STRATEGIES FOR THE DETECTION OF DESIGNER STEROIDS IN RACEHORSES

A thesis submitted for the degree of

Doctor of Philosophy

at the Australian National University

By Christopher Charles Waller

Research School of Chemistry

August 2016
Declaration

This thesis is a report of research undertaken at the Research School of Chemistry at the Australian National University from February 2013 to August 2016 under the supervision of Associate Professor Malcolm McLeod. All of the work presented within this thesis is the author’s own original work and the author has made every effort to properly acknowledge the work of others. This thesis has not been previously submitted for the award of any other degree at any university or tertiary education institution, and does not exceed 100,000 words in length.

Christopher Charles Waller

August 2016
Acknowledgements

This work would not have been possible without the help and support of so many people. I am extremely grateful to all those who have made this project possible, and made the time completing my PhD one of the most satisfying parts of my life so far.

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This thesis has been submitted by compilation. As of May 2017, sections of the work presented in this thesis have been published, submitted for review, or are currently in preparation for publication, in the following peer-reviewed journals:


6. Weththasinghe, S.; Waller, C. C.; Fam, H. L.; Stevenson, B. J.; Cawley, A. T.; McLeod M. D; *Drug Testing and Analysis* **2017**, *In vitro phase II sulfation of steroids with the liver S9 fraction employing ATP and sodium sulfate in place of PAPS* (submitted for review)

## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>δ</td>
<td>chemical shift</td>
</tr>
<tr>
<td>Δ</td>
<td>delta, change</td>
</tr>
<tr>
<td>AAS</td>
<td>anabolic androgenic steroid</td>
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<tr>
<td>ABP</td>
<td>Athlete’s Biological Passport</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine 5’-monophosphate</td>
</tr>
<tr>
<td>AORC</td>
<td>Association of Official Racing Chemists</td>
</tr>
<tr>
<td>APCI</td>
<td>atmospheric pressure chemical ionisation</td>
</tr>
<tr>
<td>APS</td>
<td>adenosine-5’-phosphosulfate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>AR CALUX</td>
<td>androgen receptor chemical activated luciferase gene expression</td>
</tr>
<tr>
<td>AU$</td>
<td>Australian dollar</td>
</tr>
<tr>
<td>BALCO</td>
<td>Bay Area Laboratory Co-Operative</td>
</tr>
<tr>
<td>CIL</td>
<td>constant ion loss</td>
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<tr>
<td>COSY</td>
<td>homonuclear correlation spectroscopy</td>
</tr>
<tr>
<td>d</td>
<td>day(s)</td>
</tr>
<tr>
<td>DIPE</td>
<td>diisopropyl ether</td>
</tr>
<tr>
<td>DMDO</td>
<td>3,3-dimethyldioxirane</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>EAAS</td>
<td>endogenous anabolic androgenic steroid</td>
</tr>
<tr>
<td>EBP</td>
<td>Equine Biological Passport</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
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<tr>
<td>EI</td>
<td>electron ionisation</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionisation</td>
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<tr>
<td>EtOAc</td>
<td>ethyl acetate</td>
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<tr>
<td>eq</td>
<td>equivalent(s)</td>
</tr>
<tr>
<td>eV</td>
<td>electron volt</td>
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<tr>
<td>furazadrol</td>
<td>[1’,2’]isoxazolo[4’,5’:2,3]-5α-androstan-17β-ol</td>
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<tr>
<td>G6P</td>
<td>glucose-6-phosphate</td>
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G6PDH | glucose-6-phosphate dehydrogenase
---|---
GC-IRMS | gas-chromatography coupled to isotope ratio mass spectrometry
GC-MS | gas-chromatography coupled to mass spectrometry
h/hr | hour(s)
hemapolin | 2α,3α-epithio-17α-methyl-5α-androst-17β-ol
HPLC | high-performance liquid chromatography
HMBC | heteronuclear multiple-bond correlation spectroscopy
HRAM | high-resolution accurate mass spectrometry
HRMS | high-resolution mass spectrometry
HSQC | heteronuclear single quantum coherence spectroscopy
IFHA | International Federation of Horseracing Authorities
IMIM | Institut Hospital del Mar d'Investigacions Mèdiques
in vitro | biological studies performed outside of a living system
in vivo | biological studies performed in a living system
IOC | International Olympic Committee
IUPAC | International Union of Pure and Applied Chemistry
J | coupling constant
LC-MS | liquid-chromatography coupled to mass spectrometry
LC-HRAM | liquid-chromatography coupled to high-resolution accurate mass spectrometry
LLOQ | lower limit of quantification
LOD | limit of detection
LRMS | low-resolution mass spectrometry
L-Selectride® | lithium tri-sec-butyliborohydride
madol | desoxymethyltestosterone, 17α-methyl-5α-androst-2-en-17β-ol
MeOH | methanol
methasterone | superdrol, 17β-hydroxy-2α,17α-dimethyl-5α-androstan-3-one
min | minute(s)
MOX-TMS | methyloxime-trimethylsilyl ether
MRM | multiple reaction monitoring
MS | mass spectrometry/mass spectrum
MS/MS, or MS^n | tandem mass spectrometry
m/z | mass-to-charge ratio
NADH | nicotinamide adenine dinucleotide
<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NIST</td>
<td>National Institute of Standards and Technology</td>
</tr>
<tr>
<td>NBS</td>
<td>N-bromosuccinimide</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>NOE</td>
<td>nuclear Overhauser effect</td>
</tr>
<tr>
<td>PAP</td>
<td>3’-phosphoadenosine-5’-phosphate</td>
</tr>
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<td>PAPS</td>
<td>3’-phosphoadenosine-5’-phosphosulfate</td>
</tr>
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<td>P. aeruginosa</td>
<td><em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td>PCC</td>
<td>pyridinium chlorochromate</td>
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<tr>
<td>ppm</td>
<td>parts-per-million</td>
</tr>
<tr>
<td>PTAB</td>
<td>phenyltrimethylammonium tribromide</td>
</tr>
<tr>
<td>R&lt;sub&gt;f&lt;/sub&gt;</td>
<td>retention factor</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature (20 °C), or retention time (minutes)</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions-per-minute</td>
</tr>
<tr>
<td>SIM</td>
<td>single ion monitoring</td>
</tr>
<tr>
<td>SRM</td>
<td>selected reaction monitoring</td>
</tr>
<tr>
<td>S/N</td>
<td>signal-to-noise</td>
</tr>
<tr>
<td>SO&lt;sub&gt;3&lt;/sub&gt;.NEt&lt;sub&gt;3&lt;/sub&gt;</td>
<td>sulfur trioxide-triethylamine complex</td>
</tr>
<tr>
<td>SO&lt;sub&gt;3&lt;/sub&gt;.py</td>
<td>sulfur trioxide-pyridine complex</td>
</tr>
<tr>
<td>SPE</td>
<td>solid phase extraction</td>
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<tr>
<td>superdrol</td>
<td>methasterone, 17β-hydroxy-2α,17α-dimethyl-5α-androstan-3-one</td>
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<tr>
<td>testosterone</td>
<td>17β-hydroxyandrost-4-en-3-one</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>THG</td>
<td>tetrahydrogestrinone, 18β-homo-17β-hydroxy-19-nor-17α-pregna-4,9,11-trien-3-one</td>
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<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
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<tr>
<td>TMS</td>
<td>trimethylsilyl/trimethylsilane</td>
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<tr>
<td>t-BuOH</td>
<td>tertiary-butanol, 2-methylpropan-2-ol</td>
</tr>
<tr>
<td>UDPGA</td>
<td>uridine diphosphate glucuronic acid</td>
</tr>
<tr>
<td>USADA</td>
<td>United States Anti-Doping Authority</td>
</tr>
<tr>
<td>UV</td>
<td>ultra-violet light</td>
</tr>
<tr>
<td>WADA</td>
<td>World Anti-Doping Agency</td>
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<tr>
<td>WAX</td>
<td>weak anion exchange</td>
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Abstract

Anabolic androgenic steroids (AAS) are a class of compounds which include the parent compound testosterone, and are well-known for their misuse as performance enhancing substances in sport. Over the last decade there has a push towards the illicit use of so-called “designer steroids” which are manufactured with the express aim of rendering these compounds undetectable by existing anti-doping methods. These compounds are readily accessible online in “dietary” or “nutritional” supplements containing compounds which have never been tested or approved as veterinary agents. Despite considerable focus in human sports, there has been limited investigation into these compounds in equine systems. As such, developing new tools to detect these compounds is of high priority.

Chapter One presents a summary of the literature detailing the metabolism of designer steroid compounds in equine systems, with an aim to identify metabolites suitable for incorporation into screening protocols by anti-doping laboratories. Future directions for anti-doping laboratories are also proposed, highlighting the importance of alternate testing matrices, improved in vitro methodologies which can faithfully replicate in vivo metabolism, and the development of untargeted screening methods which can detect new instances of steroid misuse.

Chapter Two presents new methodology for the synthesis of steroid sulfate compounds, suitable for use in analytical laboratories. Key to this synthetic methodology is the use of solid-phase extraction (SPE), a technique routinely used in anti-doping laboratories but with untapped potential in chemical synthesis. This methodology was applied to the synthesis of sixteen steroid mono-sulfate, and twelve steroid bis-sulfate reference materials, which encompass a representative range of steroid substitution patterns and configurations. The mass spectrometry behaviour of these bis-sulfate compounds has been studied, and used to develop a constant ion loss (CIL) scan method for the untargeted detection of all major classes of steroid bis-sulfate metabolites.
Chapter Three presents a study detailing the discovery of a novel anabolic agent $3\alpha/\beta$-chloro-$17\alpha$-methyl-$5\alpha$-androst-$17\beta$-ol, in samples seized by law-enforcement, and the subsequent investigations to elucidate its structure, identify the primary human and equine metabolites, and to incorporate these into routine anti-doping screening protocols. This study highlights a workflow suitable for adoption by anti-doping laboratories which allows for the development of suitable screening protocols in the event that new designer steroids are identified.

Chapter Four presents in vivo and in vitro studies of the designer steroid furazadrol ([(1',2')isoxazolo[4',5':2,3]-5α-androst-$17\beta$-ol]. Following a controlled equine in vivo administration, these investigations have identified the key urinary metabolites of furazadrol, many of which have been confirmed by comparison to synthetically-derived reference materials. These metabolites can be incorporated into anti-doping screening and confirmation procedures. Comparative in vitro studies were also undertaken alongside this work. A second study is presented in this chapter which investigates alternative conditions for in vitro phase II metabolism, and employs adenosine 5'-triphosphate (ATP) and Na$_2$SO$_4$ in place of the expensive phase II co-factor 3'-phosphoadenosine-5'-phosphosulfate (PAPS). Although the ability to replicate in vivo metabolism using in vitro methods is currently limited, it is hoped that this work will allow for the prediction of in vivo metabolism of unknown compounds solely from in vitro results.

Chapter Five presents an in vivo study of the designer steroid hemapolin (2α,3α-epithio-$17\alpha$-methyl-$5\alpha$-androst-$17\beta$-ol). Following a controlled equine in vivo administration, these investigations have identified the key urinary metabolites of hemapolin, many of which have been confirmed by comparison to an extensive library of synthetically-derived reference materials. The excretion profiles of the major enone metabolites $17\beta$-hydroxy-$17\alpha$-methyl-$5\alpha$-androst-3-en-2-one, and $17\beta$-hydroxy-$17\alpha$-methyl-$5\alpha$-androst-2-en-4-one have been established through a MRM method. These metabolites can be incorporated into anti-doping screening and confirmation procedures, allowing for the future detection of hemapolin misuse.
CHAPTER ONE

Introduction
1.1 Foreword

The following manuscript has been submitted for review in the journal Drug Testing and Analysis for their special edition entitled “Advances in Equine Anti-Doping”. This manuscript gives an introduction to anti-doping analysis, summarises some of the key techniques currently in use today, and reviews the available literature on designer steroid metabolism in equine sports. This manuscript was authored by Mr Christopher Waller and Associate Professor Malcolm McLeod. C.Waller undertook the literature review, and prepared the initial and revised drafts of the manuscript. Assoc. Prof. McLeod assisted in determining the content and scope of the review, and also assisted in revising the manuscript prior to submission.

Figure 1.1: “Designer” anabolic steroids in equine sports
Anabolic androgenic steroids (AAS) are a class of compounds which include the parent compound testosterone, and are well-known for their misuse as performance enhancing substances in sport. Over the last decade as anti-doping laboratories have developed analytical methods in order to be able to monitor for the misuse of these compounds in sport, there has been a push towards so-called “designer steroids” which are manufactured with the express aim of rendering these compounds undetectable by existing analytical methods. These compounds are readily accessible online in “dietary” or “nutritional” supplements containing compounds which have never been tested or approved as veterinary agents.

Despite considerable focus in human sports there has been limited investigation into these compounds in equine systems. In order to effectively respond to the threat of designer steroids, a detailed understanding of their metabolism is needed to identify markers and metabolites related to their misuse. In this chapter a summary of the literature detailing the metabolism of designer steroid compounds in equine systems is presented with an aim to identify metabolites suitable for incorporation into screening protocols by anti-doping laboratories. A brief exploration of the future directions of anti-doping analysis is also presented intending to highlight important areas of current and future research, and provide context to the work presented in the remaining chapters.
1.2 A review of designer anabolic steroids in equine sports
A review of designer anabolic steroids in equine sports

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Abstract:
In recent years the potential for anabolic steroid abuse in equine sports has increased due to the growing availability of “designer steroids”. These compounds are readily accessible online in “dietary” or “nutritional” supplements and contain steroidal compounds which have never been tested or approved as veterinary agents. They typically have unusual structures or substitution and as a result may pass undetected through current anti-doping screening protocols, making them a significant concern for the integrity of the industry. Despite considerable focus in human sports, until recently there has been limited investigation into these compounds in equine systems. In order to effectively respond to the threat of designer steroids, a detailed understanding of their metabolism is needed to identify markers and metabolites arising from their misuse. A summary of the literature detailing the metabolism of these compounds in equine systems is presented with an aim to identify metabolites suitable for incorporation into screening protocols by anti-doping laboratories. The future of equine anti-doping research is likely to be guided by the incorporation of alternate testing matrices into routine screening, the improvement of in vitro technologies that can faithfully replicate in vivo equine metabolism, and the improvement of instrumentation or analytical methods that allow for the development of untargeted screening, and metabolomics approaches for use in anti-doping screening protocols.
### History of doping in sport

Among the earliest known examples of doping in sport come from the ancient Greek and Roman cultures which are known to have held sporting competition in high regard. Competitors devoted themselves to winning at any cost and were said to have consumed numerous substances as a part of the strict diet and exercise regimes that accompanied preparation for these competitions. Alcohol, hallucinogenic mushrooms, leaves and syrups derived from opium-containing plants, bull urine, and raw animal testicles were favourites of these competitors. Animal sports were also quite common during this time and although records relating to the doping of animal competitors during this period are scarce, it is unlikely that they would not have been subject to the same types of treatment as their human-counterparts. Among the first known instances of doping in equine sports comes from the fifteenth century where a mixture containing anise seed, honey and red arsenic sulfide (sandarac) was reportedly given to horses as a stimulant, and later from an English regulation reported in 1666 banning the use of “exciting substances” in competitive races. The first well-documented cases of doping in equine sports however came to light during the nineteenth century and in response such substances were soon outlawed. These bans were ineffective without a means to police them however, so this lead to the rapid development of saliva-based tests to detect alkaloids such as cocaine and heroin.

With the new discovery of testosterone (17β-hydroxyandrost-4-en-3-one) and the other anabolic steroids in 1935, sports doping began to change as the effects of these drugs were quickly realised. Anabolic steroids became widely misused by athletes over the coming decades and after several deaths allegedly linked to doping, the International Olympic Committee (IOC) established a medical commission in 1967 to combat doping. Urine and blood screening became mandatory from the 1968 Olympic games and in subsequent competitions the
number of cases of doping fell as a result \(^9\). In order to more effectively combat the doping problem, the World Anti-Doping Agency (WADA) was formed in 1999 and developed a strict “Olympic Standard” drug testing regime which includes regular and random drug tests throughout the entire course of an Olympic games for all competitors. This has become a template for all anti-doping testing currently in use today \(^{10}\).

Reports of anabolic steroid misuse in equine sports date back to 1941, where an eighteen-year-old standardbred US trotter named Holloway was treated with testosterone for several months during training, and as a result reportedly regained much of his former racing ability \(^{11}\). More recently, the trainer of the US champion thoroughbred Big Brown admitted to injecting his horses with the synthetic anabolic steroid stanozolol \(\left[1',2'\right] \cdot 1'H\text{-pyrazolo}[4',5':2,3] \cdot 17\alpha\text{-methyl-5\alpha\text{-androstan}-17\beta\text{-ol}}\) during the 2008 Kentucky Derby, although stanozolol was legal in US racing at the time \(^{11}\). Big Brown won the first two races of the series under the effects of stanozolol; however, the horse failed to perform as expected in the third race where he was run steroid-free. To combat the growing problem of doping in equine sports, the Horseracing Authorities of the United States of America, France, Great Britain and Ireland jointly decided in 1961 to coordinate their resources to better manage the future of the industry, as equine sports have traditionally been governed by the local authorities in each jurisdiction \(^{12}\). In 1993 the International Federation of Horseracing Authorities (IFHA) was formed and included over 60 members. They meet annually to update the International Agreement on Breeding, Racing and Wagering, first endorsed in 1974, which outlines the recommended best practice for all jurisdictions, including protocols for how to effectively address the problem of doping in equine sports \(^{13}\).

**Anabolic androgenic steroids**

Steroids are a class of chemical compounds characterised by their tetracyclic fused ring system consisting of three cyclohexane rings (Rings A, B and C) and a cyclopentane ring (Ring D), conferring chemical stability and conformational rigidity to the molecule. Furthermore, the rings can be modified at many possible
positions, and also occur as a variety of stereochemistries and degrees of unsaturation. As a result, they occur widely in nature as lipids, hormones and other natural products. The structure of anabolic steroids is typified in the example of 17α-methyltestosterone (17β-hydroxy-17α-methylandrost-4-en-3-one) below (Figure 1).

**Figure 1: 17α-methyltestosterone showing steroid ring structure and IUPAC-recommended atom and ring labelling**

Steroid compounds can be categorised into number of subclasses, typically based on the number of carbon atoms present in their basic framework. The common core structures of most interest to anti-doping laboratories include: cholestanes (C27), which are typified by cholesterol, that are the steroid precursors from which most others are ultimately derived; cholanes (C24), which primarily consist of the steroid bile acids; pregnanes (C21), which consist of the progestogens and the corticosteroids; androstanes (C19), which form the basic framework of most androgens; and estranes (C18), which form the basic framework of some androgens, and the estrogens. Representative examples of each steroid class are shown in Figure 2.
Anabolic androgenic steroids, which include the parent compound testosterone, have legitimate uses in the medical treatment of human and animal diseases; however, they are widely-known for their misuse in sporting competition. The term anabolic androgenic steroid refers to the two distinct classes of biological activities possessed by these compounds: namely the anabolic effects and the androgenic effects, both of which are important for their natural functions. Anabolic effects promote anabolism and typically manifest as an increase in muscle strength and endurance, primarily due to increased protein synthesis, more efficient use of dietary nutrients, and increased red blood cell production. In a medical setting, these effects are of interest in the treatment of chronic wasting conditions such as the victims of severe burns and cancer patients. Androgenic effects on the other hand promote virilisation and manifest in the development and maintenance of male sex organs and secondary sex characteristics, and these effects typically find use in the treatment of various hormone deficiencies.

Based on WADA detection statistics from 2014, anabolic steroids accounted for 48% of all adverse analytical findings of banned substances in competing athletes. The main agents of misuse were stanozolol, 19-norandrosterone, and metandienone, which comprised 46% of all anabolic steroids detected. With the wide range of performance-enhancing drugs available to athletes today, it is interesting that a significant proportion that chose to dope used steroids, which suggests that they offer benefits that other types of compounds do not. Unlike...
other classes of drugs, such as stimulants, which usually offer benefits only for as long as they remain active in the body, the effects of anabolic steroids can be long-lasting. Whilst the compounds themselves may no longer be present in the body, athletes can use these compounds to train; gaining muscle mass which will be retained for a period after steroid use is discontinued, allowing athletes to keep the majority of the benefits without putting themselves at risk of being caught during in-competition screening.

**Anabolic steroids in equine sports**

In contrast to the well-documented advantages that anabolic steroids offer to human athletes, the effects of anabolic steroids in equine competitors are not well-established. A study has shown that short-term nandrolone administration increased the glycogen content of post-exercise muscle tissue, however this is in contrast with two studies that found no observable changes resulting from nandrolone administration. It has been shown that steroid administration delayed closure of epiphyseal growth plates in standardbreds, and suggested that this could lead to a potential increase to the long-term risks of injury during training. Additionally, it has been suggested that testosterone may not be involved in muscle development or maintenance in horses, as horses that experienced weight loss from maladaptation to training were observed to have similar testosterone levels compared to a control group. Aggressive behavioural changes resulting from anabolic steroid administrations have been noted which could lead to more competitive horses that perform better in training and competition, however they may also lead to injury and accidents with other horses, riders or trainers. Nonetheless, even if the evidence for anabolic steroids acting as performance-enhancing substances in horses is currently unclear, there is clear evidence that anabolic steroid misuse can result in serious animal health and welfare consequences for competing horses, riders and trainers. These concerns, as well as the overwhelming evidence of the effects of anabolic steroids in other species (including humans) more than warrant their banning in competitive equine sports by IFHA.
Designer anabolic steroids

Since the first synthesis of testosterone over 80 years ago, there has been substantial work aiming to produce steroid compounds with differing and useful biological properties. During the so-called “golden age” of steroid research during the 1950-60s, numerous potent analogues of testosterone were synthesised some of which were published\textsuperscript{28-32} whilst others were patented. The majority of these compounds however were only briefly tested for their anabolic activity and were never subsequently evaluated through clinical testing or approved for sale. As a result, they were forgotten, and then subsequently rediscovered in the past decade where they have been exploited by chemists who would seek to use the knowledge in the older steroid literature and bring these compounds to market in a clandestine fashion.

Figure 3: Structures of the first “designer” steroids: norbolethone, tetrahydrogestrinone, and madol

The term “designer steroid” can be defined as any anabolic steroid which has been prepared in such a way that it can evade detection by anti-doping laboratories. This typically involves chemical modification to the steroid core. The term itself was first coined in 2002 where Catlin et al. reported the first instance of a designer anabolic steroid compound detected in an athlete’s urine\textsuperscript{33}. This designer steroid was norbolethone (18β-homo-17β-hydroxy-19-nor-17α-pregn-4-en-3-one), a synthetic anabolic steroid compound first synthesised in 1966 and subsequently found in clinical studies to be a highly potent anabolic agent\textsuperscript{33,34}, but which had never been approved for clinical use. Two years later, the same group detected tetrahydrogestrinone (18β-homo-17β-hydroxy-19-nor-17α-pregna-4,9,11-trien-3-one; THG) another designer steroid, following analysis of a spent syringe containing an allegedly undetectable anabolic steroid which was provided
anonymously to the United States Anti-Doping Agency (USADA) by a former sporting coach. A third designer steroid, madol (17α-methyl-5α-androst-2-en-17β-ol; desoxymethyltestosterone), was also detected in a crude oily preparation received by the same laboratory in 2005. A transdermal preparation called “The Cream” was also identified, which contained a mixture of testosterone and epitestosterone in a controlled ratio. At this time the testosterone-epitestosterone ratio (T/E ratio) was an important marker used within WADA laboratories to identify samples suspected of doping with testosterone, and application of “The Cream” provided an increase in testosterone levels without altering the T/E ratio, making it harder to detect. These preparations were subsequently found in an investigation by the United States Federal Government to have been distributed by the Bay Area Laboratory Co-Operative (BALCO) alongside other anabolic steroids and performance enhancing substances in a secret program that supplied elite athletes with “undetectable drugs” to provide a competitive advantage. Although BALCO is now defunct, its legacy has changed the way anti-doping laboratories must approach the problem of anabolic steroid abuse in the world today.

Despite an increase in awareness and research to combat designer steroid misuse, their usage is becoming more widespread, in part due to the ease in which these compounds can be obtained. These compounds are typically present in “dietary” or “nutritional” supplements which are widely marketed online and often contain unusual structures and substitution patterns that may render them more difficult to detect by existing analytical methods. These supplements are also often discontinued and replaced by new products when they become detectable by anti-doping laboratories or law enforcement. These structural changes to the core steroid structure also help suppliers evade legal restrictions and penalties regarding their manufacture and sale in some jurisdictions. As a result, these designer anabolic steroids pose a number of problems. They are often prepared in a clandestine fashion and as a result there is often minimal data available detailing the purity, safety or efficacy of these products. Users are forced to rely on advice from other users reporting their outcomes in online forums, or as is common, to experiment on themselves. This environment also encourages underreporting of side-effects and health complications, often with serious consequences.
compounded by another major problem in that the labelling detailing the contents of these products is often falsified or omitted in an attempt to circumvent their control by law-enforcement\(^{40,41}\). This can hinder the work of health practitioners who are required to treat any complications that may arise from supplement usage\(^{42}\), and also prevent customs or border authorities from identifying potentially dangerous or illicit materials that may be entering their jurisdiction. With their widespread use by human athletes, it is highly likely that the problem of designer steroid misuse will find its way into the realm of equine sports as well.

**Equine steroid metabolism**

An understanding of the metabolism of anabolic steroid compounds is essential to develop methods for the detection of these compounds in equine sports. Anti-doping analysis typically requires the detection of known steroid markers and metabolites, as the parent compounds are often rapidly metabolised after administration. As such these metabolites form the basis of the majority of anti-doping screening protocols currently in use today. Such markers have historically been identified from urine, due to the relative ease of which samples can be obtained from competing animals, as well as the higher concentrations of drugs and drug metabolites that may be present. In recent times, blood samples have become another valuable biological matrix to detect steroid misuse, although this is less common due to the invasive sample collection required. Hair\(^{43-45}\), faeces\(^{46}\), and saliva\(^7\) can also be used for the detection of drug compounds, although they are not currently used routinely.

Steroid metabolism typically proceeds by two complementary pathways, named phase I and phase II metabolism. By definition these are two distinct metabolic pathways, and although commonly occur in concert, they are known to occur independently as well. Phase I metabolic processes typically involve functional group manipulations such as hydrolysis, oxidation, reduction, or hydroxylation. Metabolism primarily occurs on the steroid A- and D-rings, although if these positions are inaccessible (as is often the case in designer steroids) then the B- and C-rings can be metabolised\(^{46,47}\). Of particular interest to the equine metabolism of
anabolic steroid compounds is the tendency for C3-ketone reduction, and C16-hydroxylation to occur, particularly if the C17-position is alkylated\textsuperscript{46}. Additionally, A-ring metabolism is commonly inhibited through extended conjugation as is seen in the case of the steroid trenbolone (17\(\beta\)-hydroxyestra-4,9,11-trien-3-one) which produces multiple hydroxylated metabolites\textsuperscript{46}. The phase I metabolism of these compounds can also have an effect on their biological activity, as it has been reported in androgen bioassay studies that the metabolism of anabolic steroids can either activate or deactivate the steroid molecule\textsuperscript{41,48}.

Phase II metabolism involves the conjugation of highly polar groups to the steroid metabolites. The conjugation of small, charged or highly polar compounds to the hydrophobic steroid backbone confers an increase in the aqueous solubility of these compounds, allowing for their rapid and efficient excretion \textit{via} the urine. The two main phase II steroid conjugation pathways are glucurononylation and sulfation; steroid glucuronide metabolites form \textit{via} enzyme-mediated transfer of glucuronic acid from a uridine diphosphate glucuronic acid (UDPGA) donor, while steroid sulfate metabolites form \textit{via} enzyme-mediated transfer of sulfate from a 3'-phosphoadenosine-5'-phosphosulfate (PAPS) donor\textsuperscript{47}. Minor conjugates of other small molecules are also known including phosphate, sugars, and amino acids\textsuperscript{49,50}, although they are considered minor components in anti-doping analysis and are not routinely investigated. Although phase II conjugates are the primary components identified from urine, unconjugated metabolites can also be observed. Conjugation can also occur in the absence of phase I metabolism if metabolism is hindered, or if the compound already possesses suitable functionality\textsuperscript{47}.

\textbf{\textit{In vitro} equine steroid metabolism}

Since anti-doping analysis requires knowledge of the metabolites that indicate steroid misuse, the metabolism of each steroid must be studied in order to determine which metabolites(s) may be the most suitable markers for detection. \textit{In vivo} administration is a staple method for the study of steroid metabolism, however \textit{in vitro} methods are gaining popularity\textsuperscript{46}. Additionally, the Association of Official Racing Chemists (AORC) criteria currently allow for the use of \textit{in vitro}-
derived materials as standards for confirmatory analysis, providing additional motivation for developing in vitro systems to model equine steroid metabolism. The key concern regarding the use of in vitro methods is how accurately they reflect the metabolism observed in vivo and for this reason they typically accompany administration studies to allow comparison between in vivo and in vitro systems.

In order to model the liver, which is the principle organ involved in detoxification and metabolism of exogenous substances, preparations involving liver metabolic enzymes are amongst the most commonly used in vitro methods. Equine liver microsomes and S9 fraction are most commonly used to model equine metabolism due to their ease of use and commercial availability. These enzymes are typically supplemented with a number of biological co-factors, including nicotinamide adenine dinucleotide (NADH), or nicotinamide adenine dinucleotide phosphate (NADPH) in order to promote the metabolic reactions. Most systems use an excess of these reagents, although systems have been developed which employ co-factor regeneration, in which a NADH/NADPH-generating reaction is coupled to the NADH/NADPH-dependant metabolic reactions. Typically, the reaction of glucose-6-phosphate (G6P) with a glucose-6-phosphate dehydrogenase (G6PDH) enzyme is used, which regenerates NADH/NADPH as a by-product of oxidation. This allows for the use of a catalytic amount of these co-factors in the metabolism reaction. Recent reports have also demonstrated the practicality of using homogenised whole liver tissue to perform in vitro studies, in an effort to closely replicate in vivo metabolism. These studies have even demonstrated the ability to generate phase II metabolites without the use of expensive phase II co-factors such as UDPGA or PAPS. The limitations of this approach are reflected in the sample preparation of the whole liver extracts, as well as potential variations in the metabolic profile due to the their use of individual tissue donors. Other approaches to model equine metabolism involve microbial systems which are typically based on equine faecal bacteria, and although these are currently largely unimportant for modelling equine steroid metabolism in an anti-doping context, they may be important in other areas.
Anti-doping screening for designer anabolic steroids

Historically, steroid metabolites have been detected in analytical samples by thin layer chromatography coupled to ultra-violet detection (TLC-UV) 66. This was largely superseded by the development of high performance liquid chromatography-ultraviolet detection (HPLC-UV) which was popular until the 1980-1990's, although still finds use in specialised anti-doping applications 46. With the development of gas-chromatography coupled to mass spectrometry (GC-MS), these instruments became the method of choice in anti-doping laboratories 11. In recent years a range of other techniques have become available and occasionally find use in anti-doping laboratories alongside existing methods. Biological assays 11,67–70 can be used to detect doping directly from samples. For example, if a sample gave a positive result from an androgen receptor assay, it may indicate that anabolic agent(s) are present in the sample which would warrant further testing. In most cases, these bioassay techniques can be sufficiently general to allow for detection of certain steroid classes or highly specific for certain steroid compounds, although they often do not provide an opportunity to confirm the identity of any detected compounds 69. Building on this idea, metabolomics approaches are also gaining popularity as they allow for the high-throughput detection of minor variations of a very large number of biomarkers in response to drug administration 71, making it extremely difficult to hide instances of steroid misuse. Nuclear magnetic resonance spectroscopy (NMR) occasionally finds use in anti-doping laboratories as it can be used to unequivocally determine metabolite structure, however is severely limited in most cases as it requires extensive sample purification and large sample volumes to be effective 46.

Whilst GC-MS has been the mainstay for analytical laboratories, in recent years there has been a movement towards liquid-chromatography coupled to mass spectrometry (LC-MS) which offers several advantages over GC-MS analysis 11. GC-MS requires often laborious sample preparation including purification, hydrolysis of the phase II urinary conjugates, and chemical derivatisation to a more volatile species prior to analysis. Trimethylsilyl (TMS) ethers or other silyl derivatives are among the derivatives most widely used in laboratories due to their stability, ease
of preparation, and characteristic fragmentation patterns. In contrast, LC-MS analysis has the advantage of not requiring these preparatory steps allowing much higher throughput in sample analysis. Additionally, LC-MS allows the direct detection of intact phase II conjugates, which may offer advantages in the detection of some steroid compounds which are primarily excreted as these conjugates and may otherwise only be detected indirectly by GC-MS methods after hydrolysis and derivatisation. LC-MS is not a complete replacement for GC-MS however, as the analysis of some compounds is difficult with LC-MS, including the study of saturated steroids and steroid diols, which ionise poorly under the electrospray ionisation (ESI) and atmospheric-pressure chemical ionisation (APCI) conditions common to LC-MS systems. Additionally, the characteristic fragmentation patterns observed using electron ionisation (EI) common to GC-MS systems may often provide more diagnostic information about metabolite structure compared to the softer ionisation techniques common to LC-MS.

Recent advances in modern instrumentation which allow tandem MS experiments (MS^n) afford a greater ability to detect and identify metabolites. This is due to the ability of these systems to perform a multitude of scan types including: full scan MS, multiple reaction monitoring (MRM), product-ion or precursor-ion MS, or neutral loss experiments. This is in contrast to older technologies which often rely on full-scan and single-ion monitoring (SIM) techniques in order to achieve the required sensitivity for detection. Additionally, the recent development of affordable liquid-chromatography high resolution accurate mass spectrometry (LC-HRAM) technologies has greatly assisted anti-doping laboratories. Data obtained with these systems is of very high quality and at sufficient mass resolution that minute differences in molecular composition can be detected. The increase in sensitivity is afforded through the use of a much narrower mass window (±10-15 ppm) for mass detection compared to standard triple quadrupole detection (±1 Da). As a result, this can allow laboratories to undertake retrospective analysis of newly-identified compounds in historically acquired data, allowing detection of previously unidentified compounds.
The most recent advances in anti-doping research come from new developments in “untargeted” or “open” screening methods. Such methods typically attempt to screen for characteristic fragments, or fragmentation modes (such as characteristic neutral losses) of steroid compounds, rather than the steroid compound itself \(^{75-78}\). This would prevent minor structural changes to a molecule which results in a change of the molecular mass from rendering a compound undetectable, and highlight the need for follow-up testing to identify the new target compound. Another promising advancement is the Equine Biological Passport (EBP) \(^{79}\), which mirrors the Athlete Biological Passport (ABP) maintained by WADA for human athletes \(^{80}\). The EBP would longitudinally monitor the concentration of certain biological compounds in the horse, and allow for the detection of changes in these levels in response to anabolic steroid misuse. Such an approach would assist greatly in establishing relevant threshold levels for endogenous compounds, as well as assisting in the detection of designer anabolic steroids. Although there are a number of technical and administrative hurdles to overcome before this can be fully realised, such as identification of the relevant equine biomarkers and harmonisation of the testing protocols and data analysis between racing jurisdictions, this approach has the potential to effectively combat the misuse of drugs in equine sports. Such methods are likely to be essential to combat the rapid increase of designer steroids, and other unknown compounds into the future.

**Equine metabolism of designer steroids**

Anabolic steroids are amongst the largest class of substances used to enhance performance in competitive equine sports and as a consequence the majority of the attention has been directed towards developing methods to detect these compounds in equine systems. Such methods typically rely on screening for known metabolites and markers of steroid misuse \(^{46}\) and as such, understanding the metabolism of these compounds in equine systems is vital to developing methods for the detection of these compounds. The metabolism of all anabolic androgenic steroid compounds in horses is far beyond the scope of this review, however a substantial summary can be found in several reviews by Scarth \textit{et al} \(^{81}\) (endogenous anabolic steroids in horses and other animals), Teale \textit{et al} \(^{82}\) and
Houghton *et al* 83 (phase I and II metabolism studies of some common synthetic steroids marketed as pharmaceuticals), and Scarth *et al* 46 (a comprehensive review of drug metabolism in horses). Instead the purpose of this review is to explore recent advances in the study of designer anabolic steroids as well as other anabolic steroids present in many of the dietary supplements available online, which have not been covered in previous reviews. A summary of the metabolism of these compounds in equine systems in presented below. The designer steroids desoxyvinyltestosterone 84, and estra-4,9-diene-3,17-dione 85 have been studied in equine systems as detailed by Scarth *et al* 46, and are not included in the present review.

