol in their experiments. As reported by WADA[50],

madol available on the Internet marked, is of a different purity grade and include additional steroid compounds. This may result in a different metabolic outcome, and an unknown metabolic profile.

2.4 Steroid metabolism

Besides the processing and distribution of nutrients[36], the liver provides metabolism of exogenous compounds to prevent potential hazardous effects[57]. The liver is the first organ to encounter ingested compounds as venous blood flows directly from the stomach and intestine, through the portal vein, to the liver[55]. The liver is rich in metabolizing enzymes that can transform non polar AAS (orally administered) in two ways. These metabolic transformations are accomplished by a limited number of enzymes with a broad substrate specificity.

2.4.1 Phase I reactions

Phase I reactions involve hydroxylation, epimerization, oxidation and reduction[55]. These enzymatically catalyzed reactions alter AAS functional groups. Usually this increases the polarity and/or makes AAS more accessible to phase II metabolizing enzymes[55]. The primarily superfamily of phase I enzymes are cytocrome P450 (CYPs)[57], which hold catalytic versatility[55]. CYP enzymes catalyze the monooxygenation of a compound while reducing the other oxygen to water. Reducing equivalents derive from electrons from nicotinamide adenine dinucleotide phosphate (NADPH), transferred to CYP via the flavoprotein NADPH-cytochrome P450 reductase. Both CYP450 and the NADPH reductase are embedded in the phospholipid bilayer of endoplasmic reticulum (ER), which facilitates their interaction[55]. Epimer formation is an interesting reaction and object of discussion[12;43]. The mechanism is considered to be a sulphate conjugation, followed by nucleophilic attack by water on the labile conjugate. This do further produce a dehydration product, which in the case of methyl steroids result in a 17,17-dimethyl-18-norandrost-13-ene structure[43].

2.4.2 Phase II reactions

Phase II reactions include glucuronidation, sulphation, acetylation and conjugation with glutathione[55]. These usually increase the hydrophilisity and the excretion of a compound[57]. The main phase II reactions for AAS are glucuronidation and sulphation[29]. These reactions play an important role in conjugation and elimination of compounds with hydroxyl functional groups[57]. Glucuronidation is enzymatically catalyzed by UDP-glucuronosyltransferases (UGTs). The reaction also requires the cofactor Uridine-5'-diphospho-β-D-glucuronic acid (UDPGA)[55]. Sulphation is catalyzed by sulphotransferases (SULTs). SULTs involved in steroid metabolism are soluble and cytosolic[47], and require 3'-Phosphoadenosine-5'-phosphosulphate (PAPS) as a cofactor in the sulphonate transfer[55]. SULTs generally produce a highly water soluble ester of sulphuric acid (O-sulphonation), which inactivate the steroid ability to bind to the receptor[47]. SULT2A1 (a SULT subfamily) sulphonate a variety of substrates, including the 17β-hydroxyl group typical for steroids. Kuuranne *et al.*[29] suggested that a low cellular concentration of PAPS may limit the sulphation conjugation of steroids, a statement supported by Watkins *et al.*[55]. The SULT activity has also been reported slow. Apart from that, little or no investigation has been performed to examine the influence of PAPS availability on sulphoconjugation reactions[47].

2.4.3 Microsomes

Microsome originates from the Greek words micros (small) and soma (body). Microsomes are small cytoplasmic fractions, preferably with pieces from endoplasmic reticulum (ER), isolated with or without ribosomes[3]. Human liver microsome-preparations may prove useful in exploring ER, and when studying the liver-metabolism of foreign compounds. Micorosom-preparations can be divided in two categories: liver microsomal membranes (HLM) that contain numerous CYP and UGT enzymes, and microsomal cytosolic components (S9 fraction), were SULTs are present[2]. The level of enzymatic activity vary among humans due to environmental or/and genetic factors[55]. To avoid these variations, companies provide microsome-preparations from liver tissue from multiple donors (>100).

2.5 Metabolites

Several studies have examined AAS with emphasis on their metabolic pattern[11;41;34;37]. Based on such studies, Fragkaki *et al.*[14] did a principal component analysis (PCA) that pictured possible metabolites and alterations in the parent molecule. This provides a powerful tool capable of foreseeing metabolites for detection of prohibited compounds. The next three sections picture known and suggested metabolites concerning the three AASs mentioned.

2.5.1 Metandienon metabolites

MD metabolites emerge in phase I and II reactions during MD metabolism in the liver. Several metabolites are well described in literature[33;42;58;37], and many are today embedded in regular doping control analysis. An assortment of MD metabolites are shown in table 2.1 and figure 2.6.

TABLE 2.1: Suggested metabolites for MD, according to estimates by Frakaki *et al.*[14].

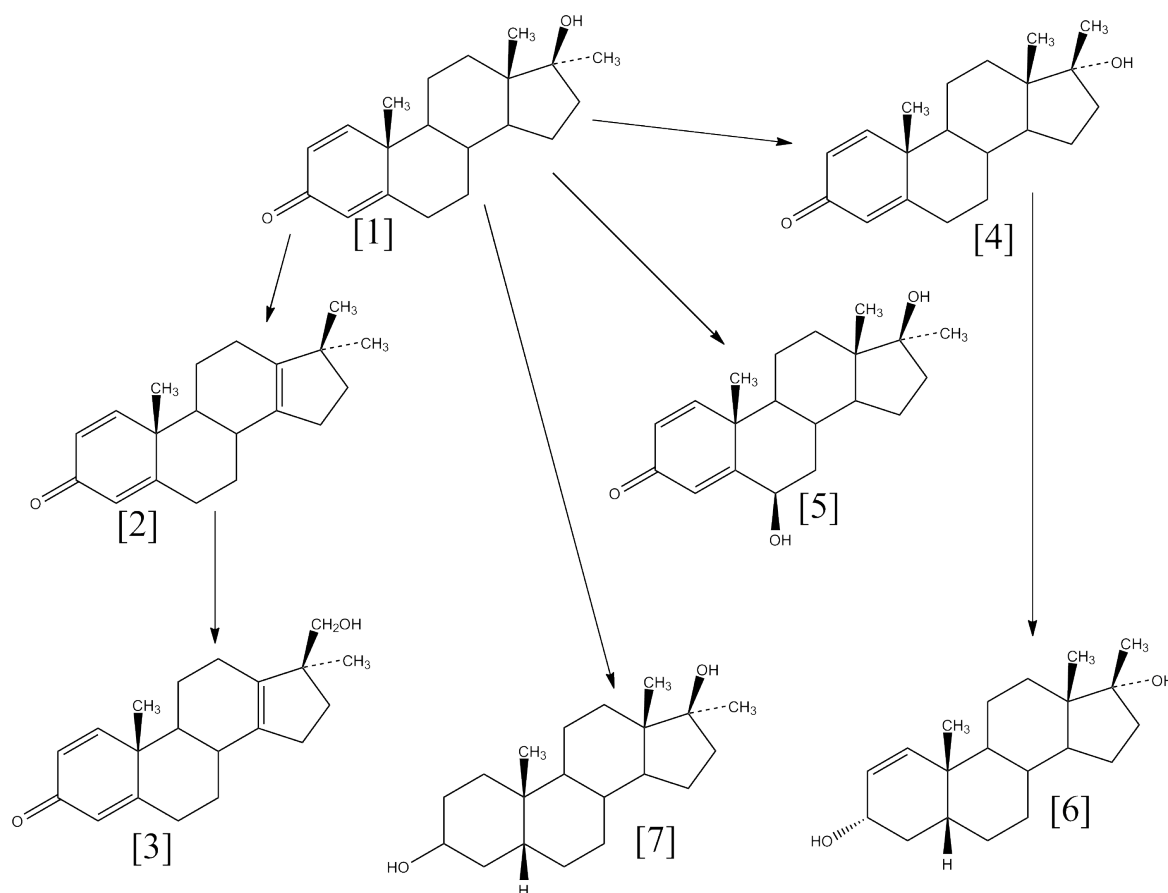| Metabolic action	Reported
C-4,5 double bond reduction to 5β-H isomer	Yes
17-epimerization	Yes
18-nor-17,17-dimethyl-13(14)-ene product	Yes
18-nor-17α-methyl,17β-hydroxymethyl,13(14)-ene product	Yes
3-keto reduction to 3α-OH isomer	Yes
C-1,2 double bond reduction	Yes
6α/β-hydroxylation	Yes
16α/β-hydroxylation	No
Double bonds at cites out of ring A reduction	No

FIGURE 2.6: MD [1] with metabolites; the 17,17-dimethyl metabolite [2], the 17β-hydroxymethyl metabolite [3], EpiMD [4], 6β-hydroxyMD [5], epimetendiol [6], and the joint (with methyltestosterone) metabolite 5β-THMT [7]. The arrows are only assumed pathways, as the metabolic pathway is yet not fully understood.

The metabolic pathways and responsible enzymes in MD metabolism are well, but not completely understood. 6β hydroxylation (B-ring metabolite) is the main route for steroids of 17β-hydroxy-17α-methyl structure[39]. Several researchers have synthesized 6β-hydroxy MD and detected it in urine after *in vivo* excretion studies[5;40;11].

The 17-epimerization was debated in late 1980s, and partially solved by Edlund *et al.*[12], who claimed that this was a non-enzymatic reaction, resulting from nucleophilic attach of the 17-sulphonate conjugate. He further argued that the labile 17-sulphonate probably also undergo dehydration that cause rearrangement of the 13-methyl group, yielding a 17,17 dimethyl metabolite depicted in figure 2.7.
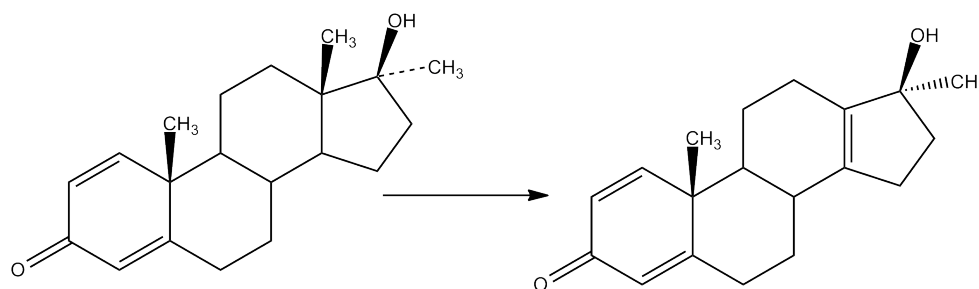
FIGURE 2.7: The rearrangement of metandienon to the 17,17-dimethyl metabolite. The rearrangement is assumed to comprise a sulphoconjugated intermediate.

Parr *et al.*[37] reported a regio- and stereo- specific hydroxylation of the 17,17-dimethyl metabolite by the enzymes CYP3A4 and CYP21. A reaction that yields the 17β-hydroxymethyl metabolite, as shown in figure 2.8.
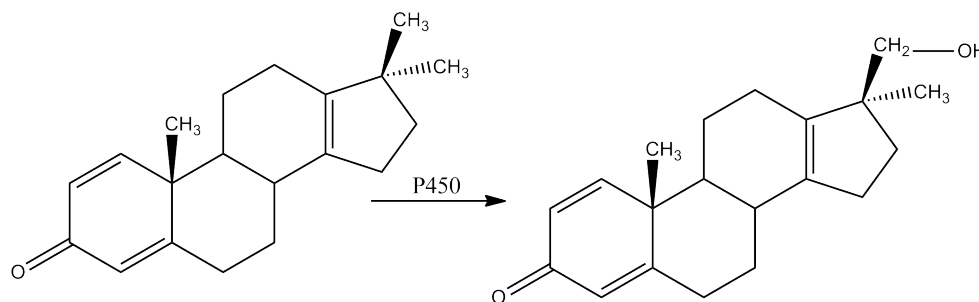

FIGURE 2.8: The enzymatic hydroxylation of the 17,17-dimethyl metabolite to the 17β-hydroxymethyl metabolite by CYP enzymes.

Metabolites from MD and their time of elimination, show inter individual variation, and depend on the metabolite in question[41]. Methods or compounds that prolong the detection window of MD and other AAS are of great interest in the field of anti-doping research. The 17β-hydroxymethyl metabolite was identified as such a long term MD metabolite when it showed to be traceable in urine 19 days post administration[58].

2.5.2   Methylnortestosterone metabolites

In accordance to Fragkaki *et al.*[16], no metabolite originating from MeNT has been discovered. Anyhow, their statistical approach (PCA) suggests that several metabolites may result from MeNT. Several metabolites were proposed in the PCA, as summarized in table 2.2.

TABLE 2.2: Suggested and/or possible metabolites for MeNT, according to the PCA by Fragkaki *et al.*[14].

Metabolic action	Reported
C-4,5 double bound reduction to 5α, 5β-H isomers	No
17-epimerization	No
18-nor-17,17-dimethyl-13(14)-ene product	No
18-nor-17α-methyl,17β-hydroxymethyl,13(14)-ene product	No
3-keto reduction to 3α-, 3β-OH isomers	No
$6\alpha/\beta$-hydroxylation	No
$16\alpha/\beta$-hydroxylation	No
Hydroxyl/keto groups at various sites oxidation/reduction to the corresponding keto/hydroxyl groups	No
Double bond(s) at sites of ring A reduction	No

2.5.3 Madol metabolites

Somewhat more information exists on madol metabolites. According to Fragkaki *et al.*[14] PCA study, many metabolites are possible for madol as well (table 2.3). The first attempt to generate madol metabolites was performed through a baboon excretion study and human liver microsomes, but no clear metabolites were found[44].

TABLE 2.3: Suggested and/or possible metabolites for madol, according to estimates by Fragkaki *et al.*[14]. *A combination of 2 and 3 hydroxylation, a 2,3,17-triol, has been reported by Gauthier *et al.*[18]. **Reported without confirmatory synthesis, and as combinations of the metabolic actions.

Metabolic action	Reported
2-hydroxylation	Yes*
17-epimerization	Yes**
18-nor-17,17-dimethyl-13(14)-ene product	Yes
18-nor-17α-methyl,17β-hydroxymethyl,13(14)-ene product	No
3-hydroxylation	Yes*and**
$6\alpha/\beta$-hydroxylation	Yes*
$16\alpha/\beta$-hydroxylation	yes
C-2,3 double bond reduction	Yes
3-hydroxyl oxidation into 3-keto (from M3)	Yes**

Recently, Gauthier *et al.*[18] discovered metabolites of madol by the use of human fresh hepatocytes and human urine reference samples. Their proposed primary metabolite of madol was 17α-methyl-$2\beta,3\alpha,17\beta$-trihydroxy-5β-androstane. In addition were traces of minor metabolites seen, synthesized, and both a 3,4-diol, and a sulphoconjugated metabolite were postulated.

Late 2012, Sobolevsky *et al.*[45] identified several madol metabolites from excretion urine samples. The detectability of observed metabolites varied up to two weeks after administration. Main metabolites are depicted in figure 2.9.
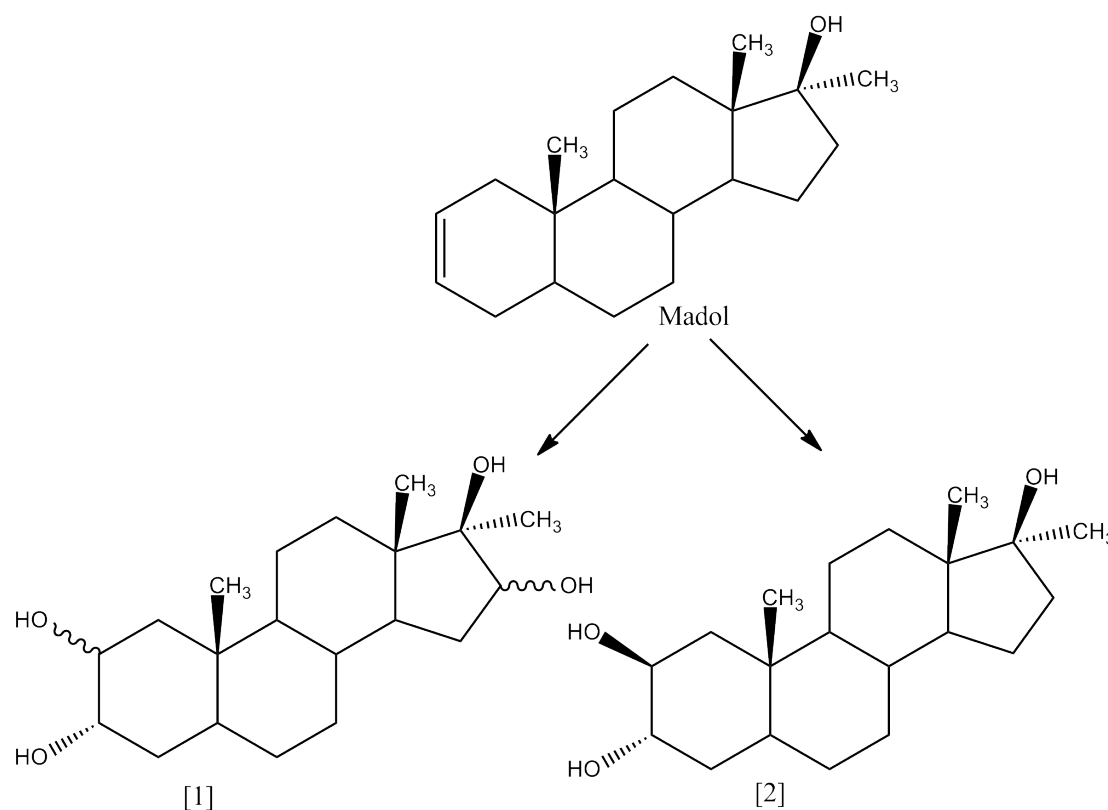
FIGURE 2.9: The molecular structure of the main madol metabolites discovered by Sobolevsky *et al.*[45]. [1] 17α-methyl-5α-androstan-$2\xi,3\alpha,16\xi,17\beta$-tetrol, and [2] 17α-methyl-5α-androstan-$2\beta,3\alpha,17\beta$-triol.