**Table 1: Summary of the equine metabolism of designer steroids**

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Phase I and Phase II metabolites</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>11-Adrenosterone (Ref 52)</td>
<td>Predominantly C3/C11/C17 mono/di/tri-reduced. Minor hydroxylated metabolites. Phase II metabolism not studied.</td>
<td><strong>Recommended analyte(s):</strong> Reduced 11-adrenosterone, 11β-hydroxyandrosterone threshold of greater than 10 µg/mL, or a 11β-hydroxyandrosterone:11β-hydroxyetiocholanolone ratio of greater than 20:1 have been suggested based on human studies. <em>In vitro</em> metabolism only. 11-Adrenosterone and its metabolites are potentially endogenous, may require threshold for detection.</td>
</tr>
<tr>
<td>ATD (Ref 52,59)</td>
<td>Predominately C17-reduced. Minor C16-hydroxylated and mono/di-reduced metabolites. C17β-sulfate and C17α-glucuronide metabolites. Mixture of both sulfate and glucuronide minor metabolites.</td>
<td><strong>Recommended analyte(s):</strong> 17β-hydroxyandrosta-1,4,6-trien-3-one, and 17β-hydroxyandrosta-4,6-dien-3-one. Elevated testosterone observed <em>in vivo</em>, suggesting aromatase inhibition Boldenone observed as an <em>in vivo</em> metabolite.</td>
</tr>
<tr>
<td>Substance</td>
<td>Identification</td>
<td>Metabolites</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>-------------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>3α-Chloro-17α-methyl-5α-androstan-17β-ol (Ref \textsuperscript{37})</td>
<td>3α-Chloro-17α-methyl-5α-androstan-16α,17β-diol identified as the sole equine metabolite. Phase II metabolism not studied.</td>
<td>Recommended analyte(s): 3α-Chloro-17α-methyl-5α-androstan-16α,17β-diol, and 17α-methyl-5α-androstan-3α,17β-diol. In vitro metabolism only. Potential elimination of C3-chloride to form madol.</td>
</tr>
<tr>
<td>Halodrol (Ref \textsuperscript{52})</td>
<td>Turinabol observed as the major metabolite. Minor C3-oxidised and hydroxylated metabolites. Phase II metabolism not studied.</td>
<td>Recommended analyte(s): Turinabol (4-chloro-17β-hydroxy-17α-methylandrosta-1,4-diene-3-one). In vitro metabolism only.</td>
</tr>
<tr>
<td>20-Hydroxyecdysone (Ref \textsuperscript{52})</td>
<td>No phase I metabolism observed. Phase II metabolism not studied.</td>
<td>Recommended analyte(s): 20-Hydroxyecdysone. In vitro metabolism only. Phase II metabolism likely to predominate in vivo.</td>
</tr>
<tr>
<td>Formestane (Ref \textsuperscript{52,53})</td>
<td>Predominately C17-reduced, with minor C3/C17 direduction. Predominately glucuronide conjugated.</td>
<td>Recommended analyte(s): Formestane, and 4,17β-dihydroxyandrost-4-en-3-one. No aromatase inhibition observed in vivo.</td>
</tr>
<tr>
<td>Furazadrol (Ref \textsuperscript{54})</td>
<td>Major in vivo metabolites: furazadrol sulfate and furazadrol glucuronide. Minor hydroxylated and, oxidised and hydroxylated metabolites Predominately sulfate conjugated.</td>
<td>Recommended analyte(s): Furazadrol, furazadrol 17-sulfate, and furazadrol 17-glucuronide.</td>
</tr>
<tr>
<td>Methasterone (Ref \textsuperscript{52})</td>
<td>Predominately C3-reduced. Minor hydroxylated metabolites. Phase II metabolism not studied.</td>
<td>Recommended analyte(s): 2α,17α-dimethyl-5α-androstan-3α/β,17β-diol. In vitro metabolism only.</td>
</tr>
</tbody>
</table>

In vitro metabolism appears limited in its ability to predict in vivo metabolism.

Δ1-Testosterone (Ref 86) | Range of mono/direduced metabolites. Minor hydroxylated and reduced metabolites. Mixture of sulfate and glucuronide conjugates. | Recommended analyte(s): Δ1-Testosterone. Some metabolites are potentially endogenous.


Trestolone (Ref 57) | 7α-methylestradiol, 7α-methylestrone, and two unidentified metabolites. Phase II metabolism not studied. | Recommended analyte(s): Trestolone, 7α-methylestradiol, and 7α-methylestrone. 

In vitro metabolism only. Aromatase inhibitor.

11-Adrenosterone (androst-4-ene-3,11,17-trione)

In humans, 11-adrenosterone is an endogenous steroid produced predominately in the adrenal cortex that exerts a mild anabolic effect 88. It has been reported as a component of dietary supplements such as 11-0XO (ErgoPharm) 88 and is marketed as a “selective cortisol modulator” rather than as an anabolic agent. It is a known inhibitor of the enzyme 11β-hydroxysteroid dehydrogenase type 1, which is required for the formation of cortisol from cortisone 88. Inhibition of cortisol biosynthesis may offer athletes a competitive advantage as cortisol itself is involved in a range of biological processes including fat and protein metabolism,
regulation of the immune system, and responding to stress. It has been banned in competition by both WADA and IFHA. The endogenous concentrations of this compound in the horse is currently unknown, however based on a previously reported human in vivo administration it has been suggested that a 11β-hydroxyandrosterone threshold of greater than 10 µg/mL, or a 11β-hydroxyandrosterone:11β-hydroxyetiocholanolone ratio of greater than 20:1, or the use of GC-IRMS may be indicative of 11-adrenosterone misuse.

The equine metabolism of 11-adrenosterone has only been studied by in vitro methods. Following metabolism with equine liver microsomes and S9 fraction, one major reduced metabolite was observed by LC-MS/MS. Minor metabolites were also observed including: one minor reduced metabolite, one direduced metabolite, one trireduced metabolite, two hydroxylated metabolites, and one reduced and hydroxylated metabolite. Metabolites were tentatively assigned structures based on analysis of their LC-HRAM product-ion spectra. An additional major reduced metabolite was observed by GC-MS/MS. Although the endogenous nature of 11-adrenosterone in humans has been established, it is currently unclear if this compound is endogenous in horses. An older study has proposed 11-adrenosterone as a metabolite of cortisol in the synovial fluid of human and equine knee joints after cortisol administration, however no studies have identified it directly. Due to its potential endogenous nature, a threshold approach may be required in order to confirm misuse of this compound.

**ATD (androsta-1,4,6-triene-3,17-dione)**

ATD is an aromatase inhibitor that has been reported as a component of dietary supplements including Attitude (SAN Nutrition) and Novedex XT (Gaspari Nutrition). Aromatase inhibitors irreversibly and covalently bind to the active site of the P450 enzyme aromatase, which is essential in the conversion of androgens into estrogens. Inhibition of this enzyme can be used to limit the endogenous conversion of androgens such as testosterone into estrogens, in turn increasing the concentration of AAS in the body. Although aromatase inhibitors have legitimate therapeutic applications such as in the treatment of human breast and ovarian cancers, they can be exploited to gain muscle mass.
as they can increase the effects of endogenous or co-administered anabolic steroids. They can also potentially alleviate some of the side effects of anabolic steroid misuse\(^{53,97}\). As a result they are banned in competition by both WADA and IFHA\(^{13,91}\).

The equine metabolism of ATD has been studied by \textit{in vitro} methods\(^{52}\). Following metabolism with equine liver microsomes and S9 fraction, two reduced metabolites (tentatively assigned as C17-isomers), and one reduced and hydroxylated metabolite (tentatively assigned as C15, or C16-hydroxylated) were identified. Minor metabolites including three direduced metabolites, three hydroxylated metabolites, five additional reduced and hydroxylated metabolites, and three hydroxylated and direduced metabolites were also observed. The structures of the major metabolites were tentatively assigned based on their LC-HRAM product-ion spectra. In addition, one of the direduced metabolites was identified as boldenone (17\(\beta\)-hydroxyandrosta-1,4-dien-3-one) by comparison to a reference material. The authors recommend analysis by LC-MS over GC-MS due to the detection of several C19-nor steroid artefacts that resulted from TMS derivatisation prior to GC-MS analysis\(^{52,98}\), and also due to the higher sensitivity of detection by LC-MS.

The metabolism of ATD has also been studied \textit{in vivo} by a controlled oral administration\(^{59}\). Following phase I metabolism, fourteen metabolites were identified by LC-HRAM analysis including: three reduced metabolites (two C17-reduced and C1-C2 reduced), three direduced metabolites (C1-C2 and C17-direduced, C1-C2 and C3-direduced, and boldenone), four reduced and hydroxylated metabolites, and four direduced and hydroxylated metabolites (two C5-C6 and C17 direduced with C16-hydroxylation, and two C1-C2 and C17-direduced and hydroxylated metabolites). A number of these were matched to reference materials. The identities of the phase I metabolites not matched to standards were tentatively assigned by analysis of the LC-HRAM product-ion spectra. The position of hydroxylation in these metabolites was not assigned where standards were not available, although the authors comment on the
presence of MS fragments at $m/z$ 149 and 167 being characteristic of D-ring hydroxylation which suggests C16-hydroxylation as a major pathway in the metabolism of these compounds. A pair of metabolites resulting from C1-C2 and C17-direduction and hydroxylation were observed that had not been previously detected in human studies. Elevated levels of testosterone were also observed which were above the threshold levels required for a positive testosterone doping result.

Phase II metabolites were observed directly as a mixture of sulfate and glucuronide conjugates, and a minority were identified by comparison to the products generated from \textit{in vitro} metabolism with homogenised horse liver. Phase II metabolites were also identified indirectly by hydrolysis of the fractionated glucuronide and sulfate metabolites. Parent ATD was excreted primarily unconjugated, whilst one C17-reduced metabolite was identified as the sulfate conjugate, and the remaining C17-reduced metabolite as the glucuronide conjugate. The authors comment that the C17-glucuronide is likely to be the C17α-stereochemistry, which agrees with observations reported by many as summarised by Scarth \textit{et al}. Sulfate conjugates were also identified for boldenone, and for the C1-C2 and C17-direduced metabolite which were tentatively assigned C17β-stereochemistry. Glucuronide conjugates were observed for the C1-C2 and C3-direduced, and both two C5-C6 and C17 direduced and hydroxylated metabolites. The remaining metabolites were observed as mixtures of both sulfate and glucuronide conjugates. Some metabolites were identified up to 77 hr post-administration, and the authors recommend 17β-hydroxyandrosta-1,4,6-trien-3-one, and 17β-hydroxyandrosta-4,6-dien-3-one as potential target analytes for screening due to their long detection windows, and commercial availability.

As a part of this study, a comparative phase I \textit{in vitro} metabolism was performed using homogenised equine liver. Twelve of the metabolites observed \textit{in vivo} were identified \textit{in vitro} in addition to both boldione (androsta-1,4-diene-3,17-dione) and epiboldenone (17α-hydroxyandrosta-1,4-dien-3-one) which were identified by
comparison to reference materials. Interestingly, elevated levels of testosterone were not observed in vitro suggesting that testosterone is not a direct metabolite of ATD but rather a result of aromatase inhibition. These results, along with the in vivo study agree well with the previously discussed in vitro study by Clarke et al. 32.

3α-Chloro-17α-methyl-5α-androstan-17β-ol

3α-Chloro-17α-methyl-5α-androstan-17β-ol is an anabolic steroid containing C3-chlorination, which was identified alongside the 3β-chloro isomer (5:2 mixture) in red-and-black capsules containing white powder seized in 2012 by law-enforcement in Queensland, Australia. This compound had not been previously reported in the literature, and it appears to be the first instance of a C3-halogenated anabolic steroid intended for doping purposes. It is currently banned in competition by both WADA and IFHA, due to structural similarity to known anabolic steroid compounds 13,91.

Owing to concerns about its chemical reactivity and potential toxicity, this compound was only studied using in vitro systems. Yeast, HEK293 and HuH7 androgen receptor bioassays have found the potency of the major 3α-chloro isomer to be similar to testosterone (87-147% potency), whilst the 3β-isomer gave much lower potency (2-9%). Acute cellular toxicity was not observed in yeast and HEK293 cell lines. In vitro metabolism studies on the 3α-isomer using human and equine liver S9 fraction identified differences in metabolism which may be useful for doping control. Equine in vitro metabolism afforded 3α-chloro-17α-methyl-5α-androstan-16α,17β-diol as the sole observed metabolite, the structure of which was matched against synthetic reference material. The relative abundance isotope pattern of \(^{35}\text{Cl}\) and \(^{37}\text{Cl}\) (3:1) confirmed retention of C3-chlorination in the equine metabolite. The stereochemistry of the C16-hydroxylation was supported by \(^1\text{H}\) NMR, and the failure to form a C16-C17 cis-acetonide derivative, with the C16α-C17α-isomer used as a control to demonstrate the efficiency of the transformation. Human in vitro metabolism afforded 17α-methyl-5α-androstane-3α,17β-diol, lacking C3-chlorination, which was not observed in the equine system. This
metabolite was confirmed by comparison to both the 3α-hydroxy and 3β-hydroxy reference materials, and is a known metabolite of a number of other methylated anabolic steroid compounds such as methyltestosterone and mestanolone (17β-hydroxy-17α-methylandrostan-3-one) 46. As such this metabolite is likely detectable by existing methods. The authors recommend laboratories monitor for the 3α-chloro-17α-methyl-5α-androstane-16α,17β-diol, and 17α-methyl-5α-androstane-3α,17β-diol metabolites in routine screening to detect the misuse of this compound.

**Halodrol (4-chloro-17α-methylandrosta-1,4-diene-3,17β-diol)**

Halodrol is a 4-chlorinated steroid structurally similar to clostebol and turinabol 46,52 which has been found a variety of dietary supplements such as Halodrol (Gaspari Nutrition), Zeus (BioArmor) and Iron Dragon (BioArmor). These supplements typically list only the 3β-hydroxy isomer on their labelling but typically contain a mixture of both stereoisomers. Halodrol alongside its analogues clostebol and turinabol, are banned in competition by both WADA and IFHA 13,91.

This equine compound has only been studied using in vitro systems 52. A mixture of halodrol isomers (3:2, α:β) was subjected to phase I metabolism using both equine liver microsomes and S9 fraction. Metabolism identified the C3-oxidised metabolite (turinabol, 4-chloro-17β-hydroxy-17α-methylandrosta-1,4-diene-3-one) and three oxidised and hydroxylated metabolites as major metabolites. Minor metabolites were observed including, two A-ring reduced and C3-oxidised metabolites, one hydroxylated metabolite, one A-ring reduced and hydroxylated metabolite, two additional oxidised and hydroxylated metabolites, one dihydroxylated metabolite, one reduced and dihydroxylated metabolite, one direduced and dihydroxylated metabolite, and two oxidised and dihydroxylated metabolites. Metabolites were tentatively assigned based on their LC-HRAM, or GC-MS/MS product-ion spectra. The parent compounds (3α/β,17-diols), and the major oxidised and hydroxylated metabolites were reported to ionise poorly under APCI.
conditions which are commonly used for LC-MS analysis, however ionised well under GC-MS (+EI) conditions after TMS derivatisation. There were minor differences reported between the two metabolism systems used, with equine liver S9 appearing to give more of the major metabolites, which the author suggested may highlight the importance of cytosolic enzymes in the metabolism of these compounds. The predominate product was the C3-oxidised metabolite, which corresponds to the synthetic anabolic steroid turinabol, which has been previously studied in vivo in the horse \textsuperscript{46,99}. A number of the key metabolites of halodrol reported in this study match those reported for the metabolism of turinabol. As such, the authors recommend that monitoring for turinabol misuse would likely be suitable for the detection of halodrol misuse.

20-Hydroxyecdysone

\textbf{(2\beta,3\beta,14\alpha,20\beta,22\alpha,25-hexahydroxy-5\beta-cholest-7-en-6-one)}

20-Hydroxyecdysone is an ecdysteroid hormone which is present naturally in numerous invertebrate and plant species. It is essential for moulting and reproduction in many arthropod species, and is also present as an insecticide in some plant species where it disrupts the development of insect pests that would feed upon them \textsuperscript{100}. Reports in the older steroid literature have suggested that ecdysteroids may exert a small anabolic effect in several mammal species \textsuperscript{100}, although more recent studies offer conflicting reports of their anabolic effects \textsuperscript{101,102}. Nonetheless, 20-hydroxyecdysone has been found in dietary supplements such as Oxybolin 250 (High-Tech Pharmaceuticals) and Ecdy-Bolin (Truly Huge Supplements). These supplements are often marketed as “natural”, “plant-based”, or “low-testosterone” alternatives to other anabolic steroid-containing body-building supplements. Whilst it is unclear whether supplements containing 20-hydroxyecdysone would offer a competitive advantage, they are currently banned in competition by both WADA and IFHA due to their structural similarity to known anabolic steroid compounds \textsuperscript{13,91}. 

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The equine metabolism of 20-hydroxyecdysone has only been studied by in vitro methods. Only the parent compound was observed after incubation with equine liver microsomes and S9 fraction. GC-MS/MS analysis afforded a complex mixture of partial derivatisation products and no characteristic fragmentation information, likely due to the multiple potential sites for silylation. It is known that complete silylation of related ecdysteroid compounds is slow, due to the hindered tertiary hydroxyl groups C14 and C20, with optimal silylation occurring only after extended reaction times. Related ecdysteroid compounds are known to be rapidly excreted with only minor metabolic changes in humans which could explain the lack of phase I metabolism observed in this study. The authors also suggest that phase I metabolism was expected to be a minor pathway compared to the phase II metabolism that would predominate in vivo and was not investigated as a part of this study. They also raise concerns over potential accidental dietary consumption through animal feed, as ecdysteroid compounds are known to be present in many plant species, potentially requiring a threshold approach for detection. The authors also observed minor levels of desoxy-, dehydro-, and hydroxy-metabolite impurities in their control in vitro incubations, suggesting the presence of these minor components in the commercial 20-hydroxyecdysone preparation. This could call into question other studies which identify these as metabolites. The authors recommend monitoring for the unconjugated parent compound, or its likely phase II conjugates for the detection of 20-hydroxyecdysone misuse.

Formestane (4-hydroxyandrost-4-ene-3,17-dione)

Formestane is a pharmaceutical aromatase inhibitor which irreversibly and covalently binds to the active site of the P450 enzyme aromatase. After an adverse analytical finding in 2011, the endogenous nature of formestane in horses was investigated after concerns were raised that it could potentially be a metabolite of androst-4-ene-3,17-dione, an intermediate in testosterone biosynthesis. Additionally, it is a potential metabolite of 4,17β-dihydroxyandrost-4-en-3-one, an anabolic steroid banned in competition by WADA and IFHA. Analysis of the data obtained during routine screening for...
269 equine urine samples showed that formestane was not present endogenously. Following this, an in vivo controlled oral administration study of formestane was undertaken. After phase I metabolism, the parent compound as well as seven metabolites were identified as follows: one reduced metabolite (4,17β-dihydroxyandrost-4-en-3-one) which was matched to a reference material, two direduced metabolites (proposed as androst-4-ene-3α,4,17β-triol, and androst-4-ene-3β,4,17β-triol) which were matched to the products derived from partial reduction of formestane with sodium borohydride, and an additional four direduced metabolites (four of the possible 3,4-dihydroxy-5-androstan-17-one metabolites) which were tentatively identified by comparison to literature data. Elevated levels of testosterone or other androgens not considered to be metabolites of formestane were not identified in this study. The structures of the phase II metabolites were determined through hydrolysis of the fractionated glucuronide and sulfate metabolites to their corresponding phase I metabolites. Formestane, 4,17β-dihydroxyandrost-4-en-3-one, and three of the 3,4-dihydroxy-5-androstan-17-one metabolites were excreted primarily as glucuronide conjugates, whilst androst-4-ene-3α,4,17β-triol, androst-4-ene-3β,4,17β-triol, and the remaining 3,4-dihydroxy-5-androstan-17-one metabolite were excreted as a mixture of glucuronide and sulfate metabolites. In addition to identifying the key metabolites, the excretion profiles of formestane and 4,17β-dihydroxyandrost-4-en-3-one were studied. Peak excretions of 40-44 µg/mL and 7-11 µg/mL respectively were observed at 5.6-6.3 hr post-administration, falling to below the limits of detection at 29 hr and 34 hr post-administration respectively. In addition, formestane was observed in plasma peaking at 6-10 hr post-administration, and falling below the limits of detection 34 hr post-administration. No other formestane metabolites were observed in equine plasma. As a part of this study, a comparative in vitro study was also undertaken. Phase I metabolism using equine liver microsomes prepared from whole liver tissue afforded the parent compound and seven metabolites, identified as follows: 4,17β-dihydroxyandrost-4-en-3-one, five direduced metabolites (androst-4-ene-3α,4,17β-triol, androst-4-ene-3β,4,17β-triol, and three of the possible 3,4-dihydroxy-5-androstan-17-one metabolites), and one trireduced metabolite. The
majority of the metabolites observed in vitro matched those observed in vivo after hydrolysis of the phase II conjugates.

A separate in vitro study has also been performed by Clarke et al, who identified a similar metabolite profile. LC-HRAM and GC-MS/MS analysis observed several hydroxylated metabolites, in addition to metabolites similar to those reported above. Metabolites were not identified by comparison to reference materials in this study, but instead were tentatively assigned by analysis of their MS data. Based on observations from both studies, the authors recommend monitoring for the parent compound, and 4,17β-dihydroxyandrost-4-en-3-one, to detect formestane misuse.

**Furazadrol ([1',2']isoxazolo[4',5':2,3]-5α-androstan-17β-ol)**

Furazadrol is a derivative of dihydrotestosterone containing an isoxazole ring fused to the steroid A-ring. It has been reported as a component of dietary supplements such as Orastan-A (Gaspari Nutrition) and Furazadrol (Axis Labs) predominately as the tetrahydropyranyl ether. In both cases, these supplements had incorrect labelling of the content information. Furazadrol has been reported to exert anabolic activity in the older literature and also in more recent yeast and human HuH7 androgen bioassays. Related isoxazole-containing anabolic steroids, including the structurally similar Danazol, are banned in competition by both WADA and IFHA.

Following an equine in vivo controlled oral administration, furazadrol was excreted primarily as the sulfate and glucuronide conjugates without phase I metabolism, which were detectable up to 24 hr post-administration by LC-HRAM analysis. Additional minor metabolites were also observed including a hydroxylated sulfate metabolite, two oxidised and hydroxylated sulfate metabolites, epifurazadrol glucuronide, and an oxidised and hydroxylated glucuronide metabolite. No unconjugated furazadrol metabolites were observed.

The identity of furazadrol 17-sulfate, furazadrol 17-glucuronide, and epifurazadrol...
17-glucuronide was confirmed by comparison to synthetic reference materials\textsuperscript{107,108}, and the identity of the other metabolites was tentatively assigned through analysis of the LC-HRAM product-ion spectra. The sites of hydroxylation for minor metabolites were not identified in this study. Further structural confirmation was performed through enzymatic hydrolysis of the fractionated sulfate and glucuronide metabolites, with analysis of the corresponding phase I metabolites as above. Hydrolysis of the sulfate fraction was achieved through use of \textit{Pseudomonas aeruginosa} arylsulfatase, which is a purified enzyme with sulfatase activity and no alternative activities as are commonly found in commercial sulfatase preparations\textsuperscript{109}. This enzyme was observed to completely hydrolyse all the \textit{in vivo} steroid sulfate metabolites identified in this study. The major urinary metabolites (furazadrol 17-sulfate and furazadrol 17-glucuronide) were quantified in equine urine, to determine the detection window and limits of detection for these analytes. The authors recommend monitoring for these analytes or their hydrolysed phase I counterpart furazadrol for the detection of furazadrol misuse.

As a part of this study, a comparative \textit{in vitro} phase I metabolism study was also undertaken using equine liver S9 fraction. A number of the metabolites identified were reported to match those observed from the \textit{in vivo} profile obtained after hydrolysis of the urinary sulfate and glucuronide conjugates. The major phase I markers were observed in this study, although the authors comment that the \textit{in vitro} study was limited in its ability to fully replicate metabolism \textit{in vivo}. Phase I metabolism identified a number of metabolites including epifurazadrol, oxidised furazadrol, eight hydroxylated metabolites, an oxidised and hydroxylated metabolite, and two dihydroxylated metabolites, which were confirmed by comparison to synthetic reference materials where available, or tentatively assigned through analysis of the LC-HRAM product-ion spectra.
Methasterone (17β-hydroxy-2α,17α-dimethyl-5α-androstan-3-one)

Methasterone is a dimethylated analogue of dihydrotestosterone, and the C17-methylated analogue of drostanolone. It has been reported in the older steroid literature to exert a strong anabolic effect in rats\textsuperscript{110,111}, and predicted to be a potent anabolic agent in a more recent computational study\textsuperscript{112}. It has been reported as a component of the dietary supplements Superdrol (Anabolic Xtreme)\textsuperscript{42}, S-drol (Black China Labs)\textsuperscript{113}, and Methasterone (Legal Gear)\textsuperscript{2}. Methylated steroids such as these typically have the advantage of being orally bioavailable at the cost of higher toxicity to the liver and kidneys\textsuperscript{46}. As a dimethylated steroid, methasterone has additional risks and has been reported to be involved in a number of serious health complications including cases of severe jaundice, and immunoglobulin A nephropathy\textsuperscript{42,114,115}. As a consequence, it is banned in competition by both WADA and IFHA\textsuperscript{13,91}.

The equine metabolism of methasterone has only been studied using \textit{in vitro} methods\textsuperscript{52}. Following metabolism with equine liver microsomes and S9 fraction, two C3-reduced metabolites were the predominate metabolites identified. Additionally, two minor hydroxylated metabolites and six minor reduced and hydroxylated metabolites were also observed. Metabolites were tentatively assigned based on their GC-MS/MS product-ion spectra as LC-MS/MS analysis afforded poor sensitivity for the target analytes. The authors consider the use of GC-MS/MS essential for the detection of the two reduced metabolites (2α,17α-dimethyl-5α-androstane-3α/β,17β-diol) which may indicate methasterone misuse.

Oxyguno (4-chloro-17β-hydroxy-17α-methylandrostan-4-ene-3,11-dione)

Oxyguno is an analogue of clostebol and 11-adrenosterone, and contains C17-methylation typical of orally bioavailable anabolic steroids\textsuperscript{46}. It has been reported as a constituent of the dietary supplement Oxyguno (Spectra Force Research)\textsuperscript{41,55,74} and has been reported in the older steroid literature.
to exert significant anabolic activity\textsuperscript{116}, as well as more recently in yeast, human HEK293, and human HuH7 androgen bioassays\textsuperscript{41}. Related 4-chloroandrost-4-ene compounds including clostebol and turinabol are banned in competition by both WADA and IFHA\textsuperscript{13,91}.

Following an equine \textit{in vivo} controlled oral administration\textsuperscript{55}, oxyguno has been reported to be excreted as a range of unconjugated, sulfate-conjugated and glucuronide-conjugated metabolites which were detectable up to 12 hr post-administration. After phase I metabolism, the parent compound as well five novel metabolites were identified as follows: two C11-reduced metabolites, a C3 and C4-direduced metabolite, a C20-hydroxylated metabolite with C3, C4 or C11-direduction, and C17-epi-oxyguno. The identities of these metabolites were tentatively assigned by analysis of their GC-MS/MS product ion spectra. The two C11-reduced metabolites and the hydroxylated and direduced metabolite were observed as glucuronide conjugates, whilst the C3 and C4-direduced metabolite was observed as both glucuronide and sulfate conjugates. The C17-epi-oxyguno metabolite was observed to arise from the sulfate conjugate. Epimerisation at the tertiary centre is a known metabolic pathway in C17-methylated steroids, and occurs \textit{via} hydrolysis of a tertiary sulfate metabolite\textsuperscript{117–119}. The tentative identities of these metabolites were established by LC-HRAM analysis of the intact conjugates, with further structural confirmation afforded by GC-MS/MS analysis of the enol-TMS ether derivatives obtained from the hydrolysis of the fractionated glucuronide and sulfate metabolites. The excretion of free oxyguno was also quantified to establish an elimination profile of the drug and determine a suitable detection window. Excretion peaked at 1-3 h and 2 h in blood and urine respectively, and fell below the limit of detection (LOD) at 7 h and 12 h respectively. The authors recommend monitoring for the parent compound, or the direduced metabolite 4-chloro-3,17β-dihydroxy-17α-methyl-5α-androstan-11-one for the detection of oxyguno misuse.

As a part of this study, a comparative \textit{in vitro} study was also undertaken. Phase I metabolism using equine liver microsomes prepared from whole liver tissue
afforded four primary metabolites including two C11-reduced metabolites, a C20-hydroxylated metabolite, and a C20-hydroxylated metabolite with C11-reduction. The tentative identities of these metabolites were established by analysis of the GC-MS product-ion spectra of the enol-TMS ether derivatives of the in vitro metabolites. The major C11-reduced metabolites were reported to be identical to the C11-reduced metabolites observed after hydrolysis of the in vivo samples. The metabolic profile generated by in vitro techniques did not agree well with the in vivo profile. Only two of the five in vivo metabolites were identified, and the recommended screening marker 4-chloro-3,17β-dihydroxy-17α-methyl-5α-androstan-11-one was not observed, suggesting this in vitro method may be limited in its ability to predict in vivo results.

**Δ1-Testosterone (17β-hydroxy-5α-androst-1-en-3-one)**

Δ1-Testosterone is a synthetic anabolic steroid which closely resembles the structure of testosterone, substituting the C4-C5 double bond for a C1-C2 double bond. It can also be viewed as a 5α-reduced form of boldenone, another anabolic steroid which is well-known for its abuse in sports. It has been reported to be a component of dietary supplements such as 1-androsterone (Advanced Muscle Science) and 1-AD (ErgoPharm) which typically contain Δ1-testosterone in addition to one or more of the following compounds: 5α-androst-1-ene-3,17-dione, 5α-androst-1-ene-3,17-diol, or 3β-hydroxy-5α-androst-1-en-17-one, all of which are metabolised to Δ1-testosterone in vivo. Δ1-Testosterone as well as the steroids listed above are all banned in competition by both WADA and IFHA.

Following an equine in vivo controlled oral administration, Δ1-testosterone has been reported to be excreted as a range of unconjugated, sulfate-conjugated and glucuronide-conjugated metabolites which were detectable up to 72 hr post-administration. After phase I metabolism, the parent compound as well as eight metabolites were identified as follows: four reduced metabolites (5α-androst-1-ene-3α,17β-diol, 5α-androst-1-ene-3β,17α-diol, 5α-androst-1-ene-3β,17β-diol, and
epiandrosterone (3β-hydroxy-5α-androstan-17-one)), three doubly reduced metabolites (5α-androstane-3β,17β-diol, 5α-androstane-3α,17α-diol, and 5α-androstane-3β,17α-diol), and one hydroxylated metabolite. The parent compound, 5α-androst-1-ene-3α,17β-diol, and the hydroxylated metabolite were found to be excreted primarily as sulfate conjugates, whilst 5α-androst-1-ene-3β,17α-diol and 5α-androst-1-ene-3β,17β-diol were excreted primarily as glucuronide conjugates. The remaining metabolites were observed as a mixture of both sulfate and glucuronide conjugates. The identities of the phase I metabolites were confirmed by comparison to synthetic reference materials where available, and the NIST spectral database, or tentatively assigned through a combination of their MS behaviour and relative elution order. Hydroxylation at C16 is a common pathway for steroid metabolism, and the authors rationalise the observed hydroxylated metabolite on this basis. The metabolites of Δ1-androgens are also known to have similar mass spectra, which may complicate analysis. The identity of the phase II metabolites was further confirmed through the hydrolysis of the fractionated glucuronide and sulfate metabolites to their corresponding phase I metabolites and their identities assigned as stated above. A number of the observed metabolites were identified up to 72 hr post-administration, however the authors comment that these are also potential in vivo metabolites for endogenous steroid compounds such as testosterone. The metabolites containing the C1-C2 double bond are characteristic of administration of Δ1-testosterone, but were observed only 2-6 hr post-administration. The authors recommend monitoring for the parent compound, which can be detected at low levels (5-9 ng/mL) up to 30 hr post-administration. Additionally, longer term detection may be possible by adopting thresholds for the endogenous metabolites, or using longitudinal monitoring of the steroid profile.

As a part of this study, a comparative in vitro study was also undertaken. Phase I metabolism using equine liver microsomes prepared from whole liver tissue afforded six metabolites: three reduced metabolites (5α-androst-1-ene-3α,17β-diol, 5α-androstane-3β,17β-diol, and epiandrosterone), two hydroxylated metabolites, and one oxidised metabolite (5α-androst-1-ene-3,17-dione). The
majority of the observed *in vitro* metabolites appear to correlate with the phase I metabolites observed after hydrolysis of the phase II metabolites observed *in vivo*.

**Tetrahydrogestrinone**

*(18β-homo-17β-hydroxy-19-nor-17α-pregna-4,9,11-trien-3-one)*

Tetrahydrogestrinone (THG) was the second “designer” steroid ever reported and was identified during the analysis of a spent syringe containing an allegedly undetectable anabolic steroid which was provided anonymously to USADA in 2004. It can be produced chemically via a one-step reduction of gestrinone, which is a legally available progestin, and was originally produced with the express intention of bypassing current screening protocols. Since its initial discovery, it has been reported in several studies to exert strong activity in yeast and mammalian AR CALUX androgen bioassays. After its discovery, it was specifically banned in competition by WADA rather than relying on the phrasing “...and other substances with a similar chemical structure or biological effect(s).” as it represented a whole new class of threat to anti-doping analysis. It is also banned by IFHA.

Following an equine *in vivo* controlled oral administration, THG has been reported to be excreted unmetabolised. An excretion profile was established for both plasma and urinary excretion. In plasma, concentrations peaked between 1-2 hr post-administration and were below the limit of detection at 24 hr post-administration. In urine, concentrations peaked at 3-6 hr post-administration and were below the limit of detection 48 hr post-administration.

In a separate *in vitro* study using equine liver microsomes and S9 fraction, it has been reported that metabolism of THG favours formation of hydroxylated metabolites. In this study, two major hydroxylated metabolites were observed, alongside one oxidised and hydroxylated metabolite. Additional minor metabolites
were observed including a reduced metabolite, a reduced and hydroxylated metabolite, a dihydroxylated and direduced metabolite, a dihydroxylated and oxidised metabolite, two dihydroxylated and reduced metabolites, and two dihydroxylated and direduced metabolites. The sites of hydroxylation, oxidation, and reduction were not identified in this study. There appears to be little difference in the metabolites observed from incubation with equine liver microsomes or S9 fraction. The minor metabolites have relative ion abundances up to three orders of magnitude less than the primary metabolites. GC-MS analysis was complicated by the presence of numerous artefactual products resulting from enol-TMS ether derivatisation. Alternate derivatisation conditions for the formation of the enol-TMS derivatives, or the use of alternate derivatives such as TMS ether or methylloxiime-TMS ether (MOX-TMS) may alleviate some of the problems associated with derivatising conjugated enone systems. Alternatively, the authors recommend LC-MS as being most suitable for the detection of THG and many of its metabolites.

In another in vitro metabolism study using equine liver microsomes and S9 fraction, the phase I metabolism and phase II glucuronylation of THG was reported. Following phase II metabolism, two hydroxylated metabolites were observed, alongside THG glucuronide, and two hydroxylated glucuronide metabolites. Metabolites were identified by analysis of the LC-MS/MS spectra. Glucuronylation of the tertiary alcohol was observed only at low levels, presumably reflecting the sterically hindered nature of this position. The positions of hydroxylation or glucuronylation were not identified in this study. Additionally, the in vitro metabolism of several related steroid 4,9,11-trienes were reported in this study. The metabolism of gestrinone (18β-homo-17β-hydroxy-19-nor-17α-pregna-4,9,11-trien-20-yn-3-one), trenbolone (17β-hydroxyestra-4,9,11-trien-3-one), and altrenogest (17β-hydroxy-17α-(prop-2-enyl)estra-4,9,11-trien-3-one, allyltenbolone) was reported but have been covered in previous reviews and are subject to routine screening in equine anti-doping laboratories. The equine metabolism of dihydrogestrinone (18β-homo-17β-hydroxy-19-nor-17α-pregna-4,9,11,20-tetraen-3-one) has not been previously reported and following in vitro metabolism, a reduced and hydroxylated metabolite was observed alongside
dihydrogestrinone glucuronide. Although reduction could occur at a number of positions, it presumably occurs at the terminal alkene to afford hydroxylated THG, as the extended conjugation in the A-C rings typically resists metabolism \(^{46}\). This compound is likely to be encountered as an impurity in preparations containing THG resulting from the incomplete the hydrogenation of gestrinone \(^{35}\). The equine metabolism of propyltrenbolone (17β-hydroxy-17α-propylestra-4,9,11-trien-3-one) has also not been previously reported and following in vitro metabolism, three hydroxylated metabolites were observed alongside propyltrenbolone glucuronide, and three hydroxylated glucuronide metabolites.