2.6 Sample preparation for analysis

Several preparing steps are required when a urine sample arrives at an accredited doping laboratory for analysis of AAS content. A low initial concentrations of AAS requires extensive purification, and increased concentration in order to be

suitable for detection. A procedure of stepwise preparation described by Bowers[4], and Schänzer *et al.*[43] is outlined next.

2.6.1 Enzymatic hydrolysis by β-glucuronidase

Many exogenic AAS are excreted as glucuronides. The enzyme β-glucuronidase can be added to cleave molecular bonds to β-D-glucuronic acids. In doping control analysis, enzymatic hydrolysis is the initial step for increasing the concentration, and prepares samples for analysis[4;32].

2.6.2 Extraction

Enzymatic hydrolysis provides non-polar metabolites that are transferable to an organic solvent by extraction. Liquid-liquid extraction is a separation method with two immiscible liquids, where the components in a mix are isolated by the preferred solubility in one of the liquids. Isolation of the solute is then performed by separation of the phases (e.g. a low temperature bath, where the aqueous layer is frozen). Non-polar AAS are extractable in the non-polar solvent tert-butylmethyleter (TBME), that allows extraction in a broad polarity range[4]. After separation, increased concentration of the AAS is achieved by solvent evaporation.

2.6.3 Derivatization

AAS metabolites bear a wide range of different structural features, including hydroxyl and keto groups[33]. These groups are unfavorable in gas chromatography analysis. To favor separation of metabolites by gas chromatography, and further detection in the mass spectrometry analysis, a trimethylsilylation of the hydroxyl and keto groups can be performed. In AAS processing, N-Methyl-N-trimethylsilyltrifluoractetamid (MSTFA), ammonium iodide (NH_4I) and ethanethiol (EtSH) are common compounds in a mixture that initiates trimethylsilylation, named derivatization[4]. Trimethylsilylation of MD by MSTFA, NH_4I and EtSH is shown in figure 2.10. Derivatization is today standard in steroid trace analysis in doping controls[48].
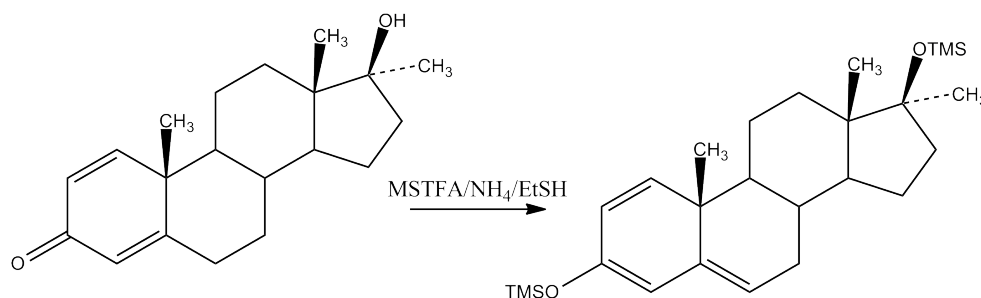
FIGURE 2.10: Trimethylsilylation of hydroxyl and keton groups of MD

2.7 Instrumental

Several analytical methods that satisfy different requirements are available. In the following sections, a brief explanation of a few analytical tools important in doping control analysis is given.

2.7.1 Gas chromatography

Gas chromatography (GC) is an instrumental method that can separate compounds in a mixture. The mixture of compounds is vaporized in a liner before entering a column. The separation happens when compounds are distributed between a stationary phase and a mobile gas phase through the column[19]. The compounds will differ in their interaction with the GC column material, resulting in different distribution between the two phases. The GC column mainly separate compounds (e.g. different AAS) by boiling point, and some on the basis of polarity. This separation results in different retention time (RT). RT can also be expressed as relative RT (RRT), for which it is relative to the RT of testosterone[38].

For doping control analysis of small molecules in urine, GC is coupled with a mass spectrometry (MS) to identify doping compounds in a sensitive and efficient way[25].

2.7.2 Mass spectrometry and tandem mass spectrometry

A mass spectrometry (MS) measures ion masses, as a mass to charge ratio (m/z), in a detector. In MS, an ion source provides a fragment pattern of a compound.

The fragment pattern is distinct, and can serve as compound identification. Electron impact (EI) is a common and strong ionization technique, frequently used in doping analysis[48]. EI applies energy, usually 70eV, to ionize and dissociate compounds into fragments. Fragments are detected, and the MS software displays a mass spectrum of ion-abundances against m/z. The dissociation pathway of a compound (e.g. derivatized AAS) results in a distinct spectrum. This spectrum provide the required data for determination of prohibited compounds in a doping control analysis[48]. When EI provides one fragmentation and ionization, the displayed MS spectrum is known as a EI mass spectrum.

In the 1970s, chemical ionization (CI) and selected ion monitoring (SIM) were introduced as additional analytical techniques[25]. CI is a more gentle ionization that provides more abundant molecular ions. SIM is performed on a single quadrupole that enables selection of specific ions in advance.

Tandem mass spectrometry (MS/MS) holds the capacity of a second fragmentation. This is provided by a collision cell known as collision induced dissociation (CID). Selected ions (precursor ions) from a EI mass spectrum are introduced to CID through a quadrupole. The CID provides a fragmentation pattern known as a product ion mass spectrum. Specific product ions from the dissociation pathway from the product ion mass spectrum may be selected through a second quadrupole. The precursor and product ion transition represents a multiple reaction monitoring (MRM) that provide superior specificity and sensitivity against background noise[31]. Even an increased specificity is met through monitoring the ratio of the identified MRM transitions within a compound. Amount of collision energy (CE) applied in CID will determine the fragmentation level. The CE may be adjusted in a method optimization to increase the sensitivity.

2.7.3   Gas chromatography tandem mass spectrometry

GC is today combined with MS or MS/MS to achieve high specificity and accuracy. It is applied in screening of AAS, and is of great importance in science[22].

2.7.4   Dissociation pathways

A EI mass spectrum reveals a dissociation pattern, that may suite as compound identification when compared to dissociation patterns of known references[48]. Fragments refer to characteristic parts of the analyzed molecule.

General and specific characteristics in a full scan.   The steroid structure gives rise to distinct steroid "fingerprint" fragments upon EI, shown as abundant peaks in a mass spectrum. Fragment ions with m/z of 73 have little diagnostic value as it derives from the loss of the $[TMS]^+$ fragment, that are abundant in all TMS-derivatives. D-ring fission between C13 and C17, and between C14 and C15, give rise to the distinct pattern of m/z of 143. This is considered as a characteristic fragment of 17α-alkylated synthetic steroids[16]

Loss of specific groups such as methyl or ethyl, is often a source of abundant intensities in an AAS mass spectrum. A m/z of 103 indicates the dissociation into the $[TMSOCH_2]^+$, with $[MW-103]^+$ also resulting as an abundant fragment. A $[MW-TMSOH-103]^+$ fragment may serve as a diagnostic characteristic[16].

Chapter 3

Experimental

The experimental was divided into four parts: establishing a detection-method, microsome incubations, microsome sample analysis, and urine sample analysis.

3.1 Establishing the detection-method

A detection-method was established by applying known reference standards of steroids and metabolites, to GC-MS(/MS) in full- and product-scan mode. First, chemicals and preparations of the steroids and MD metabolite reference standards are described. Second, the focus is upon developing the method, by retention time and MRM transitions, to detect *in vitro* synthesized metabolites.

During the pre-project for this thesis, a method for detection of MD and known metabolites by GC-MS/MS were established. Here, this existing analytical method was adjusted, by implementation of the 17,17-dimethyl metabolite. Detection of proposed methylnandrolone and madol metabolites required a different angle of methodology, due to the lack of known metabolites and not commercially available reference standards.

3.2   Reference solutions

The preparation of reference solutions was in accordance with that of the Norwegian Doping Control Laboratory. Reference standards of MD, epiMD, 6β-hydroxyMD, epimetendiol, 17,17-dimethyl metabolite were obtained from Australien Government NMI. Reference standards of: the 17α-hydroxymetyl metabolite, 5β-THMT, methylnortestosterone and madol had been obtained from: Köln, NARL Steraloids Inc. and Chiron AS respectively.

Stock solutions (1 mg/ml) were pre-made, or made by dissolving pure compound in methanol. Diluted stock solutions (50 μl, 10 ng/μl) of each component were distributed in separate tubes.

50 μl methyltestosterone (internal standard (ISTD)) was added to each tube. One tube was prepared containing a mix of all components and the ISTD.

All reference solutions in the tubes were evaporated under a stream of nitrogen (N_2) for 30 min. 100 μl MSTFA mix (described in section 2.6.3) was added to each solution, and the solutions were derivatized on a heat block (50-55 °C) for one hour. Derivatized solutions were transferred to vials with inserts, and applied to GC-MS/MS for analysis.

3.3   Instrumental setup

The instrument setup was in accordance with regular doping control analysis of AAS, with GC-MS/MS (Agilent) in split-less mode. The column was a SGE BPX5, 5 % phenyl column/95 % methyl polysilphenylene (15 m x 0.25 mm, film 0.22 μm, 5 % crosslink). Injector temperature was 280 °C. Initial column temperature was 160 °C, which was raised with 10 °C/min to 200 °C. Thereafter, the temperature was increased with 2 °C/min to 220 °C. An increase by 20 °C/min gave an end temperature of 310 °C. The flow was constant, and set to 2,4 ml/min. MSTFA was injected directly, with no pre-column. The GC-MS single quadropole had a HP ultra 1 (17 m x 0.20 mm, film 0.11 μm, 5 % crosslink) column in split mode (ratio 10:1). Injector temperature was 280 °C. Initial column temperature was 180 °C, which was raised with 3.3 °C/min to 231 °C. An increase by 30 °C/min gave an end temperature of 310 °C. The split flow was 6.0 ml/min. MSTFA was injected directly, with no pre-column.

An initial screening was performed on each reference compound and incubation solutions of methylnandrolone and madol. It was performed in full scan mode, with EI as the ionization technique. The detector area range in full scan mode was set to m/z from 50 to 550. Known fragments (from literature and data library) in the full scan were recognized, and respectively metabolite retention times were found in the chromatogram.

For each steroid, and its metabolites, one or two precursor ions were selected among the most abundant intensities in the EI mass spectrum.

A product ion scan was performed on these precursor ions. The upper range of the product ion scan was set to m/z of 5 to 10 units above the precursor ions. From the product ion mass spectrum of each precursor ion, one, two or three of the most abundant product ions were selected. The pairs of precursor ions and associated product ions were set as a MRM transitions. The MRM transition setup was tested with the reference standard mix. The MRM transitions of MeNT and its unpublished[7] proposed metabolites were applied in addition to establishing the detection-method from precursor and product ions of the incubated samples.

Several collision energies (CE) were applied to the MRM method, as an additional optimization to achieve a more specific response. CE of 5, 10, 15, 20 and 25 V were applied as trial CE for every MRM transition of MD and metabolites. CE of MeNT was set as recommended by Chundela and Große[7].

The CE that provided the greatest intensity for each MRM transition was applied to the final method. This detection-method was used to screen for metabolites as described in section 3.4.3. The selected ions, MRM transitions, and CEs are shown and discussed in table 4.1, 4.2 and 4.3 in chapter 4.

3.4 Metabolite synthesis by human liver microsomes

The assay used in this study, is consolidated on former studies[13;29] using microsomes as an *in vitro* synthesizing technique. Only small adjustments was done in comparison to the study by Kuuranne *et al.*[29]. The full procedure is presented in the following section.

3.4.1 Enzymes, substrate and working solutions

Madol, enzymes and co-substrates were obtained, while metandieon, its metabolites, methylnandrolone and ingredients for the buffer solutions were reagents mostly existing at the Norwegian Doping Control Laboratory. Microsomal and S9 fraction from human liver were purchased from BD biosolutions, and stored at -80°C. BD enzymes are two pooled mixtures of liver preparations from 150 different individual donors, to account for inter-individual variation[2]. Three co factors were purchased from Sigma: nicotinamide adenine dinucleotide phosphate (NADPH), uridine-5'-diphosglucuronic acid (UDPGA), and 3'-Phosphoadenosine-5'-phosphosulfate (PAPS). D-saccharic acid 1,4-lactone (SL) was purchased from Sigma. Buffer and working solutions were made as described in appendix and , and were placed on ice prior to the experiment.

3.4.2 *In vitro* assay

The *In vitro* enzyme assay, simulating the liver metabolism, was incubated to a total volume of 100 μl, and distributed as shown following.

The standard assay The substrates was mixed according to table 3.1 and 3.2. 10 μl steroid working solution (substrate) was added into separate eppendorf tubes. Blank samples were supplemented with 10 μl DMSO (solvent in the substrate working solutions). 50 μl incubation solution was added to each sample (60 or 70 μl for samples with only one, or without enzymes). 10 μl of solely microsomes, or 10 μl of both enzymatic preparations (HLM and S9) were added to the samples according to tables 3.1 and 3.2, and mixed carefully.

Incubation procedure Initiation of the reactions was done by adding 10 μl of both NADPH and SL to all samples. The solutions were placed in a Thermomixer Comfort (Eppendorf) at 37 °C, mixing (1000 rpm) for 4 hours in the standard assay, and as given in table 3.3 and 3.4 for the time and enzyme dependent assay. The cofactor solutions for the S9 incubated samples were prepared as described in appendix , 15 minutes prior to phase II initiation. Samples with microsomes or without enzymes were terminated after phase I reactions. Termination was done

TABLE 3.1: The sample setup of the MD metabolism study. Components mixed in the standard assay for generation of MD metabolites with HLM and S9. Each substrate was incubated in three ways: with no enzymes, with microsomes (HLM), or with both microsomes and S9 fraction from human liver.

Substrate	Enzyme(s)
Blank	HLM and S9
Metandienon	No
	HLM
	HLM and S9
Epimetandienon	No
	HLM
	HLM and S9
17,17-dimethyl metabolite	No
	HLM
	HLM and S9
Substrate mix	No

TABLE 3.2: The sample setup of the methylnortestosterone (MeNT) and madol incubations. The components were mixed in the standard assay for generation of their metabolites with HLM and S9. Each substrate was incubated in three ways, as for MD.

Substrate	Enzyme(s)
Blank	HLM and S9
Methylnortestosterone	No
	HLM
	HLM and S9
Madol	No
	HLM
	HLM and S9
Substrate mix	No

by adding 100 μl ice cold acetonitrile (ACN), vortexing, and placing on ice for 10 minutes.

Initiation of phase II reactions was achieved in the remaining samples by adding 10 μl of both UDPGA and PAPS. The samples were carefully vortexed, and placed in the thermo mixer at 37 °C for 18 hours in the standard assay, and as given in

the tables for the time and enzyme dependent assay. Termination was done with ACN as described above.

The time and enzyme dependent assay Time of incubation in the two phases was altered according to table 3.3 and 3.4.

TABLE 3.3: Time of incubation with microsomes or S9 fraction as the source of enzymes in the *in vitro* synthesis of MD metabolites. The substrates were incubated with either of the two enzyme sources (HLM or S9), and for four different points of time. A blank sample was incubated with both enzymes, but without a substrate.

Substrate	Time of incubation with HLM	Time of incubation with S9
Blank	24	19
Substrate mix	24	19
MD	2	4
	4	15
	6	17
	24	19
EpiMD	2	4
	4	15
	6	17
	24	19
Epimetendiol	2	4
	4	15
	6	17
	24	19
17,17-dimethyl metabolite	2	4
	4	15
	6	17
	24	19

TABLE 3.4: Samples with varying time of incubation with HLM and S9, in the *in vitro* synthesis of MeNT metabolites.

Substrate	Time of incubation with HLM	Time of incubation with S9
Blank		20
MeNT	1	4
	2	16
	4	18
	18	20

Isolation After termination, the sample solutions were centrifuged (Heraeus Pico 17, Thermo) with 10000 rpm for 10 minutes. Samples supernatant were transferred to clean glass test tubes, and the solvent was evaporated at 45-55 °C under a stream of N_2. 10 μl ISTD and 200 μl phosphate buffer were added to each tube.

Enzymatic hydrolysis 100 μl β-glucuronidase was added to samples transformed by phase II reactions. The samples were set at 50-55 °C for one hour for enzymatic cleavage of bonds to glucuronic acid. 0,2 ml 3M Tris-buffer was added to each sample after cooling down.

Extraction and derivatization All samples were extracted with 2 ml TBME. They were vortexed for 3 minutes each, followed by centrifugation (Hettic Rotixa 50 RS) in 10 minutes at 1500 rpm. Sample separation was performed with a freeze-bath of ethanol and dry-ice. The organic layer was transferred to clean 10 ml test tubes.

The solvent was evaporated on heat block (45-55 °C) under a stream of N_2. The inside of the tubes were poured with 0.2 ml ethanol. The ethanol was then evaporated to complete dryness of the sample, on heat block under a stream of N_2. All samples were derivatized with 40 μl MSTFA mix, and left on heat block (65-75 °C) for approximately one hour. The solutions were thereafter transferred to autosampler vials with inserts, and applied to GC-MS/MS for analysis.

3.4.3   GC-MS(/MS) analysis of metabolites

The instrument and instrument conditions were identical as when establishing the detection-method. The optimized detection-method with MRM transitions described in section 3.1 was used to detect metabolites. Data processing from the results of the synthesized metabolites, was carried out with "Agilent Masshunter Data Analysis and Reporting".

3.5   Urine sample analysis

One sample that during recent doping control tested positive for MD was screened by the method developed in section 3.1. The sample was already processed (hydrolyzed and derivatized), therefore no further processing was required. Vials with the samples were applied to the GC-MS/MS, and run with MD detection-method.

Urine samples from a MeNT excretion study performed in Kreischa, Germany, by Chundela and Große[7], had been given to the Norwegian Laboratory of Doping Analysis as a gift. The dispatch contained three urine samples collected prior, and after a short and long period of time after MeNT administration to a male volunteer. Processing of these urine samples followed the same procedure as for the incubated samples, with a few exceptions. A double set of samples was carried out, with one set of three samples going through the enzymatic hydrolysis. A second set remained in free fraction. Extraction was performed with 5 ml of TMBE. The rest of the preparation was in accordance with that described for the incubated samples. Vials with samples were applied to the GC-MS/MS with MeNT detection-method.