**Trestolone (17β-hydroxy-7α-methylestr-4-en-3-one)**

Trestolone is a C7-methylated analogue of nandrolone, which itself is a C19-norsteroid analogue of testosterone. Owing to the lack of the C19-methyl substituent, this compound is more resistant to metabolism by aromatase enzymes \(^\text{127}\), increasing its potential half-life in the body. Dietary supplements labelled to contain trestolone such as TR3ST (Olympus Labs) and 7-MENT Alpha (Wyked Labs) have become available in recent years. It has been shown in bioassays with HeLa cells transfected with the human androgen receptor \(^\text{128}\), human AR CALUX bioassays \(^\text{129,130}\) and in vivo rat models \(^\text{130–132}\) to be a potent androgen, as well as exhibit strong binding to the human progesterone receptor \(^\text{128}\). Trestolone has also been recently explored for use as a human male contraceptive as it has been shown to inhibit spermatogenesis without inducing androgen deficiency \(^\text{131,133}\). It has also been shown to inhibit equine and human steroid aromatase enzymes \(^\text{57}\). This compound is banned in competition by both WADA and IFHA \(^\text{13,91}\).

The equine metabolism of trestolone has only been studied by in vitro methods \(^\text{57}\). Following incubation of tritium labelled trestolone with equine placental microsomes, four metabolites were detected. Two of the detected metabolites were determined to be estrogenic as they were extracted in a phenolic extraction assay. Additionally, they matched TLC retention factors and GC-MS fragmentation.
with reference standards for 7α-methylestradiol, and 7α-methylestrone. The identities of the two remaining metabolites were not determined, although they were hypothesised to be intermediate compounds in androgen aromatisation, such as 1-hydroxytrestolone. These metabolites were not identified in control experiments utilising 4-hydroxyandrostenedione, a known aromatase enzyme inhibitor. Additional kinetic experiments also showed that trestolone competitively inhibited the aromatisation of androstenedione and testosterone, suggesting that it may also function as an aromatase inhibitor. As a part of this study, incubations were also performed using a purified equine testicular P450 aromatase enzyme. After metabolism, these experiments also showed the presence of 7α-methylestradiol, as well as the two intermediates identified above, which were matched to the previous experiment. The authors recommend monitoring for the parent compound, alongside 7α-methylestradiol, and 7α-methylestrone for the detection of trestolone misuse. Additionally, alteration of the endogenous steroid profile due to aromatase inhibition could likely be detected through a threshold approach, or longitudinal monitoring of the equine steroid profile.

**Future Directions**

As laboratories respond to new threats, the directions of equine anti-doping research will shift, and will vary into the future. The importance of alternate testing matrices is gaining attention, with many jurisdictions incorporating these samples into routine testing. Hair has been reported to be a potentially long-term marker of steroid misuse and these samples are easily acquired and processed in the laboratory. Blood is also becoming a more common sample matrix, and it has been suggested that the levels of drug compounds in the bloodstream may indicate the potential for pharmacological effects in vivo. Additionally, since the administered drugs can often be directly detected unmetabolised in blood or hair, the need to conduct equine metabolism studies is reduced. Urine is still likely to remain a valuable analytical matrix as it is routinely used in the majority of currently available methods, and many of the important analytical thresholds for endogenous substances have only been determined in urine.
The rapid increase in the prevalence of designer anabolic steroids, present in “dietary” and “nutritional” supplements available online containing untested and unapproved anabolic agents, is likely to pose a significant threat to the integrity of the industry if left unchecked. Due to ethical concerns over the potential detrimental effects to the health and welfare of animal subjects, in vitro techniques are rapidly gaining attention as tools to study the metabolism of these compounds. These techniques, as highlighted by multiple examples throughout this review, are rapidly improving, and can more faithfully generate a metabolic profile representative of in vivo systems which will greatly assist anti-doping analysis. Currently, AORC criteria allow for the use of in vitro-derived materials as standards for confirmatory analysis, and improvements to in vitro techniques may allow for the direct detection and confirmation of in vivo phase II metabolites by LC-MS/MS methods.

Recent improvements in untargeted and open screening methods show that these methods are also gaining popularity. These methods screen for characteristic fragments, or fragmentation modes of metabolite families rather than targeting individual drug metabolites, and they have been demonstrated to be suitable for the detection of unknown compounds in analytical samples. The recent advancements made in affordable LC-HRAM and MS technologies also have greatly assisted the translation of these methods into routine screening as they allow for the acquisition of high-quality data which may be retrospectively analysed once new compounds are identified or new methods are developed. The development of more powerful computational packages provided by manufacturers also has the potential to make metabolomics approaches practical enough to be undertaken routinely in laboratories.

The final promising advancement is the development of the Equine Biological Passport, which mirrors the Athlete Biological Passport maintained by WADA for human athletes. This approach would build a baseline profile from the routine sample analysis of all samples from an individual competing horse. Any future instances of doping would then be detected by abnormal changes between the
baseline and a subsequent sample. Although there are a number of technical and administrative hurdles to overcome before this can be realised, this approach has the potential to effectively combat the misuse of anabolic steroids, and other doping agents in equine sports now and into the future.

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In the period between when this thesis was submitted (September 2016) and the corrections to this thesis were made (May 2017), this review was accepted for publication in the journal Drug Testing and Analysis. The full citation for this work is as follows:


A copy of the full text article has been reproduced in Appendix A, with permission of John Wiley and Sons via Rightslink (License Number: 3994540395974).
1.3 Project Aims

In light of the problems designer steroids are presenting to anti-doping analysis in equine sports, this project will be focused on the development of new strategies to detect designer steroids in thoroughbred horse racing. Designer steroid compounds will be selected amongst those perceived as having a significant potential for abuse due to novel structural motifs, or due to their significant availability to athletes and trainers.

Specifically, this project aims to undertake:

1) Synthesis of designer steroid reference materials and their phase I and phase II metabolites, such as hydroxylated derivatives of the parent compound, and their corresponding glucuronide and sulfate conjugates. General method development towards these classes of compounds will also be developed throughout this project.

2) \textit{In vivo} equine metabolism studies of selected high priority designer steroid targets in thoroughbred horses, subject to ethics approval.

3) Comparative \textit{in vitro} steroid metabolism studies of designer steroids in equine systems with an aim to develop tools to replicate \textit{in vivo} results.

4) Identification of the key equine metabolites of designer steroid compounds and determining their structures by comparison to synthesised reference materials, or tentative assignment through thorough analysis of their mass spectrometry behaviour.

5) Development of suitable GC-MS, LC-MS, or other assays for the rapid analysis of designer steroids in race-day samples.
CHAPTER TWO

Steroid sulfates
2.1 Foreword

The following manuscript has been published in the journal “Steroids” and details simple protocols for the rapid synthesis and purification of steroid sulfates that are suitable for adoption by analytical laboratories. Permission has been granted by Elsevier via RightsLink for the reproduction of this publication within this thesis (License Number: 3887450060394). This publication and supporting information (112 pages containing experimental procedures, characterisation data and copies of NMR and MS spectra) was authored by Mr Christopher Waller and Associate Professor Malcolm McLeod. All experimental work was undertaken by C. Waller, alongside preparation of the initial draft of the manuscript and supporting information. Assoc. Prof. McLeod initially conceived of the project, and assisted in revising the manuscript prior to submission. A small portion of this work involving the chemical synthesis of steroid sulfates with $\text{SO}_3\cdot\text{NEt}_3$, or $\text{SO}_3\cdot\text{py}$ with purification by recrystallisation (manuscript section 2.3.3), as well as some characterisation data for these compounds has been previously reported by C. Waller in an honours thesis entitled “Enzyme preparations for the efficient hydrolysis of steroid sulfates” (ANU, 2012).

Scheme 2.1: Small-scale sulfation of a steroid with purification by solid-phase extraction (SPE)
Steroid sulfates are an important class of phase II steroid metabolites that are of growing importance to anti-doping laboratories. This is driven in part by improvements in LC-MS technologies that allow for the direct detection of phase II conjugates. In anti-doping laboratories, the analysis of phase II steroid sulfate metabolites can afford greater retrospectivity for the detection of anabolic steroid misuse, and can be used to distinguish between steroids of endogenous and exogenous origin. Although there are a range of reliable methods to analyse for phase II conjugates in both humans and animals, the range of steroid sulfate reference materials is incomplete, meaning that significant steroid sulfate markers cannot be identified or confirmed in analytical samples.

In this chapter, tools have been developed which allow synthetic access to a wide range of steroid sulfate metabolites for use in anti-doping laboratories. A simple method for the preparation of steroid sulfates has been developed which utilises SPE, a technique routinely used in anti-doping laboratories, in the key purification step. This methodology has been applied to a library of sixteen steroid compounds which encompass a representative range of steroid substitution patterns and configurations. The scope of this approach was highlighted through the sulfation of 1 µg testosterone in high yield as determined by LC-MS. This method is suitable for use in analytical laboratories and should serve to expand the availability of steroid sulfate reference materials for a range of analytical applications.
2.2 A simple method for the small scale synthesis and solid-phase extraction purification of steroid sulfates
Due to copyright restrictions from the publisher, the journal article presented in section 2.2 has been removed from the online version of this thesis. The full text article can be obtained via the ANU Library, or directly from the publisher using the link below:

In response to feedback received from the examiners of this thesis, the following comments have been made.

The use of steroid sulfate standards, as described in Chapter 2.1, are suitable for use as reference materials for a range of analytical applications. However, to serve as reference standards for quantitative analysis purposes in anti-doping laboratories around the world, these materials often require certification so the absolute concentrations of these materials can be obtained.

The use of quantitative NMR (qNMR) allows for the determination of impurities present in organic compounds, which would be required to certify these compounds for use in quantitative applications in anti-doping laboratories. Although the purity of the compounds prepared in Chapter 2.1 was determined to be >98% by analysis of the obtained NMR data, no strictly quantitative analysis was undertaken. The absence of peaks which could not be attributed to the steroid sulfate molecule was determined to be sufficient evidence of purity of these compounds. The presence of impurities not observable through NMR, such as inorganic salts, can be determined through alternate analytical methods, such as elemental composition analysis. Future work to quantify these standards through the use of a suitable qNMR standard would be highly recommended, and is currently being undertaken in the McLeod group (May 2017).
In addition to the published work presented in Chapter 2.2, the following section presents related unpublished work which is the subject of a manuscript currently in preparation. Supporting information relevant to this chapter is presented electronically alongside this thesis.

2.3 Steroid bis-sulfates

2.3.1 Introduction

According to 2014 anti-doping statistics published by WADA, the detection of the misuse of endogenous anabolic steroids (EAAS) is one of the major challenges to anti-doping laboratories. Whilst the detection of high levels of EAAS may be indicative of doping, in other cases it may simply be a result of the metabolite profile of the individual athlete. As such, there is a need for methods which allow anti-doping laboratories to take this inter-individual variation into account and still be able to detect instances of misuse. Traditionally threshold levels have been used for the detection of EAAS misuse, however these often have to be set at high levels to account for athletes with naturally high levels of EAAS. It has been recognised however that EAAS levels in the athlete remain relatively stable over time but change significantly in response to steroid administration. Therefore, the use of exogenously administered EAAS can be detected by looking for the levels and differences in the ratio of various compounds in the body of the athlete over time. These ratios form the basis of the Athlete’s Biological Passport (ABP) Steroidal Module maintained by WADA, which currently monitors seven parameters: the biological concentrations of testosterone, epitestosterone, androsterone, etiocholanolone, 5α-androstane-3α,17β-diol, and 5β-androstane-3α,17β-diol, and the testosterone/epitestosterone ratio, together with the specific gravity of the athlete’s urine sample. Whilst these seven markers are quite comprehensive, it has also been shown that monitoring for additional EAAS may improve the sensitivity of screening methods in some cases.

Even with these thresholds and ratios in place, some instances of EAAS misuse may still go undetected. It has been shown that some endogenous compounds such as 3α,6β-dihydroxy-5α-androstan-17-one 3-glucuronide, and 3α,6β-dihydroxy-5β-
androstan-17-one 3-glucuronide are resistant to the enzymatic β-glucuronidase hydrolysis routinely used in anti-doping laboratories and therefore cannot be subsequently identified by GC-MS analysis. Additionally, EAAS sulfate metabolites are currently undetectable by many routine methods as these are also resistant to β-glucuronidase hydrolysis, and alternative methods such as acid hydrolysis are typically not used as they can cause a range of complications, such as increased interference from the biological matrix, or degradation of the desired analytes, which can result in lowered sensitivity. Although sulfate metabolites represent a significant proportion of steroid metabolism in some individuals, early studies of their usefulness to anti-doping laboratories has so far been shown to be limited. Nonetheless, with the growth of LC-MS analysis in anti-doping laboratories, the direct detection of phase II conjugates is becoming more important and commonplace. Monitoring for intact endogenous steroid glucuronide and sulfate metabolites may improve the sensitivity of screening methods or provide additional criteria to confirm instances of EAAS misuse. In particular, steroid bis-conjugates may benefit greatly from this technology as they are difficult to study by existing GC-MS methods. The role of steroid bis-conjugates in vivo has been explored, and steroid bis-glucuronides, bis-sulfates, and bis-sulfate glucuronides have been found to be involved in a variety of biological processes. Their importance to anti-doping laboratories however remains unclear as to date there has not been any study of the role of steroid bis-conjugates in sports doping.

**Scheme 2.2: Potential long-term bis-sulfate metabolites of 17α-methyltestosterone**

A previous study of the in vivo human metabolism of the exogenous anabolic steroid 17α-methyltestosterone identified 17β-methyl-5α-androstane-3α,17α-diol 3-sulfate as a long-term marker of abuse, with 2-3 times the retrospectivity of
previously observed markers (Scheme 2.2)\textsuperscript{17}. Although not observed in this study, this finding implicated the existence of 17α-methyl-5α-androstan-3α,17β-diol 3,17-bis-sulfate as a metabolite of 17α-methyltestosterone. Tertiary steroid sulfates are known to hydrolyse with inversion of configuration \textsuperscript{18–20}, which suggested the presence of steroid bis-sulfates as a potential source of the observed long-term metabolite. In light of these observations, this work aimed to explore the applicability of steroid bis-sulfates to anti-doping analysis through the synthesis, and mass spectrometry analysis of a representative library of steroid bis-sulfate compounds.

2.3.2 Experimental

2.3.2.1 Materials

Chemicals and solvents including sulfur trioxide pyridine complex (SO\textsubscript{3}.py), estradiol (estra-1,3,5(10)-triene-3,17β-diol) and 1,4-dioxane (dioxane) were purchased from Sigma–Aldrich (Castle Hill, Australia) and were used as supplied unless otherwise stated. Androsterone (3α-hydroxy-5α-androst-17-one), epiandrosterone (3β-hydroxy-5α-androst-17-one), etiocholanolone (3α-hydroxy-5β-androst-17-one), 11β-hydroxyepiandrosterone (3β,11β-dihydroxy-5α-androst-17-one), 11β-hydroxyandrosterone (3α,11β-dihydroxy-5α-androst-17-one), 11β-hydroxyetiocholanolone (3α,11β-dihydroxy-5β-androst-17-one), androst-5-ene-3β,17β-diol, 16α-hydroxyandrosterone (3α,16α-dihydroxy-5α-androst-17-one) and 16α-hydroxytestosterone (16α,17β-dihydroxyandrost-4-en-3-one) were purchased from Steraloids (Newport RI, USA). 3α-Hydroxytibolone (7α-methyl-19-nor-17α-pregn-5(10)-en-20-yne-3α,17β-diol) and 3β-hydroxytibolone (7α-methyl-19-nor-17α-pregn-5(10)-en-20-yne-3β,17β-diol) were purchased from Toronto Research Chemicals (Toronto, Canada). MilliQ water was used in all aqueous solutions. \textit{N,N}-Dimethylformamide (DMF) and aqueous ammonia solution were purchased from Chem-Supply (Gillman, Australia). Formic acid was purchased from Ajax Chemicals (Auburn, Australia). Solid-phase extraction (SPE) was performed using Waters (Rydalmere, Australia) Oasis weak anion exchange (WAX) 6cc cartridges (PN 186004647).
2.3.2.2 Instruments

Melting points were determined using a SRS Optimelt MPA 100 melting point apparatus and are uncorrected. $^1$H and $^{13}$C nuclear magnetic resonance (NMR) spectra were recorded using either Bruker Avance 400 MHz or Bruker Ascend 800 MHz spectrometers at 298 K using deuterated methanol solvent. Data is reported in parts per million (ppm), referenced to residual protons or $^{13}$C in deuterated methanol solvent (CD$_3$OD: $^1$H 3.31 ppm, $^{13}$C 49.00 ppm) unless otherwise specified, with multiplicity assigned as follows: br = broad, s = singlet, d = doublet, dd = doublet of doublets, ddd = doublet of doublet of doublets, t = triplet, q = quartet, m = multiplet. Coupling constants $J$ are reported in Hertz. Low-resolution mass spectrometry (LRMS) and high-resolution mass spectrometry (HRMS) were performed using positive electron ionisation (+EI) on a Micromass VG Autospec mass spectrometer or negative electrospray ionization (–ESI) on a Micromass ZMD ESI-Quad, or a Waters LCT Premier XE mass spectrometer. Reactions were monitored by analytical thin layer chromatography (TLC) using Merck Silica gel 60 TLC plates (7:2:1 ethyl acetate: methanol: water, unless otherwise specified) and were visualised by staining with a solution of potassium permanganate [KMnO$_4$ (3 g), K$_2$CO$_3$ (20 g), NaOH (0.25 g), in H$_2$O (305 mL)] or concentrated sulfuric acid in methanol (5% v/v), with heating as required.

2.3.2.3 Synthetic methods

2.3.2.3.1 General method for the small scale steroid sulfation reaction with purification by SPE

Sulfation was performed according to literature methods 9. A solution of SO$_3$.py (10.0 mg, 62.8 mmol) in DMF (100 µL) was added to a solution of steroid (1.0 mg) in dioxane (100 µL) and the resulting solution was then stirred in a capped vial at room temperature for 4 h. The reaction was then quenched with water (1.5 mL) and subjected to purification by SPE. An Oasis WAX SPE cartridge (6 cc) was pre-conditioned with methanol (5 mL) followed by water (15 mL). The reaction mixture (1.7 mL) was then loaded onto the cartridge and eluted under a positive pressure of nitrogen at a flow rate of approximately 2 mL/min with the following solutions: formic acid in water (2% v/v, 15 mL), water (15 mL), methanol (15 mL) and saturated aqueous ammonia solution in methanol (5% v/v, 15 mL). The
methanolic ammonia fraction was concentrated *in vacuo* to yield the desired steroid bis-sulfate as the corresponding ammonium salt.

2.3.2.3.2 General method for the small scale steroid sulfation reaction with conversion determined by $^1$H NMR analysis
A steroid sulfation reaction was performed as per 2.3.2.3.1 above. A modified WAX SPE protocol eluting with only formic acid in water (2% v/v, 15 mL), water (15 mL) and saturated aqueous ammonia solution in methanol (5% v/v, 15 mL), followed by concentration of the methanolic ammonia fraction yielded a mixture containing both the starting steroid and the corresponding steroid sulfates as the ammonium salts. A 400 MHz $^1$H NMR spectrum was obtained and integration of a suitable signal (typically C3-H, and C17-H) of both steroid and steroid sulfate provided a ratio of the two compounds which was used to determine the percent conversion of the sulfation reaction. The mixture was then subjected to a second SPE purification as per 2.3.2.3.1 above to yield the desired steroid bis-sulfate in pure form as the corresponding ammonium salt.

2.3.2.3.3 General method for the small scale steroid reduction reaction with purification by SPE
A solution of steroid (5.0 mg) in methanol (100 µL) was added to a vial containing sodium borohydride (5 mg, 132 µmol). After the vigorous reaction subsided, the reaction was stirred for 4 h. The reaction was then quenched with aqueous hydrochloric acid solution (0.1 M, 1.5 mL) and subjected to purification by WAX SPE. A modified SPE protocol eluting with formic acid in water (2% v/v, 15 mL), water (15 mL) and methanol (5% v/v, 15 mL), followed by concentration of the methanol fraction afforded the desired steroid diol in pure form.
2.3.2.4 Chemical synthesis of steroid bis-sulfates

2.3.2.4.1 Androst-5-ene-3β,17β-diol 3,17-bis-sulfate, ammonium salt (1)

A solution of androst-5-ene-3β,17β-diol (5 mg, 17.2 µmol) in dioxane (500 µL) was treated with a solution of sulfur trioxide-pyridine complex (50 mg, 314 µmol, 18.2 eq) in DMF (500 µL) and purified by SPE as per 2.3.2.3.1 to yield the title compound 1 as a white solid. Performing the sulfation reaction as per 2.3.2.3.2 showed >98% conversion. Rf 0.23; δH (400 MHz): 5.40 (br s, 1H, C6-H), 4.23 (t, J 8.4 Hz, 1H, C17-H), 4.13 (m, 1H, C3-H), 2.56-0.91 (m, 19H), 1.05 (s, 3H, C18-H3), 0.82 (s, 3H, C19-H3); δc (100 MHz): 141.7 (C5), 123.0 (C6), 88.2 (C17), 79.8 (C3), 52.1, 51.7, 43.7, 40.4, 38.4, 37.8, 37.7, 33.1, 32.6, 30.0, 29.2, 24.4, 21.7, 19.8 (C19), 12.0 (C18); LRMS (+ESI): m/z 449 (5%, [C19H29O8S2]-), 351 (30%), 110 (15%), 97 (100%, [HSO4]-); HRMS (+ESI): found 449.1297, [C19H29O8S2]- requires 449.1304.

2.3.2.4.2 5α-androstane-3β,17β-diol (2)

A solution of epiandrosterone (5 mg, 17.2 µmol) in methanol (100 µL) was treated with sodium borohydride (5 mg, 132 µmol, 7.7 eq) and purified by SPE as per 2.3.2.3.3 to yield the title compound 2 as a white solid. Rf 0.59 (71% EtOAc/hexanes); mp 155-160 °C (lit 21 161-163 °C); δH (400 MHz): 3.55 (t, 8.6 Hz, 1H, C17-H), 3.51 (m, 1H, C3-H), 2.01-0.62 (m, 22H), 0.85 (s, 3H, C18-H3), 0.73 (s, 3H, C19-H3); δc (100 MHz): 82.5 (C17), 71.8 (C3), 56.1, 52.4, 46.3, 44.1, 38.9, 38.3, 38.1, 37.0, 32.9, 32.2, 30.7, 29.9, 24.3, 22.0, 12.8 (C18), 11.7 (C19), one carbon overlapping or obscured; LRMS (+EI): m/z 293 (20%), 292 (100%, [C19H32O2]+), 277 (40%), 233 (85%), 217 (70%), 215 (90%), 166 (50%), 165 (70%), 149 (55%), 123 (40%), 121 (50%), 107 (90%), 95 (60%), 93 (70%), 81 (70%), 79 (60%), 67 (70%), 55 (80%); HRMS (+EI): found 292.2401, [C19H32O2]+ requires 292.2402.
2.3.2.4.3 5α-androstane-3β,17β-diol 3,17-bis-sulfate, ammonium salt (3)

A solution of 5α-androstane-3β,17β-diol 2 (derived from epiandrosterone, 5 mg, 17.2 µmol) in dioxane (500 µL) was treated with a solution of sulfur trioxide-pyridine complex (50 mg, 314 µmol, 18.2 eq) in DMF (500 µL) and purified by SPE as per 2.3.2.3.1 to yield the title compound 3 as a white solid. Performing the sulfation reaction as per 2.3.2.3.2 showed >98% conversion. Rf 0.23; δH (400 MHz): 4.25 (m, 1H, C3-H), 4.21 (t, J 8.6 Hz, 1H, C17-H), 2.20-0.69 (m, 22H), 0.86 (s, 3H, C18-H3), 0.80 (s, 3H, C19-H3); δC (100 MHz): 88.2 (C17), 79.7 (C3), 55.8, 51.8, 46.3, 44.0, 38.2, 38.0, 36.7, 36.6, 36.4, 32.8, 29.8, 29.2, 24.4, 21.8, 12.7 (C19), 12.2 (C18), one carbon overlapping or obscured; LRMS (-ESI): m/z 451 (5%, [C19H31O8S2]-), 353 (20%), 225 (45%, [C19H30O8S2]2-), 97 (100%, [HSO4]-); HRMS (-ESI): found 451.1460, [C19H31O8S2]- requires 451.1460.

2.3.2.4.4 5α-androstane-3α,17β-diol (4)²²,²³

A solution of androsterone (5 mg, 17.2 µmol) in methanol (100 µL) was treated with sodium borohydride (5 mg, 132 µmol, 7.7 eq) and purified by SPE as per 2.3.2.3.3 to yield the title compound 4 as a white solid. Rf 0.59 (71% EtOAc/hexanes); mp 217-220 °C (lit ²² 223-224 °C); δH (400 MHz): 3.96 (br s, 1H, C3-H), 3.56 (t, J 8.6 Hz, 1H, C17-H), 2.01-0.63 (m, 22H), 0.83 (s, 3H, C18-H3), 0.73 (s, 3H, C19-H3); δC (100 MHz): 82.6 (C17), 67.2 (C3), 56.1, 52.5, 44.1, 40.4, 38.1, 37.2, 37.0, 36.8, 33.5, 32.9, 30.7, 29.7, 29.6, 24.3, 21.5, 11.7 (2 C, C18 and C19); LRMS (+EI): m/z 293 (10%), 292 (25%, [C19H32O2]+*), 277 (10%), 233 (15%), 217 (15%), 215 (20%), 166 (10%), 165 (15%), 148 (10%), 145 (20%), 121 (10%), 119 (15%), 118 (35%), 103 (80%), 91 (25%), 90 (50%), 77 (30%), 76 (100%), 67 (15%), 59 (40%); HRMS (+EI): found 292.2404, [C19H32O2]+* requires 292.2402.
2.3.2.4.5 5α-androstane-3α,17β-diol 3,17-bis-sulfate, ammonium salt (5)

A solution of 5α-androstane-3α,17β-diol 4 (derived from androsterone, 5 mg, 17.2 μmol) in dioxane (500 μL) was treated with a solution of sulfur trioxide-pyridine complex (50 mg, 314 μmol, 18.2 eq) in DMF (500 μL) and purified by SPE as per 2.3.2.3.1 to yield the title compound 5 as a white solid. Performing the sulfation reaction as per 2.3.2.3.2 showed >98% conversion. Rf 0.26; δH (400 MHz): 4.59 (br s, 1H, C3-H), 4.21 (t, J 8.5 Hz, 1H, C17-H), 2.20-0.73 (m, 22H), 0.84 (s, 3H, C18-H3), 0.80 (s, 3H, C19-H3); δc (100 MHz): 88.3 (C17), 76.4 (C3), 55.9, 51.9, 44.0, 40.8, 38.1, 36.8 (2 C), 34.7, 33.9, 32.8, 29.5, 29.2, 27.9, 24.4, 21.4, 12.2 (C19), 11.9 (C18); LRMS (-ESI): m/z 451 (10%, [C19H31O8S2]−), 353 (40%), 225 (40%, [C19H30O8S2]2−), 110 (60%), 97 (100%, [HSO4]−); HRMS (+ESI): found 451.1460, [C19H31O8S2]− requires 451.1460.

2.3.2.4.6 5β-androstan-3α,17β-diol (6) 24

A solution of etiocholanolone (5 mg, 17.2 μmol) in methanol (100 μL) was treated with sodium borohydride (5 mg, 132 μmol, 7.7 eq) and purified by SPE as per 2.3.2.3.3 to yield the title compound 6 as a white solid. Rf 0.59 (71% EtOAc/hexanes); mp 155-159 °C (lit 24 162-164 °C); δH (400 MHz): 3.58 (t, J 8.6 Hz, 1H, C17-H), 3.54 (m, 1H, C3-H), 2.03-0.99 (m, 22H), 0.96 (s, 3H, C18-H3), 0.72 (s, 3H, C19-H3); δc (100 MHz): 83.2 (C17), 72.5 (C3), 54.1, 47.2, 45.9, 45.5, 43.3, 37.2, 36.3, 36.1, 33.1, 31.7, 30.4, 28.0, 27.5, 27.2 (C19), 24.4, 13.9 (C18), one carbon overlapping or obscured; LRMS (+EI): m/z 292 (10%, [C19H32O2]2+), 274 (80%), 256 (50%), 241 (30%), 230 (40%), 217 (45%), 215 (100%), 161 (30%), 147 (40%), 133 (30%), 121 (40%), 119 (40%), 107 (70%), 95 (60%), 93 (75%), 91 (50%), 81 (70%), 79 (60%), 69 (30%), 67 (70%), 57 (30%), 55 (90%); HRMS (+ESI): found 315.2300, [C19H32O2Na]+ requires 315.2300.
2.3.2.4.7 5β-androstan-3α,17β-diol 3,17-bis-sulfate, ammonium salt (7)

A solution of 5β-androstan-3α,17β-diol 6 (derived from etiocholanolone, 5 mg, 17.2 µmol) in dioxane (500 µL) was treated with a solution of sulfur trioxide-pyridine complex (50 mg, 314 µmol, 18.2 eq) in DMF (500 µL) and purified by SPE as per 2.3.2.3.1 to yield the title compound 7 as a white solid. Performing the sulfation reaction as per 2.3.2.3.2 showed >98% conversion. Rf 0.23; δH (400 MHz): 4.28 (m, 1H, C3-H), 4.25 (t, J 8.5 Hz, 1H, C17-H), 2.22-1.00 (m, 22H), 0.96 (s, 3H, C18-H3); δc (100 MHz): 88.2 (C17), 80.4 (C3), 51.8, 44.1, 43.7, 42.0, 38.1, 37.1, 36.5, 35.7, 34.6, 29.3, 28.9, 28.2, 27.1 (C19), 24.4, 23.8, 21.4, 12.1 (C18); LRMS (-ESI): m/z 451 (5%, [C19H31O8S2]−), 353 (25%), 225 (55%, [C19H30O8S2]2−), 97 (100%, [HSO4−]); HRMS (-ESI): found 451.1463, [C19H31O8S2]− requires 451.1460.

2.3.2.4.8 11β-hydroxyepiandrosterone 3,11-bis-sulfate, ammonium salt (8)

A solution of 11β-hydroxyepiandrosterone (5.0 mg, 16.3 µmol) in dioxane (500 µL) was treated with a solution of sulfur trioxide-pyridine complex (50 mg, 314 µmol, 19.3 eq) in DMF (500 µL) and purified by SPE as per 2.3.2.3.1 to yield the title compound 8 as a white solid. Performing the sulfation reaction as per 2.3.2.3.2 showed >98% conversion. Rf 0.23; δH (400 MHz): 4.93 (br s, 1H, C11-H), 4.25 (m, 1H, C3-H), 2.47 (dd, J 8.4 Hz, 19.2 Hz, 1H, C16-H), 2.11-0.71 (m, 19H), 1.12 (s, 3H, C18-H3), 1.10 (s, 3H, C19-H3); δc (100 MHz): 222.4 (C17), 79.7 (C3), 79.5 (C11), 59.5, 54.1, 47.3, 37.3, 36.9, 36.7, 36.2, 35.9, 32.4, 29.5, 29.0, 22.5, 15.9 (C18), 14.9 (C19), two carbons overlapping or obscured; LRMS (-ESI): m/z 465 (10%, [C19H29O9S2]−), 383 (30%), 367 (25%), 232 (40%, [C19H28O9S2]2−), 97 (100%, [HSO4−]); HRMS (-ESI): found 465.1259, [C19H29O9S2]− requires 465.1253.
2.3.2.4.9 11β-hydroxyandrosterone 3,11-bis-sulfate, ammonium salt (9)

A solution of 11β-hydroxyandrosterone (5.0 mg, 16.3 µmol) in dioxane (500 µL) was treated with a solution of sulfur trioxide-pyridine complex (50 mg, 314 µmol, 19.3 eq) in DMF (500 µL) and purified by SPE as per 2.3.2.3.1 to yield the title compound 9 as a white solid. Performing the sulfation reaction as per 2.3.2.3.2 showed >98% conversion. Rf 0.23; δH (400 MHz): 4.97 (br s, 1H, C11-H), 4.59 (br s, 1H, C3-H), 2.46 (dd, J 8.5, 17.8 Hz, 1H, C16-H), 2.05-0.81 (m, 19H), 1.13 (s, 3H, C18-H3), 1.09 (s, 3H, C19-H3); δc (100 MHz): 222.4 (C17), 76.4 (C3), 76.2 (C11), 59.7, 54.3, 41.7, 37.0, 36.9, 36.2, 34.1, 32.7, 32.5, 32.3, 28.6, 27.7, 22.5, 16.0 (C18), 14.0 (C19), one carbon overlapping or obscured; LRMS (-ESI): m/z 465 (5%, [C19H29O9S2]-), 367 (30%), 232 (15%, [C19H28O9S2]2-), 110 (30%), 97 (100%, [HSO4]-); HRMS (-ESI): found 465.1273, [C19H29O9S2]- requires 465.1253.

2.3.2.4.10 11β-hydroxyetiocholanolone 3,11-bis-sulfate, ammonium salt (10)

A solution of 11β-hydroxyetiocholanolone (5.0 mg, 16.3 µmol) in dioxane (500 µL) was treated with a solution of sulfur trioxide-pyridine complex (50 mg, 314 µmol, 19.3 eq) in DMF (500 µL) and purified by SPE as per 2.3.2.3.1 to yield the title compound 10 as a white solid. Performing the sulfation reaction as per 2.3.2.3.2 showed >98% conversion. Rf 0.23; δH (400 MHz): 4.31 (m, 1H, C3-H), 2.47 (dd, J 8.7, 18.5 Hz, 1H, C16-H), 2.10-1.05 (m, 19H), 1.22 (s, 3H, C18-H3), 1.12 (s, 3H, C19-H3), C11-H proton obscured by solvent; δc (100 MHz): 222.4 (C17), 80.4 (C3), 76.2 (C11), 55.1, 54.0, 46.4, 45.7, 37.0, 36.2, 35.8, 35.7, 34.4, 32.7, 29.1, 27.0, 26.6, 22.6, 15.9, one carbon overlapping or obscured; LRMS (-ESI): m/z 465 (1%, [C19H29O9S2]-), 367 (15%), 232 (30%, [C19H28O9S2]2-), 110 (30%), 97 (100%, [HSO4]-); HRMS (-ESI): found 465.1252, [C19H29O9S2]- requires 465.1253.
### 2.3.2.4.11 16α-hydroxyandrosterone 3,16-bis-sulfate, ammonium salt (11)

A solution of 16α-hydroxyandrosterone (5.0 mg, 16.3 µmol) in dioxane (500 µL) was treated with a solution of sulfur trioxide-pyridine complex (50 mg, 314 µmol, 19.3 eq) in DMF (500 µL) and purified by SPE as per 2.3.2.3.1 to yield the title compound 11 as a white solid. Performing the sulfation reaction as per 2.3.2.3.2 showed >98% conversion. \( R_f \) 0.30; \( \delta_H \) (400 MHz): 4.94 (d, \( J \) 8.0 Hz, 1H, C16-H), 4.60 (br s, 1H, C3-H), 2.25 (dd, \( J \) 8.0, 16.0 Hz, 1H, C15-H), 2.00-1.07 (m, 19H), 0.96 (s, 3H, C18-H), 0.86 (s, 3H, C19-H); \( \delta_C \) (100 MHz): 216.3 (C17), 77.7 (C16), 76.3 (C3), 55.7, 50.1, 40.8, 36.9, 36.3, 34.6, 33.8, 32.8, 31.8, 30.7, 29.2, 27.8, 20.8, 14.6 (C18), 11.8 (C19), one carbon overlapping or obscured; LRMS (-ESI): \( m/z \) 465 (2%, \([C_{19}H_{29}O_9S_2]\)), 427 (20%), 383 (10%), 369 (20%), 232 (100%, \([C_{19}H_{28}O_9S_2]^2\)), 110 (10%), 97 (20%, \([HSO_4^-]\)), 80 (30%); HRMS (-ESI): found 465.1258, \([C_{19}H_{29}O_9S_2]\) \( \text{requires} \) 465.1253.