Chapter 4

Results and discussion

In this chapter, the results from this thesis, although closely related, are put in three sections according to their origin. Firstly the results used in establishing the detection-method, are briefly described. Then the results from the *in vitro* synthesis of metabolites is presented. Last, results of further method- and incubation-optimization are presented and discussed.

Metabolites produced in the *in vitro* synthesis of all three steroids, were recognized with the developed detection-method. The time of incubation was adjusted, and specific cofactors (NADPH, PAPS, UDPGA) were selected. This made it possible to differ, and briefly examine, the enzymatic reactions.

4.1   Establishing the detection-method

An optimized instrumental method is necessary for detection of metabolites. The procedure for establishing such a method, was described in section 2.7. Results from establishing the detection-method for one metabolite per steroid are presented next. The approach of detecting metabolites through the distinct retention time (RT) and by multiple reaction monitoring (MRM), applied to all metabolites originating from every parent compound. A flow chart describing the route of which the results were utilized in establishing the detection-method, is shown in figure 4.1.
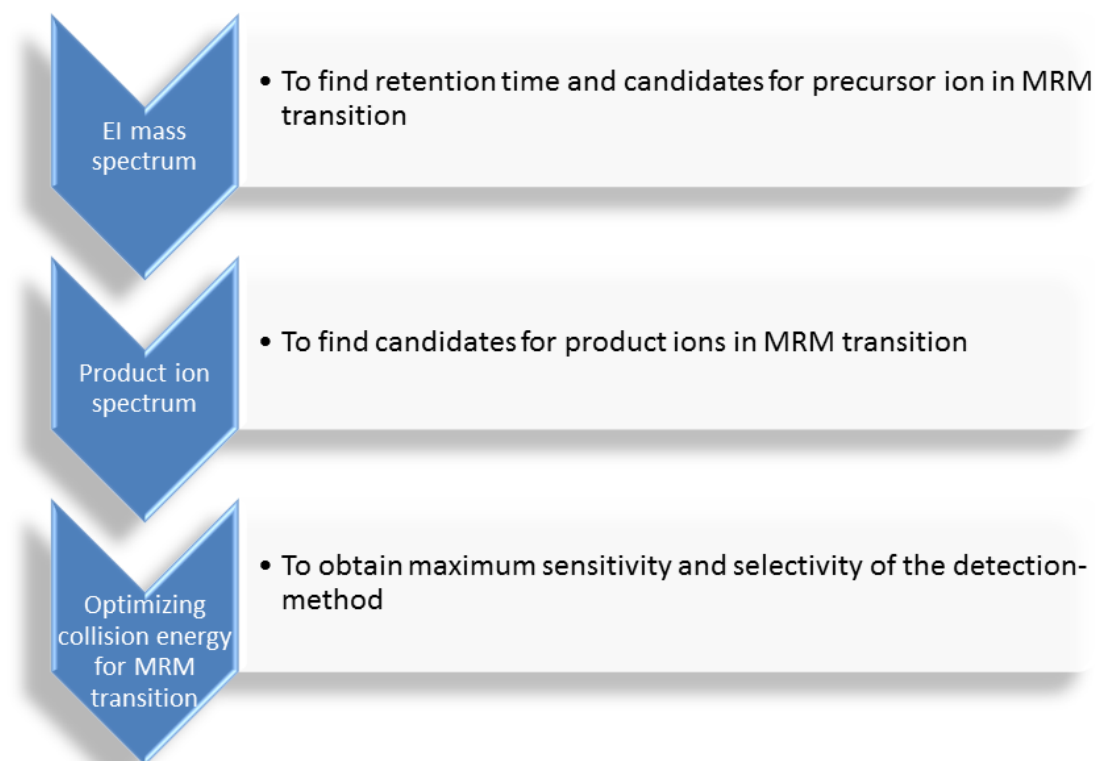
FIGURE 4.1: Flow chart describing how results of the *in vitro* synthesis, or accessible reference compounds were utilized in establishing the detection-method. The retention time and MRM transitions were combined into a selective and sensitive detection-method.

4.1.1 Metandienon

All the MD metabolites in this study are commercially available. This makes establishing a detection-method easily feasible. Modification of the existing method (from the pre-project), made detecting of the 17,17-dimethyl metabolite possible.

Retention time Isolation of the 17,17-dimethyl metabolite by gas chromatographic separation, resulted in a peak at a distinct retention time (RT). RT of the metabolite was 8.1 minutes (figure 4.2). RT is an instrument specific response, and vary with column and chromatographic conditions (pressure, temperature etc.).

The derivatized 17,17-dimethyl metabolite, being a mono-TMS, had the lowest mass among the examined MD metabolites. An increase in mass, will often give an increase in boiling point. A reduced boiling point favors faster passage in the type of column used in this study. On the basis of this, the 17,17-dimethyl
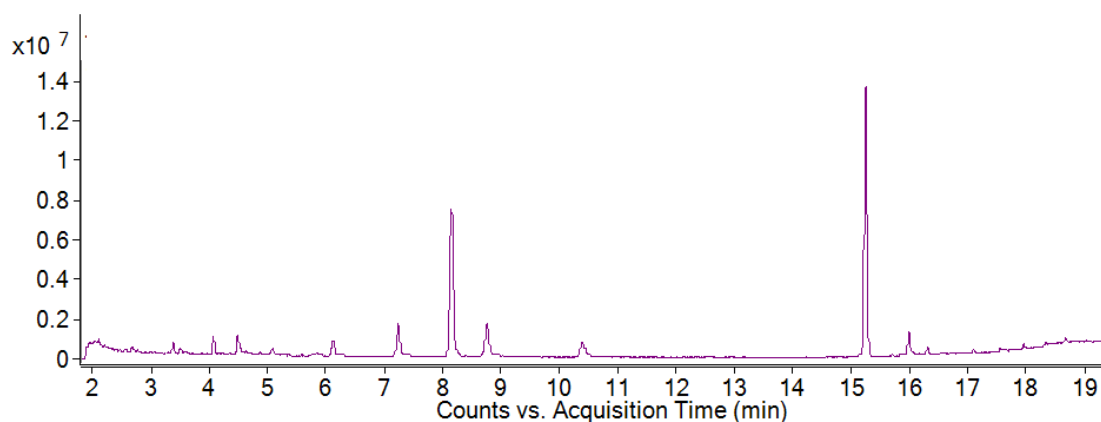
FIGURE 4.2: Chromatogram of metandienon 17,17-dimethyl metabolite. 8.1 minutes is observed as the retention time for this metabolite from the reference sample. A major peak is also seen at 15,2 minutes, which corresponds to the internal standard (ISTD), methyltestosterone.

metabolite was expected to have an earlier RT than the other MD metabolites. The RT of 8.1 minutes was implemented into the existing detection-method.

The mass spectrum An EI mass spectrum of a compound can be found from a full-scan (m/z 50-550) of the entire sample at a distinct RT. The mass spectrum of the 17,17-dimethyl metabolite (mono-TMS) at 8.1 minutes, is depicted in figure 4.3. A fragment of m/z 354 is consistent with the molecular weight (mono-TMS) of the 17,17-dimethyl metabolite, and are abundant in the spectrum.
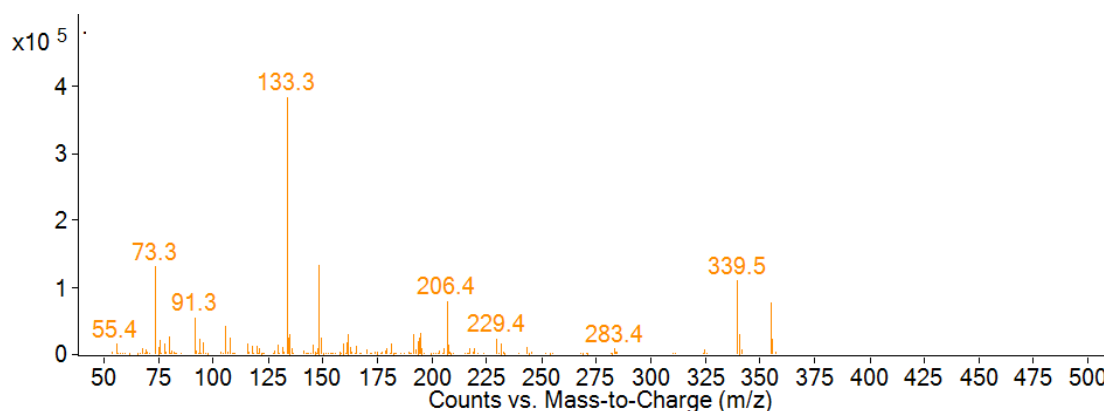
FIGURE 4.3: EI mass spectrum at 8.1 minutes, of the 17,17-dimethyl metabolite (mono-TMS). The ions m/z 339 and 354 were selected as candidates for precursor ions.

In selecting precursor ions from the spectrum, emphasis was aimed at fragments of considerable signal intensities. This usually increases the specificity. Based on the mass spectrum of 17,17-dimethyl metabolite, m/z 339 and 354 were selected

as candidates for precursor ions. Mass spectra of the other MD metabolites are not presented in this study, as they were presented in the pre-project.

Product ion mass spectra Upon the second fragmentation, the product ion mass spectra were generated. The trimethylsilylated 17,17-dimethyl metabolite had rich EI product ion mass spectra (figure 4.4).
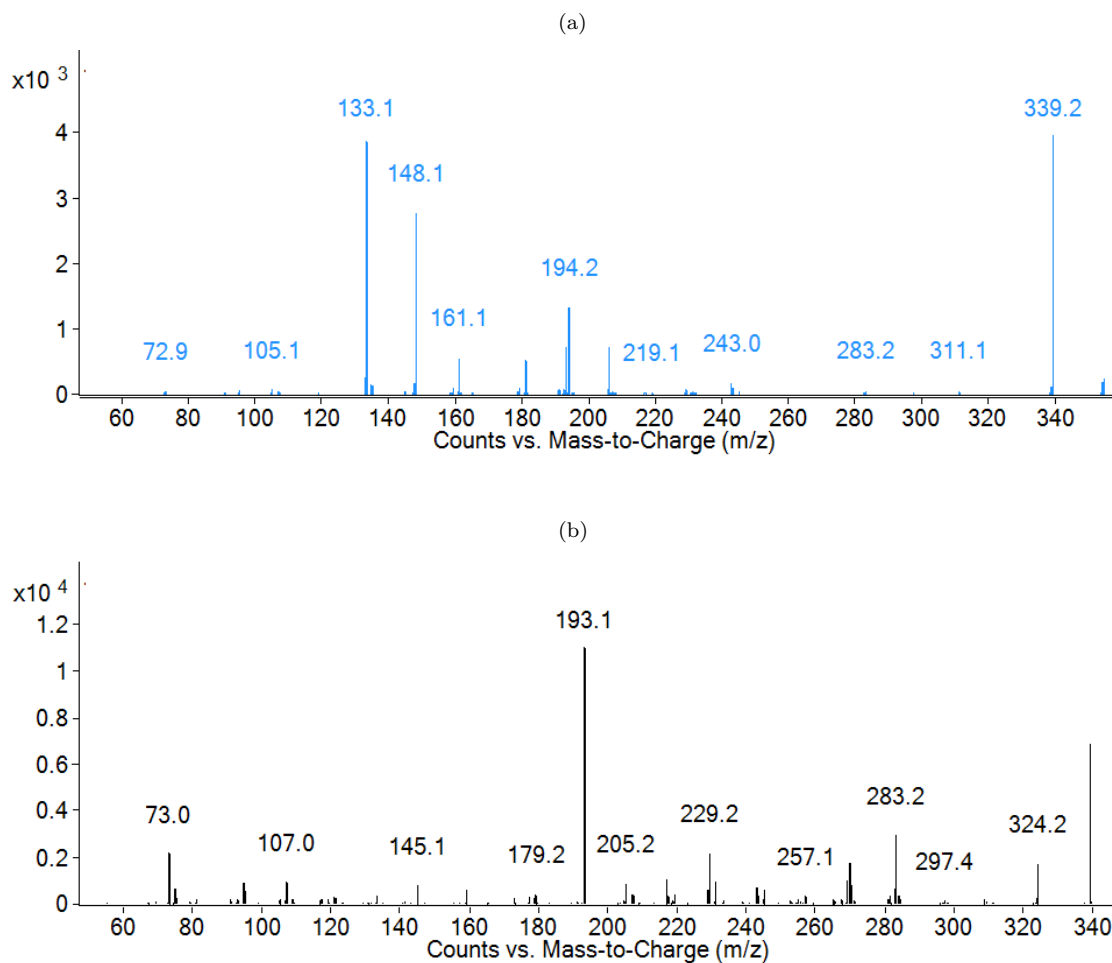
FIGURE 4.4: Product ion mass spectra at 8.1 minutes of the 17,17-dimethyl metabolite: a) precursor ion m/z 354, b) precursor ion m/z 339. Ions m/z 339, 148, 133 in a), and m/z 193 in b), were selected as potential product ions for MRM transitions.

Lower masses precursor ions (m/z<100) does often result in less specific fragments during product ion formation. The steroid nuclei resemblance among AAS gives reason to believe that several lower masses precursor/product ions are mutual for many AASs. Unique ions from the spectra, provides a strong detection-method.

According to this, product ions were selected: m/z 193 from precursor ion m/z 339, and m/z 339, 148 and 133 from precursor ion m/z 354.

A similar approach was applied to the other MD metabolites during the pre-project, thus will not be presented here.

Multiple reaction monitoring A precursor-product ion may function as a characteristic transition (MRM), as explained during theory chapter. Based on the mass spectra, four MRM transitions were suggested for confirmation of the 17,17-dimethyl metabolite. Abundance ratios of the MRMs were part of the identification criteria together with RT.

A positive result (detection) of the 17,17-dimethyl metabolite reference standard was observed in the MRM chromatogram (figure 4.5). The MRM chromatogram pictures the chromatographic and mass spectrometric data in a combined peak.

The detection-method turned out to be specific and sensitive. Other ion-pairs of the 17,17-dimethyl metabolite may also function as MRM transitions, but this was not further investigated here.
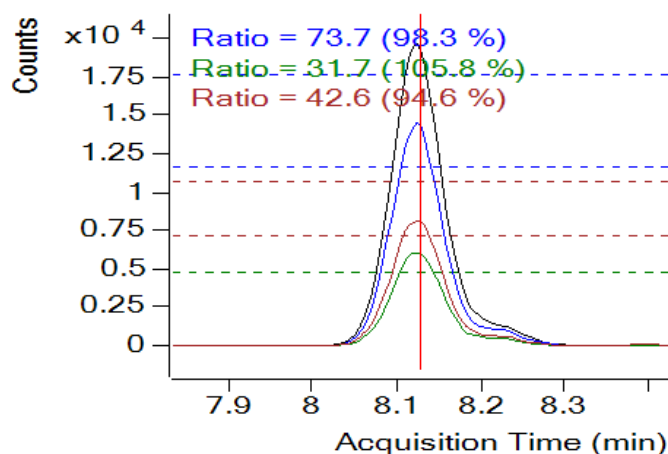
FIGURE 4.5: The MRM chromatogram of the 17,17-dimethyl metabolite. The peaks indicate the abundance of each transition at that RT. The color of the curve corresponds to a transition. Black = 339 → 193, blue = 354 → 133, green = 354 → 148, and red = 354 → 339. The ratio percentage score above the chromatogram indicate the match relative to the ratio observed in a reference.

The collision energy (CE) was ramped from 5 to 25 eV to obtain maximum sensitivity and selectivity of the MRM transitions. Results of the ramped CE on abundance of the MRM transitions of the 17,17-dimethyl metabolite can be found in appendix , but the optimal values are presented next.

The chromatographic and mass spectrometric results from establishing the detection-method for MD and its metabolites, are reported in table 4.1.

TABLE 4.1: Chromatographic and mass spectrometric data for TMS-derivatives of MD and its metabolites.

Component	Retention time (min)	Precursor ion (MW)	Product ion (MW)	CE (eV)	Ratio
Metandienon (MD)	15.1	444	206	15	
			339	15	30.2
		339	283	10	20.0
			229	15	9.7
EpiMD	13.4	444	206	15	
			339	15	42.4
		339	283	15	50.0
			229	15	22,2
6β-hydroxyMD	16.2	517	229	25	
			337	15	9.0
			429	20	11.0
Epimetendiol	9.6	358	301	15	
			343	15	50.0
		448	358	5	5.5
			194	10	2.5
17β-hydroxymethyl metabolite	13.4	339	193	15	
		442	339	10	26.5
			236	10	17.0
			133	25	34.0
5β-THMT	12.8	270	255	10	
			213	10	75.2
		435	345	5	100.0
			255	15	36.3
17,17-dimethyl metabolite	8.1	339	193	15	
		354	339	10	45.0
			148	10	30.0
			133	25	75.0

4.1.2 Methylnortestosterone

It is desirable to include MeNT metabolites in the screening at the laboratory to detect possible MeNT misuse. Metabolites of MeNT are not commercially available. Thus, a modified approach was utilized in establishing the detection method. Establishing the detection-method was performed based on the *in vitro* synthesized samples. One of the observed metabolites is presented here as an illustration of how these results contributed in establishing the detection-method. This applied to all the metabolites, but full results on metabolites from the *in vitro* synthesis, are given later, in section 4.2.

The mass spectrum The EI mass spectrum of the trimethylsilylated MeNT metabolite M1 is displayed in figure 4.6. Spectra of the "design steroid" metabolites were captured from a GC-MS single quadropole, as this instrument proved more sensitive than the GC-MS/MS was in full-scan mode.
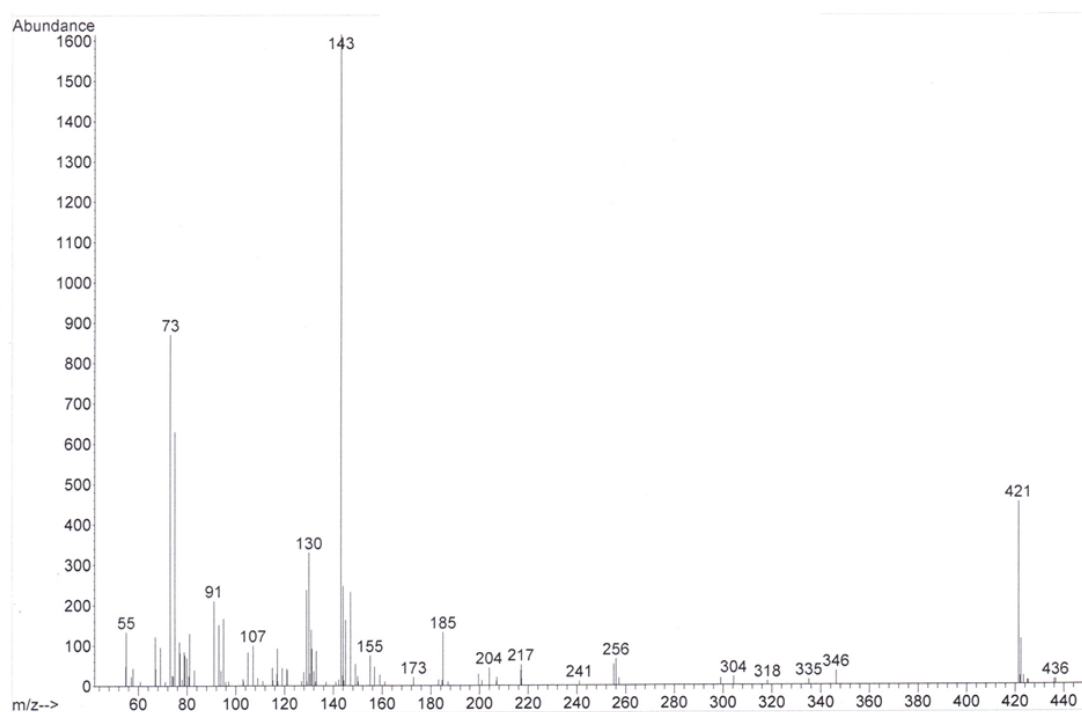
FIGURE 4.6: Mass spectrum (GC-EI-MS) of MeNT M1 at 10.5 minutes, displaying the possible molecular ion m/z 436. The ions m/z 421 and 256 were selected as candidates for precursor ions.

The initial focus when establishing the detection-method, was the search for TMS derivatives of $[MW]^+$ and $[MW-15]^+$. The mass-to-change ratio of a probable $[MW]^+$ (m/z 436) show an increase of 4 units compared to MeNT. Structural assignment of MeNT M1 was rendered from the this GC-MS scan, but is outlined later together with discussions regarding dissociation pathway and characteristic fragments (section 4.2). Based on the mass spectrum results, m/z 421 and 256 were selected as candidates for precursor ions.

Product ion mass spectra Product ion mass spectra of precursor ions m/z 421 and m/z 256, are depicted in figure 4.7. Product ions of substantial signal intensity were in accordance with those presented in an unpublished study[7].
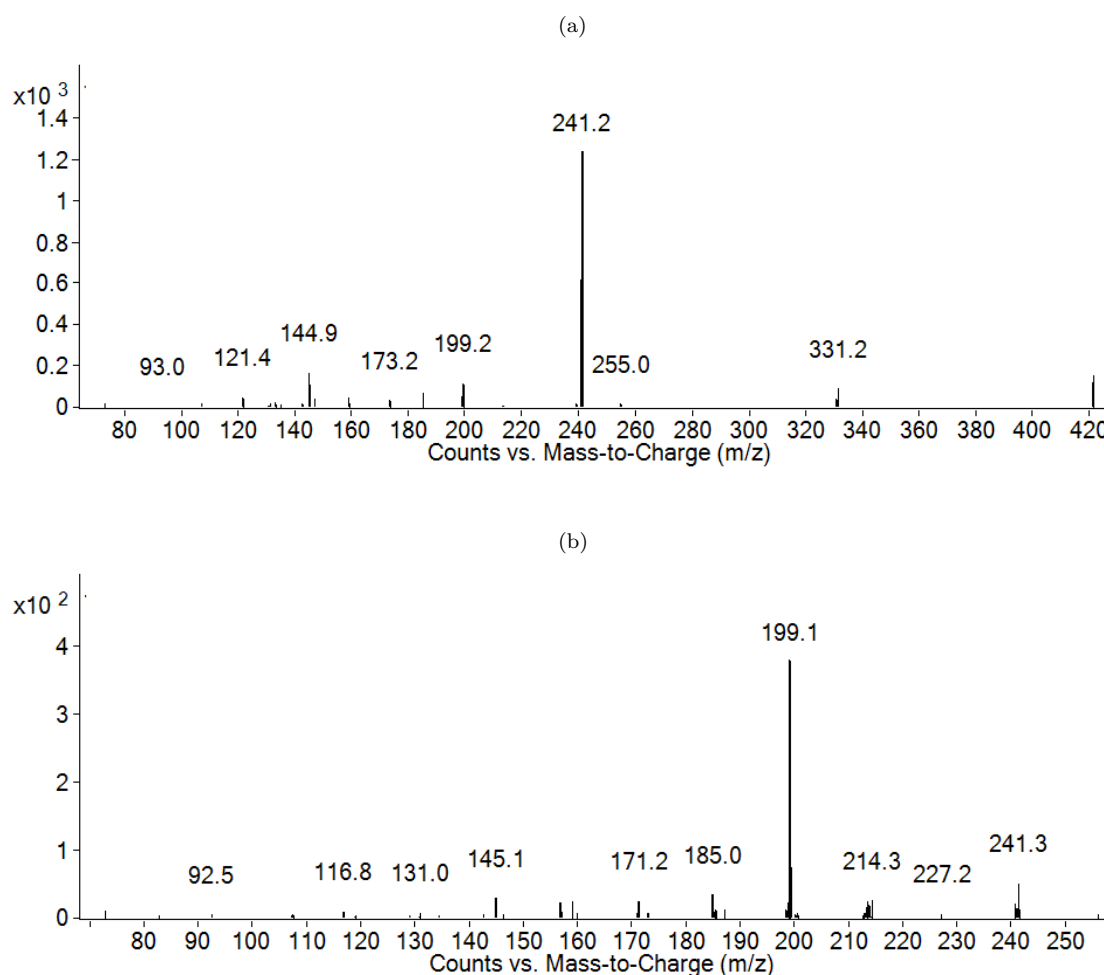
FIGURE 4.7: Product ion mass spectra of precursor ions: a) m/z 421 and b) m/z 256. The ions m/z 241 from m/z 421, and m/z 199 and 213 from m/z 256, were selected as candidates for MRM transitions.

Product ions of m/z 241 from precursor ion m/z 421, and m/z 199 and 213 from precursor ion m/z 256 were selected as candidates for MRM transitions.

Retention time Since few publications have reported about MeNT metabolites, neither published their RT, the RT was discovered by chromatographic interpretations of the MRM transitions. The MRM transitions (presented in the next paragraph) were captured from the above described mass spectra, and a poster of an unpublished excretion study on MeNT[7].
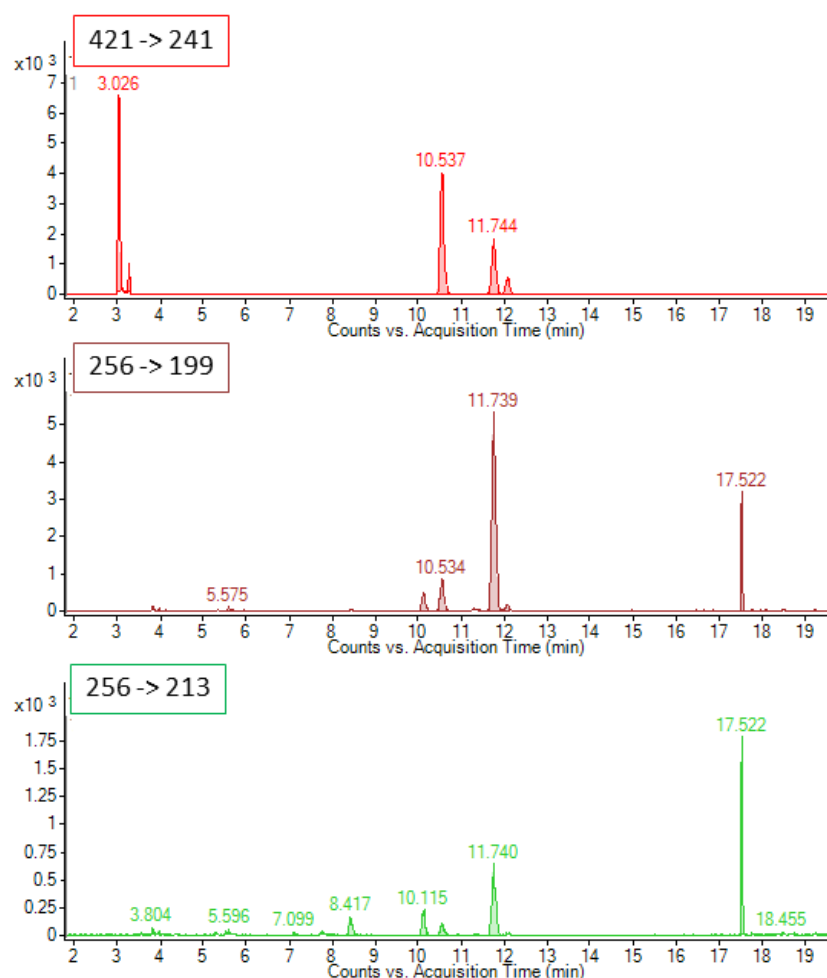
FIGURE 4.8: Chromatograms of selected MRM pairs of MeNT M1. Compared with the chromatogram of a non-incubated sample and a MeNT reference standard, RT of 10.5 and 11.7 may indicate MeNT M1. Upper panel show m/z 421 → 241, mid panel show m/z 256 → 199, and lower panel show m/z 256 → 213. All these were MRM transitions comprised for confirmation of MeNT M1 in the detection-method.

The peaks at 10.5 and 11.7 minutes in the chromatograph in figure 4.8 comprises all the MRM transitions selected for MeNT M1. This may indicate that MeNT M1 has an epimeric compound eluting shortly after. This epimer (MeNT M2) is described in section 4.2.

Multiple reaction monitoring By monitoring the response of the ion transitions, a selective method for detection of MeNT is suggested. The chromatographic and mass spectrometric results utilized in establishing the detection-method, are summarized in table 4.2.

TABLE 4.2: Chromatographic and mass spectrometric results of MeNT and its suggested metabolites.

Component	Retention time (min)	Precursor ion (MW)	Product ion (MW)	CE (eV)	Ratio
Methyl-nortestosterone	14.7	432	287	17	
			285	15	20.0
			300	15	14.0
MeNT M1	10.9	421	241	15	
		256	199	8	23.1
			213	12	3.6
MeNT M2	12.1	421	241	15	
		256	143	8	62.0
			213	12	35.7
MeNT M3	16.7	520	415	20	
			287	17	20.5
			194	30	10.2
MeNT M4	5.7	331	241	8	
			143	35	2.1
		256	143	20	1.0

4.1.3 Madol

Recent reports have described some madol metabolites[44;18]. Still, reference standards of the metabolites are not commercially available. A similar approach as

for MeNT was employed in establishing the detection-method. Only one metabolite is presented as illustration of establishing the detection-method of the madol metabolites.

The mass spectrum EI mass spectrum of the trimethylsilylated possible madol metabolite M1, is displayed in figure 4.9.
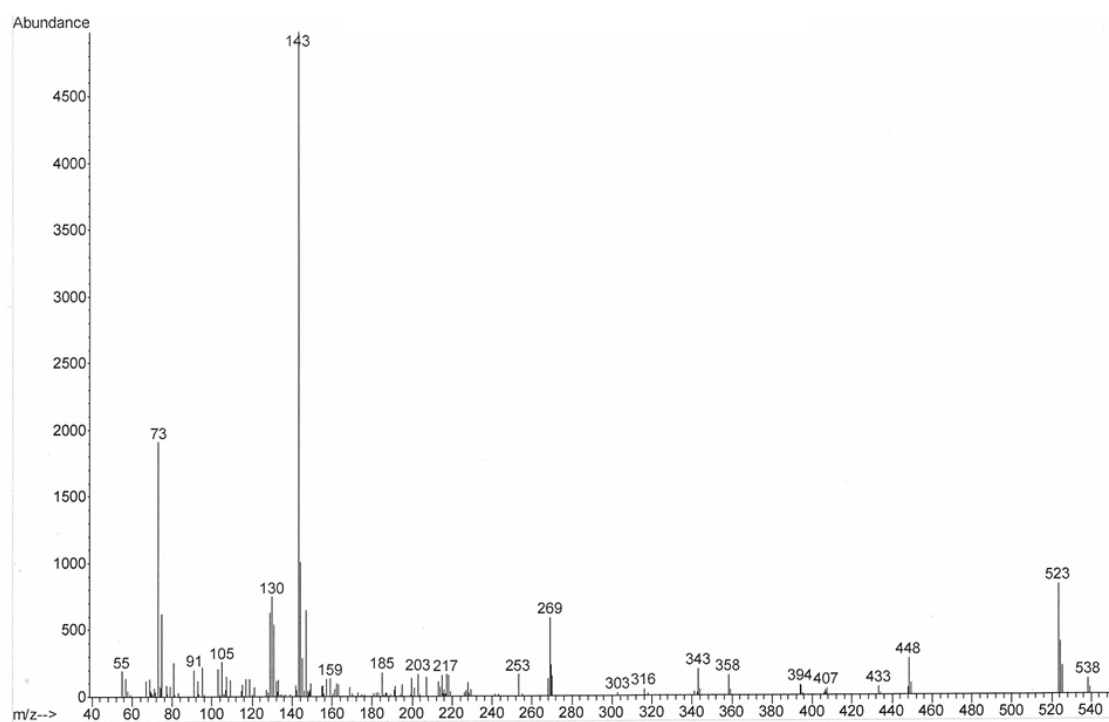
FIGURE 4.9: Mass spectrum (GC-EI-MS) at 15.0 minutes, possibly of madol M1 metabolite.

The spectrum comprises both a possible $[MW]^+$ of m/z 538, and the $[MW-15]^+$ of m/z 523 ions. This is considerably larger than madol, further described in section 4.2 Abundant signals were observed at m/z 523 and m/z 269, and selected as candidates for further fragmentation.

Product ion mass spectra Product ion mass spectra of m/z 523 and m/z 269 (figure 4.10) displayed rich spectra with intense ions at m/z 343 and 253 for precursor ion m/z 523, and m/z 213 and 159 for precursor ion m/z 269. Sobolevsky et. al.[45] used product ions m/z 343 and 253 from m/z 523, as sufficient recognition

of madol M1. The same ions were chosen from these results. By extending the method with two additional product ions, the intent was to enhance the selectivity.
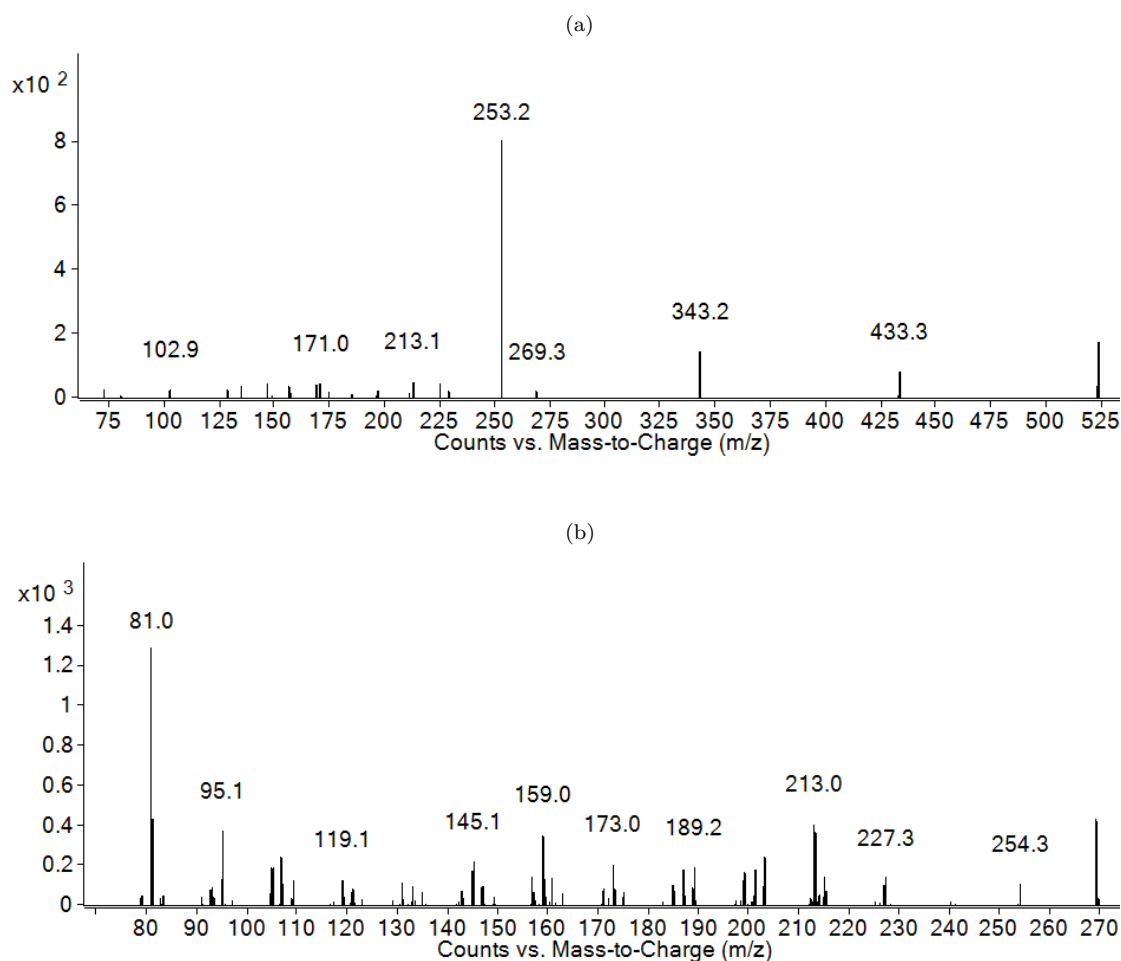
FIGURE 4.10: Product ion mass spectra of precursor ion: a) m/z 523, and b) m/z 269. Candidates for product ions for MRM transitions were m/z 253 and 343 from m/z 523, and m/z 213 and 159 from m/z 269.