### 2.3.2.4.12 16α-hydroxytestosterone 16,17-bis-sulfate, ammonium salt (12)

A solution of 16α-hydroxytestosterone (5.0 mg, 16.4 µmol) in dioxane (500 µL) was treated with a solution of sulfur trioxide-pyridine complex (50 mg, 314 µmol, 19.1 eq) in DMF (500 µL) and purified by SPE as per 2.3.2.3.1 to yield the title compound 12 as a white solid. Performing the sulfation reaction as per 2.3.2.3.2 showed >98% conversion. \( R_f \) 0.31; \( \delta_H \) (400 MHz): 5.72 (s, 1H, C4-H), 4.79 (t, \( J \) 6.0 Hz, 1H, C16-H), 4.32 (d, \( J \) 5.8 Hz, 1H, C16-H), 2.53-0.99 (m, 17H), 1.00 (s, 3H, C18-H); 0.92 (s, 3H, C19-H); \( \delta_C \) (100 MHz): 202.4 (C3), 175.1 (C5), 124.2 (C4), 93.2 (C17), 84.5 (C16), 55.1, 43.7, 40.0, 37.8, 36.7, 36.3, 34.7, 33.8, 32.7, 32.1, 21.3, 17.7 (C19), 12.9 (C18), one carbon overlapping or obscured; LRMS (-ESI): \( m/z \) 463 (5%, \([C_{19}H_{27}O_9S_2]\)), 383 (10%), 365 (10%), 231 (100%, \([C_{19}H_{26}O_9S_2]^2\)), 97 (20%, \([HSO_4^-]\)); HRMS (-ESI): found 463.1100, \([C_{19}H_{27}O_9S_2]\) \( \text{requires} \) 463.1097.
2.3.2.4.13 17β-estradiol 3,17-bis-sulfate, ammonium salt (13)

A solution of 17β-estradiol (5.0 mg, 18.4 µmol) in dioxane (500 µL) was treated with a solution of sulfur trioxide-pyridine complex (50 mg, 314 µmol, 17.1 eq) in DMF (500 µL) and purified by SPE as per 2.3.2.3.1 to yield the title compound 13 as a white solid. Performing the sulfation reaction as per 2.3.2.3.2 showed >98% conversion. 

Rf 0.23, δH (400 MHz): 7.23 (d, J 8.4 Hz, 1H, C1-H), 7.08–6.98 (m, 2H, C2-H and C4-H), 4.31 (t, J 8.0 Hz, 1H, C17-H), 2.89–2.83 (m, 2H, C6-H2), 2.40–1.15 (m, 13H), 0.78 (s, 3H, C18-H3); δC (100 MHz): 151.8, 138.8, 137.6, 127.0, 122.5, 119.8, 88.8 (C17), 51.7, 49.5, 45.5, 39.6, 36.7, 32.8, 30.5, 27.6, 27.0, 22.5, 14.3 (C18); LRMS (-ESI): m/z 431 (10%, [C18H23O8S2-]), 351 (30%), 333 (5%), 215 (20%, [C18H22O8S2-]), 175 (20%), 110 (30%), 97 (60%), 80 (100%); HRMS (-ESI): found 431.0840, [C18H23O8S2-] requires 431.0834.

2.3.2.4.14 3α-hydroxytiibolone 3,17-bis-sulfate, ammonium salt (14)

A solution of 3α-hydroxytiibolone (1.0 mg, 3.18 µmol) in dioxane (100 µL) was treated with a solution of sulfur trioxide-pyridine complex (10 mg, 62.8 µmol, 19.7 eq) in DMF (100 µL) and purified by SPE as per 2.3.2.3.1 to yield the title compound 14 as a white solid. Performing the sulfation reaction as per 2.3.2.3.2 showed 96% conversion. 

Rf 0.25; δH (400 MHz): 4.43 (m, 1H, C3-H), 2.95 (s, 1H, C21-H), 2.62–1.15 (m, 20H), 0.94 (s, 3H, C18), 0.79 (d, J 7.0 Hz, 3H, C19-H3); δC (100 MHz): 129.8 (C10), 125.2 (C5), 87.9 (C20), 85.4 (C17), 77.0 (C21), 76.8 (C3), 50.2, 45.6, 42.9, 41.2, 39.7, 38.8, 37.0, 34.4, 30.9, 28.6, 27.7, 26.5, 23.3, 14.5 (C18), 13.1 (C22); LRMS (-ESI): m/z 473 (5%, [C21H25O8S2-]), 393 (5%, C21H22O5S-), 375 (30%), 236 (100%, [C21H22O8S2-]), 97 (95%, [HSO4-]); HRMS (-ESI): found 473.1307, [C21H25O8S2-] requires 473.1304.
2.3.2.4.15 3β-hydroxytibolone 3,17-bis-sulfate, ammonium salt (15)

A solution of 3β-hydroxytibolone (3.0 mg, 9.54 µmol) in dioxane (500 µL) was treated with a solution of sulfur trioxide-pyridine complex (50 mg, 314 µmol, 32.9 eq) in DMF (500 µL) and purified by SPE as per 2.3.2.3.1 to yield the title compound 15 as a white solid. Performing the sulfation reaction as per 2.3.2.3.2 showed 93% conversion. Rf 0.46; δH (800 MHz): 4.68 (m, 1H, C3-H), 2.94 (s, 1H, C21-H), 2.62 (ddd, J 4.1 Hz, 12.2 Hz, 15.4 Hz, 1H, C16-H), 2.45 (ddd, J 5.6 Hz, 9.7 Hz, 14.7 Hz, 1H, C16-H), 2.33 (m, 1H, C1-H), 2.27 (dd, J 4.2 Hz, 16.5 Hz, 1H, C6ax-H), 2.23-2.16 (m, 2H, C4-H2), 2.06-1.98 (m, 2H, C2eq-H and C11-H), 1.89-1.79 (m, 4H, C1-H, C7-H, C12-H and C14-H), 1.72-1.63 (m, 4H, C2ax-H, C9-H, C12-H and C15-H), 1.56 (dd, J 1.6 Hz, 16.8 Hz, 1H, C6eq-H), 1.48 (ddd, J 2.6 Hz, 11.2 Hz, 13.4 Hz, 1H, C8-H), 1.38 (m, 1H, C15-H), 1.19 (ddd, J 3.6 Hz, 13.0 Hz, 16.6 Hz, 1H, C11-H), 0.94 (s, 3H, C18), 0.79 (d, J 6.9Hz, 3H, C19-H3); δc (200 MHz): 129.9 (C5 or C10), 123.7 (C5 or C10), 87.9 (C17), 85.5 (C20), 76.9 (C21), 75.1 (C3), 50.1 (C13), 45.7 (C14), 42.9 (C8), 41.1 (C9), 40.0 (C6), 37.8 (C1, C2 or C4), 37.0 (C16), 34.4 (C12), 28.9 (C7), 28.6 (C1, C2 or C4), 26.3 (C11), 23.8 (C1, C2 or C4), 23.3 (C15), 14.0 (C18), 13.3 (C19); LRMS (-ESI): m/z 473 (5%, [C21H29O8S2]+), 393 (5%, C21H29O5S)2+, 375 (30%), 236 (90%, [C21H28O8S2]2+), 97 (100%, [HSO4]-), 80 (20%); HRMS (-ESI): found 473.1305, [C21H29O8S2]- requires 473.1304.
2.3.3 Results and Discussion

2.3.3.1 Synthesis of steroid bis-sulfates

The chemical synthesis of steroid bis-sulfate compounds was achieved using previously reported methodology. The treatment of the steroid diol with an excess of sulfur trioxide-pyridine (SO$_3$-py) cleanly afforded the desired steroid bis-sulfate in high conversion (93 to >98%) after purification by WAX SPE. These reaction conditions have proved to be quite general, and have afforded a library of twelve steroid bis-sulfates, as highlighted below (Scheme 2.3, and Table 2.1).

Scheme 2.3: Small-scale sulfation and purification of steroid bis-sulfates

The synthesis of bis-sulfates 1, 3, 5, and 7-12 which contain only secondary alcohols proceeded smoothly to afford the desired bis-sulfates as the sole observed products. Conducting the sulfation reaction of androst-5-ene-3β,17β-diol in the presence of limiting (1 eq) SO$_3$-py provided a mixture of C3-mono sulfated, and C17-mono sulfated products suggesting no discernible difference between the reactivity at the C3 and C17 positions. Based on these observations it was expected that the C11, and C16 positions would display similar reactivity. Of interest was the reaction of 16α-hydroxytestosterone which proceeded smoothly to afford the desired 16,17-bis-sulfate 12 in high conversion (>98%) which was expected to be problematic due to the steric hindrance resulting from sulfation at the two adjacent reaction sites.
Table 2.1: Synthesis of steroid bis-sulfates with purification by SPE

<table>
<thead>
<tr>
<th>Entry</th>
<th>Steroid bis-sulfate</th>
<th>Conversion (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Entry</th>
<th>Steroid bis-sulfate</th>
<th>Conversion (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1" alt="Steroid bis-sulfate 1" /></td>
<td>&gt;98</td>
<td>10</td>
<td><img src="image10" alt="Steroid bis-sulfate 10" /></td>
<td>&gt;98</td>
</tr>
<tr>
<td>3</td>
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<td>11</td>
<td><img src="image11" alt="Steroid bis-sulfate 11" /></td>
<td>&gt;98</td>
</tr>
<tr>
<td>5</td>
<td><img src="image5" alt="Steroid bis-sulfate 5" /></td>
<td>&gt;98</td>
<td>12</td>
<td><img src="image12" alt="Steroid bis-sulfate 12" /></td>
<td>&gt;98</td>
</tr>
<tr>
<td>7</td>
<td><img src="image7" alt="Steroid bis-sulfate 7" /></td>
<td>&gt;98</td>
<td>13</td>
<td><img src="image13" alt="Steroid bis-sulfate 13" /></td>
<td>&gt;98</td>
</tr>
<tr>
<td>8</td>
<td><img src="image8" alt="Steroid bis-sulfate 8" /></td>
<td>&gt;98</td>
<td>14</td>
<td><img src="image14" alt="Steroid bis-sulfate 14" /></td>
<td>96%</td>
</tr>
<tr>
<td>9</td>
<td><img src="image9" alt="Steroid bis-sulfate 9" /></td>
<td>&gt;98</td>
<td>15</td>
<td><img src="image15" alt="Steroid bis-sulfate 15" /></td>
<td>93%</td>
</tr>
</tbody>
</table>

<sup>a</sup>Conversion based on 400 MHz <sup>1</sup>H NMR integration
2.3.3.2 Synthesis of estradiol 3,17-bis-sulfate

The sulfation of estradiol proceeded smoothly to afford bis-sulfate 13 in >98% conversion, which is in contrast to previous observations. Sulfation at the aromatic position was expected to be reduced (based on electronic considerations), due to the reduced reactivity of the phenolic hydroxyl groups over alkyl hydroxyl groups. The study described in Chapter 2.2 required the use of chlorosulfonic acid to achieve sulfation at the C3 position of estrone, while the present study achieved sulfation under much milder conditions. The sulfation of estrone and estradiol has also been reported under similar mild conditions. It has been hypothesised that wet or impure sources of SO$_3$.py may be the culprit in the earlier reactions, as the effects from poor quality reagents may be amplified on the milligram-scale. The reaction of estradiol (5 mg) under standard conditions as per section 2.3.2.3.1, with the addition of water (100 µL) at the start of the reaction, resulted in a mixture of estradiol 17-sulfate, and estradiol 3,17-bis-sulfate. The decomposition of SO$_3$.py in the presence of water would likely yield a solution of dilute sulfuric acid and pyridine, which could be implicated in these observations. It is currently unknown if the earlier observed product distribution occurred as a result of the hydrolysis of the phenolic sulfate after the sulfation reaction had taken place, or as a result of an incomplete reaction due to decomposition of the reagent before the sulfation reaction could occur.

2.3.3.3 Synthesis of tertiary steroid bis-sulfates

The sulfation of 3α/β-hydroxytibolone to give bis-sulfates 14 and 15 was expected to be more difficult as both of these compounds possess a tertiary alcohol. Previous results have shown that tertiary hydroxyl groups remain untouched under these reaction conditions, although this may have been in part to the same problems that have been discussed immediately above in section 2.3.3.2. Additionally, C17-tertiary steroid sulfates have been observed to decompose under mildly acidic conditions, rearranging to give 17,17-dimethylandrost-13-enes, as well as C17-epimerisation, which have also been observed as steroid metabolites in vivo.
Whilst tertiary steroid sulfates have been previously used to access C17-epimers of existing steroid compounds 20, attempts to isolate 17α-methyltestosterone 17-sulfate were met with success only when care was taken to avoid exposure to excess air or moisture. This was achieved through the use of dry, distilled solvents, and a nitrogen atmosphere throughout the reaction. These conditions were then applied to the synthesis of bis-sulfates 14 and 15. Owing to the steric congestion afforded by the adjacent alkynyl substituent, the reaction afforded a mixture of C3-mono and C3,C17-bis sulfated products, although the bis-sulfates were still observed in high conversions of 96% and 93% respectively. There was no evidence to suggest epimerisation at C17, or elimination to the corresponding 7α,17β-dimethyl-18,19-dinor-17α-pregn-5(10),13-dien-20-yn-3-ol 3-sulfates. This was surprising, as the propargylic cation that would result from sulfate elimination was expected to be highly stable 18, however the stability of these tertiary sulfates may reflect the differences in the steric demand of a smaller C17-alkyne substituent over a C17-methyl substituent, or other unknown factors.
2.3.3.4 NMR analysis of steroid bis-sulfates

During this study we found that the majority of the bis-sulfate compounds prepared had little to no characterisation data present in the literature and as a result they were characterised by $^1$H and $^{13}$C NMR after their preparation. NMR analysis was simplified due to their similarity to data previously reported for related steroid mono-sulfates. For the secondary alcohols subjected to sulfation, we observed a strong downfield shift of the protons at the reaction site. A shift of 0.63-0.84 ppm was observed for the C3 protons, a shift of 0.63 ppm was observed for the C11 protons, a shift of 0.64-0.77 ppm was observed for the C16 protons, and a shift of 0.65-0.93 ppm was observed for the C17 protons. The proximity of the two sulfate groups to each other had an effect on the magnitude of the downfield shift observed, as expected due to electronic considerations. The smallest downfield shifts were observed when the sulfate groups were far apart such as for 3,17 bis-sulfates (0.63, and 0.65 ppm for C3, and C17 respectively), whilst the largest downfield shifts was observed for 16α-hydroxytestosterone 16,17-bis-sulfate (0.77 ppm, and 0.93 ppm for C16, and C17 respectively) which contain sulfate groups on adjacent carbons. For the sulfation of estradiol to give bis-sulfate 13, a strong downfield shift of 0.51-0.54 ppm for the ortho C2, and C4 protons was observed alongside a smaller shift of 0.20 ppm for the C1 proton. These observations were broadly consistent with previously reported observations. The sulfation of 3α/β-hydroxytibolone to give bis-sulfates 14 and 15 proceeded with a downfield shift of 0.69 ppm for the C3 proton, and a very small shift of 0.06 ppm for the C21 alkyne proton.

2.3.3.5 Full scan mass spectrometry behaviour of steroid bis-sulfates

In addition to the synthesis of bis-sulfates 1, 3, 5, and 7-15, the mass spectrometry behaviour of these compounds was investigated in full-scan with -ESI. In the example spectrum below (Figure 2.1), the mass spectrum of bis-sulfate 1 displays in-source fragmentation and peaks at mass-to-charge ratio (m/z) 449 (5%, [M-H]), 351 (30%, [M-2H-HSO$_4$]~), 224 (15%, [M-2H]~2), and 97 (100%, [HSO$_4$]~), where M refers to the neutral compound, C$_{19}$H$_{30}$O$_8$S$_2$. Additional fragmentations observed in some cases include loss of [SO$_3$]* (m/z 80), and loss of [HSO$_4$]~ (m/z 81). For the
majority of these bis-sulfate compounds, the major peak associated with the intact molecule in full scan was the di-anion \([\text{M-2H}]^{2-}\), while the mono-anion \([\text{M-H}]^-\) normally ranged between 5-45%. The exception to this was bis-sulfate 12 which ionised preferentially as the mono-anion. This preference presumably arises from the charge repulsion that results from the two negatively-charged sulfate groups being in close proximity, which decreases the stability of the di-anion. Bis-sulfates 1, 3, 5, 7-11, and 13-15 where the sulfate groups were further apart had a lower proportion of the mono-anion in their full scan spectra, and ionised preferentially as the di-anion.

**Figure 2.1: Full scan (-ESI) mass spectrometry behaviour of androst-5-ene-3β,17β-diol 3,17-bis-sulfate (1)**

2.3.3.6 MS/MS behaviour of steroid bis-sulfates

In addition to the full scan studies undertaken at the ANU, the MS/MS behaviour of bis-sulfates 1, 3, 5, 7, 9-12, and 15 was also studied on a triple quadrupole instrument (Table 2.2). This work was undertaken by Ms Argitxu Esquivel, Ms
Georgina Balcells, and Dr Óscar Pozo at the Institut Hospital del Mar d’Investigacions Mèdiques (IMIM, Barcelona, Spain). After selecting the di-anion as the precursor ion, the resulting MS/MS product ion spectra of the above compounds were dominated by the presence of two major ions. The base peak in most cases corresponded to \([\text{HSO}_4^-] (m/z \ 97)\), alongside a fragment ion \([\text{M-2H-} \text{HSO}_4^-]\), which was generated from the loss of the \([\text{HSO}_4^-]\) anion, and depended on the molecular mass of the bis-sulfate under study. This resulted in an increase of \(m/z\) of the observed fragment, and appeared to be general to most bis-sulfate compounds. A general mechanism for this fragmentation pathway is proposed below (Scheme 2.5).

### Table 2.2: MS/MS product ion behaviour of bis-sulfates 1, 3, 5, 7, 9-12, and 15, at 20 eV collision energy

<table>
<thead>
<tr>
<th>Bis-sulfate</th>
<th>Product ion scan selecting ([\text{M-2H}]^2) as the precursor ion</th>
<th>Base peak ((m/z))</th>
<th>([\text{M-2H-} \text{HSO}_4^-] (m/z))</th>
<th>Other ions (&gt;10%) ((m/z))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>97 (100%)</td>
<td>351 (25%)</td>
<td>335 (18%)</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>97 (100%)</td>
<td>353 (27%)</td>
<td>337 (10%)</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>97 (100%)</td>
<td>353 (81%)</td>
<td>80 (20%)</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>97 (100%)</td>
<td>353 (40%)</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>97 (100%)</td>
<td>367 (27%)</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>97 (100%)</td>
<td>367 (24%)</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>97 (100%)</td>
<td>367 (2%)</td>
<td>80 (48%), 81 (25%), 369 (48%)</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>97 (100%)</td>
<td>365 (58%)</td>
<td>80 (45%), 285 (17%)</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>97 (100%)</td>
<td>375 (9%)</td>
<td>80 (53%)</td>
</tr>
</tbody>
</table>

**Scheme 2.5:** Proposed fragmentation pathway of androst-5-ene-3β,17β-diol 3,17-bis-sulfate (1), based on observed MS/MS behaviour.
Although these two peaks were the most prominent ions, a significant fragment ion corresponding to \([\text{SO}_3]^*\) (m/z 80) was also observed for bis-sulfates 5, 11, 12, and 15 (20-53% abundance, Table 2.2). This ion has been previously observed for some steroid mono-sulfate compounds, most commonly for compounds containing a phenolic, or tertiary sulfate group \(^9,^{27}\). Additionally, for bis-sulfate 11 which has a sulfate group adjacent to a carbonyl group at C17, a prominent ion was observed corresponding to the consecutive loss of \([\text{SO}_3]^*\) and \([\text{CH}_3]^*\). A feasible pathway for this fragmentation is shown below (Scheme 2.6).

**Scheme 2.6: Proposed fragmentation pathway for the consecutive losses of \([\text{SO}_3]^*\) and \([\text{CH}_3]^*\) for 16α-hydroxyandrosterone 3,16-bis-sulfate (11).**

![Proposed fragmentation pathway for the consecutive losses of \([\text{SO}_3]^*\) and \([\text{CH}_3]^*\) for 16α-hydroxyandrosterone 3,16-bis-sulfate (11).](image)

The observed ion fragmentation patterns were used to develop an open screening protocol for the untargeted detection of steroid bis-sulfate compounds. Two approaches were considered for the detection of bis-sulfate compounds: a precursor ion scan method selecting m/z 97 as the product ion, and a constant ion loss (CIL) method based on the constant loss of the \([\text{HSO}_4]^-\) anion. The precursor ion scan method was easily employed by common MS/MS software packages, and allowed for selection of m/z 97 in the second quadrupole, with scanning for precursor ions in the first quadrupole. The detection of sulfate metabolites by precursor ion methods has been previously reported\(^{27-29}\).
The CIL method was more difficult to employ as the product ion $m/z$ depended upon the $m/z$ of the precursor ion, as described below.

\[ \text{Precursor ion (m/z)} = \text{Product ion (m'/z')} + \text{CIL (m''/z'')} \]

As a result, the mass of the product ion for a given CIL, was given as shown below:

\[ m'/z' = \frac{[(m/z) \times z] - [(m''/z'') \times z'']}{z - z''} \]

The change in $m/z$ ($\Delta m/z$) observed during the CIL was given as shown below:

\[ \Delta m/z = \frac{z'' \times [(m/z) - (m''/z'')]}{z - z''} \]

In the current application the CIL is [HSO₄]⁻ ($m''/z''$ 97), $z''=1$, and the precursor ion is [M-2H]²⁻; $z=2$. The theory predicted a positive $\Delta m/z$ (ie. an increase in $m/z$) for any CIL where the $m/z$ of the precursor ion is greater than the constant ion. This agreed with experimental observations.

Although the $m/z$ of the product ion could be calculated, a software tool was not available to perform the CIL method on currently available instrumentation. As such, a MRM method was developed which included a calculated SRM transition for each precursor ion/product ion pair arising from CIL. Based on the known molecular masses of endogenous steroid compounds and their metabolites (250-400 Da), the bis-sulfate SRM transitions selected were restricted to the range $m/z$ 199 to $m/z$ 274. A MRM method containing 75 transitions in this range was then used for the untargeted detection of steroid bis-sulfates in human urine. Although
the transitions selected were predetermined, this approach was considered untargeted as the MRM method covered the entire expected mass range of endogenous steroid bis-sulfates.

In order to test the suitability of this method to detect steroid bis-sulfates, the method was applied to human urine samples spiked with bis-sulfates 1, 3, 5, 8, 9, and 11-13. The extraction recovery of these compounds was determined by comparing the bis-sulfate responses in urine samples spiked with bis-sulfate both before and after sample processing. Additional experiments were also performed to identify the limits of detection of these analytes by the precursor ion, and CIL methods. Urine samples spiked with various concentrations of bis-sulfates were analysed by both analytical methods, and the lowest concentration with a signal-to-noise (S/N) ratio greater than 3 was selected as the limit of detection (LOD). These results are shown in Table 2.3. Extraction recovery from human urine was 44-85%, with a relative standard deviation of 4-20%. Limits of detection were 2-20 ng/mL for the CIL method, compared to 10-50 ng/mL for the precursor ion method. In general, the CIL strategy showed a lower LOD than the precursor ion scan approach. The increase in sensitivity can be explained by the higher specificity of the transitions selected. The use of transitions involving increases of m/z have been reported to be more specific in the detection of other multiply charged analytes such as peptides 30.

Table 2.3: Extraction recoveries and LODs obtained for the bis-sulfates reference materials 1, 3, 5, 8, 9, 11-13 (PI: precursor ion).

<table>
<thead>
<tr>
<th>Bis-sulfate</th>
<th>Extraction</th>
<th>CIL</th>
<th>PI scan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recovery (%)</td>
<td>RSD (%)</td>
<td>Precursor ion (m/z)</td>
</tr>
<tr>
<td>1</td>
<td>55</td>
<td>7</td>
<td>224</td>
</tr>
<tr>
<td>3</td>
<td>53</td>
<td>25</td>
<td>225</td>
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<tr>
<td>5</td>
<td>56</td>
<td>20</td>
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<td>8</td>
<td>83</td>
<td>4</td>
<td>232</td>
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<td>9</td>
<td>85</td>
<td>11</td>
<td>232</td>
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<td>11</td>
<td>56</td>
<td>16</td>
<td>232</td>
</tr>
<tr>
<td>12</td>
<td>84</td>
<td>11</td>
<td>231</td>
</tr>
<tr>
<td>13</td>
<td>44</td>
<td>10</td>
<td>215</td>
</tr>
</tbody>
</table>
The endogenous bis-sulfate profile was evaluated by analysis of the urine samples collected from healthy volunteers. Ethical approval for this study was granted by the Comité Ètic d'Investigació Clínica (CEIC, Parc de Salut Mar, Barcelona, Spain, and all subjects participating in the study gave their written informed consent prior to inclusion. In the majority of the samples, several peaks were detected by the CIL method. Peaks were observed at the theoretical m/z for several classes of endogenous compounds including: estrogens like estriol bis-sulfate (m/z 223 → m/z 349) and estradiol bis-sulfate (m/z 215 → 333); androgens like androstanediol bis-sulfate (m/z 225 → m/z 353) and androstenediol bis-sulfate (m/z 224 → m/z 351); progestogens like pregnanediol bis-sulfate (m/z 239 → m/z 381) and pregnenediol bis-sulfate (m/z 238 → m/z 379); and corticosteroids like tetrahydrocortisol (m/z 262 → m/z 427). For many transitions multiple peaks were identified, suggesting the presence of regio-, stereo-, or constitutional isomers. The observation of bis-sulfate metabolites belonging to several classes of steroid metabolites suggests that bis-sulfation is a common metabolic pathway for steroid compounds. The identity of some of the detected bis-sulfates was confirmed by comparison of the urine samples to reference materials. Bis-sulfate 1 was observed to match an endogenous metabolite by comparison of both retention time and relative abundances of the selected ion transitions, by both analytical methods. Additionally, endogenous metabolites were confirmed by comparison to 16-hydroxydehydroepiandrosterone bis-sulfate, and 5-pregnenediol bis-sulfate reference materials available at the IMIM laboratories.

2.3.4 Conclusions
Utilising a small-scale sulfation protocol with purification by solid-phase extraction, twelve steroid bis-sulfate compounds have been prepared for study. These compounds have not been previously prepared, and have been characterised by 1H NMR, 13C NMR, and mass spectrometry studies. These compounds were determined to be suitable for use as reference materials in analytical laboratories. A unique mode of fragmentation, corresponding to anion loss of [HSO₄]⁻ (m/z 97) which results in an increase of m/z has been identified, and formed the basis of a constant ion loss scan method for the untargeted detection of bis-sulfate metabolites of anabolic steroids. This methodology could
also potentially be used for the detection of bis-sulfate metabolites of other drug classes. This method proved more sensitive for steroid bis-sulfate metabolites over the alternative precursor ion method, which also detects steroid mono-sulfate metabolites. The developed CIL approach allowed for the untargeted detection of endogenous bis-sulfate metabolites at the low ng/mL range. The detection of endogenous bis-sulfate metabolites confirms that this metabolic pathway is common to all classes of endogenous steroid compounds. This method will hopefully be suitable for the detection of all classes of steroid bis-conjugates such as bis-glucuronides, mixed bis-sulfate-glucuronides, or other multiply-charged metabolites, broadening the utility of this method for use in anti-doping laboratories. It is hoped in future that this methodology can be applied to study the endogenous steroid profile and how it changes in response to EAAS misuse, and to study the formation of steroid bis-sulfates that result from the administration of synthetic anabolic steroids, including designer steroids.

2.3.5 References


In the period between when this thesis was submitted (September 2016) and the corrections to this thesis were made (May 2017), this work was accepted for publication in the journal Analytical Chemistry. The full citation for this work is as follows:


A copy of the full text article has been reproduced in Appendix A. Reprinted with permission. Copyright 2017 American Chemical Society.
CHAPTER THREE

3α/β-chloro-17α-methyl-5α-androstan-17β-ol
**Publication:**


### 3.1 Foreword

The following manuscript has been published in the journal “*Drug Testing and Analysis*” and details the discovery of a novel anabolic agent intended for doping purposes in samples seized by law-enforcement, and the subsequent investigations to elucidate its structure, identify the primary human and equine metabolites, and to incorporate these into routine anti-doping screening protocols. Permission has been granted by John Wiley and Sons via RightsLink for the reproduction of this publication within this thesis (License Number: 3887470777611). This publication and supporting information (15 pages containing experimental procedures, and characterisation data) was authored by Dr Adam Cawley, Dr Karen Blakey, Mr Christopher Waller, Associate Professor Malcolm McLeod, Dr Sue Boyd, Dr Alison Heather, Dr Kristine McGrath, Professor David Handelsman, and Dr Anthony Willis. This collaboration resulted through the contribution of all authors, and was coordinated by Dr Cawley. The specific contributions of C. Waller are listed below:

- The synthesis of 3α-chloro-17α-methyl-5α-androstan-17β-ol, 3β-chloro-17α-methyl-5α-androstan-17β-ol, 3α-chloro-17β-methyl-5α-androsta-16α,17α-diol, and 3α-chloro-17α-methyl-5α-androsta-16α,17β-diol reference materials.
- Full characterisation of all reference materials and intermediates produced.
- Human and equine *in vitro* metabolism studies, including the identification and confirmation of the major metabolites.
- Preparation of pages 9-15 of the supporting information, which contains experimental procedures, and characterisation data.
The detection of novel anabolic agents intended for doping purposes remains a significant problem in both human and equine sports. These “designer” steroids frequently contain chemical modifications which may prevent their identification by both law-enforcement and anti-doping laboratories. The greatest hurdle to responding effectively is often obtaining intelligence regarding the availability of new designer steroids, and therefore identifying the most likely candidates for misuse.

In this chapter, a suite of tools has been deployed to assist anti-doping laboratories in responding to the threat of designer steroids. The manuscript presented describes a study in which an unknown sample was seized by law-enforcement, and after extensive structural analysis was determined to contain a 5:2 mixture of 3α-chloro-17α-methyl-5α-androstan-17β-ol, and 3β-chloro-17α-methyl-5α-androstan-17β-ol. Although new to science, the proposed structures were confirmed through the individual chemical synthesis of both isomers present in the mixture, which then allowed for subsequent investigations using androgen bioassays and in vitro metabolism. The data obtained from these studies was then used in order to develop and evaluate routine anti-doping screening protocols to allow for detection of these compounds in human and equine urine.

This study highlights a workflow suitable for adoption by anti-doping laboratories that allows for the development of screening protocols in the event that new designer steroids are identified.
3.2 Detection and metabolic investigations of a novel designer steroid: 3-chloro-17α-methyl-5α-androstan-17β-ol
Due to copyright restrictions from the publisher, the journal article presented in section 3.2 has been removed from the online version of this thesis. The full text article can be obtained via the ANU Library, or directly from the publisher using the link below:

CHAPTER FOUR

Furazadrol
[1’,2’]isoxazolo[4’,5’:2,3]-5α-androstan-17β-ol
4.1 Foreword

The following manuscript has been published in the "Journal of Pharmaceutical and Biomedical Analysis" and details an investigation into the in vivo and in vitro metabolism of the designer steroid furazadrol in thoroughbred racehorses, with an aim to identify metabolites relevant to anti-doping laboratories. Permission has been granted by Elsevier via RightsLink for the reproduction of this publication within this thesis (License Number: 3887470374721). This publication and supporting information (58 pages containing experimental procedures, characterisation data and copies of NMR and MS spectra) was authored by Mr Christopher Waller, Dr Adam Cawley, Dr Craig Suann, Mr Paul Ma, and Associate Professor Malcolm McLeod. All experimental work with the exception of the in vivo administration was undertaken by C. Waller, alongside preparation of the initial draft of the manuscript. Dr Suann performed the in vivo administration and collected the biological samples. P. Ma expressed and purified the Escherichia coli β-glucuronylsynthase enzyme, and prepared the α-D-glucuronyl fluoride which were used to prepare the glucuronide reference materials. Dr Cawley, and Assoc. Prof. McLeod initially conceived of the project, assisted with interpretation of the LC-MS/MS data, and assisted in revising the manuscript prior to submission.

The detection of designer anabolic steroids remains a problem for anti-doping laboratories, and understanding the metabolism of these compounds is essential in order to develop methods which can identify them in biological samples. The designer steroid furazadrol ([1′,2′]isoxazolo[4′,5′:2,3]-5α-androstan-17β-ol), which is the focus for this chapter, is found in many "dietary" or "nutritional" supplements available online. The presence of an isoxazole ring fused to the steroid core changes how this compound is metabolised in vivo compared to
traditional anabolic steroids, and understanding the metabolism of this compound would provide information useful to anti-doping laboratories.

In this chapter the equine metabolism of furazadrol was explored with an aim to provide information regarding the metabolism of this compound for use by anti-doping laboratories. Synthetic furazadrol was administered to a thoroughbred racehorse, and the key urinary metabolites were identified in post-administration samples.

**Figure 4.1: The major urinary metabolites observed following furazadrol administration**

![Chemical structures](image)

The major metabolites observed after furazadrol administration (furazadrol 17-sulfate, and furazadrol 17-glucuronide) were identified by comparison to synthetically prepared reference materials, and quantified to establish an excretion profile and determine suitable limits of detection. Epifurazadrol glucuronide was also identified, alongside minor metabolites including hydroxylated furazadrol, and hydroxylated and oxidised furazadrol, present as the sulfate and glucuronide conjugates. The phase II metabolites were subjected to enzymatic hydrolysis by *E. coli* β-glucuronidase and *P. aeruginosa* arylsulfatase to further confirm the identity of the corresponding phase I metabolites. A comparative *in vitro* study was also undertaken, which identified all but two of the minor *in vivo* phase I metabolites. These investigations have identified the key urinary metabolites of furazadrol following oral administration, which can be incorporated into anti-doping screening and confirmation procedures.
4.2 *In vivo* and *in vitro* metabolism of the designer anabolic steroid furazadrol in thoroughbred racehorses
Due to copyright restrictions from the publisher, the journal article presented in section 4.2 has been removed from the online version of this thesis. The full text article can be obtained via the ANU Library, or directly from the publisher using the link below:

In addition to the published work presented in Chapter 4.2, the following related unpublished work is also presented. Supporting information relevant to this chapter is presented electronically alongside this thesis.

4.3 \textit{In vitro} phase II metabolism

4.3.1 Introduction

As has been highlighted throughout this thesis so far, one of the primary problems with developing methods for the detection of designer steroids in horses is a lack of data detailing how these compounds are metabolised in equine systems. In order to successfully develop methods that allow for the detection of these compounds, metabolites of the parent drug must usually be targeted, and these have to be determined experimentally. These metabolites have been traditionally identified through \textit{in vivo} studies, although the use of \textit{in vitro} technologies is rapidly gaining acceptance in anti-doping laboratories. \textit{In vitro} experiments have the advantage of being technically simpler to perform, as well as mitigating many of the ethical concerns regarding animal health and safety. These concerns are particularly important in the case of designer steroids since a large number of these compounds are brought to market in a clandestine fashion, and the majority of these compounds do not have any available data regarding their purity, safety, or efficacy. As a result, \textit{in vivo} studies are often hard to justify on ethical grounds. Although phase I steroid metabolism has been studied extensively by \textit{in vitro} methods, the same cannot be said for the study of phase II metabolism. This is problematic as phase II metabolism is an important aspect of steroid metabolism.