Retention time   A peak at 15.0 minutes in the chromatograph in figure 4.11 comprises all the MRM transitions selected for madol M1. Searching the literature was useful during this process, since some publications had reported mass spectra of madol metabolites[38;18;45]. The MRM transitions was captured from both the spectra after the *in vitro* synthesis, and from published spectra on madol metabolites. A peak considerably smaller than at 15.0 minutes, but including all the MRM transitions is observed at 16.2 minutes. This was not further examined during this study.
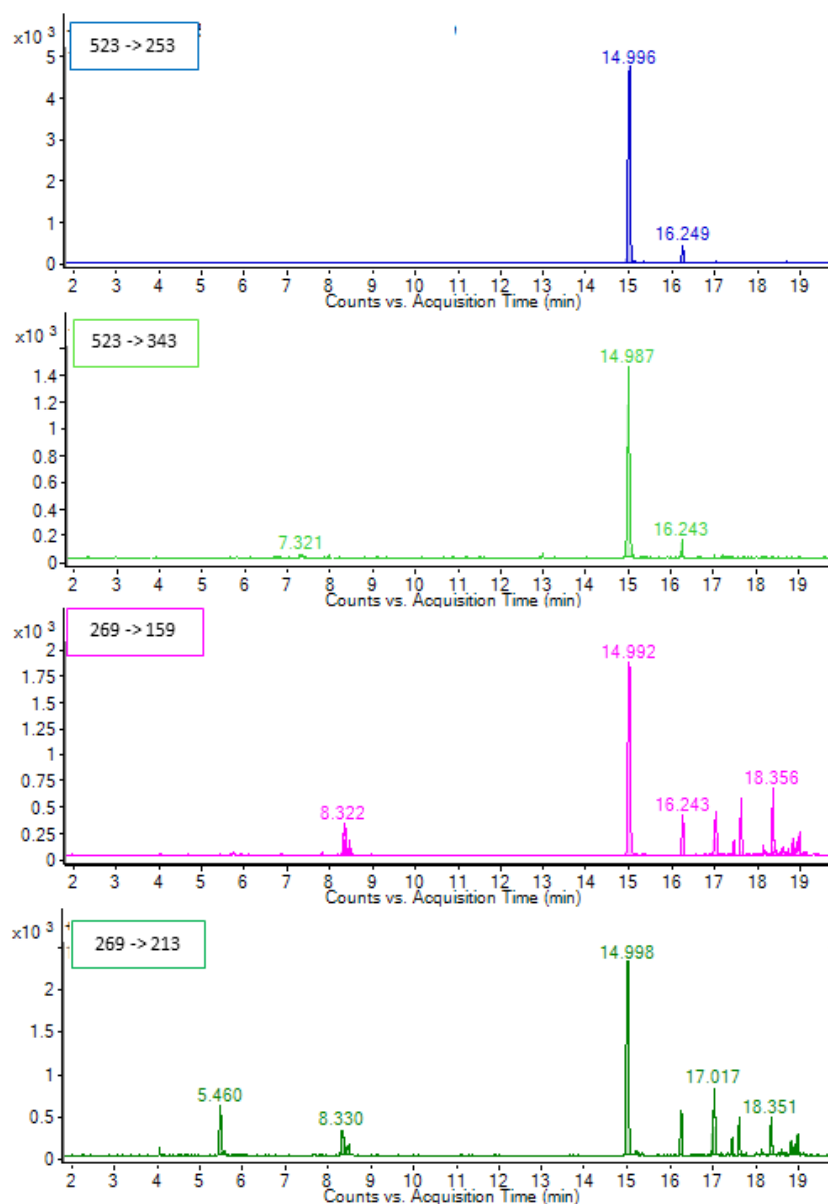
FIGURE 4.11: Chromatograms of suggested MRM pairs for the madol metabolite. RT of 15.0 minutes may indicate madol M1, as it was common for all the transitions, but nonexistent in the blank and substrate samples.

Multiple reaction monitoring The detection-method of madol M1 was established by assembling the RT, MRM, CE and MRM ratios. The chromatographic and mass spectrometric results of madol and its suggested metabolites are summarized in table 4.3. Most MD and MeNT metabolites function well with a CE of 15 eV, as seen in their detection-method. CE of 15 eV was therefore used in the madol detection-method.

TABLE 4.3: Chromatographic and mass spectrometric results of madol and suggested metabolites.

Component	Retention time (min)	Precursor ion (MW)	Product ion (MW)	CE (eV)	Ratio
Madol	8.4	345	201	15	
			147	15	34.0
			131	15	6.8
Madol M1	15.0	523	253	15	
			343	15	30.0
		269	213	15	51.8
			159	15	39.5
Madol M2	14.1	446	431	15	
		431	193	15	50.0
			179	15	50.0

4.2 *In vitro* synthesized metabolites

The *in vitro* incubation successfully produced metabolites of both a familiar anabolic steroid and the "designer steroids". The AAS screening at the Norwegian Doping Control Laboratory may incorporate MeNT and madol metabolites after this thesis submission.

4.2.1 Metandienon

In vitro incubations of either MD, epiMD, or the 17,17-dimethyl metabolite generated four metabolites: 6β-hydroxy metandienon, epiMD, 17,17-dimethyl metabolite, and the 17β-hydroxymethyl metabolite. Generation of epimetendiol or 5β-THMT were nonexistent from these experiments, either did incubation with epimetendiol result in any metabolites.

6β-hydroxy metandienon

In vitro incubation of MD yielded 6β-hydroxy MD (figure 4.12), both in the pre-project, and during this thesis. It stands to reason, since 6β-hydroxylation is considered the main metabolic route in MD metabolism[12]. As 6β-hydroxy MD was

generated in the pre-project, and has been reported in several excretion studies, it is reasonable to believe that such hydroxylation is an uncomplicated metabolic pathway during MD metabolism.
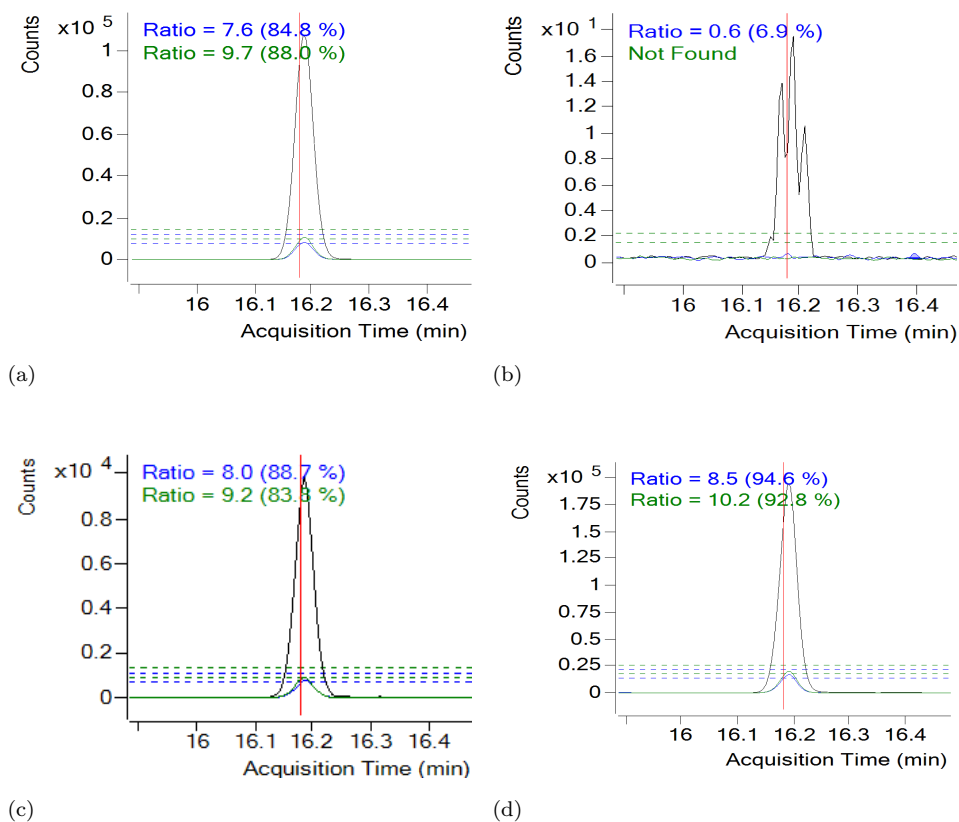
FIGURE 4.12: The MRM chromatogram verifying/refuting the existence of 6β-metandienon in: a reference standard solution a), a matrix sample (HLM, S9 and co-factors) b), the *in vitro* incubated sample c), urine that tested positive for metandienon usage by regular doping screening d).

Epimetandienon

In contrast to the pre-project, where no epimerization was seen, epiMD was synthesized *in vitro* from MD (figure 4.13) in this thesis. An effect presumably caused by an increased availability of SULT[43]. This is reasonable as the concentration of PAPS was increased (compared to the pre-project) in the incubation assay. A hypothesis supported by earlier studies using microsomes. For instance Kuuranne *et al.*[29], whom stated that a low PAPS concentration probably was a limiting factor for the yield of their metabolites.
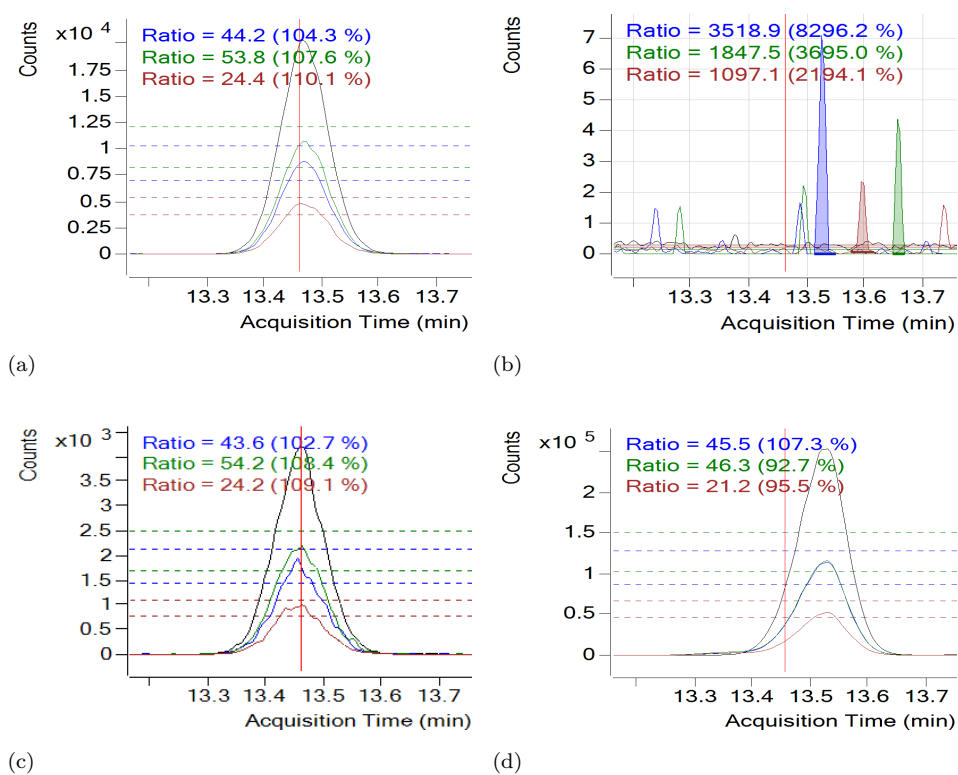
FIGURE 4.13: The MRM chromatogram verifying/refuting the existence of epimetandienon in: a reference solution a), a matrix sample (HLM, S9 and co-factors) b), the *in vitro* incubated sample c), urine that tested positive for metandienon use in regular doping screening d).

17,17-dimethyl metabolite

In vitro generation of the 17,17-dimethyl metabolite from MD has been argued as demanding in preceding reports[58]. Here, the 17,17-dimethyl metabolite was synthesized *in vitro*, from either MD, or epiMD (figure 4.14). This implies that epiMD is a precursor in the generation of the 17,17-dimethyl metabolite. Thus, microsomes with additives (co-factors) seem to comprise enzymes and factors necessary for this conversion.
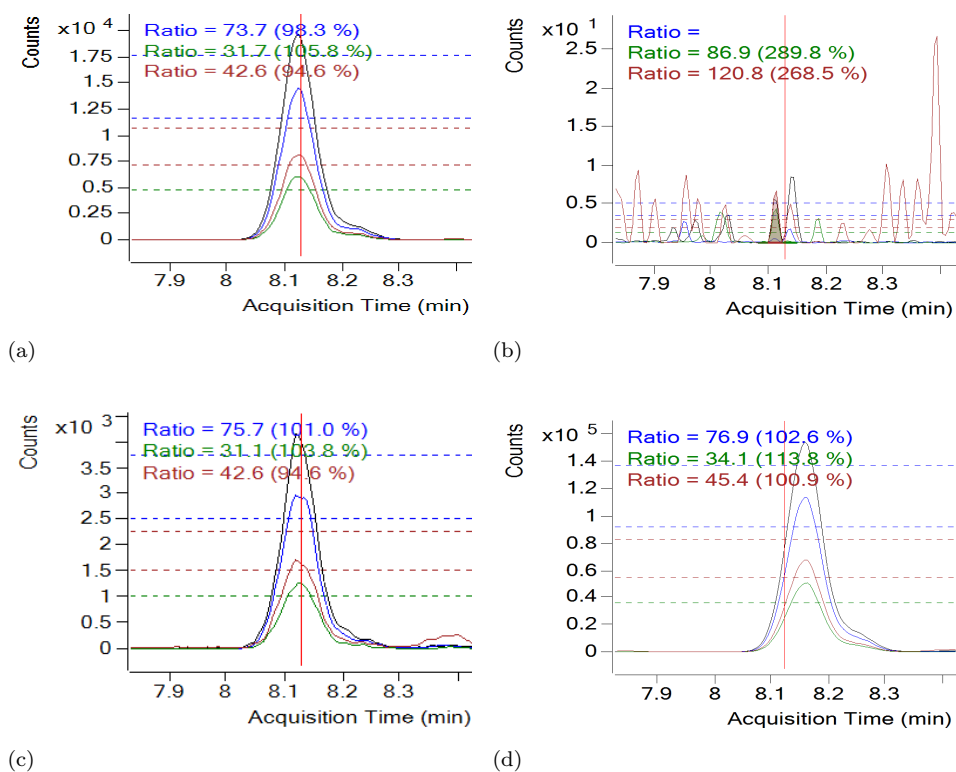
FIGURE 4.14: The MRM chromatogram verifying/refuting the existence of the 17,17-dimethyl metabolite in: a reference solution a), a matrix sample (HLM, S9 and co-factors) b), the *in vitro* incubated sample c), urine that tested positive for metandienon use in regular doping screening d).

17β-hydroxymethyl metabolite

Zöllner *et al.*[58], used the 17,17-dimethyl metabolite to produce the 17β-hydroxymethyl metabolite. By doing so, they avoided the demanding conversion of MD to the 17,17-dimethyl metabolite, and were able to examine the enzymatic reactions behind. In this study, 17β-hydroxymethyl metabolite was also synthesized *in vitro* from the 17,17-dimethyl metabolite (figure 4.15), but not from MD. Referring to the above paragraph (17,17-dimethyl metabolite), generation of 17β-hydroxymethyl metabolite should be feasible from MD at optimal incubation conditions. It is of interest to be capable of synthesizing this metabolite. Reference material of this metabolite is not commercially available, but if embedded in the screening, the time window of detection of MD is increased[41].
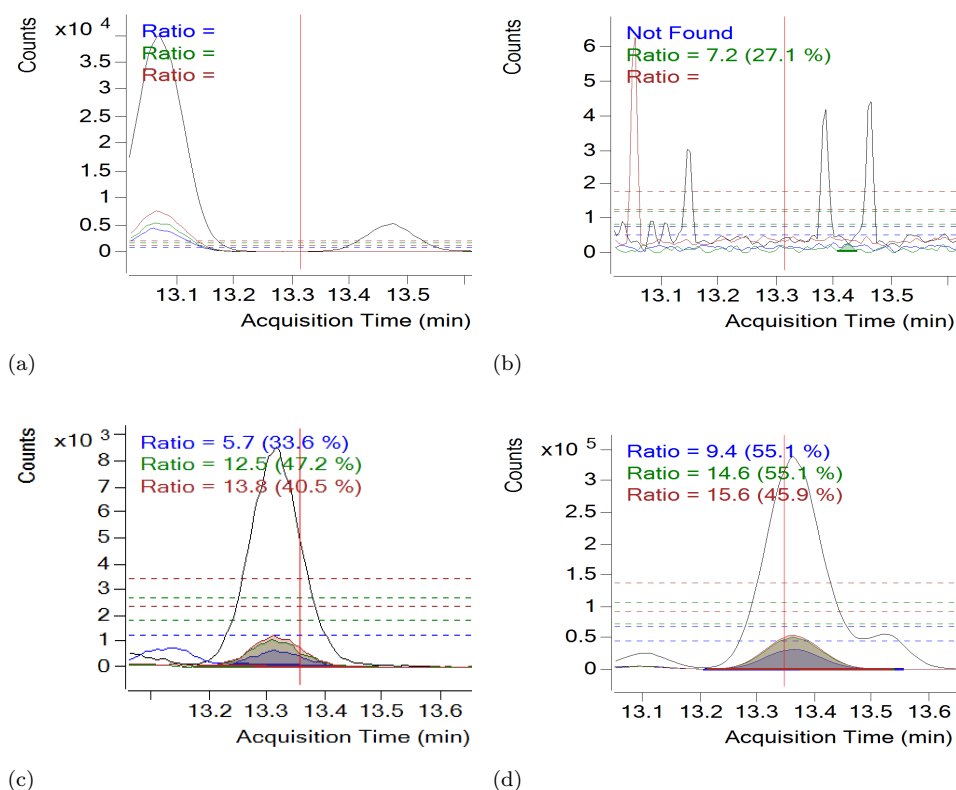
FIGURE 4.15: The MRM chromatogram verifying/refuting the existence of the 17β-hydroxymethyl metabolite in: a sample of the 17α-hydroxymethyl metabolite reference (used to establish the MRMs, with only a deviating RT) a), a matrix sample (HLM, S9 and co-factors) b), the *in vitro* incubated sample c), urine that tested positive for metandienon usage in regular doping screening d). In spite of irregularities of the ratio, are both sample c) and d) considered as positive results

4.2.2 Methylnortestosterone

Three metabolites of methylnortestosterone (MeNT) were observed after the *in vitro* incubations. Detection and structural assignment of a metabolite is demanding. The *in vitro* synthesized metabolites should share a similar core-structure as the parent compound and other steroids, and thus hold EI fragmentation similarities with previously characterized steroid and steroid metabolites. Effort during this study, was therefore invested in searching through mass spectra of the incubated samples, looking for characteristic ions for establishing a method, alongside detecting likely metabolites and assigning their structure.

The epimeric metabolites

The first two observed metabolites, MeNT M1 and M2, had almost similar mass spectra and MRM transitions, but differed in their RT. This is typical for epimeric compounds[43], as their conformation will retain them different through the column of the gas chromatographic separation.

Molecular structure of MeNT M1 and M2 A part of the molecular structure (A- and B-ring) of MeNT is identical to among others: nandrolone, norethandrolone, norbolethone and mibolerone. Former studies on their metabolism have revealed typical metabolic patterns. Schänzer[39] reviewed those studies in 1996, and based on this information, a 5-α/β-reduction, followed by reduction of the 3-keto group, seems likely for MeNT. It is emphasized that variations in the D-ring structure can influence the reductase activity. Regardless of this, A-ring reduced metabolites would be expected for MeNT.

A structural assignment can be facilitated based on spectral interpretations[20;16;21]. The mass spectrum of MeNT M1 was presented when establishing the detection-method, figure 4.6. The mass spectrum of MeNT M2 at 12.1 minutes is quite similar, and depicted in figure 4.16. In both scans, several mutual characteristics are prominent. The EI fragmentation of these metabolites seem to generate an ion of considerable intensity at m/z 143, and abundant fragments at m/z 436, 421, 256, and 130.

The per-TMS derivative of MeNT has a molecular weight of 432. An ion of four mass equivalents more is visible in the metabolite spectrum. The m/z 436 fragment
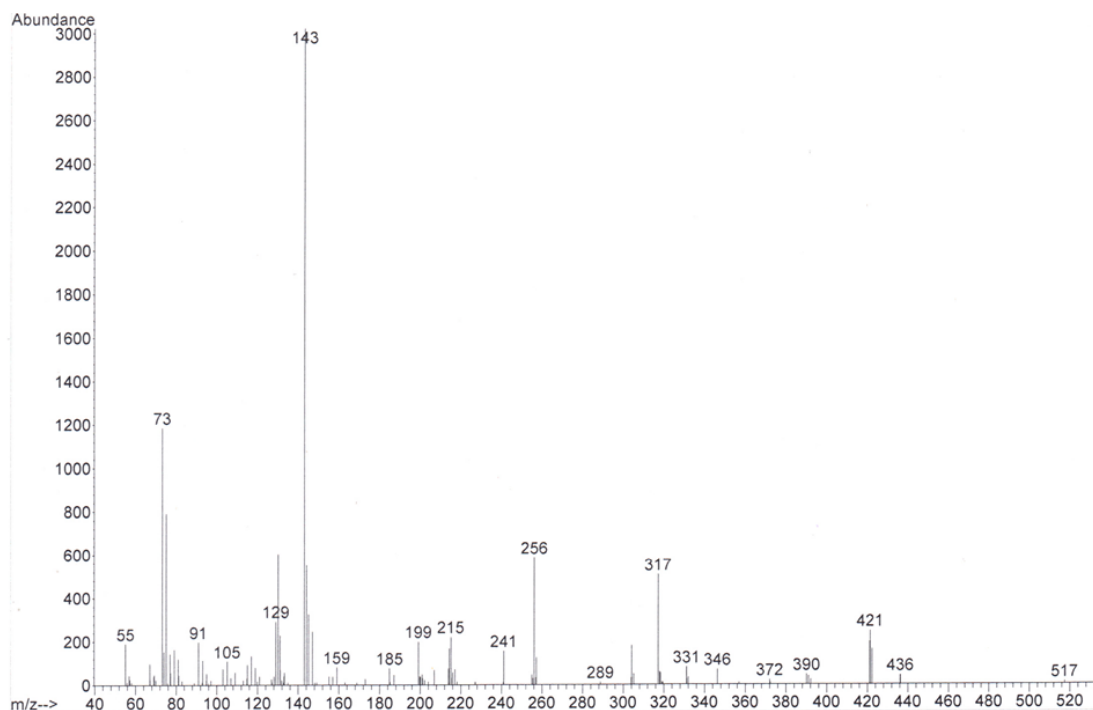
FIGURE 4.16: Mass spectrum (GC-EI-MS) at 12.1 minutes, possibly of MeNT M2.

is possibly the intact trimethylsilylated M1/M2 ion, and the low abundance is supported in an unpublished poster[7]. A gain of 4 *Da* may indicate a reduction of double bonds in the parent compound, leading to four additional H-atoms. A reduction of double bonds in MeNT, is only possible in the A- and/or B-ring. Ions at m/z 421, are probably [MW-15]$^+$, resulting from the loss of a methyl group. [MW-15]$^+$ has been used as a diagnostic characteristic[16], but does not reveal much structural information.
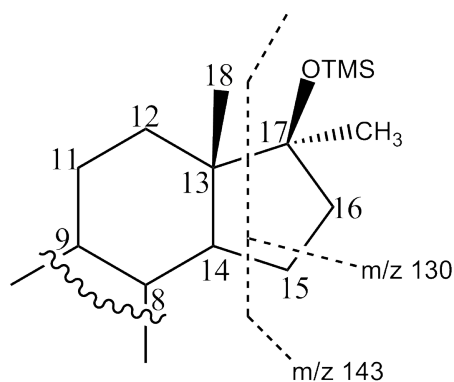
FIGURE 4.17: The molecular structure of the characteristic m/z 143 and 130 fragments, indicative of a 17α-alkylated steroid structure.

Ion at m/z 143 is due to fission of bonds in the D-ring of 17-methyl steroids (figure 4.17)[16;48], and is often abundant together with m/z 130. Their abundance in the EI mass spectra (figure 4.6 and 4.16) fortify the assumption that MeNT kept the 17α-alkylated structure intact during the metabolic reactions.

The C-19 methyl group has a remarkable effect on the fragmentation pattern[16]. Norethandrolon is a 19-nor steroid with a similar A and B ring structure as MeNT. In the mass spectra of norethandrolone 5α/β reduced metabolites are m/z 129 fragment abundant.
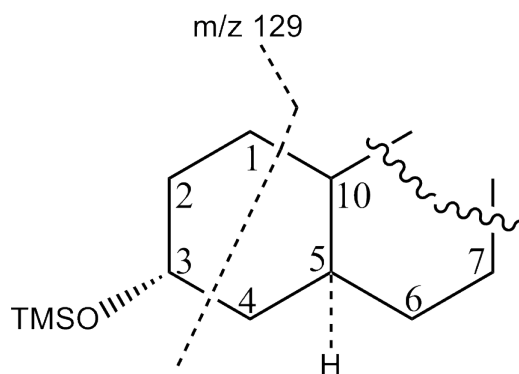
FIGURE 4.18: The molecular structure of the characteristic m/z 129 ion, proving the resemblance in the A-ring of MeNT M1/M2 and 5α/β-reduced metabolites of norethandrolone.

This ion is deemed to consist of A-ring carbons according to Fragkaki *et al.*[16]. The m/z 129 (figure 4.18) is abundant in the mass spectra of the first MeNT metabolites, although difficult to spot compared to m/z 130 and 143, providing considerable intensity. Due to all these mass spectrometric features, 17α-methyl-5α/β-estrane-3α,17β-diols (figure 4.19) are proposed as structures of the epimeric MeNT metabolites.

NMR is more reliable as a method of structure determination. Neither the referred poster, nor this study, used NMR to confirm the metabolites. This might be an idea for further studies for determining the MeNT M1/M2 structure.
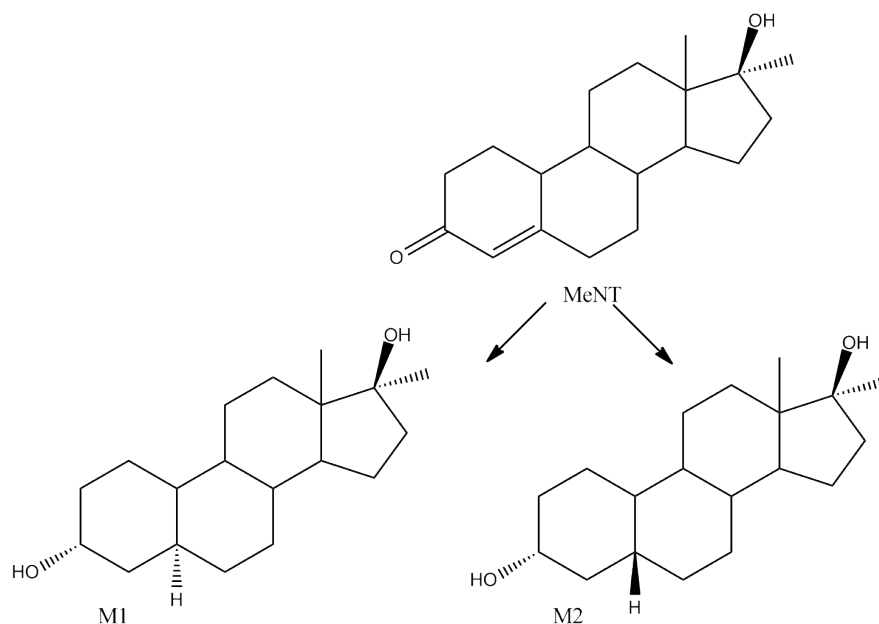
FIGURE 4.19: Suggested molecular structures of MeNT M1 and M2.

The verification of MeNT M1 and M2 Incubation of MeNT yielded 17α-methyl-$5\alpha/\beta$-estrane-$3\alpha,17\beta$-diols ($5\alpha/\beta$-estrane-diol metabolites) during this thesis. A positive result for the $5\alpha/\beta$-estrane-diols are depicted in figure 4.20 and 4.21.

In figure 4.20, the matrix sample (HLM, S9 and co-factors) responded to the MRM transitions at 10.9 minutes. The only evidence of nonexistent MeNT M1 in this sample is the deviant MRM ratio.
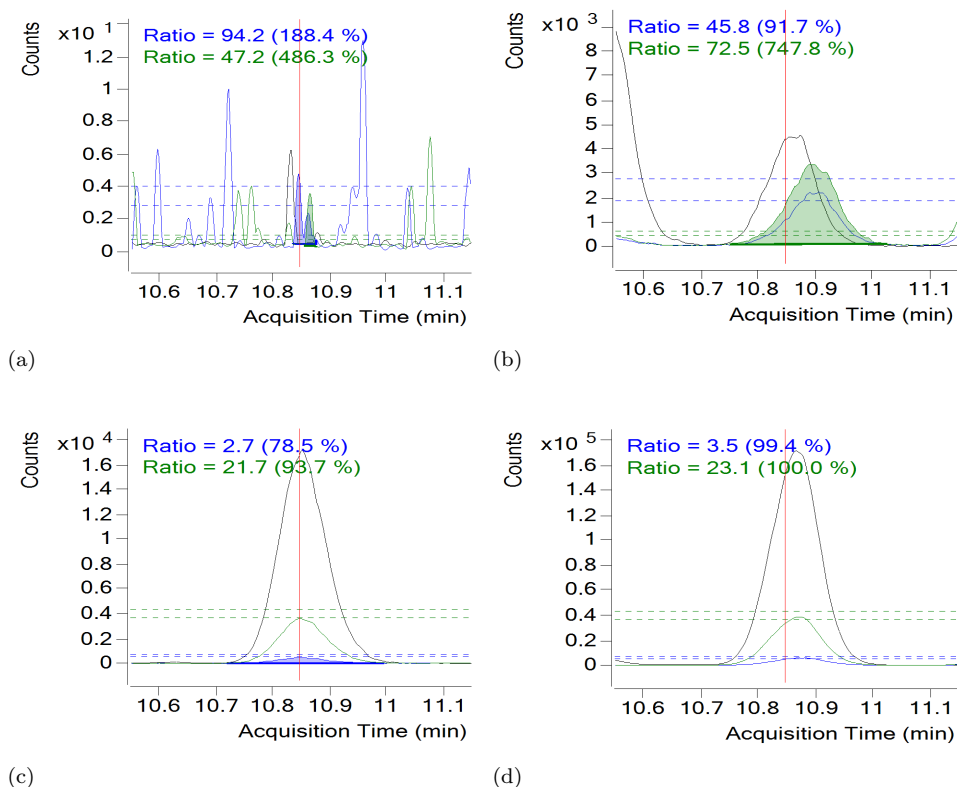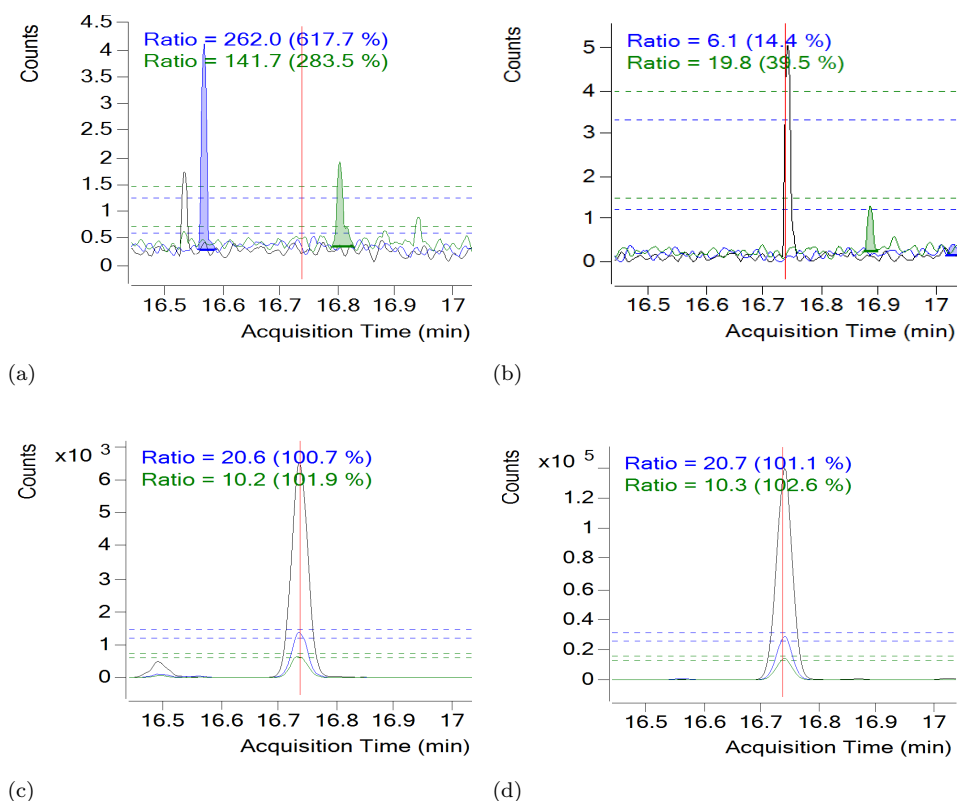
FIGURE 4.20: The MRM chromatogram verifying/refuting the existence of the 5α-estrane-diol metabolite in: a sample of nonincubated MeNT a), a matrix sample (HLM, S9 and co-factors) b), the *in vitro* incubated sample c), urine from a MeNT excretion study d). The deviant MRM ratio of b) is amplified by the colored area. This is an interference possibly caused by matrix compounds responding to the selected transitions.
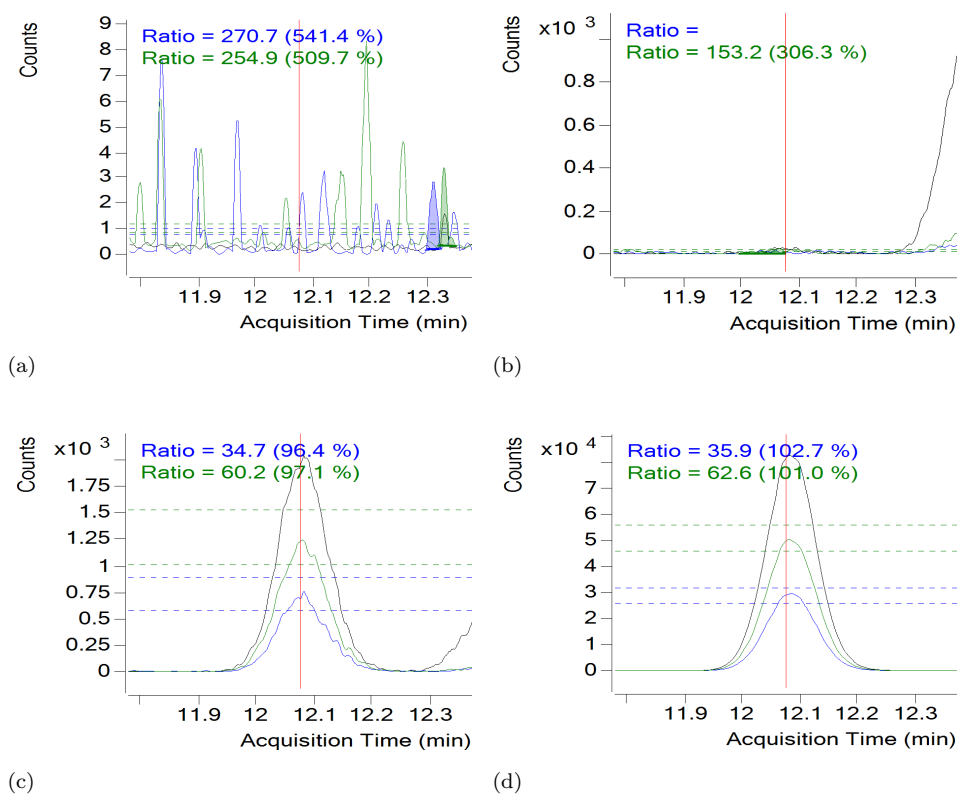
FIGURE 4.21: The MRM chromatogram verifying/refuting the existence of the 5β-estrane-diol metabolite in: a sample of nonincubated MeNT a), a matrix sample (HLM, S9 and co-factors) b), the *in vitro* incubated sample c), urine from a MeNT excretion study d).