The conjugation of polar sulfate or glucuronic acid donor groups to the hydrophobic steroid backbone increases their aqueous solubility, and facilitates their excretion from the body via the urine. These phase II metabolites are important markers for drug detection, and monitoring directly for these metabolites can offer advantages such as reduced sample preparation, and increased detection windows for some analytes. Although \textit{in vitro} systems are sometimes able to predict the major metabolites of steroid metabolism, there is a need for systems which can faithfully replicate \textit{in vivo} metabolism as the accuracy of these systems is often questioned, and can limit how these methods are used to study steroid metabolism. This is particularly important in equine sports, as
current AORC criteria allow for the use of in vitro-derived materials as reference materials in confirmatory analysis \(^4\), so systems that can better replicate in vivo metabolism can be used to detect instances of steroid misuse.

*In vitro* technologies typically make use of enzymatic products derived from liver tissue, as the liver is the primary organ involved in detoxification of xenobiotic compounds \(^2,5^{–11}\). Equine liver microsomes and S9 fraction are among the most popular choices; however, they often require supplementation with expensive uridine diphosphate glucuronic acid (UDPGA), and 3’-phosphoadenosine-5’-phosphosulfate (PAPS) co-factors in order to study phase II metabolism, and as a result studies are typically limited to phase I metabolism. There have been some reports detailing the study of phase II steroid metabolism using *in vitro* systems \(^9,12\), but to date these have not been widely adopted by laboratories.

Although phase II metabolites can be generated *in vitro* through the use of the co-factors UDPGA and PAPS, these reagents have a number of limitations that frequently prohibit their usage. The use of UDPGA (AU$100 for 25 mg, Sigma Aldrich \(^13\)), and PAPS (AU$2790 for 25 mg, Sigma-Aldrich \(^14\)) are limited by their chemical instability and prohibitive costs, and as a consequence a number of systems have been developed which allow for the study of phase II metabolism using alternative methods.

One of the primary methods used to overcome the prohibitive cost of PAPS is to use it in a catalytic quantity in conjugation with a PAPS-regeneration system. These systems regenerate PAPS *in situ* through use of various enzymatic cascades \(^15^{–17}\). Since PAPS is an essential co-factor required for phase II sulfation *in vivo*, a number of the PAPS-regeneration systems mimic its biosynthesis, as shown in Scheme 4.1. It is known that PAPS is generated *in vivo* in two enzymatic steps from adenosine triphosphate (ATP); the enzyme ATP sulfurylase first catalyses the sulfation of ATP to generate adenosine-5’-phosphosulfate (APS), and APS is subsequently phosphorylated by APS kinase to generate PAPS \(^18\). During the transfer of sulfate to the target molecule, sulfotransferase enzymes liberate 3’-
phosphoadenosine-5′-phosphate (PAP) which is then dephosphorylated by PAP-nucleotidase to generate adenosine 5′-phosphate (AMP). This is then converted back to ATP, allowing the cycle to begin again. A number of enzymatic and whole-cell systems have been developed which include these enzymes to regenerate PAPS as it is consumed during the reaction.

**Scheme 4.1: In vivo regeneration of PAPS**

Since PAPS is generated *in vivo* from ATP, it was reasoned that the enzymes required to generate PAPS may already be present in the liver S9 fraction, and could be used to generate PAPS *in vitro* in the presence of ATP and a suitable sulfate source. This would allow for phase II sulfation using vastly cheaper reagents (ATP AU$79 for 1000 mg, Sigma Aldrich). Optimal conditions for *in vitro* phase II steroid sulfation were previously developed by Ms Ling Fam during an undergraduate research project in the McLeod Group, and were achieved when using ATP (16 mM), Na₂SO₄ (8 mM), and MgCl₂ (16 mM) in place of PAPS (80 µM) with a reaction time of 16-24 h. With these conditions in hand, we aimed
to investigate the *in vitro* phase II metabolism of the designer steroids furazadrol, and superdrol.

### 4.3.2 Experimental

#### 4.3.2.1 Materials

Chemicals and solvents including lithium tri-sec-butylborohydride (L-Selectride®) solution in anhydrous tetrahydrofuran (THF), anhydrous *N,N*-dimethylformamide (DMF), magnesium chloride, sulfur-trioxide pyridine complex (SO₃.py), tertiary-butanol (t-BuOH), glucose-6-phosphate (G6P), NAD-dependant glucose-6-phosphate dehydrogenase (G6PDH) from *Leuconostoc mesenteroides*, adenosine triphosphate (ATP), 3’-phosphoadenosine-5’-phosphosulfate (PAPS), nicotinamide adenine dinucleotide phosphate (NADP), and uridine diphosphate glucuronic acid (UDPGA) were purchased from Sigma–Aldrich (Castle Hill, Australia), and were used as supplied unless otherwise stated. Nicotinamide adenine dinucleotide (NAD) was purchased from Amresco (Solon, USA). Formic acid was purchased from Ajax Chemicals (Auburn, Australia). 1,4-Dioxane (dioxane) was purchased from Merck (Darmstadt, Germany). Superdrol (methasterone, 17β-hydroxy-2α,17α-dimethyl-5α-androstan-3-one) was purchased from the National Measurement Institute (North Ryde, Australia). Equine liver S9 fraction was purchased from XenoTech (Kansas City, USA). Solid-phase extraction (SPE) was performed using Waters (Rydalmere, Australia) Oasis WAX 6cc cartridges (PN 186004647), or Waters Sep-Pak C18 (3 cc, 500 mg) cartridges (PN WAT020805) as specified. *Escherichia coli* glucuronylsynthase, and α-D-glucuronyl fluoride were prepared according to literature methods ²¹. NADP-dependant G6PDH was recombinantly expressed from *E. coli* according to a previously reported method ²².

#### 4.3.2.2 Furazadrol reference materials

A range of furazadrol reference materials were employed to aid the identification of phase I and phase II metabolites. These were furazadrol ([1’,2’]isoxazolo[4’,5’:2,3]-5α-androstan-17β-ol) F, isofurazadrol ([1’,2’]isoxazolo-[4’,3’:2,3]-5α-androstan-17β-ol) IF, epifurazadrol ([1’,2’]isoxazolo[4’,5’:2,3]-5α-androstan-17α-ol) EF, oxidised furazadrol ([1’,2’]isoxazolo[4’,5’:2,3]-5α-androstan-17β-ol) OXF, furoxazadrol ([1’’,2’’]isoxazolo[5’,6’:4’,5’]5α-androstan-17β-ol) XOF, and superdrol ([1’’,2’’]isoxazolo[5’,6’:4’,5’]5α-androstan-17β-ol) G.
17-one) OIF, oxidised isofurazadrol ([1',2']isoxazolo[4',3':2,3]-5α-androstan-17-one) OIF, furazadrol 17-sulfate FS, isofurazadrol 17-sulfate IFS, epifurazadrol 17-sulfate EFS, furazadrol 17-glucuronide FG, isofurazadrol 17-glucuronide IFG, and epifurazadrol 17-glucuronide EFG. These were prepared as previously reported 2,23.

4.3.2.3 Superdrol reference materials

A range of superdrol reference materials were employed to aid the identification of phase I and phase II metabolites. Superdrol S was sourced commercially, however the remaining materials were prepared synthetically, including: 2α,17α-dimethyl-5α-androstan-3α,17β-diol 16, 2α,17α-dimethyl-5α-androstan-3β,17β-diol 17, 2α,17α-dimethyl-5α-androstan-3α,17β-diol 3-sulfate 3α-RSS, 2α,17α-dimethyl-5α-androstan-3β,17β-diol 3-sulfate 3β-RSS, and 2α,17α-dimethyl-5α-androstan-3β,17β-diol 3-glucuronide 3β-RSG. These were prepared as outlined below. Copies of the 1H NMR, 13C NMR, and +EI LRMS or -ESI LRMS where appropriate are included electronically in the supporting information for this chapter.

4.3.2.3.1 2α,17α-Dimethyl-5α-androstan-3α,17β-diol (16)

A solution of superdrol (1.0 mg, 3.14 µmol) in methanol (80 µL) was treated with a solution of L-Selectride® in anhydrous THF (1.0 M, 20 µL, 20 µmol, 6.4 eq), and stirred at room temperature for 2 h. The reaction was quenched with aqueous hydrochloric acid (2 M, 1 mL) and purified by SPE. A C18-SPE cartridge was pre-conditioned with methanol (3 mL) and water (3 mL), and the reaction mixture was then loaded. The sample was washed with water (2 mL) until neutral, and eluted with methanol (2 mL). Concentration of the methanol fraction afforded the title compound 16 in >98% conversion as determined by 400 MHz 1H NMR analysis 23. Rf 0.55 (40% EtOAc/hexanes); δH (400 MHz, CDCl3): 3.77 (m, 1H, C3-H), 1.82-0.68 (m, 22H), 1.20 (s, 3H, C20-H3), 0.93 (d, J 6.9 Hz, 3H, C21-H3), 0.84 (s, 3H, C18-H3), 0.80 (s, 3H, C19-H3), 2 x OH not observed; δc (100 MHz, CDCl3): 81.9 (C17), 70.9 (C3), 54.6, 50.9, 45.7, 41.0, 39.2, 39.0, 36.7, 36.6, 36.4, 31.9 (2 peaks), 31.8, 28.3, 26.0 (C20), 23.4, 20.1, 18.6 (C21),
14.2 (C18), 12.4 (C19); LRMS (+EI): \( m/z \) 320 (25%, \([C_{21}H_{36}O_2]^+\)), 305 (45%), 245 (70%), 231 (85%), 179 (80%), 163 (55%), 135 (45%), 121 (100%), 107 (70%), 95 (70%), 81 (70%), 79 (40%), 55 (65%); HRMS (+EI): found 320.2715, \([C_{21}H_{36}O_2]^+\) requires 320.2715.

4.3.2.3.2 2α,17α-Dimethyl-5α-androstane-3β,17β-diol (17)

A solution of superdrol (1.0 mg, 3.14 µmol) in methanol (80 µL) was treated with sodium borohydride (1 mg, 26.4 µmol, 8.4 eq) and stirred at room temperature for 2 h. The reaction was quenched with aqueous hydrochloric acid (2 M, 1 mL) and purified by SPE as outlined in section 4.3.2.3.1. Concentration of the methanol fraction afforded a 3:1 mixture containing the title compound 17, alongside the 3α-isomer 16 in >98% total conversion as determined by 400 MHz \(^1\)H NMR analysis. Data are reported for the 3β-isomer where appropriate. \( R_F \) 0.54 (40% EtOAc/hexanes); \( \delta_H \) (400 MHz, CDCl\(_3\)): 3.13 (dt, \( J \) 4.8 Hz, 10.4 Hz, 1H, C3-H), 1.82-0.58 (m, 22H), 1.20 (s, 3H, C20-H), 0.98 (d, \( J \) 6.4 Hz, 3H, C21-H), 0.84 (s, 6H, C18-H, and C19-H), 2 x OH not observed; \( \delta_C \) (100 MHz, CDCl\(_3\)): 81.9 (C17), 70.9 (C3), 54.6, 50.9, 46.6, 45.7, 45.6, 39.2, 38.2, 36.7, 36.4, 35.8, 31.9, 31.8, 28.5, 26.0 (C20), 23.4, 21.1, 19.0 (C21), 14.2 (C18), 13.3 (C19); LRMS (+EI): \( m/z \) 320 (40%, \([C_{21}H_{36}O_2]^+\)), 305 (60%), 245 (60%), 231 (80%), 179 (80%), 163 (55%), 135 (50%), 121 (100%), 107 (70%), 95 (70%), 81 (70%), 55 (70%); HRMS (+EI): found 320.2711, \([C_{21}H_{36}O_2]^+\) requires 320.2715.

4.3.2.3.3 2α,17α-Dimethyl-5α-androstane-3α,17β-diol 3-sulfate, ammonium salt (3α-RSS)

Sulfation was performed according to a literature method with minor modifications. A solution of 2α,17α-dimethyl-5α-androstane-3α,17β-diol 16 (derived from 1.0 mg superdrol, assumed 3.14 µmol) in DMF (2 mL) was treated with \( \text{SO}_3 \text{py} \) (10 mg, 62.8 µmol, 20.0 eq) and stirred at room temperature for 4 h. The reaction was quenched by addition of water (5 mL) and was subjected to purification by SPE as per Chapter 2.2, manuscript section 2.3.1), affording the title compound 3α-RSS in >98% total conversion. \( R_F \) 0.51
(7:2:1 EtOAc:MeOH:H$_2$O); $\delta$H (400 MHz, CD$_3$OD): 4.41 (m, 1H, C3-H), 2.51-0.68 (m, 21H), 1.01 (s, 3H), 0.98 (d, J 7.0 Hz, 3H, C21-H$_3$), 0.87 (s, 3H), 0.81 (s, 3H); LRMS (-ESI): m/z 399 (20%, [C$_{21}$H$_{35}$O$_5$S]$^-$), 381 (100%), 110 (55%), 97 (90%); HRMS (-ESI): found 399.2209, [C$_{21}$H$_{35}$O$_5$S]$^-$ requires 399.2205.

4.3.2.3.4 2α,17α-Dimethyl-5α-androstane-3β,17β-diol 3-sulfate, ammonium salt (3β-RSS)

A solution of 2α,17α-dimethyl-5α-androstane-3β,17β-diol 17 (3:1 β:α mixture derived from 1.0 mg superdrol, assumed 3.14 µmol) in DMF (500 µL) was treated with SO$_3$.py (10 mg, 62.8 µmol, 20.0 eq), followed by purification by WAX SPE as outlined in section 4.3.2.3.3, to afford a 3:1 mixture containing the title compound 3β-RSS, alongside the 3α-isomer 3α-RSS in >98% conversion. Data are reported for the 3β-isomer where appropriate. Rf 0.49 (7:2:1 EtOAc:MeOH:H$_2$O); $\delta$H (400 MHz, CD$_3$OD): 3.86 (dt, J 4.9 Hz, 10.8 Hz, 1H, C3-H), 2.63 (m, 1H), 2.03-0.68 (m, 20H), 1.50 (s, 3H, C20-H$_3$), 1.01 (d, J 6.4 Hz, 3H, C21-H$_3$), 0.92 (s, 3H, C18-H$_3$), 0.88 (s, 3H, C19-H$_3$); LRMS (-ESI): m/z 399 (90%), 381 (100%), 239 (10%), 97 (90%); HRMS (-ESI): found 399.2221, [C$_{21}$H$_{35}$O$_5$S]$^-$ requires 399.2205.

4.3.2.3.5 2α,17α-Dimethyl-5α-androstane-3β,17β-diol 3-glucuronide, ammonium salt (3β-RSG)

Glucuronylation was performed according to literature methods. A solution of 2α,17α-dimethyl-5α-androstane-3β,17β-diol (derived from 5.0 mg superdrol, assumed 15.7 µmol) in t-BuOH (2.26 mL) was treated with a solution of α-D-glucuronyl fluoride (21 mg, 98.5 µmol, 6.3 eq) in sodium phosphate buffer (50 mM, pH 7.5, 18.34 mL), followed by a solution of glucuronylsynthase (10.2 mg/mL, 450 µL) in aqueous sodium phosphate buffer/glycerol solution (50% v/v, 50 mM, pH 7.4). The reaction was incubated for 48 h at 37 ºC, and was then subjected to purification by WAX SPE as outlined in section 4.3.2.3.3 to afford the title compound 3β-RSG in 51% conversion. Rf 0.41 (7:2:1 EtOAc:MeOH:H$_2$O);
\( \delta_H \) (400 MHz, CD\textsubscript{3}OD): 4.40 (d, \( J \) 7.8 Hz, 1H, C22-H), 3.53-3.35 (m, 4H, C3-H, C24-H, C25-H, and C26-H), 3.20 (t, \( J \) 8.5 Hz, 1H, C23-H), 1.81-0.65 (m, 20H), 1.18 (s, 3H, C20-H\textsubscript{3}), 0.99 (d, \( J \) 6.2 Hz, 3H, C21-H\textsubscript{3}), 0.87 (s, 3H, C18-H\textsubscript{3}), 0.83 (s, 3H, C19-H\textsubscript{3}); \( \delta_C \) (100 MHz, CD\textsubscript{3}OD): 179.9 (C27), 100.9 (C22), 82.5 (C3, or C17), 82.3 (C3, or C17), 78.0 (C24), 76.8 (C26), 75.1 (C23), 73.8 (C25), 55.9, 52.1, 48.1, 46.9, 46.3, 39.3, 37.6, 37.3, 34.6, 34.5, 33.1, 33.0, 29.7, 26.1 (C20), 24.3, 22.0, 19.6 (C21), 14.7 (C18), 13.5 (C19); LRMS (-ESI): \( m/z \) 495 (100%), 82 (10%), 61 (10%); HRMS (-ESI): found 495.2964, [C\textsubscript{27}H\textsubscript{43}O\textsubscript{8}]\textsuperscript{-} requires 495.2963.

### 4.3.2.4 Analytical methods

#### 4.3.2.4.1 Furazadrol LC-MS analysis

LC-MS analysis for furazadrol was performed as previously reported \(^2\). Positive mode liquid chromatography-high resolution accurate mass (LC-HRAM) spectrometry analysis was undertaken using a Thermo Fisher Scientific (Bremen, Germany) Ultimate 3000 HPLC coupled to an Q Exactive Hybrid Quadrupole Orbitrap mass spectrometer equipped with a Waters SunFire C18 column (100 x 2.1 mm, 3.5 \( \mu \)m) eluting with a gradient consisting of the following mobile phases, A: 0.1% formic acid in water, B: 0.1% formic acid in methanol, gradient: 0-1 min A-B (95:5 v/v), 1-15 min A-B (95:5 v/v) to A-B (5:95 v/v), 15-19 min A-B (5:95 v/v), 5 min re-equilibration, flow rate 0.4 mL min\(^{-1}\). Unconjugated steroids and steroid glucuronides were monitored for the proton adduct ([M+H]\textsuperscript{+}) using HESI in positive full scan mode at a resolution of 70,000 (FWHM). Negative mode LC-HRAM spectrometry analysis was undertaken using a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer equipped with a Phenomenex (Torrance CA, USA) Gemini C18 column (50 mm x 2 mm, 5 \( \mu \)m), eluting with a gradient consisting of the following mobile phases, A: aqueous ammonium acetate (0.01 M, pH 9.0), B: 0.1% acetic acid in acetonitrile, gradient: 0-2 min A-B (99:1 v/v), 2-8.5 min A-B (99:1 v/v) to A-B (20:80 v/v), 2.7 min re-equilibration, flow rate 0.5 mL/min. Steroid glucuronide and sulfate conjugates were monitored for the anion ([M-H]) using HESI in negative full-scan or targeted MS/MS mode at a resolution of 70,000 (FWHM).
4.3.2.4 Superdrol LC-MS analysis

LC-MS analysis for superdrol was performed using an Agilent 1290 Infinity II LC system coupled to an Agilent 6545 Q-TOF mass spectrometer equipped with a Phenomenex Gemini C18 column (50 mm x 2 mm, 5 µm). Injections were resolved using the gradient outlined for negative mode LC-MS analysis in section 4.3.2.4.1.

4.3.2.5 In vitro phase II metabolism

4.3.2.5.1 In vitro phase II metabolism using PAPS

In vitro phase II metabolism was performed by modification to a known literature method. A solution containing steroid (120 µM, 250 µL) in sodium phosphate buffer (100 mM, pH 7.4) and methanol (0.4%) was treated in order with the following solutions: aqueous magnesium chloride (1.0 M, 2.3 µL), aqueous G6P (100 mM, 37.5 µL), aqueous NAD (50 mM, 15 µL), aqueous NADP (50 mM, 15 µL), aqueous NAD-dependant G6PDH (40 units/mL, 12.5 µL), aqueous NADP-dependant G6PDH (40 units/mL, 12.5 µL), aqueous PAPS (1.6 mM, 22.5 µL), additional aqueous magnesium chloride in association with PAPS (1.0 M, 2.5 µL), aqueous UDPGA (610 µM, 31 µL), water (74 µL), and equine liver S9 fraction (20 mg/mL, 25 µL). The final solution (500 µL) was then incubated in an open tube with agitation for 16 h at 37 °C. The reaction was quenched with acetonitrile (1 mL), centrifuged (2000 rpm, 5 min) to pellet solids, and the supernatant was decanted. Concentration of the supernatant under a stream of nitrogen at 60 °C afforded a residue which was reconstituted in methanol-water (5:95 v/v, 200 µL) and transferred to a sealed vial for subsequent LC-MS analysis as per section 4.3.2.4. Control experiments excluding PAPS, UDPGA, both UDPGA and PAPS, all phase I co-factors, equine liver S9 fraction, and steroid respectively were performed alongside the above reaction, with addition of water or buffer as required to maintain a constant final reaction volume and buffer concentration.

4.3.2.5.2 In vitro phase II metabolism using ATP and Na₂SO₄

In vitro metabolism with ATP and sodium sulfate was performed as per section 4.3.2.5.1 with the following substitutions: the addition of PAPS solution was replaced by addition of aqueous ATP (250 mM, 32 µL), aqueous sodium sulfate
(100 mM, 40 µL), and additional aqueous magnesium chloride (1.0 M, 5.5 µL). The volume of water added was reduced to maintain a constant final reaction volume (500 µL). Control experiments analogous to those reported in section 4.3.2.5.1 were also performed alongside this reaction.

4.3.4 Results and Discussion

4.3.4.1 Synthesis of steroid reference materials

The synthesis of the furazadrol reference materials used in this study has been discussed in Chapter 4.2. The chemical synthesis of superdrol reference materials has not been previously reported, although some of these materials have been tentatively identified in previous in vitro metabolism studies. The reduction of superdrol with sodium borohydride gave rise to a mixture of 3α/β-alcohol isomers 16 and 17, predominately favouring the 3β-isomer 17. Axial addition to cyclohexanone systems by small hydride donors is well-known, and selectively affords the equatorial alcohol. In the case of superdrol however, the adjacent equatorial C2α-methyl substituent partially blocks approach of the incoming nucleophile on the α-face, and as a result the reduction is less selective, affording a 3:1 mixture of products. On the other hand, reduction with L-Selectride®, which is a bulky reducing agent, exclusively gave rise to the 3α-alcohol isomer 16. The addition of hydride occurs exclusively on the β-face as this minimises the steric interaction between the reducing agent, and the ring substituents. Performing these reactions on the milligram-scale proceeded smoothly but unfortunately did not allow these isomers to be readily separated. As a result, the 3β-isomer 17 was prepared with a minor 3α-impurity 16 which was carried through subsequent reaction steps. The pure 3α-isomer 16 was used as a control to identify the presence of products derived from the 3β-isomer 17.

Mono-sulfation of the 3α/β,17β-diols to give 3α-RSS, and mixed 3α-RSS/3β-RSS proceeded smoothly, and in high yield, using established methodology. Surprisingly, there appeared to be no difference in the reactivity of the two isomers even in the presence of the bulky C2 methyl substituent. On the other hand, enzymatic glucuronylation was problematic as only the 3β-isomer
underwent the reaction. This was highlighted by the reaction of a mixture of 3α/β-hydroxy isomers 16/17, which afforded 2α,17α-dimethyl-5α-androstane-3β,17β-diol 3-glucuronide 3β-RSG as the sole product after purification by SPE. Unreacted 16 was isolated from the reaction mixture. The selectivity for the glucuronylation of 3β-steroid alcohols using this enzyme has been documented 21, and presumably reflects the substrate binding within the enzyme active site. Alternative methods for the synthesis of the 3α-hydroxy isomer, 3α-RSG (2α,17α-dimethyl-5α-androstane-3α,17β-diol 3-glucuronide), such as the chemical Koenigs-Knorr method 26, were not attempted for this substrate due to the expense and limited availability of 16.

4.3.4.2 In vitro phase II metabolism of superdrol

In vitro metabolism systems are a useful alternative when in vivo studies are not possible due to ethical or financial constraints. One of the key criticisms of in vitro methods is their purported inability to adequately reflect the in vivo metabolic profile. To investigate these issues, the phase II equine metabolism of the designer steroids superdrol, and furazadrol was investigated. Metabolism data were examined using mass filters for predicted metabolites formed from up to three metabolic transformations including oxidation, reduction and hydroxylation, with or without subsequent sulfation or glucuronylation. Metabolite peaks were identified where exact masses were observed within ±10 ppm of the predicted mass, and by comparison with control experiments. Metabolites were also matched against synthesised reference materials where available. The phase II equine metabolism of superdrol has not been previously reported, however previous studies have reported the phase I metabolism of superdrol using equine 5, and human 24 in vitro systems, which will be used for comparison to the present study.

Metabolism of superdrol with UDPGA, ATP, Na₂SO₄, and equine liver S9 fraction as per section 4.3.2.5.2 afforded a range of metabolites (Table 4.1). Phase I metabolism presumably afforded a number of steroid diol, or triol metabolites which are known to poorly ionise under +ESI conditions 1,27, and were
subsequently not detected by LC-HRAM analysis. Major phase II metabolites were also observed including 3β-RSS, and a reduced superdrol glucuronide metabolite G2, the former of which was matched to the corresponding reference material. Minor phase II metabolites were observed including two hydroxylated superdrol sulfate metabolites (S1, S2), a reduced and hydroxylated superdrol metabolite (S3), a superdrol glucuronide (G1), 3β-RSG, and two reduced and hydroxylated superdrol metabolites (G3, G4). The phase I metabolism appears to be quite simple, and the phase II conjugates observed appear to correlate well with the phase I metabolites previously reported for the equine and human in vitro studies 5,24. Both 3β-RSS and 3β-RSG were confirmed as major and minor metabolites respectively by comparison to the corresponding reference materials.

Interestingly however, 3α-RSS was not observed as a metabolite by comparison to reference materials. The tendency for C3β-reduction in the horse is well-known, as is the preference for sulfation to predominate in phase II, as reflected by 3β-RSS in the metabolite profile above. The formation of the C3β-sulfate is known to be favoured in horses, compared to the C3α-glucuronide that predominates in human metabolism 1. This is reflected in the present in vitro profile where sulfate metabolites are among the most intense peaks observed. The observation of G2, the presumed 3α-hydroxy isomer, as a major metabolite was unexpected and this metabolite is reasoned to form from 16, which is not subject to sulfation.

The metabolism of superdrol appears to be simple however (Figure 4.2), and the reduced superdrol phase II metabolites appear to be the most abundant. This also mirrors observations made in previous phase I in vitro studies 5,24. As such, these metabolites would likely be among the major metabolites observed in vivo. Additionally, these compounds are also easily prepared from the parent compound superdrol, and so should be available as reference materials to assist in confirmation 21,23. As such, it is recommended that anti-doping laboratories monitor for 3β-RSS, and 2α,17α-dimethyl-5α-androstan-3α,17β-diol 3-glucuronide (assumed to correspond to G2), or their phase I counterparts 17, and 16, until such time as a comparative in vivo study can be undertaken.
The use of *in vitro* methods to study phase II metabolism is currently limited by the requirements for expensive phase II co-factors, such as PAPS. This work has demonstrated that that ATP/Na₂SO₄ can be used to generate *in vitro* sulfate metabolites, and can serve as a viable alternative to study phase II metabolism. This methodology will now be used to study the phase II metabolism of the designer steroid furazadrol, with comparison to the previously reported *in vivo* study described in Chapter 4.2.
Table 4.1: In vitro phase II equine metabolism of superdrol

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Precursor ion and MS/MS fragments (m/z) (% of base peak), [collision energy] (^a)</th>
<th>RT (min) (^a)</th>
<th>Precursor ion</th>
<th>Theoretical m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>3β-RSS</td>
<td>399.2219 (95%), 96.9607 (100%), [40 eV]</td>
<td>5.53 (^b)</td>
<td>[M-H]⁻</td>
<td>399.2211</td>
</tr>
<tr>
<td>hydroxylated superdrol sulfate S1</td>
<td>413.1998 (60%), 96.9599 (100%), [40 eV]</td>
<td>5.03</td>
<td>[M-H]⁻</td>
<td>413.2003</td>
</tr>
<tr>
<td>hydroxylated superdrol sulfate S2</td>
<td>413.1997 (65%), 96.9597 (100%), [40 eV]</td>
<td>5.68</td>
<td>[M-H]⁻</td>
<td>413.2003</td>
</tr>
<tr>
<td>reduced and hydroxylated superdrol sulfate S3</td>
<td>415.2170 (100%), 96.9616 (50%), [40 eV]</td>
<td>5.03</td>
<td>[M-H]⁻</td>
<td>415.2160</td>
</tr>
<tr>
<td>superdrol glucuronide G1</td>
<td>493.2789 (40%), 317.2486 (60%), 175.0272 (20%), 113.0241 (40%), 85.0292 (90%), 75.0089 (100%), [40 eV]</td>
<td>5.11</td>
<td>[M-H]⁻</td>
<td>493.2807</td>
</tr>
<tr>
<td>3β-RSG</td>
<td>495.2954 (25%), 113.0244 (55%), 85.0296 (90%), 75.0089 (100%), [40 eV]</td>
<td>4.99 (^c)</td>
<td>[M-H]⁻</td>
<td>495.2963</td>
</tr>
<tr>
<td>reduced superdrol glucuronide G2</td>
<td>495.2966 (50%), 113.0247 (50%), 85.0296 (90%), 75.0090 (100%), [40 eV]</td>
<td>5.14</td>
<td>[M-H]⁻</td>
<td>495.2963</td>
</tr>
<tr>
<td>reduced and hydroxylated superdrol glucuronide G3</td>
<td>511.2906 (50%), 335.2587 (25%), 113.0245 (50%), 85.0293 (90%), 75.0088 (100%), [40 eV]</td>
<td>4.90</td>
<td>[M-H]⁻</td>
<td>511.2913</td>
</tr>
<tr>
<td>reduced and hydroxylated superdrol glucuronide G4</td>
<td>511.2904 (95%), 113.0246 (80%), 85.0293 (100%), 75.0089 (95%), [40 eV]</td>
<td>5.28</td>
<td>[M-H]⁻</td>
<td>511.2913</td>
</tr>
</tbody>
</table>

\(^a\)From targeted MS/MS data acquisition on the Agilent LC-MS/MS instrument using conditions specified for negative mode analysis (section 4.3.2.4.1). \(^b\)Matched against mixed 3α-RSS and 3β-RSS reference material. \(^c\)Matched against 3β-RSG reference material

4.3.4.3 In vitro phase II metabolism of furazadrol with PAPS

The designer steroid furazadrol was the next compound to be studied. The data obtained from this study were compared to the recently reported phase II in vivo, and phase I in vitro equine metabolism studies of furazadrol, as described in Chapter 4.2 ². The in vitro metabolism of furazadrol using equine liver S9 fraction, UDPGA, and PAPS, afforded a range of metabolites, as shown below (Tables 4.2,
and 4.3). For reference, the numbering system for the minor metabolites M1-M16 in this section is different to that reported in Chapter 4.2, but correlations will be highlighted where appropriate.

Table 4.2: In vitro equine metabolism of furazadrol – Phase I

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Precursor ion and MS/MS fragments (m/z) (% of base peak), [collision energy] A</th>
<th>RT (min) A</th>
<th>Precursor ion</th>
<th>Theoretical m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>316.2270 (10%), 173.1325 (10%), 159.1168 (15%), 145.1012 (25%), 119.0857 (30%), 105.0702 (50%), 84.0048 (100%), [60 eV]</td>
<td>14.15 B, G, H, I, J, K</td>
<td>[M+H]^+</td>
<td>316.2271</td>
</tr>
<tr>
<td>IF</td>
<td>316.2271 (45%), 288.2323 (15%), 201.1639 (15%), 187.1482 (20%), 159.1168 (30%), 145.1013 (60%), 105.0702 (100%), [60 eV]</td>
<td>13.76 B, C, G, H, I, J, K</td>
<td>[M+H]^+</td>
<td>316.2271</td>
</tr>
<tr>
<td>EF</td>
<td>316.2272 (15%), 171.1170 (10%), 143.0856 (30%), 105.0702 (40%), 84.0449 (100%), [60 eV]</td>
<td>14.32 D, H, I, J, K</td>
<td>[M+H]^+</td>
<td>316.2271</td>
</tr>
<tr>
<td>OF</td>
<td>314.2115 (10%), 197.1325 (10%), 159.1170 (20%), 145.1013 (45%), 105.0702 (60%), 84.0449 (100%), [60 eV]</td>
<td>13.68 E, I, J, K</td>
<td>[M+H]^+</td>
<td>314.2115</td>
</tr>
<tr>
<td>OIF</td>
<td>314.2115 (30%), 286.2163 (20%), 199.1482 (20%), 185.1326 (30%), 157.1012 (40%), 119.0857 (60%), 105.0702 (100%), [60 eV]</td>
<td>13.41 E, F, I, J, K</td>
<td>[M+H]^+</td>
<td>314.2115</td>
</tr>
<tr>
<td>hydroxylated furazadrol M1</td>
<td>332.2220 (80%), 197.1328 (10%), 161.1325 (20%), 145.1012 (35%), 105.0702 (50%), 84.0448 (100%), [50 eV]</td>
<td>11.27 I</td>
<td>[M+H]^+</td>
<td>332.2220</td>
</tr>
<tr>
<td>hydroxylated furazadrol M2</td>
<td>332.2220 (50%), 197.1327 (10%), 145.1013 (20%), 105.0702 (30%), 84.0449 (100%), [50 eV]</td>
<td>11.48 I, J, K</td>
<td>[M+H]^+</td>
<td>332.2220</td>
</tr>
<tr>
<td>hydroxylated furazadrol M3</td>
<td>332.2220 (30%), 201.1639 (5%), 171.1168 (10%), 145.1013 (20%), 105.0702 (25%), 84.0449 (100%), [50 eV]</td>
<td>11.63 I, J, K</td>
<td>[M+H]^+</td>
<td>332.2220</td>
</tr>
<tr>
<td>hydroxylated furazadrol M4</td>
<td>332.2220 (90%), 304.2270 (30%), 185.1326 (10%), 145.1012 (20%), 108.0811 (100%), [50 eV]</td>
<td>11.91 G, I, J, K</td>
<td>[M+H]^+</td>
<td>332.2220</td>
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<tr>
<td>hydroxylated furazadrol M5</td>
<td>332.2220 (50%), 211.1484 (10%), 171.1169 (20%), 145.1013 (40%), 105.0702 (50%), 84.0448 (100%), [50 eV]</td>
<td>12.16 I, K</td>
<td>[M+H]^+</td>
<td>332.2220</td>
</tr>
<tr>
<td>hydroxylated furazadrol M6</td>
<td>332.2219 (50%), 145.1012 (30%), 105.0702 (30%), 84.0448 (100%), [50 eV]</td>
<td>12.28 K</td>
<td>[M+H]^+</td>
<td>332.2220</td>
</tr>
<tr>
<td>Compound</td>
<td>M/Z Formula</td>
<td>M/Z Formula Details</td>
<td>Retention</td>
<td>M/Z Formula Details</td>
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<tr>
<td>--------------------------------</td>
<td>-----------------</td>
<td>------------------------------</td>
<td>-----------</td>
<td>---------------------</td>
</tr>
<tr>
<td>hydroxylated furazadrol M7</td>
<td>332.2228 (100%), 304.2270 (15%), 185.1325 (15%), 145.1011 (25%), 105.0701 (35%), [50 eV]</td>
<td>12.47 K</td>
<td>[M+H]+</td>
<td>332.2220</td>
</tr>
<tr>
<td>hydroxylated furazadrol M8</td>
<td>332.2220 (70%), 211.1480 (10%), 171.1169 (15%), 145.1012 (25%), 105.0702 (40%), 84.0448 (100%), [60 eV]</td>
<td>12.81 I, J, K</td>
<td>[M+H]+</td>
<td>332.2220</td>
</tr>
<tr>
<td>hydroxylated furazadrol M9</td>
<td>332.2221 (80%), 215.1432 (10%), 197.1325 (20%), 145.1013 (30%), 84.0449 (100%), [50 eV]</td>
<td>13.12 I, K</td>
<td>[M+H]+</td>
<td>332.2220</td>
</tr>
<tr>
<td>oxidised and hydroxylated furazadrol M10</td>
<td>330.2063 (100%), 288.1949 (15%), 199.1484 (25%), 159.1169 (55%), 84.0448 (100%), [60 eV]</td>
<td>10.35 J</td>
<td>[M+H]+</td>
<td>330.2064</td>
</tr>
<tr>
<td>oxidised and hydroxylated furazadrol M11</td>
<td>330.2060 (15%), 300.1957 (75%), 240.2684 (10%), 201.1273 (15%), 143.0855 (15%), 105.0702 (25%), 84.0448 (100%), [50 eV]</td>
<td>12.03 K</td>
<td>[M+H]+</td>
<td>330.2064</td>
</tr>
<tr>
<td>oxidised and hydroxylated furazadrol M12</td>
<td>330.2063 (45%), 288.1955 (15%), 185.1326 (15%), 145.1013 (30%), 84.0448 (100%), [60 eV]</td>
<td>12.16 I, K</td>
<td>[M+H]+</td>
<td>330.2064</td>
</tr>
<tr>
<td>dihydroxylated furazadrol M13</td>
<td>348.2158 (20%), 183.1165 (5%), 157.1008 (15%), 112.0392 (25%), 84.0446 (100%), [50 eV]</td>
<td>8.92 I, K</td>
<td>[M+H]+</td>
<td>348.2169</td>
</tr>
<tr>
<td>dihydroxylated furazadrol M14</td>
<td>348.2156 (25%), 209.1313 (5%), 159.1165 (10%), 105.0698 (20%), 84.0446 (100%), [50 eV]</td>
<td>9.75 J</td>
<td>[M+H]+</td>
<td>348.2169</td>
</tr>
<tr>
<td>dihydroxylated furazadrol M15</td>
<td>348.2157 (25%), 183.1162 (5%), 145.1007 (15%), 112.0392 (30%), 84.0446 (100%), [50 eV]</td>
<td>10.24 I, K</td>
<td>[M+H]+</td>
<td>348.2169</td>
</tr>
<tr>
<td>dihydroxylated furazadrol M16</td>
<td>348.2159 (20%), 237.1633 (5%), 197.1319 (10%), 155.0850 (15%), 105.0699 (25%), 84.0446 (100%), [50 eV]</td>
<td>10.78 I, K</td>
<td>[M+H]+</td>
<td>348.2169</td>
</tr>
</tbody>
</table>