The hydroxylated metabolite MeNT M3

One hydroxylated metabolite was recognized after incubation of MeNT. The understanding for such statement is described next.

Molecular structure of MeNT M3 EI mass spectrum of a possible third MeNT metabolite (M3) is depicted in figure 4.22.
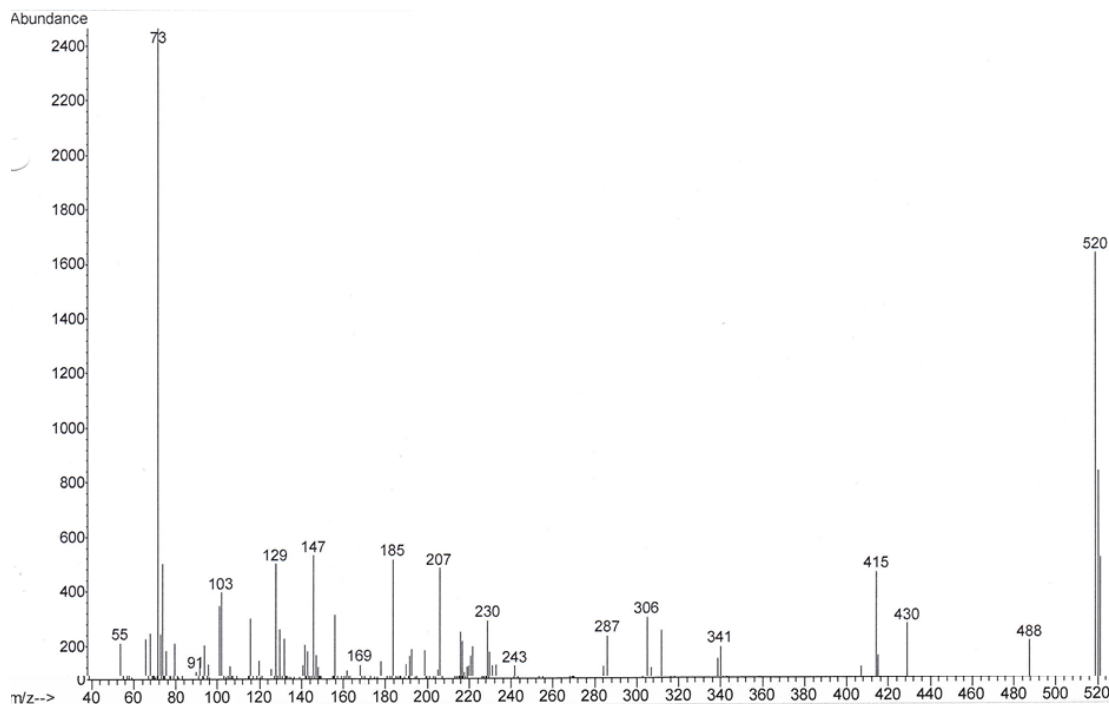
FIGURE 4.22: Mass spectrum (GC-EI-MS) at 16.7 minutes, possibly of MeNT M3.

The EI mass spectrum display molecular ions at m/z 520, 430, 415, 287 and 194. Compared to MeNT, m/z 520 is consistent with an additional TMS-group, and a loss of a proton. This might indicate hydroxylated MeNT as a metabolite. Ions m/z 287 and 194 shows structural resemblance with the mass spectrum of MeNT. The presence of m/z 194 are of structural interest. It is considered typical for the nandrolone A-ring, where it indicates a 3-keto-4-ene structure of 19-nor steroids[16;48] (figure 4.23).

The characteristic m/z 143 is not appearent in figure 4.22. An useful indication that the D-ring structure has been subject to changes during the metabolism of generating a third metabolite. Some D-ring alteration are comprehensive for the
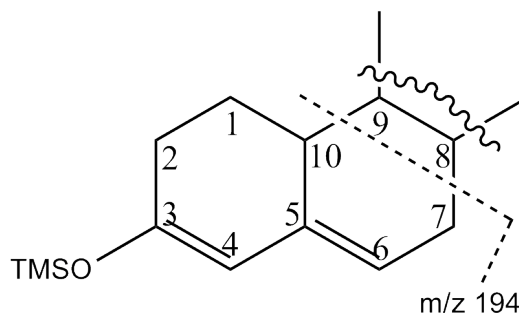
FIGURE 4.23: The molecular structure of the characteristic m/z 194 ion, illustrating the structural resemblance in the A- and B- ring of MeNT M3, with that of MeNT and nandrolone before metabolism.

metabolism of 3-keto-4-ene steroids, mainly regarding the C-16, C-17 or C-18 position[15]. 16-hydroxylation is a well-known metabolic pathway in familiar steroids[40]. Fragmentation of a 16-hydroxylatet metabolite gives rise to characteristic fragments of m/z 218 and 231 (figure 4.24).
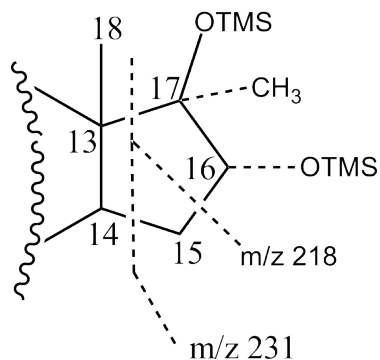
FIGURE 4.24: Characteristic D-ring dissociation pathways, yielding ions of m/z 218 and 231 upon fragmentation of trimethylsilylated 16,17-dihydroxy-17-methyl steroids.

Based on these spectral interpretations, 16z,17β-dihydroxy-17α-methylestr-4-en-3-one was put forward as the structure of MeNT M3 (figure 4.25).
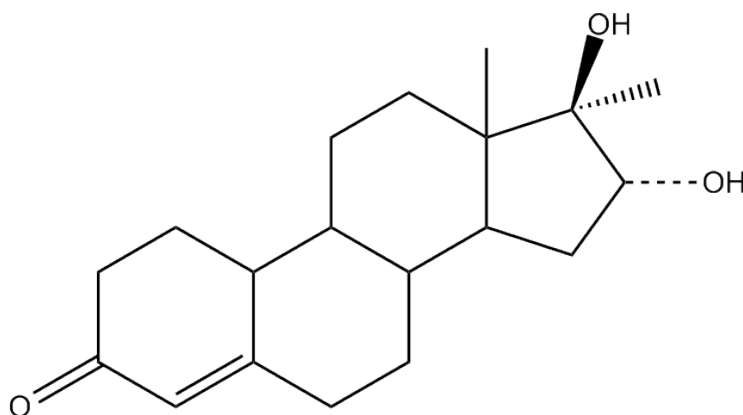
FIGURE 4.25: The proposed structure of the third MeNT metabolite.

The verification of MeNT M3 Incubation of MeNT resulted in the generation of 16z,17β-dihydroxy-17α-methylestr-4-en-3-one (16-hydroxy MeNT). Detection of this metabolite post incubation, and in urine, are depicted in figure 4.26, together with a sample of non-incubated MeNT .

FIGURE 4.26: The MRM chromatogram verifying/refuting the existence of the 16-hydroxy MeNT in: a sample of non-incubated MeNT a), a matrix sample (HLM, S9 and co-factors) b), the *in vitro* incubated sample c), urine from a MeNT excretion study d).

Conjugation state When analyzing the urine samples, no MeNT metabolites were found in the free fraction. After hydrolysis, all MeNT metabolites described here, were detected. This is an indication that MeNT metabolites are excreted as glucoronides (conjugates). The analogue $5\alpha/\beta$ reduced nandrolone metabolites, have all been determined as conjugates in preceding excretion studies[42].

4.2.3 Madol

A recent study suggested MRMs of five potential metabolites[45], while Gauthier *et al.*[18] suggested four metabolites, produced by human fresh hepatocytes. In screening the *in vitro* incubated samples of madol for potential metabolites, one metabolite was observed in this study. Since confirmatory synthesis was not performed, the structure of the metabolites was not confirmed.

Molecular structure of madol M1 The EI mass spectrum of the possible madol M1 was shown in figure 4.9. EI fragmentation of this metabolite seemed to generate molecular ions of considerable intensity at m/z 523 and 146, and abundant fragments at m/z 448, 394 and 269. The per-TMS derivative of madol has the molecular weight of 360. An m/z 538 ion have additional 178 *Da*, which corresponds to two additionally attached $[TMSO]^+$ groups . This is probably the intact trimethylsilyated madol M1 ion with additional diols. The dissociation is in conformity with that of Gauthier *et al.*[18]. Ion m/z 523, is most likely $[MW-CH_3]^+$. A 17-methyl steroid structure is supported by the m/z 143 and 130 fragment[16;48]. Abundant ions at m/z 129 are not solely connected to the A-ring, as described for MeNT metabolites. A D-ring fragment of the same signal has been described for 17,19-dimethyl steroids[48], as the C-19 methyl influences the fragment formation. The m/z 117 and 147 ions have been seen in mass spectra of vicinal diols[40]. Vicinal diols have previously been suggested and confirmed by NMR analysis, at position C-2 and C-3 for madol M1[18]. The structure in figure 4.27 result from the spectral interpretations, and is consistent with former research on madol metabolites[45;18;44].

Until recently, only A-ring alterations were confirmed for madol[18]. The 17α-methyl, and the 17β-hydroxy group seemed to persist during metabolism, even though metabolic action has been proposed in positions C-6, C-16 and C-17[15]. In late 2012, urine from an excretion study was partly evaluated, and a 18-nor-17,17-dimethyl and 2,3,16,17-tetrol structure were described[45].
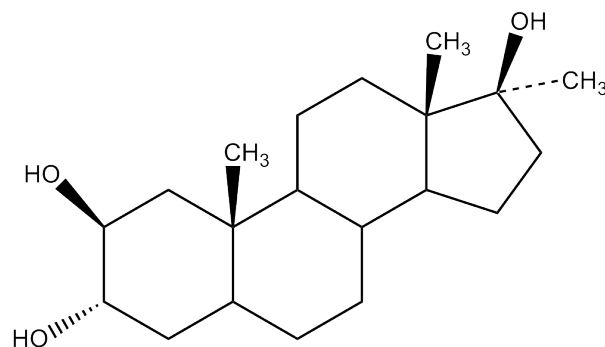
FIGURE 4.27: The proposed structure of madol M1, 17α-methyl-2β,3α,17β-trihydroxy-5α-androstane (2,3,17-trihydroxy madol).

The verification of madol M1   2,3,17-trihydroxy madol was identified after *in vitro* incubation of the parent compound, when screened through the established detection-method (figure 4.28). This metabolite has a longer window for detection than madol, and can be determined approximately one week post administration[45]. Implementation of 2,3,17-trihydroxy madol in the AAS screening at the Norwegian Doping Control Laboratory will better the detectability, as madol itself only is detectable for 2 days[44].

(a)   (b)
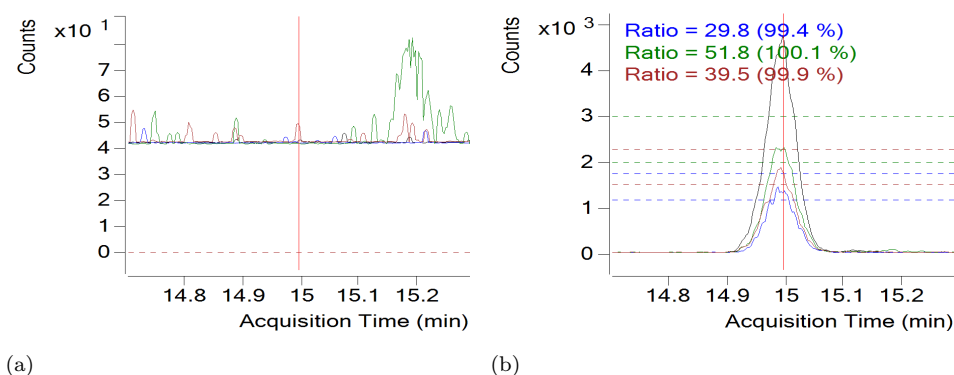
FIGURE 4.28: The MRM chromatograms verifying/refuting the existence of 2,3,17-trihydroxy madol in: a sample of madol (not incubated) a), *in vitro* incubated madol b).

Interestingly, the MRMs chosen in this study, proved more specific toward 2,3,17-trihydroxy madol, than those reported by Sobolevsky *et al.*[45]. Only two transitions per metabolite were implemented in their detection-method. For confirmation purposes, WADA encourage more than two transitions[52]. By implementing additional ion transitions, the specificity may increase. This might contribute to distinguish between 2,3,17-trihydroxy madol and pregnanediol, mentioned to co-eluate with 2,3,17-trihydroxy madol[45].

Madol M2   A trace, possibly from a second metabolite, was observed, though with a weak signal intensity. An EI mass spectrum at 14.1 minutes, of this suggested metabolite, is depicted in figure 4.29.
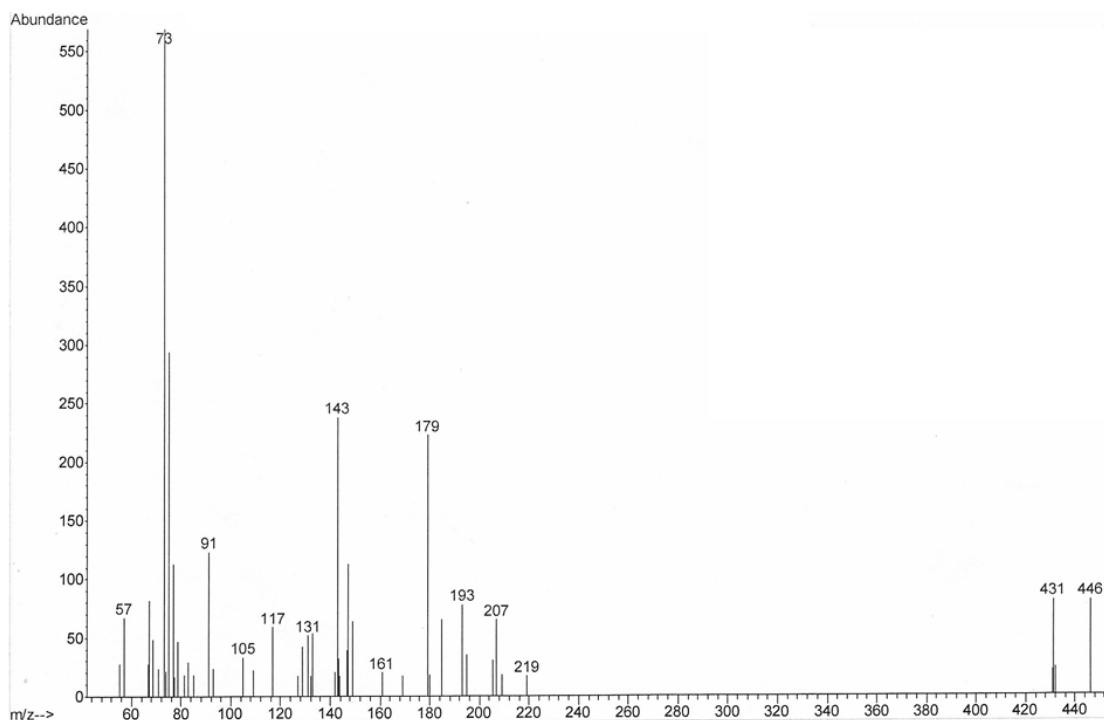
FIGURE 4.29: Mass spectrum (GC-EI-MS), possibly of a second madol metabolite.

It was not possible to verify the existence of a second madol metabolite in this study. The MRM might not be correct or sensitive enough, or the microsomes were unable to generate additional metabolite. By comparing the mass spectrum to those reported by Gauthier *et al.*[18], similarities were seen with the suggested metabolite 17β-hydroxy-17α-methyl-5α-androstan-2-en-4-one.

4.3   Evaluation of the assay

The incubation conditions were evaluated with respect to time and to distinguish the metabolic reactions. Some tendencies and assumptions might be drawn from this evaluation. This section further address ways to optimize the incubation assay in future studies.

4.3.1 Metandienon

The optimal incubation time for generation of metabolites seemed to be diverse between the metabolites. Welsch *et al.*[56] suggested that a long incubation time would reverse the metabolite synthesis, a phenomena observed in this study. The optimal time of incubation was seen in the range of 2-24 hours, depending on the metabolite and the responsible enzymes in question.

6β-hydroxy metandienon   6β-hydroxy MD was the only metabolite solely produced from MD with microsomes and NADPH. The *in vitro* assay provided both CYPs (in microsomes) and necessary co-factors for hydroxylation. Thus removing NADPH, and only incubating with S9, UDPGA and PAPS, gave no result of 6β-hydroxy MD. The optimal time of incubation was 24 hours. This was the endpoint in the experimental setup of the time-dependent assay. It is therefore unknown whether 24 hours is the true optimal time, or if between 6 and 24, or after 24 hours is optimal. It is reasonable that 6β-hydroxy MD was the only metabolite found in the pre-project. Metabolites requiring only CYPs and NADPH were probably the only operative reactions, and MD was the only incubated compound.

Epimetandienon   Incubation of MD resulted in the formation of epiMD when S9 and PAPS were available for the epimerization. By incubating in 15 hours, the most adequate response was observed. It is noteworthy to implement that already after 4 hours, epiMD had been generated. As no samples were gathered between 4 and 15 hours, its optimum might be somewhere in between. Samples incubated with microsomes and NADPH gave no epimerization. This indicates that the sulphate conjugation is the reaction pathway of which the rearranged epiMD is generated, as described by Schänzer *et al.*[43].