*From targeted MS/MS data acquisition on the Q Exactive instrument using conditions specified for positive mode analysis (section 4.3.2.4.1). \(^{a}\)Matched with mixed F and IF reference material. \(^{b}\)Matched with IF reference material. \(^{c}\)Matched with mixed EF and EIF reference material. \(^{d}\)Matched with mixed OF and OIF reference material. \(^{e}\)Matched with OIF reference material. \(^{f}\)Matched with metabolite identified in in vivo samples after P. aeruginosa arylsulfatase hydrolysis. \(^{g}\)Matched with metabolite identified in in vivo samples after E. coli β-glucuronidase hydrolysis. \(^{h}\)Matched with metabolite identified in previously reported phase I in vitro study (Ref 2). \(^{i}\)Metabolite observed from in vitro metabolism with UDPGA and PAPS. \(^{j}\)Metabolite observed from in vitro metabolism with UDPGA, ATP, and Na₂SO₄.
### Table 4.3: *In vitro* equine metabolism of furazadrol – Phase II

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Precursor ion and MS/MS fragments (m/z) (% of base peak), [collision energy] (^A)</th>
<th>RT (min) (^A)</th>
<th>Precursor ion</th>
<th>Theoretical m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>FS</td>
<td>394.1624 (10%), 96.9573 (100%), [60 eV]</td>
<td>6.27</td>
<td>B, G, H, I</td>
<td>[M-H] 394.1683</td>
</tr>
<tr>
<td>IFS</td>
<td>394.1624 (20%), 96.9573 (100%), [60 eV]</td>
<td>6.13</td>
<td>B, G, H, I</td>
<td>[M-H] 394.1683</td>
</tr>
<tr>
<td>hydroxylated furazadrol sulfate S1</td>
<td>410.1636 (30%), 330.2068 (30%), 96.9589 (100%), [40 eV]</td>
<td>5.07</td>
<td>H, I</td>
<td>[M-H] 410.1632</td>
</tr>
<tr>
<td>hydroxylated furazadrol sulfate S2</td>
<td>410.1633 (100%), 364.1584 (40%), 96.9589 (50%), [40 eV]</td>
<td>5.13</td>
<td>G, H, I</td>
<td>[M-H] 410.1632</td>
</tr>
<tr>
<td>hydroxylated furazadrol sulfate S3</td>
<td>410.1637 (100%), 334.1987 (10%), 96.9586 (50%), [40 eV]</td>
<td>5.21</td>
<td>H, I</td>
<td>[M-H] 410.1632</td>
</tr>
<tr>
<td>hydroxylated furazadrol sulfate S4</td>
<td>410.1644 (60%), 330.2069 (15%), 96.9589 (100%), [40 eV]</td>
<td>5.41</td>
<td>H, I</td>
<td>[M-H] 410.1632</td>
</tr>
<tr>
<td>hydroxylated furazadrol sulfate S5</td>
<td>410.1638 (75%), 96.9588 (100%), [40 eV]</td>
<td>5.72</td>
<td>H, I</td>
<td>[M-H] 410.1632</td>
</tr>
<tr>
<td>hydroxylated furazadrol sulfate S6</td>
<td>410.1571 (55%), 328.1861 (10%), 96.9574 (100%), [60 eV]</td>
<td>5.89</td>
<td>H, I</td>
<td>[M-H] 410.1632</td>
</tr>
<tr>
<td>oxidised and hydroxylated furazadrol sulfate S7</td>
<td>408.1481 (20%), 328.1907 (25%), 96.9589 (100%), [40 eV]</td>
<td>5.41</td>
<td>H</td>
<td>[M-H] 408.1475</td>
</tr>
<tr>
<td>oxidised and hydroxylated furazadrol sulfate S8</td>
<td>408.1479 (100%), 96.9589 (50%), 79.9561 (50%), [40 eV]</td>
<td>5.79</td>
<td>G, I</td>
<td>[M-H] 408.1475</td>
</tr>
<tr>
<td>FG</td>
<td>492.2589 (70%), 316.2269 (90%), 199.1479 (20%), 141.0181 (35%), 113.0234 (45%), 84.0447 (100%), [40 eV]</td>
<td>13.96</td>
<td>D, G, I, L, H, I</td>
<td>[M+H] (^+) 492.2592</td>
</tr>
<tr>
<td>IFG</td>
<td>492.2583 (15%), 316.2266 (70%), 288.2319 (30%), 187.1480 (20%), 141.0181 (40%), 113.0234 (55%), 85.0287 (100%), [50 eV]</td>
<td>13.46</td>
<td>D, E, G, H, I</td>
<td>[M+H] (^+) 492.2592</td>
</tr>
<tr>
<td></td>
<td>Molecular Formula</td>
<td>Precursor Ions (m/z)</td>
<td>Retention Time (min)</td>
<td>Charge State</td>
</tr>
<tr>
<td>-----</td>
<td>-------------------</td>
<td>----------------------</td>
<td>----------------------</td>
<td>--------------</td>
</tr>
<tr>
<td><strong>EFG</strong></td>
<td>492.2588 (50%), 316.2268 (55%), 298.2160 (100%), 217.0315 (20%), 159.0285 (10%),</td>
<td>14.35</td>
<td>[M+H]^+</td>
<td>492.2592</td>
</tr>
<tr>
<td>hydroxylated furazadrol glucuronide G1</td>
<td>508.2538 (40%), 332.2220 (20%), 304.2266 (20%), 199.1476 (10%), 141.0181 (55%), 84.0448 (100%), [40 eV]</td>
<td>10.57</td>
<td>[M−H]^−</td>
<td>508.2541</td>
</tr>
<tr>
<td>hydroxylated furazadrol glucuronide G2</td>
<td>508.2539 (35%), 332.2217 (70%), 197.1318 (10%), 136.0617 (100%), [40 eV]</td>
<td>11.16</td>
<td>[M−H]^−</td>
<td>508.2541</td>
</tr>
<tr>
<td>hydroxylated furazadrol glucuronide G3</td>
<td>508.2544 (10%), 332.2216 (100%), 296.2005 (10%), 184.0732 (15%), [30 eV]</td>
<td>11.37</td>
<td>[M−H]^−</td>
<td>508.2541</td>
</tr>
<tr>
<td>hydroxylated furazadrol glucuronide G4</td>
<td>508.2534 (45%), 332.2213 (50%), 293.2103 (10%), 197.1322 (10%), [40 eV]</td>
<td>12.64</td>
<td>[M+H]^+</td>
<td>508.2541</td>
</tr>
<tr>
<td>hydroxylated furazadrol glucuronide G5</td>
<td>508.2525 (55%), 332.2220 (40%), 296.2001 (10%), 184.0732 (15%), [30 eV]</td>
<td>13.47</td>
<td>[M+H]^+</td>
<td>508.2541</td>
</tr>
<tr>
<td>hydroxylated furazadrol glucuronide G6</td>
<td>508.2534 (25%), 332.2216 (75%), 296.2005 (10%), 197.1326 (10%), [40 eV]</td>
<td>13.53</td>
<td>[M+H]^+</td>
<td>508.2541</td>
</tr>
<tr>
<td>oxidised and hydroxylated furazadrol glucuronide G7</td>
<td>506.2390 (10%), 136.0618 (100%), [30 eV]</td>
<td>11.29</td>
<td>[M+H]^+</td>
<td>506.2385</td>
</tr>
<tr>
<td>oxidised and hydroxylated furazadrol glucuronide G8</td>
<td>506.2386 (35%), 330.2063 (65%), 294.1851 (30%), 136.0618 (100%), [30 eV]</td>
<td>12.28</td>
<td>[M+H]^+</td>
<td>506.2385</td>
</tr>
<tr>
<td>oxidised and hydroxylated furazadrol glucuronide G9</td>
<td>506.2376 (55%), 330.2059 (100%), 294.1849 (40%), 242.1537 (10%), 141.0181 (50%), [30 eV]</td>
<td>12.34</td>
<td>[M+H]^+</td>
<td>506.2385</td>
</tr>
<tr>
<td>oxidised and hydroxylated furazadrol glucuronide G10</td>
<td>506.2393 (30%), 330.2058 (55%), 300.1955 (70%), 201.1275 (20%), 136.0618 (100%), [40 eV]</td>
<td>12.47</td>
<td>[M+H]^+</td>
<td>506.2385</td>
</tr>
</tbody>
</table>

*From targeted MS/MS data acquisition on the Q Exactive instrument using conditions specified for positive mode or negative mode analysis as specified (section 4.3.2.4.1). Matched against mixed FS and IFS reference material. Matched against IFS reference material. Matched against mixed FG and IFG reference material. Matched against IFG reference material. Matched against mixed EFG and episoisofurazadrol 17-glucuronide reference material. Matched with in vivo metabolite (Ref 2). Metabolite observed from in vitro metabolism with UDPGA and PAPS. Metabolite observed from in vitro metabolism with UDPGA, ATP, and Na₂SO₄.
Major phase I metabolites were observed including \textbf{F}, \textbf{IF}, \textbf{EF}, \textbf{OF}, and \textbf{OIF} which were matched to reference materials. Additionally, a number of unidentified minor phase I metabolites were observed by positive mode LC-HRAM analysis including seven hydroxylated metabolites (\textbf{M1-M5}, \textbf{M8}, \textbf{M9}), two oxidised and hydroxylated metabolites (\textbf{M10}, \textbf{M12}), and four dihydroxylated metabolites (\textbf{M13-M16}). Of these metabolites, \textbf{F}, \textbf{IF}, \textbf{EF}, \textbf{OF}, \textbf{OIF}, \textbf{M2-M4}, and \textbf{M8} were identified as matches with our previously reported phase I study by comparison of their retention times and mass spectrometry behaviour. The extent of phase I metabolism in the present study appeared to be greater than the previously reported phase I study \cite{2}. The present study used a combination of NADH and NADPH co-factors, in contrast to the previous study which used only NADH, which could explain the minor increase in hydroxylated metabolites identified in the present study. Metabolite \textbf{M4} was also observed to match a phase I metabolite (matches \textbf{M3} in Chapter 4.2) which was observed after \textit{P. aeruginosa} arylsulfatase hydrolysis of \textit{in vivo} samples. Major phase II metabolites were also observed including \textbf{FS}, \textbf{IFS}, \textbf{FG}, \textbf{IFG}, and \textbf{EFG} which were matched to reference materials. \textbf{EFS} was not observed by comparison to reference material, which also reflects observations from the \textit{in vivo} study. Additionally, a number of minor unidentified phase II metabolites were observed including six hydroxylated furazadrol sulfate metabolites (\textbf{S1-S6}), one oxidised and hydroxylated furazadrol sulfate metabolite (\textbf{S7}), five hydroxylated furazadrol glucuronide metabolites (\textbf{G1-G4}, \textbf{G6}), and two oxidised and hydroxylated furazadrol glucuronide metabolites (\textbf{G9}, \textbf{G10}). The overall number of phase II metabolites agreed well with the number of observed phase I metabolites. Of these metabolites, \textbf{FS}, \textbf{IFS}, \textbf{FG}, \textbf{IFG}, \textbf{EFG}, and \textbf{S2} (matches \textbf{S1} in Chapter 4.2) were identified as matches with the previously reported \textit{in vivo} study.

A comparison of the study previously reported in Chapter 4.2 with the present study is outlined below (Table 4.4). Briefly, the major phase I metabolites \textbf{F}, \textbf{IF}, \textbf{EF}, \textbf{OF}, and \textbf{OIF} have been identified in both studies. The phase I profile of the minor metabolites in these studies also appears to be similar, with only minor differences observed. The major phase II metabolites \textbf{FS}, \textbf{IFS}, \textbf{FG}, \textbf{IFG}, and \textbf{EFG} have been identified in both studies (Table 4.5), and the lack of \textbf{EFS} is common to both \textit{in vivo}
and in vitro systems. The in vitro studies appear to generate significantly more minor phase II metabolites compared to those observed in vivo.

### Table 4.4: Comparison of in vivo and in vitro (UDPGA and PAPS) metabolism of furazadrol

<table>
<thead>
<tr>
<th>Metabolic transformation</th>
<th>Chapter 4.2 Phase I in vitro</th>
<th>Chapter 4.3 Phase I in vitro</th>
<th>Chapter 4.2 Phase II in vivo</th>
<th>Chapter 4.3 Phase II in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>furazadrol (major)</td>
<td>F, IF, EF</td>
<td>F, IF, EF</td>
<td>FS, IFS, FG, IFG, EFG</td>
<td>FS, IFS, FG, IFG, EFG</td>
</tr>
<tr>
<td>oxidised furazadrol (major)</td>
<td>OF, OIF</td>
<td>OF, OIF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hydroxylated furazadrol (minor)</td>
<td>(x 8)</td>
<td>(x 7) M1-M5, M8, M9</td>
<td>sulfate (x 1)</td>
<td>sulfate (x 6) S1-S6 glucuronide (x 5) G1-G4, G6</td>
</tr>
<tr>
<td>oxidised and hydroxylated furazadrol (minor)</td>
<td>(x 1)</td>
<td>(x 2) M10, M12</td>
<td>sulfate (x 2) glucuronide (x 2)</td>
<td>sulfate (x 1) S7 glucuronide (x 2) G9, G10</td>
</tr>
<tr>
<td>dihydroxylated furazadrol (minor)</td>
<td>(x 2)</td>
<td>(x 4) M13-M16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 4.5: Comparison of in vivo and in vitro (UDPGA, ATP, and Na₂SO₄) metabolism of furazadrol

<table>
<thead>
<tr>
<th>Metabolic transformation</th>
<th>Chapter 4.2 Phase I in vitro</th>
<th>Chapter 4.3 Phase I in vitro</th>
<th>Chapter 4.2 Phase II in vivo</th>
<th>Chapter 4.3 Phase II in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>furazadrol (major)</td>
<td>F, IF, EF</td>
<td>F, IF, EF</td>
<td>FS, IFS, FG, IFG, EFG</td>
<td>FS, IFS, FG, IFG, EFG</td>
</tr>
<tr>
<td>oxidised furazadrol (major)</td>
<td>OF, OIF</td>
<td>OF, OIF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hydroxylated furazadrol (minor)</td>
<td>(x 8)</td>
<td>(x 8) M2-M9</td>
<td>sulfate (x 1)</td>
<td>sulfate (x 6) S1-S6 glucuronide (x 2) G3, G5</td>
</tr>
<tr>
<td>oxidised and hydroxylated furazadrol (minor)</td>
<td>(x 1)</td>
<td>(x 2) M11, M12</td>
<td>sulfate (x 2) glucuronide (x 2)</td>
<td>sulfate (x 1) S8 glucuronide (x 3) G7, G8, G10</td>
</tr>
<tr>
<td>dihydroxylated furazadrol (minor)</td>
<td>(x 2)</td>
<td>(x 3) M13, M15, M16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.3.4.4 Comparison of PAPS and ATP/Na$_2$SO$_4$ for the generation of phase II \textit{in vitro} sulfate metabolites

Due to the high cost to purchase PAPS we elected to investigate conditions which would allow for comparable sulfation using more readily available reagents, as discussed in the introduction. Optimal conditions for \textit{in vitro} phase II steroid sulfation were previously developed by Ms Ling Fam during an undergraduate research project in the McLeod Group\textsuperscript{20}, and were employed for the phase II \textit{in vitro} metabolism of furazadrol.

The phase II \textit{in vitro} metabolism of furazadrol using UDPGA, ATP and Na$_2$SO$_4$ proceeded similarly to metabolism using UDPGA and PAPS (Tables 4.3, and 4.3). Major phase I metabolites were observed including F, IF, EF, OF, and OIF, which were matched to reference materials. Additional minor phase I metabolites were observed, including eight hydroxylated metabolites (M2-M9), two oxidised and hydroxylated metabolites (M11, M12), and three dihydroxylated metabolites (M13, M15, M16). Of these metabolites, F, IF, EF, OF, OIF, M2-M4, and M8 were identified as matches with the phase I study reported in Chapter 4.2\textsuperscript{2}. Metabolite M4 (matches M3 in Chapter 4.2) was also observed to match the phase I metabolite which was identified after \textit{P. aeruginosa} arylsulfatase hydrolysis of the \textit{in vivo} samples. A comparison of the phase I metabolites observed using both PAPS, and ATP/Na$_2$SO$_4$ \textit{in vitro} systems indicated that a majority of the metabolites were common. The major phase I metabolites F, IF, EF, OF, and OIF were common to both systems. The minor phase I metabolites also appeared to correlate well between systems (M2-M5, M8, M9, M12, M13, M15, and M16 were common), with a few exceptions. The number of phase I metabolites observed did not appear to change significantly in control experiments where phase II co-factors were excluded, suggesting that phase II metabolism was occurring after phase I metabolism had occurred, and was not altering the phase I profile.

Major phase II metabolites were also observed including FS, IFS, FG, IFG, and EFG which were matched to reference materials. EFS was not observed by comparison to the reference material. Additionally, minor phase II metabolites were observed
including six hydroxylated furazadrol sulfate metabolites (S1-S6), one oxidised and hydroxylated furazadrol sulfate metabolite (S8), two hydroxylated furazadrol glucuronide metabolites (G3, G5), and three oxidised and hydroxylated furazadrol glucuronide metabolites (G7, G8, G10). The overall number of phase II metabolites agreed well with the number of observed phase I metabolites. Of these metabolites, FS, IFS, FG, IFG, EFG, and S8 (matches S3 in Chapter 4.2) were identified as matches with the previously reported in vivo study.

A comparison of the phase II metabolites observed using both PAPS and ATP/Na$_2$SO$_4$ in vitro systems (Tables 5, and 6) also indicated that a majority of the metabolites were common. The major phase II metabolites FS, IFS, FG, IFG, and EFG were common to both systems. In both instances EFS was not observed, which agreed with observations from the in vivo study. Of particular interest are metabolites S2, and S8, which were both observed in the previous in vivo study (matches S1, and S3 in Chapter 4.2 respectively). Metabolite S8 was not formed in the corresponding in vitro study employing PAPS, in contrast to S2 which was only observed with PAPS, suggesting that ATP/Na$_2$SO$_4$ may be a better choice for generating some in vivo metabolites.

Although the majority of the sulfate metabolites were also common to both systems, the minor phase II metabolites appeared to vary between systems, particularly for the glucuronides (S1-S6, G3, G10 were common). The differences in the glucuronide profile could potentially be attributed to the different sulfation reagents used in these systems, and this issue was not explored in the present study. In the experiment utilising PAPS, the co-factors are added at the start of the metabolism reaction, and PAPS is then consumed by the reaction, or decays in solution over time. This results in in a burst of phase II metabolism towards the beginning of the metabolism reaction, which levels off relatively quickly. This is in contrast to the experiments utilising ATP/Na$_2$SO$_4$ in which there is an initial lag to phase II metabolism as PAPS has to be generated in situ. Once PAPS is generated however, there is significantly more phase II metabolism occurring, and the
resulting metabolism reaction lasts for a longer period until all the ATP is consumed.

It appears that the substitution of PAPS with ATP/Na₂SO₄ is capable of generating comparable in vitro metabolic profiles, and can serve as a cheaper alternative for the generation of phase II in vitro sulfate metabolites. The major metabolites FS, IFS, FG, IFG, and EFG, which represent the most useful markers for anti-doping screening were all detected at much higher levels (ion counts ~10⁷-10⁸) than the corresponding minor phase II metabolites in these studies (ion counts ~10⁵-10⁶). On the other hand, the minor in vivo metabolites S₂ and S₈ would likely be difficult to distinguish from the other minor phase II metabolites observed in vitro.

In the absence of in vivo data, the major metabolites would likely be considered the most important metabolites and targeted by anti-doping laboratories, however in more complicated systems it may be difficult to distinguish between major and minor metabolites, and as such limit the ability to predict the predominate in vivo metabolites. Although it could be argued from these results that these conditions are capable of generating an adequate representation of the in vivo profile, further work could be targeted towards refining these conditions, allowing for the unambiguous determination of the entire in vivo profile solely from in vitro results. This is particularly important as current AORC criteria currently allow for the use of in vitro-derived materials as reference materials in confirmatory analysis⁴, and being able to accurately reflect the in vivo profile using in vitro techniques could allow additional instances of anabolic steroid misuse to be confirmed through the use of these reference materials.

4.3.5 Conclusions

In vitro technologies offer an opportunity to study the metabolism of steroid compounds that may not otherwise be able to be studied in vivo due to ethical or financial constraints. The data gained from these studies may assist anti-doping laboratories in developing protocols to allow detection of compounds that may otherwise pass undetected through routine screening. The in vitro phase II metabolism of the designer steroids furazadrol, and superdrol has been
investigated. The observed metabolites of superdrol have been recommended as suitable screening markers until a comparative in vivo study can be undertaken. The observed metabolites of furazadrol have been shown to correlate well with the metabolites observed in a previously reported in vivo study. Alternate conditions for in vitro phase II sulfation employing ATP/Na$_2$SO$_4$ in place of the expensive phase II co-factor PAPS were also explored, and in the case of the designer steroid furazadrol was found to offer comparable metabolism. The ability of these systems to replicate in vivo results has been shown through the identification of the major equine in vivo metabolites of furazadrol in the phase II in vitro study. Although some of the minor in vivo metabolites were also identified in this work, the ability of these systems to fully replicate in vivo metabolism for the minor metabolites is currently limited, and it is hoped that future work will afford the ability to accurately predict the in vivo metabolism of unknown compounds solely from in vitro results.

4.3.6 Acknowledgements

The author would like to thank Ms Ling Fam for optimising the conditions for phase II steroid metabolism with ATP/Na$_2$SO$_4$, and Mr Nurul Zakaria and Dr Bradley Stevenson for providing the recombinantly expressed NADP-dependant G6PDH, and β-glucuronylsynthase enzymes used in this chapter.

4.3.7 References

(20) Fam, H. L. Development of in vitro methods to study phase II metabolism, Australian National University: Canberra, Australia, 2015.
CHAPTER FIVE

Hemapolin
2α,3α-epithio-17α-methyl-5α-androstan-17β-ol
This chapter presents unpublished work which is the subject of a manuscript currently in preparation. Supporting information relevant to this chapter is presented electronically alongside this thesis.

5.1 *In vivo* metabolism of the designer anabolic steroid hemapolin in thoroughbred racehorses

5.1.1 Introduction

Hemapolin (2α,3α-epithio-17α-methyl-5α-androstan-17β-ol, called H in this work) is unique in that it possesses a 2,3-episulfide; a motif that is not often encountered by chemists due to its rarity in nature, chemical instability, the unpleasant odour often associated with its compounds or decomposition products, as well as a general lack of reliable methodology for its synthesis \(^1\). Hemapolin is also a designer steroid which has been detected in “dietary” supplements such as those marketed by Black China Labs \(^2\), and StarMark Laboratories \(^3\). It is also marketed as a component of the supplements EPISTANE™ (Innovative Body Enhancement), and P-PLEX™ (Competitive Edge Labs), however a study has reported that these supplements labelled to contain H instead contain the related compounds: 2β,3β-epithio-17α-methyl-5α-androstan-17β-ol, madol (M, desoxymethyltestosterone, 17α-methyl-5α-androst-2-en-17β-ol), and 17α-methyl-5α-androst-3-en-17β-ol \(^4\). Hemapolin has also been reported to exert significant anabolic activity in yeast and human HuH7 androgen bioassays \(^3\), as well as predicted activity in recent computational QSAR models \(^5\). Hemapolin and related epithio-containing steroids are banned in competition by the World Anti-Doping Agency (WADA), and the International Federation of Horseracing Authorities (IFHA) as they have similar chemical structures and biological effects to other banned AAS \(^6,7\).

The metabolism of H has not been previously reported, highlighting the need for this work. To this end, the equine metabolism of this compound was investigated through use of an *in vivo* drug administration study, with major metabolites matched against an extensive library of synthetically derived reference materials. This metabolic profiling study provides anti-doping laboratories with the
information required to establish routine screening methods which allow for the detection of H misuse in horses.

5.1.2 Experimental

5.1.2.1 Materials

Chemicals and solvents including \(N\)-bromosuccinimide (NBS), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), 4-toluenesulfonyl chloride (TsCl), pyridinium chlorochromate (PCC), anhydrous \(N,N\)-dimethylformamide (DMF), phenyltrimethylammonium tribromide (PTAB), sulfur-trioxide pyridine complex (SO\(_3\).py), \(N\)-methyl-\(N\)-(trimethylsilyl)trifluoroacetamide (MSTFA), dithiothreitol (DTT), and \(n\)-dodecane were purchased from Sigma–Aldrich (Castle Hill, Australia), and were used as supplied unless otherwise stated. Aqueous perchloric acid solution, formic acid, and pyridine were purchased from Ajax Chemicals (Auburn, Australia). 1,4-Dioxane (dioxane), ethyl acetate (EtOAc), methanol (MeOH), petroleum spirit (bp 40–60 °C, hexanes), and diisopropyl ether (DIPE), were purchased from Merck (Darmstadt, Germany). Aqueous hydrogen peroxide solution was purchased from Chem-Supply (Gillman, Australia). Epiandrosterone (3β-hydroxy-5α-androstan-17-one), mestanolone (17β-hydroxy-17α-methyl-5α-androstan-3-one), and 17α-methyltestosterone (17β-hydroxy-17α-methylandrost-4-en-3-one) were purchased from Steraloids (Newport RI, USA). 16,16,17-d\(_3\)-Testosterone, 16,16,17-d\(_3\)-testosterone 17-glucuronide, and 16,16,17-d\(_3\)-testosterone 17-sulfate were purchased from the National Measurement Institute (North Ryde, Australia). NBS was recrystallised from water and dried thoroughly under vacuum before use. Tetrahydrofuran (THF) was distilled from sodium wire before use. TsCl was purified before use according to literature methods. 3,3-Dimethylidioxirane (DMDO) was prepared as a solution in acetone according to literature methods and titrated immediately before use. MilliQ water was used in all aqueous solutions. Solid-phase extraction (SPE) was performed using Waters (Rydalmere, Australia) Oasis WAX 6cc cartridges (PN 186004647), Waters Oasis WAX (60 mg, 3 cc, 30 µm) cartridges (PN 186002490), Waters Sep-Pak C18 (3 cc, 500 mg) cartridges (PN WAT020805), UCT (Bristol, USA) Xtrackt (200 mg, 3 mL) cartridges (PN XRDH203), or Agilent (Santa Clara, USA) Bond-Elute NEXUS (60 mg, 3 mL) cartridges (PN 12103101) as specified.
5.1.2.2 Instruments

Melting points were determined using a SRS (Sunnyvale CA, USA) Optimelt MPA 100 melting point apparatus and are uncorrected. Optical rotations were determined using a Perkin-Elmer (Waltham, USA) Model 343 polarimeter (sodium D line, 298 K) in the indicated solvents. $^1$H and $^{13}$C nuclear magnetic resonance (NMR) spectra were recorded using either Bruker Ascend 400 MHz or Bruker Ascend 800 MHz spectrometers at 298 K using deuterated chloroform or deuterated methanol solvent. Data is reported in parts per million (ppm), referenced to residual protons or $^{13}$C in deuterated chloroform (CDCl$_3$: $^1$H 7.26 ppm, $^{13}$C 77.16 ppm) or methanol (CD$_3$OD: $^1$H 3.31 ppm, $^{13}$C 49.0 ppm) unless otherwise specified, with multiplicity assigned as follows: br = broad, s = singlet, d = doublet, dd = doublet of doublets, ddd = doublet of doublet of doublets, dt = doublet of triplets, t = triplet, q = quartet, m = multiplet. Coupling constants $J$ are reported in Hertz. Low-resolution mass spectrometry (LRMS) and high-resolution mass spectrometry (HRMS) for reference material characterisation were performed using positive electron ionisation (+EI) on a Micromass VG Autospec mass spectrometer or negative electrospray ionization (–ESI) on a Micromass ZMD ESI-Quad, or a Waters LCT Premier XE mass spectrometer. Reactions were monitored by analytical thin layer chromatography (TLC) using Merck Silica gel 60 TLC plates using solvents as specified and were visualised by staining with a solution of concentrated sulfuric acid in methanol (5% v/v), with heating as required.
5.1.2.3 Hemapolin reference materials

An extensive range of hemapolin reference materials were employed to aid the identification of phase I, and phase II metabolites. These were hemapolin H, madol M, 17β-hydroxy-17α-methyl-5α-androst-2-en-1-one E1, 17β-hydroxy-17α-methyl-5α-androst-3-en-2-one E2, 17β-hydroxy-17α-methyl-5α-androst-1-en-3-one E3, 17β-hydroxy-17α-methyl-5α-androst-2-en-4-one E4, 17α-methyltestosterone E5, 17α-methyl-5α-androst-2-ene-1α,17β-diol D1, 17α-methyl-5α-androst-2-ene-1β,17β-diol D2, 17α-methyl-5α-androst-3-ene-2α,17β-diol D3, 17α-methyl-5α-androst-3-ene-2β,17β-diol D4, 17α-methyl-5α-androst-1-ene-3α,17β-diol D5, 17α-methyl-5α-androst-1-ene-3β,17β-diol D6, 17α-methyl-5α-androst-2-ene-4α,17β-diol D7, 17α-methyl-5α-androst-2-ene-4β,17β-diol D8, 17α-methylandrost-4-ene-3α,17β-diol D9, 17α-methylandrost-4-ene-3β,17β-diol D10, hemapolin S-oxide HS, and madol 17-sulfate MS. The structures of these compounds are outlined in the supporting information, together with experimental procedures, characterisation data, and copies of the $^1$H NMR, $^{13}$C NMR, and +EI LRMS or -ESI LRMS where appropriate.
5.1.2.3.1 Epiandrosterone 3-tosylate (18)\textsuperscript{11-13}

A solution of epiandrosterone (10.075 g, 34.7 mmol) in pyridine (100 mL) was added to a flask containing TsCl (10.043 g, 52.7 mmol, 1.5 eq) and was stirred at room temperature for 16 h. The reaction was then diluted with water (500 mL) and the solid that formed collected by filtration. The crude tosylate was then purified by recrystallisation from acetone to afford the title compound 18 (14.202 g, 92\%) as small white needles. Rf 0.56 (40\% EtOAc/hexanes); mp 159-162 °C (lit \textsuperscript{12} 164-165 °C); [\alpha]\textsuperscript{25}D +50 (c 1, CHCl\textsubscript{3}) (lit \textsuperscript{11} [\alpha]\textsuperscript{25}D +46 [CHCl\textsubscript{3}]); \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}): 7.79 (d, J 8.2 Hz, 2H, C21-H), 7.32 (d, J 8.2 Hz, 2H, C22-H), 4.41 (m, 1H, C3-H), 2.44 (s, 3H, C24-H\textsubscript{3}), 2.43 (dd, J 8.8 Hz, 19.4 Hz, 1H, C16-H), 1.94-0.61 (m, 21H), 0.84 (s, 3H, C18-H\textsubscript{3}), 0.80 (s, 3H, C19-H\textsubscript{3}); \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}): 221.1 (C17), 144.5 (C20), 134.8 (C23), 129.8 (C21), 127.7 (C22), 82.3 (C3), 54.3, 51.4, 47.8, 44.9, 36.8, 35.9, 35.5, 35.0, 34.9, 31.6, 30.8, 28.4, 28.2, 21.8, 21.7 (C24), 20.5, 13.9 (C18), 12.2 (C19); LRMS (+EI): m/z 444 (15\%, [C\textsubscript{26}H\textsubscript{36}O\textsubscript{4}S]\textsuperscript{+}), 273 (20\%), 272 (100\%), 257 (25\%), 218 (95\%), 190 (30\%), 172 (45\%), 161 (25\%), 147 (20\%), 122 (20\%), 108 (45\%), 107 (70\%), 93 (35\%), 91 (100\%), 79 (40\%), 67 (25\%), 55 (25\%); HRMS (+EI): found 444.2340, [C\textsubscript{26}H\textsubscript{36}O\textsubscript{4}S]\textsuperscript{+} requires 444.2334.
5.1.2.3.2 5α-androst-2-en-17-one (19)\textsuperscript{14–16}

A solution of epiandrosterone 3-tosylate 18 (4.874 g, 11.0 mmol) in DBU (25 mL, 167 mmol, 15.2 eq) under a nitrogen atmosphere was brought to reflux and stirred for 16 h. The reaction was diluted with ethyl acetate (200 mL) and washed with a solution of aqueous hydrochloric acid (2 M, 2 x 200 mL). The aqueous phase was then re-extracted with additional ethyl acetate (2 x 200 mL). The combined organic extract was then washed with water (200 mL), saturated aqueous sodium chloride solution (200 mL), dried with anhydrous magnesium sulfate and concentrated\textit{ in vacuo}. Purification of the crude residue by column chromatography (10\% EtOAc/hexanes) afforded the \textit{title compound} 19 (1.494 g, 50\%) as a 95:5 inseparable mixture of Δ\textsuperscript{2,3} and Δ\textsuperscript{3,4} alkene isomers. Data is reported for the major isomer where appropriate. R\textsubscript{f} 0.74 (40\% EtOAc/hexanes); mp 96-103 °C (lit \textsuperscript{14} 92-96 °C); [α]\textsuperscript{25}D +163 (c 0.9, CHCl\textsubscript{3}) (lit \textsuperscript{15} +153 [CHCl\textsubscript{3}]);\textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}): 5.64-5.55 (m, 2H, C\textsubscript{2}-H and C\textsubscript{3}-H), 2.44 (dd, J 9.1 Hz, 18.9 Hz, 1H, C\textsubscript{16}-H), 2.11-0.73 (m, 19H), 0.87 (s, 3H, C\textsubscript{18}-H\textsubscript{3}), 0.78 (s, 3H, C\textsubscript{19}-H\textsubscript{3}); \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}): 221.4 (C17), 125.7 (2 peaks, C2 and C3), 54.2, 54.5, 47.7, 41.4, 39.7, 35.8, 35.1, 34.8, 31.6, 30.7, 30.2, 28.4, 21.8, 20.2, 13.7 (C18), 11.7 (C19); LRMS (+EI): 272 (75\%, [C\textsubscript{19}H\textsubscript{28}O]\textsuperscript{+}), 219 (25\%), 218 (100\%), 190 (40\%), 161 (25\%), 147 (15\%), 122 (20\%), 106 (20\%), 93 (20\%), 91 (25\%), 79 (20\%), 67 (15\%), 55 (15\%); HRMS (+EI): found 272.2139, [C\textsubscript{19}H\textsubscript{28}O]\textsuperscript{+} requires 272.2140.
A solution of 5α-androst-2-en-17-one 19 (1.027 g, 3.77 mmol, 95:5 inseparable mixture of Δ²⁻³ and Δ³⁻⁴ alkene isomers) in dry THF (13 mL) under an atmosphere of nitrogen was treated with a solution of methylmagnesium bromide in diethyl ether (3.0 M, 7.0 mL, 21.0 mmol, 5.6 eq) and stirred at room temperature 16 h. The reaction was diluted with ethyl acetate (100 mL) and washed with saturated aqueous ammonium chloride solution (100 mL), water (100 mL), saturated aqueous sodium chloride solution (100 mL), dried with anhydrous magnesium sulfate and concentrated in vacuo. The crude residue was purified by column chromatography (30% EtOAc/hexanes) to afford the title compound M (834 mg, 77%) as a 95:5 inseparable mixture of Δ²⁻³ and Δ³⁻⁴ alkene isomers. Data is reported for the major isomer where appropriate. Rf 0.59 (40% EtOAc/hexanes); mp 145-150 °C (lit 17154-155 °C); [α]₂⁵_D +53 (c 0.8, CHCl₃); ¹H NMR (800 MHz, CDCl₃): 5.60 (m, 1H, C₃⁻H), 5.58 (m, 1H, C₂⁻H), 1.94 (m, 1H, C₁⁻H), 1.86 (m, 1H, C₄⁻H), 1.80 (ddd, J 3.1 Hz, 11.6, Hz, 12.8 Hz, 1H, C₁₆⁻H), 1.74-1.65 (m, 4H, C₁⁻H, C₄⁻H, C₁₆⁻H, and C₇⁻H), 1.59-1.52 (m, 2H, C₁₅⁻H, and C₁₁⁻H), 1.48 (m, 1H, C₁₂⁻H), 1.42 (m, 1H, C₆⁻H), 1.39-1.33 (m, 3H, C₅⁻H, C₈⁻H, and C₁₁⁻H), 1.28-1.23 (m, 2H, C₁₅⁻H, and C₁₂⁻H), 1.21 (s, 3H, C₂₀⁻H₃), 1.18-1.10 (m, 2H, C₁₄⁻H, and C₆⁻H), 0.86 (m, 1H, C₇⁻H), 0.85 (s, 3H, C₁₈⁻H₃), 0.77 (s, 3H, C₁₉⁻H₃), 0.68 (m, 1H, C₉⁻H), OH not observed; ¹³C NMR (200 MHz, CDCl₃): 126.0 (2 peaks, C₂ and C₃), 81.9 (C₁₇), 54.3 (C₉), 50.8 (C₁₄), 45.7 (C₁₃), 41.7 (C₅), 40.0 (C₁), 39.1 (C₁₆), 36.7 (C₈), 34.9 (C₁₀), 31.8 (C₁₂), 31.7 (C₇), 34.5 (C₄), 28.8 (C₆), 25.9 (C₂₀), 23.4 (C₁₅), 20.7 (C₁₁), 14.1 (C₁₈), 11.9 (C₁₉); LRMS (+EI): 288 (55%, [C₂₀H₃₂O⁺]+·), 270 (30%), 255 (30%), 234 (65%), 230 (100%), 216 (75%), 215 (60%), 201 (25%), 176 (70%), 161 (55%), 147 (55%), 135 (30%), 108 (40%), 107 (40%), 106 (75%); HRMS (+EI): found 288.2455, [C₂₀H₃₂O⁺]+· requires 288.2453.
5.1.2.3.4 17α-methyl-5α-androstan-3β,17β-diol (20)\textsuperscript{19-21}

A solution of \textit{epiandrosterone} (2.020 g, 6.95 mmol) in dry THF (58 mL) under an atmosphere of nitrogen was treated slowly with a solution of methylmagnesium bromide in diethyl ether (3.0 M, 10.0 mL, 30.0 mmol, 4.3 eq) and stirred at room temperature for 18 h. The reaction was diluted with ethyl acetate (100 mL), washed with saturated aqueous ammonium chloride solution (100 ml), water (100 mL), saturated aqueous sodium chloride solution (100 mL), dried with anhydrous magnesium sulfate and concentrated \textit{in vacuo}. The crude residue was purified by column chromatography (40% EtOAc/hexanes) to afford the \textit{title compound} 20 (1.738 g, 82%) as a white solid. \textit{Rf} 0.57 (40% EtOAc/hexanes), mp 164-167 °C (lit\textsuperscript{19} 165-168 °C); \([\alpha]\textsuperscript{25}D +20 \text{ (c 0.7, CHCl}_3\text{) (lit}\textsuperscript{19} [\alpha]\textsuperscript{25}D +20 [CHCl}_3\text{]}); \textit{1H NMR} (400 MHz, CDCl\textsubscript{3}): 3.59 (m, 1H, C3-H), 1.82-0.61 (m, 22H), 1.21 (s, 3H, C20-H\textsubscript{3}), 0.85 (s, 3H, C18-H\textsubscript{3}), 0.83 (s, 3H, C19-H\textsubscript{3}), 2 x OH not observed; \textit{13C NMR} (100 MHz, CDCl\textsubscript{3}): 81.9 (C20), 71.5 (C3), 54.6, 50.8, 45.1, 39.2, 38.3, 37.2, 36.5, 35.7, 32.0, 31.8, 31.7, 28.8, 25.9 (C20), 23.4, 21.0, 14.1 (C18), 12.5 (C19), one carbon overlapping or obscured; LRMS (+EI): \textit{m/z} 306 (35%, [C\textsubscript{20}H\textsubscript{34}O\textsubscript{2}]\textsuperscript{+•}), 291 (75%), 273 (100%), 255 (45%), 233 (50%), 215 (50%), 201 (10%), 165 (45%), 147 (40%), 121 (40%), 107 (65%), 93 (50%), 81 (45%), 67 (35%), 55 (30%); HRMS (+EI): found 306.2556, [C\textsubscript{20}H\textsubscript{32}O\textsubscript{2}]\textsuperscript{+•} requires 306.2559.
A solution of 17α-methyl-5α-androstan-3β,17β-diol 20 (1.738 g, 5.67 mmol) in chloroform (60 mL) was added to a flask containing PCC (6.137 g, 28.5 mmol, 5.0 eq) and silica gel (6.334 g) and the resulting slurry was stirred at room temperature for 18 h. The reaction was filtered and the solid residue washed thoroughly with diethyl ether (10 x 25 mL). The combined organic extract was then washed with aqueous hydrochloric acid solution (2 M, 2 x 200 mL), water (2 x 200 mL), saturated aqueous sodium chloride solution (200 mL), dried with anhydrous magnesium sulfate and concentrated in vacuo. The crude residue was purified by column chromatography (40% EtOAc/hexanes) to afford the title compound 21 (1.228 g, 71%) as a pale yellow solid. Rf 0.65 (40% EtOAc/hexanes); mp 189-191 °C (lit 189-192 °C); [α]$_D$ +24 (c 0.7, CHCl$_3$) (lit 18 $[α]_D$ +10.5 [CHCl$_3$]); $^1$H NMR (400 MHz, CDCl$_3$): 2.44-0.61 (m, 21H), 1.21 (s, 3H, C$_{20}$-H$_3$), 0.88 (s, 3H, C$_{18}$-H$_3$), 0.69 (s, 3H, C$_{19}$-H$_3$), OH not observed; $^{13}$C NMR (100 MHz, CDCl$_3$): 212.1 (C3), 81.8 (C17), 54.0, 50.7, 47.0, 45.7, 44.9, 39.1, 38.8, 38.3, 36.5, 35.9, 31.8, 31.6, 29.0, 26.0 (C20), 23.4, 21.3, 14.2 (C18), 11.7 (C19); LRMS (+EI): m/z 304 (30%, [C$_{20}$H$_{32}$O$_2$]$^+$), 289 (70%), 271 (100%), 247 (65%), 231 (65%), 215 (30%), 175 (30%), 163 (50%), 123 (40%), 107 (35%), 93 (35%), 81 (35%), 67 (35%), 55 (35%); HRMS (+EI): found 304.2408, [C$_{20}$H$_{32}$O$_2$]$^+$ requires 304.2402.
5.1.2.3.6 17β-hydroxy-17α-methyl-5α-androst-1-en-3-one (E3) 21-23

A solution of 17β-hydroxy-17α-methyl-5α-androstan-3-one (21) (994 mg, 3.26 mmol) in THF (30 mL) was treated with phenyltrimethylammonium tribromide (1.329 g, 3.56 mmol, 1.1 eq) and stirred at room temperature for 60 min. The reaction was diluted with ethyl acetate (100 mL) and the organic extract washed with saturated aqueous sodium bicarbonate solution (100 mL), water (100 mL), saturated aqueous sodium chloride solution (100 mL), dried with anhydrous magnesium sulfate and concentrated in vacuo to afford 2α-bromo-17β-hydroxy-17α-methyl-5α-androstan-3-one which was used without further purification. Rf 0.46 (40% EtOAc/hexanes); 1H NMR (400 MHz, CDCl3): 4.75 (dd, J 6.3 Hz, 13.4 Hz, 1H, C2-H), 2.65 (dd, J 6.4 Hz, 12.8 Hz, 1H, C4-H), 2.44-0.74 (m, 19H), 1.21 (s, 3H, C20-H3), 1.11 (s, 3H, C19-H3), 0.87 (s, 3H, C18-H3), OH not observed. The crude product (1.292 g, assumed 3.37 mmol) was then dissolved in DMF (35 mL) and the solution was transferred to a dry flask containing lithium bromide (473 mg, 5.45 mmol, 1.6 eq) and lithium carbonate (438 mg, 5.93 mmol, 1.8 eq) under a nitrogen atmosphere. The solution was then brought to reflux and stirred for 18 h. After cooling to room temperature, the reaction was diluted with ethyl acetate (200 mL) and the organic extract was washed with aqueous hydrochloric acid solution (2 M, 2 x 200 mL), water (2 x 200 mL), saturated aqueous sodium chloride solution (200 mL), dried with anhydrous magnesium sulfate and concentrated in vacuo. The crude residue was purified by column chromatography (40% EtOAc/hexanes) to afford the title compound E3 (423 mg, 41 % over two steps) as a pale yellow solid. Rf 0.36 (40% EtOAc/hexanes); mp 150-153 °C (lit 22 154-155 °C); [α]25D +31 (c 0.6, CHCl3) (lit 22 [α]25D +30 [CHCl3]); 1H NMR (800 MHz, CDCl3): 7.14 (d, J 10.2 Hz, 1H, C1-H), 5.85 (d, J 10.2 Hz, 1H, C2-H), 2.37 (dd, J 14.3 Hz, 17.7 Hz, 1H, C4-H), 2.22 (dd, J 4.0 Hz, 17.7 Hz, 1H, C4-H), 1.92 (m, 1H, C5-H), 1.84-1.80 (m, 2H, C12-H, and C16-H), 1.76-1.71 (m, 2H, C7-H, and C16-H), 1.59-1.55 (m, 2H, C11-H, and C15-H), 1.53 (dd, J 4.0 Hz, 10.4 Hz, 11.1 Hz, 1H, C8-H), 1.49-1.38 (m, 3H, C6-H2, and C12-H), 1.33 (dd, J 4.1 Hz, 12.7 Hz, 12.7 Hz, 1H, C11-H), 1.30-1.23 (m, 2H, C14-H, and C15-H), 1.22 (s, 3H, C20-H3), 1.03 (s, 3H, C19-H3), 0.99-0.91 (m, 2H, C7-H, and C9-H), 0.89 (s, 3H, C18-H3), OH not observed; 13C NMR (200 MHz, CDCl3): 200.3 (C3), 158.5 (C1), 127.6 (C2), 81.7 (C17), 50.8 (C14), 50.2 (C9), 45.8 (C13), 44.6 (C5), 41.1 (C4), 39.2 (C10), 39.1
(C16), 36.7 (C8), 31.7 (C11), 31.2 (C7), 27.7 (C6), 26.0 (C20), 23.3 (C15), 21.1 (C12), 14.3 (C18), 13.2 (C19); LRMS (+EI): $m/z$ 302 (30%, $[\text{C}_{20}\text{H}_{30}\text{O}_2]^+$), 284 (10%), 269 (85%), 245 (30%), 200 (10%), 163 (45%), 149 (35%), 134 (35%), 122 (100%), 107 (60%), 91 (50%), 79 (45%), 67 (25%), 55 (25%); HRMS (+EI): found 302.2242, $[\text{C}_{20}\text{H}_{30}\text{O}_2]^+$ requires 302.2246.
A solution of 17β-hydroxy-17α-methyl-5α-androst-1-en-3-one E3 (238 mg, 786.9 µmol) in methanol (4 mL) was cooled to 0 °C (ice-water bath) and treated with a solution of aqueous sodium hydroxide (5.0 M, 240 µl, 1.20 mmol, 1.5 eq) followed by a solution of aqueous hydrogen peroxide (30% w/v, 900 µL, 7.94 mmol, 10.1 eq) and stirred at 0 °C for 2h, then at room temperature for 3 h. The reaction was diluted with water (50 mL) and extracted with chloroform (3 x 50 mL). The combined organic extract was dried with anhydrous magnesium sulfate and concentrated in vacuo. The crude residue was purified by column chromatography (40% EtOAc/hexanes) to afford the title compound 22 (30 mg, 12%) as an off-white solid. Rf 0.40 (40% EtOAc/hexanes); mp 145-150 °C (lit 24 138-142 °C); [α]D25 +67 (c 0.3, CHCl3) [lit 24 [α]D25 +37 [CHCl3]]; 1H NMR (400 MHz, CDCl3): 3.52 (d, J 4.1 Hz, 1H, C2-H), 3.23 (d, J 4.1 Hz, 1H, C1-H), 2.27 (dd, J 4.6 Hz, 18.6 Hz, 1H, C4-H), 2.10-0.88 (m, 17H), 1.23 (s, 3H, C20-H3), 0.91 (s, 3H, C19-H3), 0.88 (s, 3H, C18-H3), OH not observed; 13C NMR (100 MHz, CDCl3): 206.0 (C3), 81.8 (C17), 61.2 (C1), 56.2 (C2), 50.6, 49.0, 45.7, 40.3, 39.0, 36.9, 36.5, 34.4, 31.6, 31.0, 27.3, 26.0 (C20), 23.4, 21.4, 14.2 (C18), 11.3 (C19); LRMS (+EI): m/z 318 (5%, [C20H30O3]+*), 303 (55%), 285 (100%), 261 (60%), 245 (40%), 215 (15), 201 (15%), 187 (15%), 173 (20%), 163 (25%), 147 (25%), 133 (20%), 121 (30%), 107 (40%), 91 (50%), 79 (40%), 67 (30%), 55 (25%); HRMS (+EI): found 318.2204, [C20H36O3]+• requires 318.2195.
5.1.2.3.8 17α-methyl-5α-androst-2-en-1α,17β-diol (D1) 21

A solution of 1α,2α-epoxy-17β-hydroxy-17α-methyl-5α-androstan-3-one 22 (27 mg, 84.9 µmol) in hydrazine monohydrate (1.0 mL, 20.6 mmol, 242 eq) was stirred at room temperature for 90 min. The reaction was diluted with water (10 mL) and extracted with chloroform (3 x 10 mL). The combined organic extract was dried with anhydrous magnesium sulfate and concentrated in vacuo. The crude residue was purified by column chromatography (40% EtOAc/hexanes) to afford the title compound D1 (20 mg, 77%) as a yellow-brown solid. Rf 0.39 (40% EtOAc/hexanes); mp 101-104 °C; 1H NMR (400 MHz, CDCl₃): 5.87-5.78 (m, 2H, C2-H and C3-H), 3.71 (d, J 4.4 Hz, 1H, C1-H), 2.41-0.89 (m, 18H), 1.21 (s, 3H, C20-H₃), 0.86 (s, 3H, C18-H₃), 0.73 (s, 3H, C19-H₃), 2 x OH not observed; 13C NMR (100 MHz, CDCl₃): 130.7 (C2 or C3), 127.8 (C2 or C3), 81.8 (C17), 69.8 (C1), 50.6, 46.2, 45.4, 39.0, 38.9, 36.4, 34.8, 31.5, 32.2, 30.7, 28.5, 25.9 (C20), 23.3, 20.3, 13.9 (C18), 11.4 (C19); LRMS (+EI): m/z 304 (10%, [C₂₀H₃₂O₂]⁺), 286 (25%), 271 (25%), 246 (30%), 234 (25%), 201 (15%), 176 (40%), 161 (40%), 147 (25%), 135 (15%), 119 (15%), 105 (45%), 91 (45%), 79 (25%), 70 (15%), 55 (10%); HRMS (+EI): found 304.2402, [C₂₀H₃₂O₂]⁺ requires 304.2402.
5.1.2.3.9 17β-hydroxy-17α-methyl-5α-androst-2-en-1-one (E1) 21

A solution of 17α-methyl-5α-androst-2-en-1α,17β-diol D1 (15 mg, 49.3 µmol) in chloroform (500 µL) was added to a flask containing PCC (55 mg, 255 µmol, 5.2 eq) and silica gel (53 mg) and the resulting slurry was stirred at room temperature for 18 h. The reaction was diluted with diethyl ether (10 mL), filtered and the solid residue washed thoroughly with additional diethyl ether (10 x 5 mL). The combined organic extract was then washed with aqueous hydrochloric acid solution (2 M, 2 x 50 mL), water (2 x 50 mL), saturated aqueous sodium chloride solution (50 mL), dried with anhydrous magnesium sulfate and concentrated in vacuo. The crude residue was purified by column chromatography (40% EtOAc/hexanes) to afford the title compound E1 (10 mg, 67%) as a pale yellow solid. Rf 0.48 (40% EtOAc/hexanes); mp 148-153 ºC; 1H NMR (400 MHz, CDCl3): 6.65 (dt, J 3.9 Hz, 10.0 Hz, 1H, C3-H), 5.80 (dt, J 2.1 Hz, 10.1 Hz, 1H, C2-H), 2.44 (m, 1H, C4-H), 2.17-2.13 (m, 2H, C4-H and C5-H), 1.87-0.79 (m, 15H), 1.23 (s, 3H, C20-H3), 1.08 (s, 3H, C19-H3), 0.88 (s, 3H, C18-H3), OH not observed; 13C NMR (100 MHz, CDCl3): 206.6 (C1), 145.2 (C3), 129.0 (C2), 81.9 (C17), 50.8, 47.6, 47.5, 45.8, 43.6, 38.9, 37.6, 31.9, 31.4, 30.5, 28.3, 26.1 (C20), 23.5, 23.3, 14.5 (C18), 10.7 (C19); LRMS (+EI): m/z 302 (10%, [C20H30O2]•), 284 (20%), 269 (100%), 245 (70%), 201 (10%), 161 (15%), 122 (45%), 109 (40%), 91 (30%), 79 (15%), 55 (10%); HRMS (+EI): found 302.2247, [C20H30O2]• requires 302.2246.
5.1.2.3.10 17β-hydroxy-17α-methyl-5α-androst-3-en-2-one (E2)

A solution of 17α-methyl-5α-androst-2-en-17β-ol M (989 mg, 3.43 mmol) in dioxane (20 mL) was treated with a solution of NBS (681 mg, 3.82 mmol, 1.1 eq) in water (20 mL). An aqueous solution of perchloric acid (70% w/w, 540 µL, 3.87 mmol, 1.1 eq) was then added dropwise and the reaction was stirred at room temperature for 18 h. The reaction was diluted with water (100 mL), extracted with chloroform (4 x 100 mL) and the organic extract was then dried with anhydrous magnesium sulfate and concentrated in vacuo to afford 3α-bromo-17α-methyl-5α-androstan-2β,17β-diol which was used without further purification. Rf 0.55 (40% EtOAc/hexanes); 1H NMR (400 MHz, CDCl3): 4.33 (br s, 1H, C3-H), 4.22 (br s, 1H, C2-H), 2.20-0.67 (m, 21 H), 1.21 (s, 3H, C20-H3), 1.02 (s, 3H, C19-H3), 0.84 (s, 3H, C18-H3), 2 x OH not observed.

The crude product (1.206 g, assumed 3.13 mmol) was then dissolved in chloroform (31 mL), treated with PCC (3.375 g, 15.7 mmol, 5.0 eq) and silica (3.457 g) and stirred at room temperature for 18 h. The reaction was then filtered and the solid residue washed with diethyl ether (10 x 25 mL). The combined organic extract was then washed with aqueous hydrochloric acid solution (2 M, 2 x 100 mL), water (2 x 200 mL), saturated aqueous sodium chloride solution (200 mL), dried with anhydrous magnesium sulfate and concentrated in vacuo to afford 3α-bromo-17β-hydroxy-17α-methyl-5α-androstan-2-one which was used without further purification. Rf 0.58 (40% EtOAc/hexanes); 1H NMR (400 MHz, CDCl3): 4.29 (br s, 1H, C3-H), 2.83 (d, J 13.5 Hz, 1H, C1-H), 2.30 (d, J 13.5 Hz, 1H, C1-H), 2.14-0.75 (m, 18 H), 1.21 (s, 3H, C20-H3), 0.84 (s, 3H, C19-H3), 0.75 (s, 3H, C18-H3), OH not observed.

The crude product (962 mg, assumed 2.51 mmol) was then dissolved in DMF (25 mL) and the solution was transferred to a dry flask containing lithium bromide (335 mg, 3.85 mmol, 1.5 eq) and lithium carbonate (272 mg, 3.68 mmol, 1.5 eq) under a nitrogen atmosphere. The solution was then brought to reflux and stirred for 18 h. After cooling to room temperature, the reaction was diluted with ethyl acetate (200 mL) and the organic extract was washed with aqueous hydrochloric acid solution (2 M, 2 x 200 mL), water (2 x 200 mL), saturated aqueous sodium chloride solution (200 mL), dried with anhydrous magnesium sulfate and concentrated in vacuo. The crude residue was purified by column chromatography (40% EtOAc/hexanes) to afford an 83:17 mixture containing the title compound E2 and E3 (381 mg, 37%
over three steps) as a pale yellow solid. Data is reported for the major isomer where appropriate. Rf 0.37 (40% EtOAc/hexanes); [α]$_{25}^{20}$ +70 (c 0.7, CHCl$_3$); $^1$H NMR (400 MHz, CDCl$_3$): 6.56 (d, $J$ 9.9 Hz, 1H, C4-H), 5.96 (d, $J$ 9.8 Hz, 1H, C3-H), 2.56 (d, $J$ 16.0 Hz, 1H, C1-H), 2.06 (d, $J$ 18.2 Hz, 1H, C1-H), 2.64-0.67 (m, 16H), 1.21 (s, 3H, C20-H$_3$), 0.89 (s, 3H, C19-H$_3$), 0.85 (s, 3H, C18-H$_3$), OH not observed; $^{13}$C NMR (100 MHz, CDCl$_3$): 200.3 (C2), 154.2 (C4), 128.7 (C3), 81.7 (C17), 53.6, 52.2, 50.6, 47.2, 45.8, 41.1, 39.0, 35.6, 31.8, 31.6, 26.6, 26.0 (C20), 23.3, 20.7, 14.2 (C18), 12.9 (C19); LRMS (+EI): m/z 302 (65%, [C$_{20}$H$_{30}$O$_2$]+), 284 (45%), 269 (100%), 245 (45%), 229 (45%), 161 (35%), 147 (25%), 121 (55%), 107 (55%), 91 (45%), 79 (45%), 55 (30%); HRMS (+EI): found 302.2242, [C$_{20}$H$_{30}$O$_2$]+ requires 302.2246.
5.1.2.3.11 3α,4α-epoxy-17β-hydroxy-17α-methyl-5α-androstan-2-one (23)

A solution of 17β-hydroxy-17α-methyl-5α-androst-3-en-2-one E2 (381 mg, 1.26 mmol, 83:17 mixture containing E2 and E3) in methanol (6 mL) was cooled to 0 °C (ice-water bath) and treated with a solution of aqueous sodium hydroxide (5.0 M, 380 µl, 1.90 mmol, 1.5 eq) followed by a solution of aqueous hydrogen peroxide (30% w/v, 1.4 mL, 9.79 mmol, 9.8 eq) and stirred at 0 °C for 2h, then at room temperature for 3 h. The reaction was diluted with water (50 mL) and extracted with chloroform (3 x 50 mL). The combined organic extract was dried with anhydrous magnesium sulfate and concentrated in vacuo. The crude residue was purified by column chromatography (40% EtOAc/hexanes) to afford the title compound 23 (141 mg, 36%) as a white solid. Rf 0.41 (40% EtOAc/hexanes); mp 98-103 °C; [α]$_{25}^{D}$ +53 (c 0.7, CHCl$_3$); $^1$H NMR (400 MHz, CDCl$_3$): 3.21 (d, J = 3.8 Hz, 1H, C3-H), 3.10 (d, J = 3.7 Hz, 1H, C4-H), 2.29 (d, J = 13.8 Hz, 1H, C1-H), 2.19 (d, J = 13.8 Hz, 1H, C1-H), 1.88-0.76 (m, 16H), 1.21 (s, 3H, C20-H$_3$), 0.83 (s, 6H, C18-H$_3$ and C19-H$_3$), OH not observed; $^{13}$C NMR (100 MHz, CDCl$_3$): 207.3 (C2), 81.6 (C17), 61.3 (C4), 54.9 (C3), 52.6, 50.4, 48.5, 47.4, 45.8, 43.1, 39.0, 35.6, 31.5, 31.4, 26.0 (C20), 25.6, 23.3, 21.0, 14.9 (C19), 14.2 (C18); LRMS (+EI): m/z 318 (40%, [C$_{20}$H$_{30}$O$_{3}$]+•, 303 (100%), 285 (100%), 260 (50%), 245 (65%), 215 (20%), 201 (20%), 175 (35%), 161 (25%), 147 (30%), 133 (30%), 121 (30%), 105 (35%), 91 (45%), 81 (35%), 71 (50%), 55 (30%); HRMS (+EI): found 318.2191, [C$_{20}$H$_{30}$O$_{3}$]+• requires 318.2195.
5.1.2.3.12 17α-methyl-5α-androst-2-en-4α,17β-diol (D7) 21

A solution of 3α,4α-epoxy-17β-hydroxy-17α-methyl-5α-androstan-2-one 22 (119 mg, 373.7 µmol) in hydrazine monohydrate (4.0 mL, 82.2 mmol, 220 eq) was stirred at room temperature for 90 min. The reaction was diluted with water (50 mL) and extracted with chloroform (3 x 50 mL). The combined organic extract was dried with anhydrous magnesium sulfate and concentrated in vacuo. The crude residue was purified by column chromatography (40% EtOAc/hexanes) to afford the title compound D7 (43 mg, 38%) as an off-white solid. Rf 0.47 (40% EtOAc/hexanes); mp 99-102 °C; 1H NMR (400 MHz, CDCl₃): 5.72-5.60 (m, 2H, C2-H and C3-H), 3.78 (m, 1H, C4-H), 2.02-0.70 (m, 18H), 1.21 (s, 3H, C20-H₃), 0.85 (s, 3H, C19-H₃), 0.83 (s, 3H, C18-H₃), 2 x OH not observed; 13C NMR (100 MHz, CDCl₃): 129.2 (C2 or C3), 127.7 (C2 or C3), 81.7 (C17), 70.8 (C4), 53.9, 50.6, 50.5, 45.4, 39.6, 39.0, 36.6, 36.2, 31.6, 31.1, 25.8 (C20), 24.3, 23.2, 20.3, 13.9 (C18), 12.7 (C19); LRMS (+EI): m/z 304 (40%, [C₂₀H₃₂O₂]⁺), 286 (40%), 268 (30%), 253 (30%), 228 (30%), 213 (25%), 216 (15%), 161 (40%), 147 (40%), 133 (30%), 119 (40%), 105 (90%), 91 (100%), 79 (50%), 67 (35%), 55 (40%); HRMS (+EI): found 304.2404, [C₂₀H₃₂O₂]⁺ requires 304.2402.
5.1.2.3.13 17β-hydroxy-17α-methyl-5α-androst-2-en-4-one (E4) 21

A solution of 17α-methyl-5α-androst-2-en-4α,17β-diol D7 (22 mg, 72.2 µmol) in chloroform (700 µL) was added to a flask containing PCC (79 mg, 366.5 µmol, 5.1 eq) and silica gel (93 mg) and the resulting slurry was stirred at room temperature for 18 h. The reaction was diluted with diethyl ether (10 mL), filtered and the solid residue washed thoroughly with additional diethyl ether (10 x 5 mL). The combined organic extract was then washed with aqueous hydrochloric acid solution (2 M, 2 x 50 mL), water (2 x 50 mL), saturated aqueous sodium chloride solution (50 mL), dried with anhydrous magnesium sulfate and concentrated in vacuo. The crude residue was purified by column chromatography (40% EtOAc/hexanes) to afford the title compound E4 (15 mg, 69%) as an off-white solid. Rf 0.50 (40% EtOAc/hexanes); mp 130-133 °C; 1H NMR (400 MHz, CDCl3): δ 6.79 (ddd, J 2.3 Hz, 6.0 Hz, 10.1 Hz, 1H, C2-H), 5.98 (dd, J 3.0 Hz, 10.1 Hz, 1H, C3-H); 2.49-0.79 (m, 18H), 1.21 (s, 3H, C20-H3), 0.87 (s, 3H, C19-H3), 0.85 (s, 3H, C18-H3), OH not observed; 13C NMR (100 MHz, CDCl3): 201.3 (C4), 146.6 (C2), 128.8 (C3), 81.6 (C17), 55.8, 54.2, 50.4, 45.4, 40.7, 40.3, 39.0, 35.7, 31.5, 30.4, 25.8 (C20), 23.2, 20.7, 20.3, 13.9 (C18), 13.2 (C19); LRMS (+EI): m/z 302 (10%, [C20H30O3]+•), 287 (100%), 269 (60%), 245 (55%), 229 (25%), 217 (10%), 173 (10%), 163 (30%), 147 (25%), 135 (25%), 121 (60%), 107 (40%), 91 (35%), 79 (40%), 67 (25%), 55 (25%); HRMS (+EI): found 302.2238, [C20H30O3]+• requires 302.2246.
5.1.2.3.14 2α,3α-episulfanyl-17α-methyl-5α-androstan-17β-ol (HS)

A dry flask containing 2α,3α-epithio-17α-methyl-5α-androstan-17β-ol H (30 mg, 93.6 µmol) under a nitrogen atmosphere was treated dropwise with a solution of DMDO in acetone (0.098 M, 5.0 mL, 490 µmol, 5.2 eq) and stirred at room temperature for 90 min. The reaction was diluted with ethyl acetate (10 mL), washed with aqueous sodium metabisulfite solution (10% w/v, 10 mL), water (10 mL), saturated aqueous sodium chloride solution (10 mL), dried with anhydrous magnesium sulfate and concentrated in vacuo. The crude residue was purified by column chromatography (20% EtOAc/hexanes, silica pre-treated with NEt₃ (5% v/v)) to afford the title compound HS (27 mg, 86%) as a white solid. Rf 0.17 (40% EtOAc/hexanes); mp 135-140 °C; [α]²⁵D -5 (c 0.4, CHCl₃); ¹H NMR (400 MHz, CDCl₃): 3.24 (dt, J 3.5 Hz, 10.5 Hz, 1H, C₃-H), 3.11 (dd, J 5.6 Hz, 10.8 Hz, 1H, C₂-H), 2.45 (dd, J 10.5 Hz, 16.0 Hz, 1H, C₁-H), 2.18 (dd, J 4.3 Hz, 15.5 Hz, 1H, C₄-H), 1.80-0.33 (m, 18H), 1.17 (s, 3H, C₂₀-H₃), 0.81 (s, 3H, C₁₈-H₃), 0.78 (s, 3H, C₁₉-H₃), OH not observed; ¹³C NMR (100 MHz, CDCl₃): 81.7 (C₂₀), 53.8, 53.5 (C₂ or C₃), 51.3 (C₂ or C₃), 50.6, 45.4, 40.6, 39.0, 36.6, 35.6, 33.3, 31.6, 31.1, 28.9, 25.9 (C₂₀), 25.7, 23.3, 20.5, 14.0 (C₁₈), 12.7 (C₁₉); LRMS (+EI): m/z 336 (10%, [C₂₀H₃₂O₂S]⁺), 288 (40%, [C₂₀H₃₂O]⁺), 270 (30%), 255 (100%, [C₁₉H₂₇]⁺), 234 (40%), 230 (55%), 216 (55%), 201 (20%), 176 (45%), 161 (45%), 147 (40%), 121 (25%), 107 (45%), 93 (45%), 81 (40%), 55 (30%); HRMS (+EI): found 336.2123, [C₂₀H₃₂O₂S]⁺ requires 336.2123.
Sulfation was performed by minor modification to a known literature procedure \(^{25}\). A solution of 17α-methyl-5α-androst-2-en-17β-ol \(\text{M} \) (3.0 mg, assumed 10.4 µmol) in dioxane (500 µL) was treated with a solution of sulfur trioxide-pyridine complex (50 mg, 314 µmol, 30.2 eq) in DMF (500 µL) and stirred at room temperature for 4 h. The reaction was diluted with water (2 mL) and subjected to purification by SPE. An Oasis WAX SPE cartridge (6 cc) was preconditioned with methanol (3 mL) followed by water (15 mL). The reaction mixture (3 mL) was then loaded onto the cartridge and washed with a solution of formic acid in water (2% v/v, 15 mL), water (15 mL) and methanol (15 mL). Elution with saturated aqueous ammonia solution in methanol (5% v/v, 15 mL) afforded the title compound \(\text{MS} \) as a 95:5 inseparable mixture of \(\Delta^2\) and \(\Delta^3\) alkene isomers with >98% conversion \(^{25}\). Data is reported for the major isomer where appropriate. 

\[ R_f \text{ 0.55 (7:2:1 EtOAc:MeOH:H}_2\text{O) \}; 1}^\text{H NMR (400 MHz, CD}_3\text{OD)} : 5.61-5.50 (m, 2H, C2-H and C3-H), 2.63 (ddd, \( J \text{ 3.4 Hz, 12.4 Hz, 14.8 Hz, 1H, C16-H), 1}\text{.98-0.70 (m, 19H), 1}\text{.50 (s, 3H, C20-H3), 0}\text{.93 (s, 3H, C18-H3), 0}\text{.80 (s, 3H, C19-H3); 1}^\text{3C NMR (100 MHz, CD}_3\text{OD)} : 126.9 (C2 or C3), 126.7 (C2 or C3), 94.4 (C17), 55.6, 50.3, 48.3, 43.0, 41.0, 37.7, 36.0, 35.8, 33.0, 32.8, 31.4, 29.9, 24.5, 23.3, 21.6, 15.2 \text{(C18), 12.1 (C19); LRMS (-ESI): 367 (100\%, [C}_{20}\text{H}_{31}\text{O}_{4}\text{S}^-), 111 (15\%), 97 (10\%, [HSO}_4^-); HRMS (-ESI): found 367.1944, [C}_{20}\text{H}_{31}\text{O}_{4}\text{S}^- \text{requires 367.1943.} \]
5.1.2.3.16 General procedure for the small scale Luche reduction of enones (D1-D10)

Small scale reduction was performed using modified Luche conditions. A solution of steroid enone (1.0 mg, 3.31 µmol) and cerium chloride heptahydrate (10 mg, 26.8 µmol, 8.1 eq) in methanol (100 µL) was treated slowly with sodium borohydride (1 mg, 26.4 µmol, 8.0 eq) on ice. The resulting white slurry was then stirred at room temperature for 15 min. The reaction was quenched with aqueous hydrochloric acid (2 M, 1 mL) and subjected to purification by SPE. A Waters Sep-Pak C18 SPE cartridge was pre-conditioned with methanol (3 mL) and water (3 mL). The sample was loaded, washed with water (3 mL) and eluted with methanol (3 mL). The methanol fraction was concentrated in vacuo to afford the desired steroid diol, typically as a mixture of α- and β-isomers. Enones E1-E5 were subjected to these conditions to afford mixtures containing diols D1-D10, which were characterised by GCMS analysis as per section 5.1.2.4.1.