17,17-dimethyl metabolite   Incubation of MD or epiMD with UDPGA and co-factors generated the 17,17-dimethyl metabolite. The optimal incubation time was 17 hours for MD, and 4 hours for epiMD. This logic relationship follows from the above (epiMD) reasoning. EpiMD seem to be the intermediate between MD and 17,17-dimethyl metabolite, and is necessary for the reaction of rearrangement to occur. It seems to be a rapid but reversible reaction under the proper circumstances, due to the optimal time of incubation seen for epiMD. This metabolite

was produced in all the incubations of both MD and epiMD, but was absent in incubation solely comprising microsomes and NADPH.

17β-hydroxymethyl metabolite 17β-hydroxymethyl metabolite generated a sufficient response in the detection-method after incubation of the 17,17-dimethyl metabolite. The optimal incubation condition included two hours of incubation with microsomes and NADPH. It remains a challenge to determine whether the reaction is further optimized at an earlier point. The formation drop was remarkable within 4 hours, suggesting a reversible reaction during microsome incubations.

4.3.2 Methylnortestosterone

The generated of all MeNT metabolites required microsomes and NADPH for their formation in this study. The optimal time for maximal generation, varied from 1-18 hours. Even though microsomes and NADPH were required, glucuronidation is probably true for all metabolites at the accessible cites provided during phase I reactions. This is according to the lack of metabolites observed in the urine free fraction.

5α-estrane-diol metabolite The maximum response of the 5α-estrane-diol metabolite was observed after 1 hour of incubation. Whether the reaction is optimal at an earlier point is unknown, the metabolite response dropped with a factor of 3 within two hours of additional incubation. Reduction is a phase I reaction that seems to proceed rapidly for MeNT, with available CYPs and NADPH reducing equivalents.

5β-estrane-diol metabolite 5β-estrane-diol metabolite was not observed from the optimization incubations. It was nevertheless generated during a 4 hour incubation comprising S9 fraction and NADPH. This indicates that the two reduced epimers follow different pathways in MeNT metabolism. This stands to reason as the α- and β- reductase are individual enzymes located at different spots in the hepatocytes. 5α reduced metabolites is usually more abundant than 5β. As anticipated, neither of the reduced metabolites was generated from incubations with S9, UDPGA and PAPS.

16-hydroxy metabolite Microsome incubation of MeNT with NADPH resulted in the 16-hydroxy metabolite. The time of incubation seemed to reach an optimum at 18 hours, but traces were detected from 4 hours. It is unknown whether the reaction is further optimal after 18 hours, as this was the final point in the time dependent incubation assay. Hydroxylation is a CYP reaction, but accordingly more demanding than the 4-5 reduction, judged upon these results.

4.3.3 Madol

No evaluation regarding time of incubation of madol was performed. This is suggested in future work, and thus might reveal additional metabolites that faded out with time spent in the standard incubation assay. The main metabolite of madol was seen from incubation of microsomes with NADPH, suggesting encountering parts of the phase I metabolism.

Chapter 5

Conclusion

The established detection-method of a distinct retention time and MRM transitions of a steroid metabolite, was sufficient to detect: steroids and metabolites in a reference mix, from *in vitro* synthesized samples, and from urine samples. Microsomes showed promising as a technique to synthesize metabolites *in vitro*. 6β-hydroxyMD, epiMD and the 17,17-dimethyl metabolite was generated from incubated MD. The last two was probably due to sufficient SULT availability. The MD 17β-hydroxymethyl metabolite was generated from the 17,17-dimethyl MD metabolite, and is of great interest since it is considered as a long term metabolite of MD. Incubation of MeNT resulted in three metabolites: the $5\alpha/\beta$ reduced MeNT and a 16-hydroxy MeNT. A 2,3,17-trihydroxy madol was observed after incubation of the parent steroid. The detection-method of MeNT and madol might be implemented in the screening at the Norwegian Doping Control Laboratory after this thesis submission. The optimal time of incubation varied among the steroids. The most prominent enzymatic reactions seemed to be CYP mediated reactions, but other reactions were observed as well. Future trials with microsomes may focus on other AAS with unknown metabolic pathways, in order to identify and incorporate new metabolites in the doping screening.

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AAS	Anabolic androgenic steroids
ACN	Acetonitrile
AR	Androgen receptor
CE	Collision energy
CI	Chemical ionization
CID	Collision induced dissociation
CYP	Cytochrome P450 superfamily
DMSO	Dimethyl sulfoxide
EI	Electron ionization
ER	Endoplasmic reticulum
EtSH	Etanethiol
eV	Electron volt
GC	Gas chromatography
HLM	Human liver microsomes
IOC	International Olympic Committee
ISTD	Internal standard
m/z	Mass-to-charge ratio
MD	Metandienon
MeNT	Methylnortestosterone
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MSTFA	N-Methyl-N-trimethylsilyltrifluoroacetamidet
MW	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate
PAPS	3'-Phosphoadenosine-5'-phosphosulfate
PCA	Principal component analysis
RT	Retention time
RRT	Relative retention time
S9	S9-fraction from human liver
SIM	Selected ion monitoring
SL	D-Saccharic acid 1,4-lactone monohydrate
SULT	Sulfotransferase
TBME	Tert-butyl methyl ether
TMS	Trimethylsilyl
UDPGA	Uridine 5'-diphospho-glucuronosyltransferase
UGT	UDP-glucuronosyltransferase
WADA	The Word Anti-Doping Agency

Appendix

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Buffer solutions

Following appendix shows the buffer solutions used in the experimental assay. All buffer solutions were made once, and kept in the refrigerator between experiments.

TABLE 1: Buffer solutions for the experimental assay.

Buffer solution	K_2HPO_4	KH_2PO_4	$MgCl_2$ (in phosphate buffer)
Molecular weight (g/mol)	174,1	136,1	95,2
Weight (g)	1,75	1,36	0,00475
Dissolvent (ml)	10,0	10,0	10,0
Concentration (M)	1.0	1,0	0,005

Phosphate buffer 0,005 M phosphate buffer was made by mixing 100 μl 1M KH_2PO_4 and 400 μl 1M K_2HPO_4, and dilute with milli-Q water to a total volume of 10 ml.

Incubation buffer Incubation buffer was made by mixing 4,8 mg of $MgCl_2$, 400 μl 1M K_2HPO_4, and 100 μl 1M KH_2PO_4, and dilute with milli-Q water to a total volume of 10 ml. The buffer solutions were placed in the refrigerator before and between experiments.

Working solutions

Following appendix shows the working solutions used in the experimental assay. Several synthesis were performed, all of which followed the experimental setup described in section 3.4.2.

The substrate working solution (100μM) of each steroid was made once, by dissolving the amount (mg) shown in table 2 in equal volume (ml) of DMSO to a concentration of 1.0 mg/ml. 150 μl 1 mg/ml solution was diluted with DMSO to a total volume of 5 ml, making up the 0.1 M solution. Working solutions of epiMD and epimetendiol were made from stock solutions "in-house". Since these were 1 mg/ml in ethanol, 30 μl were evaporated under N_2. 1 ml DMSO was added to each dry sample, making up the 100 μM working solution. The solutions were stored in a cold-storage chamber between experiments.

TABLE 2: Working solutions of the steroid substrates. All substrates were dissolved in equal amount (ml) DMSO as weigth (mg) of substrate.

	MW (g/mol)	Weight (mg)
MD	300.4	0.81
17,17-dimethyl metabolite	285.4	0.84
MeNT	288.4	1.41
Madol	288.5	1.40

NADPH (50mM) was made by mixing 10.0 ($\pm$0.2) mg NADPH with 240 μl incubation buffer in an eppendorf tube, and vortexed carefully. SL (50mM) was made by mixing 3.0 ($\pm$0.2) mg SL with 250 μl phosphate buffer in an eppendorf tube, and vortexed carefully. The solutions were then placed on ice. Solution of UDPGA and PAPS were prepared 15 minutes prior to incubation of phase II reactions. 3.3 ($\pm$0.2) mg UDPGA was mixed with 100 μl incubation buffer in an

eppendorf tube. 0.25 ($\pm$0.1) mg PAPS was mixed with 1000 μl incubation buffer in an eppendorf tube.

Chemicals and solutions

Chemicals and solutions (in appendix and) used during this study are presented below. All metabolites were pure ($\geq$95%), and the solvents were of pro-analyse quality:

MD, metabolites and ISTD:

MD = 17β-hydroxy-17α-methylandrosta-1,4-dien-3-one
17α-hydroxymethyl metabolite = 17α-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one
EpiMD = 17α-hydroxy-17β-methylandrosta-1,4-dien-3-one
6β-hydroxyMD = 6β,17β-dihydroxy-17α-methylandrosta-1,4-dien-3-one
Epimetendiol = 17β-methyl-5β-androsta-1-ene-3α,17α-diol
5β-THMT = 17α-methyl-5β-androstane-3α,17α-diol
Madol = 17α-methyl-17β-hydroxy-5α-androst-2-ene
MeNT = 17β-hydroxy-17α-methylestr-4-en-3-one

Solvents:

Ethanol
Methanol
Milli-Q water
DMSO
Phosphate buffer
Incubation buffer
Tris-buffer
TBME

Other reagents:

Human liver microsomes

S9 fraction from human liver

SL

β-glucuronidase

ACN

MSTFA mix:

- MSTFA

- NH$_4$

- EtSH

Heptan

Establishing the detection-method

Full results of establishing the detection-method towards MeNT and Madol follows:

- The EI mass spectra of metabolites: MeNT and Madol (figure 1).

- Product ion mass spectra of selected precursor ions from the EI mass spectra: MeNT with precursor ion m/z 432, and madol with precursor ion m/z 345 (figure 2).

- The intensity distribution after varying the CE MRM transition of 17,17-dimethyl metabolite of MD is shown in figure 3.
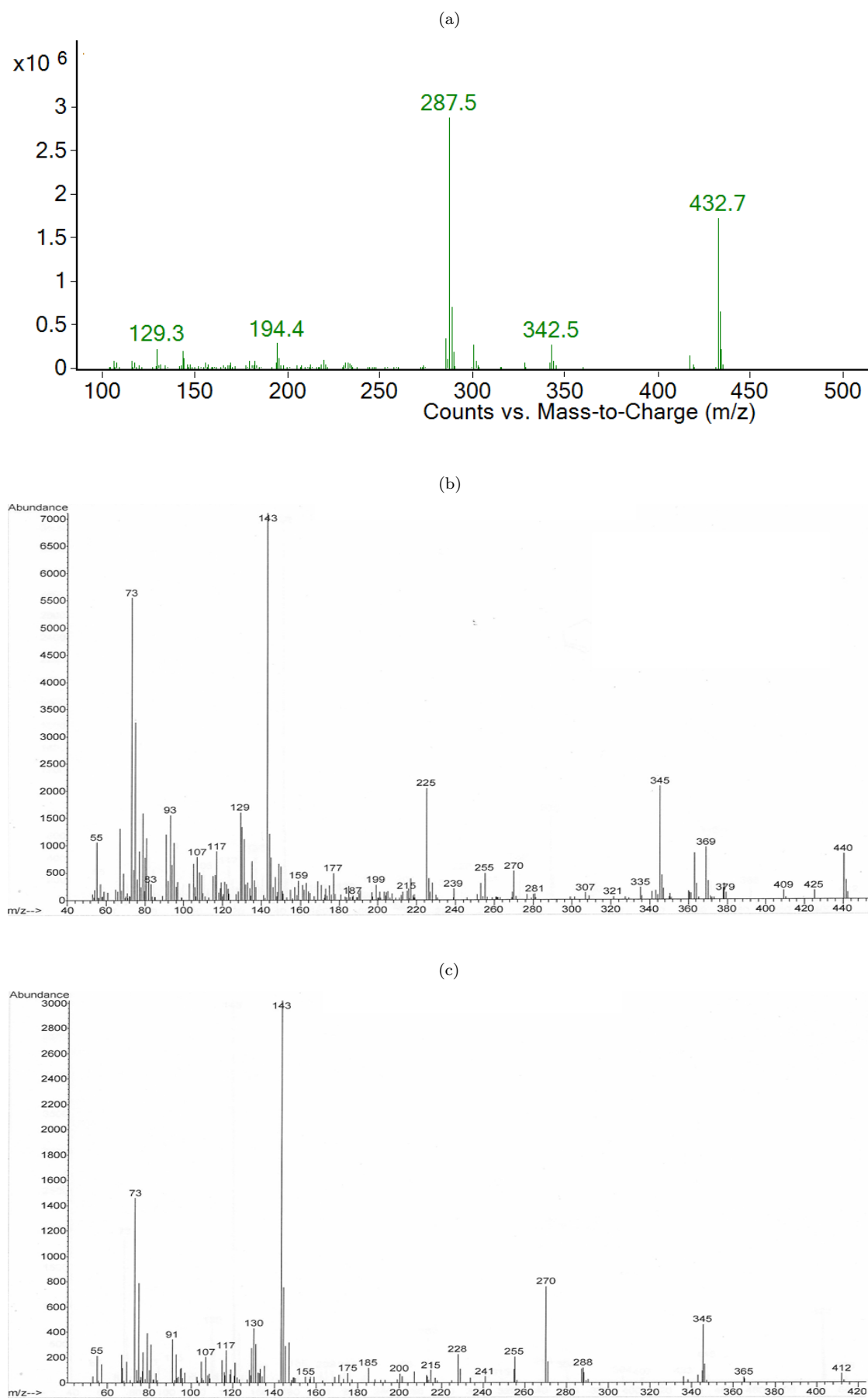
(a)

(b)

(c)

FIGURE 1: Mass spectra (GC-EI-MS) of: MeNT a), madol b). The madol
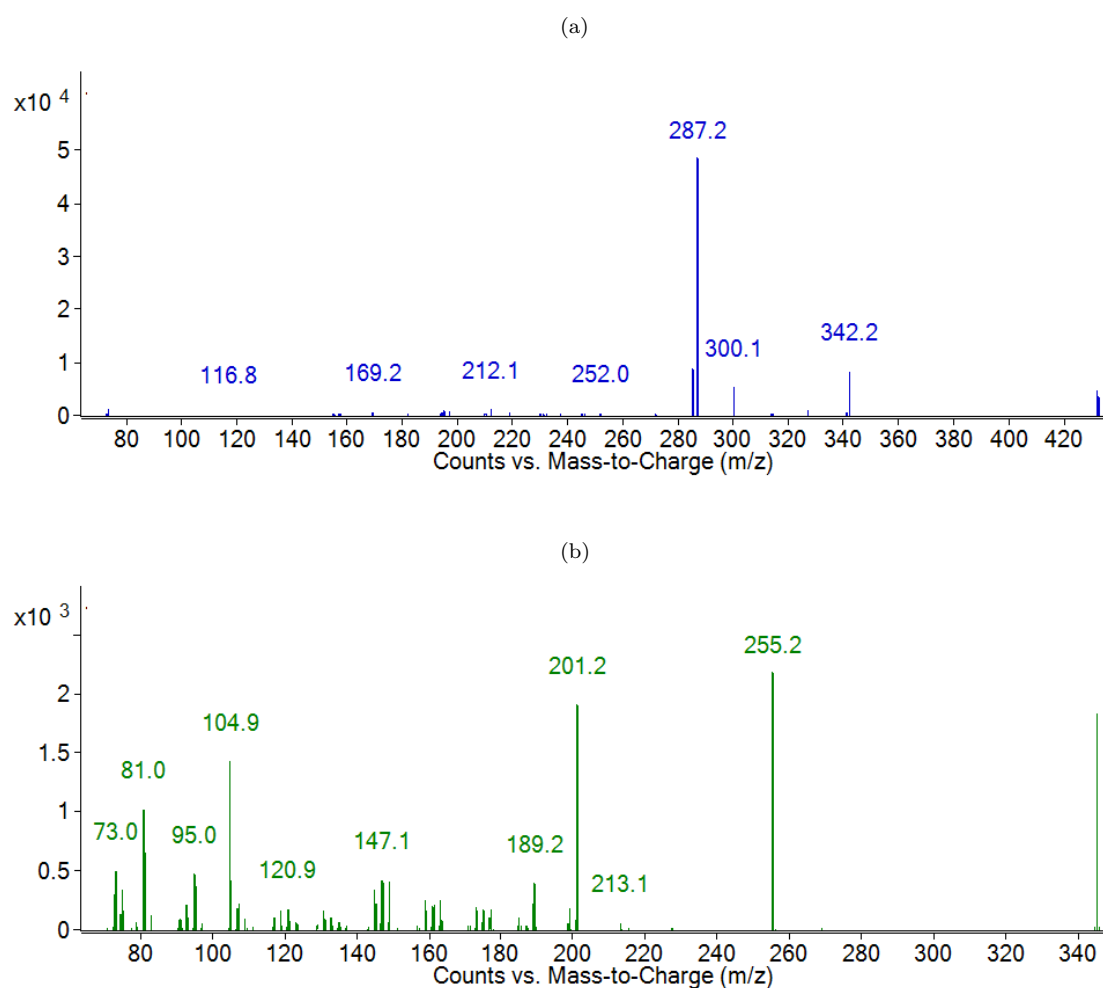isomer, eluting shortly after madol (tail) is shown in c)

(a)

(b)

FIGURE 2: Product ion mass spectra of precursor ions: m/z 432 of MeNT a),
m/z 345 of madol b).

17,17-dimethyl metabolite	Quantifier (339.2 -> 193.1) Results	Qualifier (354.3 -> 339.2) Results	Qualifier (354.3 -> 148.0) Results	Qualifier (354.3 -> 133.0) Results
Collision Energy	Area	Area	Area	Area
5	103796	126030	171286	51187
10	171711	181430	192708	117811
15	205497	175629	132668	201886
20	190448	119550	64040	257418
25	141353	59251	24683	264648
30	91612	23190	8667	254724

FIGURE 3: The intensity distribution with different CE, for the MRMs of the
TMS-derivative of 17,17-dimethyl metabolite.