5.1.2.4 Analytical methods

5.1.2.4.1 GC-MS/MS Analysis

Positive mode gas chromatography-tandem mass spectrometry (GC-MS/MS) analysis was undertaken on an Agilent 7890A GC system coupled to an Agilent 7000 GC/MS Triple Quadrupole mass spectrometer equipped with an Agilent HP-5MS Ultra Inert column (30 m x 250 µm x 0.25 µm). Helium was used as a carrier gas with a constant flow rate of 1.2 mL/min. Injection volumes of 2-5 µL were resolved in pulsed splitless mode. The injector temperature was set to 260 °C, and the MSD transfer line was set to 300 °C. The oven temperature commenced at 180 °C with a hold time of 0.2 min, followed by a 5 °C/min ramp to 235 °C, followed by a 15 °C/min ramp to 265 °C, followed by a 25 °C/min ramp to 300 °C, and a final hold time of 10 min (total run time 24.6 min). The solvent delay was set to 6.5 min. Steroids were derivatised as the enol trimethylsilyl (TMS) derivative using MSTFA/NH4I/DTT (1000:2:4 v/w/w, 50 µL) at 80 °C for 60 min, reconstituted in n-dodecane (50 µL) prior to analysis, and monitored for the radical cation ([M]+*) using +EI.
5.1.2.4.2 LC-HRAM Analysis

Positive mode liquid chromatography-high resolution accurate mass (LC-HRAM) analysis was undertaken using an Agilent 1290 Infinity II LC system coupled to an Agilent 6545 Q-TOF mass spectrometer equipped with a Waters Sun-Fire C18 column (100 mm x 2.1 mm, 3.5 µm) eluting with a gradient consisting of the following mobile phases, A: 0.1% formic acid in water, B: 0.1% formic acid in methanol, gradient: 0-1 min A-B (95:5 v/v), 1-15 min A-B (95:5 v/v) to A-B (5:95 v/v), 15-19 min A-B (5:95 v/v), 5 min re-equilibration, flow rate 0.4 mL min⁻¹. Unconjugated steroids and steroid glucuronides were monitored for the proton adduct ([M+H]+) using HESI in positive full scan or targeted MS/MS mode.

Negative mode LC-HRAM analysis was undertaken on the same instrument using a Phenomenex (Torrance CA, USA) Gemini C18 column (50 mm x 2 mm, 5 µm), eluting with a gradient consisting of the following mobile phases, A: aqueous ammonium acetate (0.01 M, pH 9.0), B: 0.1% acetic acid in acetonitrile, gradient: 0-2 min A-B (99:1 v/v), 2-8.5 min A-B (99:1 v/v) to A-B (20:80 v/v), 2.7 min re-equilibration, flow rate 0.5 mL min⁻¹. Steroid glucuronide and sulfate conjugates were monitored for the anion ([M-H]-) using HESI in negative full-scan or targeted MS/MS mode.

5.1.2.5 In vivo equine metabolism study

5.1.2.5.1 Animal administration

Animal administration was approved by the Racing NSW Animal Care and Ethics Committee. A sample of synthetic hemapolin (200 mg) was administered orally as a suspension in water by way of a nasogastric tube to a thoroughbred gelding (21 years old, 580 kg), with samples of blood and urine collected at 0, 1, 2, 4, 6, 8, 12, and 24 h post-administration, and daily thereafter up to 7 d post administration. The samples were stored at −20 °C until required for analysis.

5.1.2.5.2 Extraction of blood samples

The blood samples were centrifuged (3000 rpm, 10 min) and the plasma was decanted into new vials. Unused plasma was stored frozen at -20 °C. An aliquot of plasma (2 mL) was treated with d₃-testosterone (50 ng/mL) as an internal
standard, and then an aqueous solution of trichloroacetic acid (10% w/v, 67 µL). The sample was centrifuged (3000 rpm, 10 min), the supernatant was treated with sodium phosphate buffer (0.1 M, pH 7.4, 2 mL), and then purified by SPE. A UCT Xtrackt cartridge was pre-conditioned with methanol (2 mL) and water (2 mL). The sample was loaded and washed with a solution of aqueous acetic acid (0.1 M, 2 mL), and eluted with a solution of ethyl acetate/n-hexane (60% v/v, 3 mL). Concentration under a stream of nitrogen at 60 °C afforded a residue which was reconstituted in methanol-water (5:95, 200 µL) and transferred to a sealed vial for subsequent LC-HRAM analysis as per section 5.1.2.4.2.

5.1.2.5.3 Extraction of urine samples for LC-HRAM analysis
An aliquot of urine (2 mL) was treated with sodium phosphate buffer (0.1 M, pH 7.4, 2 mL), and centrifuged (3000 rpm, 10 min) to pellet solids. The supernatant was decanted, treated with d₅-testosterone (100 ng/mL urine), d₅-testosterone 17-glucuronide (100 ng/mL), and d₅-testosterone 17-sulfate (100 ng/mL), and purified by SPE. A Waters WAX (3 cc) cartridge was preconditioned with methanol (2 mL) and water (2 mL). The sample was loaded and washed with an aqueous solution of sodium hydroxide (0.1 M, 2 mL), sodium phosphate buffer (0.1 M, pH 7.4, 2 mL), and water (2 mL), and eluted with a solution of methanol-ethyl acetate-diethylamine (25:25:1 v/v/v, 2 mL). Concentration under a stream of nitrogen at 60 °C afforded a residue which was reconstituted in methanol-water (5:95, 200 µL) and transferred to a sealed vial for subsequent LC-HRAM analysis as per section 5.1.2.4.2.

5.1.2.5.4 Extraction of the urine samples for GC-MS/MS analysis
An aliquot of urine (3 mL) was adjusted manually to pH 5.0-5.5 with aqueous hydrochloric acid solution (10% w/v), and treated with d₅-testosterone (50 ng/mL) as an internal standard. An Agilent NEXUS SPE cartridge was pre-conditioned with methanol (3 mL) and water (3 ml). The sample was loaded and washed with water (3 mL), a solution of aqueous sodium hydroxide (0.1 M, 3 mL), additional water (3 mL), and eluted with methanol (3 mL). Concentration under a stream of nitrogen at 60 °C afforded a residue which was reconstituted in a
solution of acetyl chloride in methanol (1.0 M, 500 µL) and heated in a capped tube at 60 °C for 10 min. The hydrolysis reaction was quenched with a solution of aqueous sodium hydroxide (2 M, 3 mL), and extracted with DIPE (3.5 mL) for 30 min. Concentration of the ether layer under a stream of nitrogen at 60 °C afforded a residue which was derivatised as the TMS enol ether, and subjected to GC-MS/MS analysis as per section 5.1.2.4.1.

5.1.3 Results and Discussion
5.1.3.1 Synthesis of hemapolin and reference materials
As H was required on large scale (200 mg) and in high purity, a laboratory synthesis was undertaken. Synthetic H was prepared from epiandrosterone in 6 synthetic steps in 6% overall yield through a novel synthetic strategy. The experimental procedures, characterisation data, and copies of the 1H NMR, 13C NMR, and +EI LRMS or -ESI LRMS are included in the supporting information for this chapter, but have been excluded from the final electronic version of this thesis due to concerns over the potential for the misuse of this information in the illicit manufacture of new designer steroids. Requests for this information from legitimate parties can be made to Associate Professor Malcolm McLeod. Enones E1-E4 were prepared from M, mestanolone, or epiandrosterone as described below. Briefly, bromination of mestanolone 21 with PTAB cleanly afforded the C2-bromide, which was eliminated by treatment with LiBr and Li2CO3 in anhydrous DMF to afford E3 (Scheme 5.1) 23. Subsequent treatment of E3 with hydrogen peroxide under basic conditions afforded epoxide 22, which was subjected to Wharton conditions to selectively generate diol D1 21,28. This diol was oxidised with PCC to afford E1, which was subsequently reduced under modified Luche conditions to afford a mixture containing diols D1, and D2 26. Enone E2 (Scheme 5.2) was prepared from M by formation of the bromohydrin, followed by oxidation to the bromoketone, and subsequent elimination using the conditions outlined above. Luche reduction of E2 afforded a mixture of diols D3 and D4. A small amount (~15%) of E3 formed alongside E2, presumably resulting from the formation of the undesired C2-bromohydrin, which was carried through the subsequent steps. Treatment of E2 with hydrogen peroxide afforded epoxide 23 in pure form after column chromatography, which was converted to diol D7 under
Wharton conditions as outlined above. Subsequent oxidation afforded \( \text{E4} \), which was reduced as above to afford a mixture containing diols \( \text{D7} \) and \( \text{D8} \). The \(^1\text{H} \) NMR analysis of the alkene protons in deuterated chloroform agreed with the proposed structures of the enone reference materials: enone \( \text{E1} \) produced a pair of doublet of triplets at 6.65 ppm (\( \text{C3-H, } J=10.1 \) Hz, \( J=3.9 \) Hz) and 5.80 ppm (\( \text{C2-H, } J=10.1 \) Hz, \( J=2.1 \) Hz); \( \text{E2} \) produced a pair of doublets at 6.56 ppm (\( \text{C4-H, } J=9.8 \) Hz) and 5.96 ppm (\( \text{C3-H, } J=9.8 \) Hz); \( \text{E3} \) produced a pair of doublets at 7.14 ppm (\( \text{C1-H, } J=10.2 \) Hz) and 5.85 ppm (\( \text{C2-H, } J=10.2 \) Hz); \( \text{E4} \) produced a doublet of doublet of doublets at 6.79 ppm (\( \text{C2-H, } J=10.1 \) Hz, \( J=6.0 \) Hz, \( J=2.3 \) Hz), and a doublet of doublets at 5.98 ppm (\( \text{C3-H, } J=10.1 \) Hz, \( J=3.0 \) Hz) and; \( \text{E5} \) displayed a singlet at 5.73 ppm (\( \text{C4-H} \)). These signals distinguish between the different chemical environments of the steroid A-ring, and matched the expected multiplet splitting patterns.

**Scheme 5.1: Synthesis of enones E1, and E3, and their corresponding diols**
Diols D5, and D6 were obtained by reduction of E3 (Scheme 5.1), while diols D9, and D10 were obtained by reduction of E5 (not shown). Based on observations reported by Okano et al for a related epithio-steroid, we also expected a hemapolin sulfoxide metabolite HS, which was afforded in low yield by treatment of H with hydrogen peroxide in acetic acid. In our hands M was observed as the major product of this reaction, so alternate milder conditions for the synthesis of HS were investigated. Treatment of H with DMDO effected the mild generation of HS as the major product (Scheme 5.3). This compound proved unstable at room temperature, decomposing slowly to generate M over the course of several days. However, storage at lower temperatures (<0 °C) resulted in minimal decomposition over longer periods of time. Madol 17-sulfate MS was prepared from M in high conversion (>98%) using established conditions (Scheme 5.3).
5.1.3.2 *In vivo* equine metabolites

To date, there have been no reported studies detailing the metabolism of H in equine systems. To address this, an *in vivo* administration study was undertaken in which a synthetically prepared sample of hemapolin (200 mg) was administered to a thoroughbred gelding, with collection of blood, and urine samples occurring up to 7 d post-administration. Sample preparation employed SPE using UCT Xtrackt, Waters Oasis WAX, or Agilent NEXUS cartridges as specified in section 5.1.2.5, to afford extracts suitable for LC-HRAM and GC-MS/MS analysis. The LC-HRAM data were examined using mass filters for predicted metabolites formed from up to three metabolic transformations including oxidation, reduction and hydroxylation, with or without subsequent sulfation or glucuronylaion. Metabolite peaks were identified where exact masses were observed within ±10 ppm of the predicted accurate mass. For GC-MS/MS data, the metabolites were identified by full scan or product ion spectra, and comparison to a urine blank. Metabolites identified by LC–HRAM or GC-MS/MS were matched against synthesised reference materials where available.
5.1.3.3 Analysis of blood metabolites

Positive mode LC-HRAM analysis showed that H was metabolised extensively in the A-ring (Table 5.2). Analysis of blood samples showed two oxidised and hydroxylated madol phase I metabolites (E2, and E4), which were confirmed against synthetic reference materials. Under our LC conditions we observed that the reference materials for enones E2, and E3 had identical retention times, although E2 displayed a prominent ion at m/z 227 in common with the observed metabolite. In contrast, E3 displayed a prominent ion at m/z 201 which was not common with the observed metabolite. A previous study of the equine in vitro metabolism of M (used as a 3:1 mixture of Δ2-3 and Δ3-4 alkene isomers) has been reported which identified E3 as the major equine metabolite by GC-MS/MS analysis after TMS derivatisation. However, reference materials for E1, E2, or E4 were not examined in this study, and as a result the identities of the equine metabolites could have inadvertently been misassigned. Alternatively, there could be subtle differences in the metabolism of M in vitro compared to the metabolism of H in vivo. In the present study, a pure isomer of H was used which affords greater certainty over the metabolic fate of this compound. No matches were observed for reference materials H, M, E1, E3, E5, D1-D10, or HS in the blood
samples by positive mode LC-HRAM analysis. Additionally, no phase II metabolites were observed in the blood samples.

Analysis of the data obtained from the blood samples by positive mode LC-HRAM analysis with an aim to identify metabolites containing an intact episulfide based on knowledge of their +ESI fragmentation, and by comparison to H reference material, were unsuccessful. Additionally, searching for intact sulfoxide metabolites such as HS was similarly unsuccessful. It has been proposed that epithio-steroids can be oxidised to the corresponding sulfoxides, which can then undergo dethionylation, and then subsequent phase I metabolism in vivo. Episulfides and sulfoxides are known to be chemically unstable, and as a result dethionylation is likely to be a facile process which could occur enzymatically, or even under the ambient physiological conditions in the horse. As such, it is not surprising that no metabolites were observed to contain an intact episulfide, or sulfoxide, as upon ingestion H is likely to be rapidly metabolised to the corresponding alkene M, which could then be further metabolised. Oxidation of M to generate the enone metabolites is likely to occur via one of two pathways. Oxidation of M by a cytochrome P450-mediated process could afford an epoxide, which could be opened to afford an allylic alcohol, with subsequent oxidation to the corresponding enone. In the present study which employs isomerically pure H, such a pathway would be expected to result in enones E2 and E3 (Figure 5.2).

**Figure 5.2: Proposed cytochrome P450-mediated epoxidation of M leading to enones E2 and E3**
Table 5.1: *In vivo* equine metabolites observed by GC-MS/MS

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Precursor ion and MS/MS fragments (m/z) (% of base peak), [collision energy]A</th>
<th>RT (min)</th>
<th>Precursor ion</th>
<th>Theoretical m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>E4</td>
<td>446 (70%), 431 (100%), 195 (70%), 179 (50%), 149 (40%), [5 eV]</td>
<td>13.96</td>
<td>[M]*</td>
<td>446.3</td>
</tr>
<tr>
<td>E2</td>
<td>446 (100%), 356 (10%), 251 (15%), 207 (20%), 179 (15%), [5 eV]</td>
<td>14.16</td>
<td>[M]*</td>
<td>446.3</td>
</tr>
<tr>
<td>hydroxylated madol M1</td>
<td>448 (100%), 433 (25%), 358 (50%), 343 (50%), 297 (50%), 284 (75%), 268 (60%), 253 (70%), 246 (45%), 215 (90%), 195 (60%), 162 (30%), 147 (25%), [10 eV]</td>
<td>11.42</td>
<td>[M]*</td>
<td>448.3</td>
</tr>
<tr>
<td>hydroxylated madol M2</td>
<td>448 (15%), 231 (100%), 217 (5%), 173 (5%), 143 (10%), [10 eV]</td>
<td>13.61</td>
<td>[M]*</td>
<td>448.3</td>
</tr>
<tr>
<td>hydroxylated madol M3</td>
<td>448 (25%), 231 (100%), 172 (5%), [10 eV]</td>
<td>13.79</td>
<td>[M]*</td>
<td>448.3</td>
</tr>
<tr>
<td>hydroxylated madol M4</td>
<td>448 (15%), 433 (55%), 358 (40%), 343 (25%), 268 (25%), 226 (25%), 213 (20%), 197 (20%), 182 (20%), 149 (15%), 143 (100%), 117 (15%), 73 (25%), [10 eV]</td>
<td>13.96</td>
<td>[M]*</td>
<td>448.3</td>
</tr>
<tr>
<td>hydroxylated madol M5</td>
<td>448 (35%), 433 (10%), 143 (100%), [5 eV]</td>
<td>14.09</td>
<td>[M]*</td>
<td>448.3</td>
</tr>
<tr>
<td>hydroxylated madol M6</td>
<td>448 (100%), 433 (30%), 358 (70%), 343 (45%), 301 (30%), 268 (60%), 253 (75%), 240 (30%), 226 (40%), 215 (60%), 183 (45%), 159 (30%), 143 (70%), 123 (15%), 106 (15%), [10 eV]</td>
<td>14.52</td>
<td>[M]*</td>
<td>448.3</td>
</tr>
<tr>
<td>dihydroxylated madol M7</td>
<td>536 (100%), 231 (45%), 202 (15%), 143 (20%), [5 eV]</td>
<td>15.29</td>
<td>[M]*</td>
<td>536.4</td>
</tr>
<tr>
<td>dihydroxylated madol M8</td>
<td>536 (100%), 446 (10%), 341 (20%), 251 (20%), 196 (50%), 156 (50%), 143 (10%), [5 eV]</td>
<td>15.75</td>
<td>[M]*</td>
<td>536.4</td>
</tr>
<tr>
<td>dihydroxylated madol M9</td>
<td>536 (100%), 341 (25%), 266 (15%), 231 (20%), 212 (10%), 196 (60%), 181 (25%), 156 (30%), 143 (15%), 117 (70%), [10 eV]</td>
<td>16.71</td>
<td>[M]*</td>
<td>536.4</td>
</tr>
<tr>
<td>dihydroxylated and reduced madol M10</td>
<td>538 (100%), 523 (5%), 453 (45%), 439 (50%), 349 (90%), 259 (35%), 233 (5%), 197 (5%), 169 (5%), 144 (20%), 73 (40%) [10 eV]</td>
<td>16.70</td>
<td>[M]*</td>
<td>538.4</td>
</tr>
<tr>
<td>trihydroxylated madol M11</td>
<td>626 (15%), 231 (100%), 143 (10%), [10 eV]</td>
<td>15.75</td>
<td>[M]*</td>
<td>626.4</td>
</tr>
<tr>
<td>trihydroxylated madol M12</td>
<td>626 (15%), 231 (100%), 143 (10%), [10 eV]</td>
<td>16.71</td>
<td>[M]*</td>
<td>626.4</td>
</tr>
</tbody>
</table>

AFrom targeted MS/MS data acquisition on the Agilent GC instrument using conditions specified for positive mode analysis (section 2.4.1). B Metabolite observed in urine sample analysis. C Matched against E4 reference material. D Matched against E2 reference material. E Matched against mixed.
Table 5.2: *In vivo* equine metabolites observed by LC-HRAM

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Precursor ion and MS/MS fragments (m/z) (% of base peak), [collision energy] (^A)</th>
<th>RT (min)</th>
<th>Precursor ion</th>
<th>Theoretical m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E4</strong></td>
<td>303.2326 (100%), 285.2214 (55%), 267.2117 (35%), 187.1479 (15%), 145.1015 (30%), 95.0855 (15%), 95.0859 (90%), 69.0702 (35%), [20 eV]</td>
<td>12.84 B, C, D</td>
<td>[M+H]+ (^*)</td>
<td>303.2315</td>
</tr>
<tr>
<td><strong>E2</strong></td>
<td>303.2351 (55%), 285.2221 (60%), 267.2118 (100%), 245.1898 (20%), 227.1803 (95%), 187.1479 (50%), 135.1170 (50%), 121.0653 (85%), 95.0859 (90%), 69.0702 (35%), [20 eV]</td>
<td>12.98 B, C, E</td>
<td>[M+H]+ (^*)</td>
<td>303.2315</td>
</tr>
</tbody>
</table>

**madol sulfate S1**

| 367.1957 (100%), 79.9572 (15%), [20 eV] | 5.24 c | [M-H]- | 367.1949 |

**hydroxylated madol sulfate S2**

| 383.1929 (60%), 79.9575 (100%), [10 eV] | 4.63 c | [M-H]- | 383.1898 |

**hydroxylated madol sulfate S3**

| 383.1894 (100%), 96.9574 (40%), [10 eV] | 6.89 c | [M-H]- | 383.1898 |

**dihydroxylated madol sulfate S4**

| 399.1875 (100%), 96.9579 (50%), [30 eV] | 4.96 c | [M-H]- | 399.1847 |

**dihydroxylated and reduced madol sulfate S5**

| 401.2028 (100%), 96.9579 (75%), [40 eV] | 5.00 c | [M-H]- | 401.2003 |

**dihydroxylated and reduced madol sulfate S6**

| 401.2012 (100%), 223.972 (35%), 96.9579 (90%), 79.9574 (20%), [10 eV] | 5.35 c | [M-H]- | 401.2003 |

**trihydroxylated madol sulfate S7**

| 415.1789 (100%), 96.9597 (90%), [30 eV] | 4.27 c | [M-H]- | 415.1796 |

**trihydroxylated madol sulfate S8**

| 415.1791 (100%), 96.9598 (50%), [30 eV] | 4.68 c | [M-H]- | 415.1796 |

**hydroxylated madol glucuronide G1**

| 481.2765 (100%), 305.2446 (25%), [10 eV] | 14.82 c | [M+H]+ \(^*\) | 481.2796 |

**oxidised and hydroxylated madol glucuronide G2**

| 479.2636 (10%), 429.3012 (25%), 303.2316 (100%), 177.1136 (15%), 133.0876 (50%), 89.0603 (95%), [20 eV] | 14.20 c | [M+H]+ \(^*\) | 479.2639 |

\(^A\)From targeted MS/MS data acquisition on the Agilent LC instrument using conditions specified for positive mode or negative mode analysis (section 2.4.2). \(^*\)Metabolite observed in blood sample analysis. \(^\circ\)Metabolite observed in urine sample analysis. \(^\diamond\)Matched against E4 reference material. \(^\diamond\)Matched against E2 reference material.
In light of the observed metabolites \textbf{E2} and \textbf{E4}, an alternate pathway was proposed which could explain the observed equine metabolite distribution. Mechanistically, P450 enzymes perform hydroxylation through a sequence involving hydrogen atom abstraction to form a carbon-centred radical, followed by rapid hydroxyl radical recombination. Abstraction of a hydrogen atom at C4 would result in an allylic radical which could then undergo radical recombination at C2 or C4, resulting in allylic alcohols \textbf{D3/D4}, or \textbf{D7/D8} which could be subsequently oxidised to the corresponding enones \textbf{E2} and \textbf{E4} respectively \textsuperscript{30,34–36}. Evidence for the allylic rearrangement required to access the double-bond isomerised hydroxylated metabolites has been reported \textsuperscript{34,36}. In the present study, abstraction of the hydrogen at C4 would result in enones \textbf{E2} and \textbf{E4} (Figure 5.3), whilst the alternative abstraction of the hydrogen atom at C1 would result in enones \textbf{E1}, and \textbf{E3}. The exclusive formation of enones \textbf{E2} and \textbf{E4} as the equine metabolites could potentially be explained by the steric environment surrounding C4, as this position is less hindered than the environment at C1, due to the adjacent methyl group at C10. Alternatively, this selectivity could be rationalised through cytochrome P450 enzyme-substrate interactions.

\textbf{Figure 5.3: Proposed cytochrome P450-mediated hydroxylation of M leading to enones E2 and E4}

\[ \text{Scheme showing proposed pathway for E2 and E4 formation from M.} \]

5.1.3.4 Analysis of phase I urinary metabolites

Positive mode ESI LC-HRAM analysis showed two oxidised and hydroxylated madol phase I metabolites, which were identical to those found in the blood samples (Table 5.2). These metabolites were confirmed against synthetic reference
materials E2, and E4. No matches were observed for reference standards H, M, E1, E3, E5, D1-D10, or HS in the urine samples by positive mode LC-HRAM analysis.

Analysis by EI GC-MS/MS also identified E2 and E4 as the major urinary metabolites and these were also matched to reference materials (Table 5.1). Under the GC-MS/MS conditions employed, enones E2 and E3 were separated chromatographically, which allowed identification of the equine metabolite E2. A combination of retention time, and the presence of an ion at m/z 179 which was common to the observed metabolite and E2 reference material, and the absence of ions at m/z 201 and m/z 145 which were observed only in the E3 reference material, were used to confirm the identity of the equine metabolite against reference materials. The ion counts for the urinary metabolites (~10²) were substantially lower than those of the reference materials (~10⁴), due to their low urinary concentration, which led to differences in the ion profiles of the observed metabolites and reference materials (Figure 5.4, and 5.5). Although there were differences in the number of ions observed, the major ions for the urinary metabolites were still identified in the reference materials, and were within the Association of Official Racing Chemists (AORC) mass spectrometry criteria 37. Analysis by GC-MS/MS also identified a number of additional metabolites which were not observed by LC-HRAM analysis. A number of the observed metabolites were steroid diols, or triols, which are known to be difficult to ionise under +ESI conditions common to LC analysis 38,39. However, as the sample preparation involved acid hydrolysis of the phase II steroid conjugates, only the corresponding phase I metabolites were observed. In addition to the major enone metabolites, other major metabolites observed by GC-MS/MS analysis included four hydroxylated madol metabolites (M1-M3, M6). Minor metabolites were observed including two hydroxylated madol metabolites (M4 and M5), three dihydroxylated madol metabolites (M7-M9), one dihydroxylated and reduced metabolite (M10), and two trihydroxylated metabolites (M11 and M12).

The minor metabolites M4 and M5 were both observed to match reference materials by retention time for both D3/D4, and D7/D8, preventing their
unambiguous identification. The retention time for the observed metabolite M₄ (RT 13.96 min) closely matched one peak in both the mixed D₃/D₄ and D₇/D₈ reference materials. The absence of metabolite M₄ ions at m/z 358 and m/z 149, and the presence of an additional ion at m/z 240 in the corresponding peak of the D₃/D₄ reference material, suggested a closer match with the D₇/D₈ reference material. As the 4α-hydroxy isomer D₇ was prepared in pure form, and matches with the second eluting peak (RT 14.07 min) of the mixed D₇/D₈ reference material, the identity of M₄ was tentatively assigned as the 4β-hydroxy isomer D₈.

In contrast, the metabolite M₅ (RT 14.09 min) afforded only a few fragment ions, none of which could be used to differentiate between the mixed D₃/D₄ and D₇/D₈ reference materials. It is likely that the low urinary concentration of this minor metabolite affords poor sensitivity in the observed GC-MS/MS data, preventing its unambiguous assignment. However, since both of the enones (E₂, and E₄) were observed as major metabolites, it is also likely that the corresponding diol metabolites D₃, D₄, D₇, and D₈ would also be present. Additionally, as the D₃/D₄ reference material was prepared as a mixture, it would not be possible to assign the stereochemistry of these products to a particular metabolite peak. No reference materials were available for the major metabolites M₂ and M₃, however these were tentatively assigned as a pair of 16α/β-hydroxylated madol metabolites, based on the observed m/z 231 fragment ion, which has been previously reported to be characteristic of 16-hydroxylated-17-methylated steroids 40. This fragment ion was also observed for minor metabolites M₇, M₉, M₁₁, and M₁₂, which were also tentatively assigned to contain 16-hydroxylation. No matches were observed for reference standards H, M, E₁, E₃, E₅, D₁-D₂, D₅-D₆, D₉-D₁₀, or HS in the urine samples by GC-MS/MS.

Unsurprisingly, subjecting H reference material to GC-MS/MS analysis resulted in decomposition of the episulfide on heating or derivatisation to generate M, however as no metabolites were observed to contain an intact episulfide, or sulfoxide by LC-HRAM analysis, it is unlikely that these metabolites would be present in the urine samples.
Figure 5.4: Extracted ion chromatograms showing targeted GC-MS/MS spectra (5 eV) of a) E2 identified in the 4 h urine, b) E2 reference material

a)

b)
Figure 5.5: Extracted ion chromatograms showing targeted GC-MS/MS spectra (5 eV) of a) E4 identified in the 4 h urine, b) E4 reference material

5.1.3.5 Analysis of phase II urinary metabolites

Positive and negative mode LC-HRAM analysis identified a number of phase II conjugates that corresponded to the phase I metabolites described in the previous sections (Table 5.2). Analysis of the urine samples identified one madol sulfate metabolite S1, two hydroxylated madol sulfates S2 and S3, one dihydroxylated madol sulfate S4, two dihydroxylated and reduced madol sulfates S5 and S6, two trihydroxylated madol sulfates S7 and S8, one hydroxylated madol glucuronide G1, and one oxidised and hydroxylated madol glucuronide G2. The reference material for MS did not match the retention time for S1, even though there is only one potential site for sulfation. Additionally, comparison of the LC-HRAM spectrum of S1 to MS showed that S1 did not exhibit the expected [HSO₄]⁻ fragment of m/z 96.9601, but instead afforded the [SO₃]⁻ fragment m/z 79.9572. Tertiary steroid sulfates such as MS have been observed to hydrolyse with inversion of configuration to generate the epimeric 17α-hydroxy compounds, which could

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potentially then be re-sulfated. As such, these metabolites have been tentatively assigned as a 17-epi-madol sulfate. Unfortunately, there was no reference material available for the corresponding 17-epi-madol compound to confirm this assignment.

Although a number of the key phase II metabolites were observed by LC-HRAM analysis, a number of the major phase I metabolites were only observed by GC-MS/MS analysis. While the use of HRAM affords a higher degree of confidence for the detected metabolites, which is particularly important at very low metabolite concentrations, the inability to detect phase I steroid diols and triols by LC-HRAM is a major limitation for screening. In light of the advantages afford by both methods of analysis, the use of both LC-HRAM and GC-MS/MS in conjunction with each other is recommended for the detection of H misuse in equine sports.

5.1.3.6 Excretion profiling of the major equine metabolites

One of the aims of this study was to identify potential metabolites to incorporate into anti-doping screening protocols. The excretion of the major enone and diol metabolites, as determined by GC-MS/MS analysis, was established through a response ratio plot of metabolite signal intensity relative to d₃-testosterone as an internal standard. In order to enhance sensitivity, and assist in the translation to a routine screening protocol, a series of multiple reaction monitoring (MRM) transitions were identified from the most intense ion peaks observed in the product ion spectra. The excretion profiles of the enone metabolites E2, and E4, were established, in addition to the diol metabolites M1-M6. The excretion profile of E4 is shown below (Figure 5.6), and the excretion profiles of E2, and M1-M6 are included in the supporting information.
The enone metabolite E4 was observed up to 72 h post-administration by monitoring for the MRM transitions 431 → 179, and 446 → 431, in contrast to E2 which was observed up to 12 h post-administration by monitoring for the transition 446 → 179. For the diol metabolites M2 and M3, the most sensitive transitions appeared to be 448 → 231, which was observed up to 48 h post-administration, and 448 → 143, which was observed up to 8 h post-administration. The remaining metabolites M1, M4-M6 were observed by monitoring for the transition 448 → 143, and were all observed up to 8 h post-administration. The AORC mass spectrometry criteria requires the use of at minimum three fragment ions at unit mass resolution, or two fragment ions at HRAM resolution in order to confirm a metabolite and as such E4, M2, and M3 appeared to be the most suitable metabolites for screening purposes.

5.1.4 Conclusions
Designer steroids such as hemapolin (H) have the potential to harm the integrity of sporting competition if left unchecked. The metabolism of hemapolin was investigated in equine systems for the first time by an in vivo administration study. The enone metabolites 17β-hydroxy-17α-methyl-5α-androstan-3-en-2-one E2, and 17β-hydroxy-17α-methyl-5α-androstan-2-en-3-one E4, presumably derived from cytochrome P450-mediated hydroxylation and oxidation of M, were detected in equine blood and urine, the latter being observed up to 3 d post-administration.
The hydroxylated madol metabolites M1-M6 were also identified, with M2 and M3 observed up to 48 h post-administration by MRM methods. Minor metabolites resulting from dihydroxylation, dihydroxylation and reduction, and trihydroxylation were also observed, alongside a number of phase II sulfate and glucuronide conjugates. These metabolites were matched against an extensive library of synthetically derived reference materials where available. The excretion of a number of the key enone and diol metabolites was profiled. These investigations have identified the key metabolites resulting from hemapolin administration which can be incorporated into routine anti-doping screening and confirmation protocols.

5.1.5 Future work

As a number of aspects of this work remain incomplete, there are several avenues for future work on this project. A number of the key metabolites have been tentatively identified in this study based on analysis of their mass spectrometry behaviour, as reference materials for many of the metabolites were unavailable. Providing access to synthetic reference materials for these metabolites could unambiguously confirm their identities. The major phase I metabolites M2 and M3 were proposed as a pair of 16α/β-hydroxylated madol isomers by GC-MS/MS analysis, based on the observation of a characteristic fragment ion of m/z 231. These compounds could potentially be confirmed through preparation of the corresponding C16-hydroxylated reference materials. Access to the C16α-hydroxylated isomer could be afforded through a similar protocol as described in Chapter 3.2 for the synthesis of 3α-chloro-17α-methyl-5α-androstan-16α,17β-diol. Access to the C16β-hydroxylated isomer could be afforded through alternative methods, such as the protocol reported by Hungerford et al.

In addition to M2 and M3, the metabolites M4 and M5 have been only tentatively assigned. Mixed reference materials for D3/D4 and D7/D8 had similar retention times, and ion fragmentation patterns compared with the observed metabolite, and as a result their identities were not able to be unambiguously determined. The low sensitivity of the detected metabolites resulting from their low urinary
concentration also contributed to this problem. The preparation of these reference materials on a larger scale may facilitate their separation by column chromatography, which could allow for pure materials to assist in the identification of these metabolites. Furthermore, the observed dihydroxylated and reduced madol metabolite \textbf{M10} could match \textit{17a-methyl-5a-androstan-2\beta,3\alpha,17\beta-triol}, which has been reported as a major metabolite for the \textit{in vitro} human metabolism of \textbf{M} \textsuperscript{21}. Although there are expected to be differences in the metabolism observed in this study compared to the present study, determining the identity of this metabolite may prove useful for screening purposes. The synthesis of isomeric compounds of this type could provide reference materials which could be used to shed light on the identity of \textbf{M10}. Additionally, synthetic access to a variety of reference materials for metabolites \textbf{M2-M5}, and \textbf{M10} may also allow for preparation of their corresponding phase II sulfate and glucuronide conjugates, allowing for the unambiguous assignment of other metabolites.

In addition to the problems surrounding the identity of some of the phase I metabolites, there also remains an issue with the identity of the observed phase II madol sulfate metabolite \textbf{S1}. The reference material for \textbf{MS} was not observed to match the retention time for \textbf{S1}, even though there is only one potential site for sulfation. It is known that tertiary alcohols from C17-methylated steroids can undergo epimerisation \textit{in vivo} at C17 via the sulfate conjugate \textsuperscript{50-52}, which could potentially then be re-sulfated. A similar process could also be occurring during the preparation of the \textbf{MS} reference material, to generate the epimeric madol compound. In light of the complicated nature of the reactivity of this metabolite, additional study is needed to unambiguously determine the identity of this metabolite. Standards of both \textbf{M} and \textit{17-epi-madol} should be prepared, to test how they behave under the sulfation conditions, and to ultimately provide reference materials which can be used for the assignment of \textbf{S1}.

Finally, additional work needs to be undertaken to fully profile the excretion of the key equine metabolites. Although the excretion of several of the major enone (\textbf{E2} and \textbf{E4}), and diol (\textbf{M1-M6}) metabolites has been undertaken using MRM methods,
these only qualitatively profile the excretion of these metabolites. In addition, a number of these profiles make use of only one MRM transition, which is inadequate under AORC criteria for confirming these compounds as metabolites. With the provision of suitable reference materials, the excretion of the major metabolites $E4$, $M2$ and $M3$ should be quantified in equine urine, as this will allow determination of suitable limits of detection, and quantification which would prove invaluable for routine screening purposes. At present, only $E4$ has an available reference material, however even the quantification of this metabolite alone would provide valuable information for anti-doping screening, and should be undertaken.

5.1.6 Acknowledgements
The author would like to thank the staff at the Australian Racing Forensic Laboratory (Sydney, Australia) for technical assistance with LC-MS, and GC-MS analysis.

5.1.7 References
CHAPTER SIX

Conclusions and Future Work
6.1 Conclusions

The work presented throughout this thesis highlights a number of strategies that can be employed for the detection of designer steroids in racehorses. This final chapter aims to summarise the main conclusions drawn from the work presented, and how they contribute to the area of anti-doping research.

The literature reviewed in Chapter One highlights the tools currently available to anti-doping laboratories. This review has highlighted the strengths and weaknesses of current anti-doping strategies, and identified the areas that currently require additional focus. Although anti-doping laboratories are equipped with a variety of tools to identify instances of designer steroid misuse, they are often limited by a lack of information detailing the metabolism of these compounds in equine systems. A number of studies detailing the metabolism of designer steroids have been reported in recent years, and have identified some metabolites and markers to assist in their detection. However, the vast majority of currently available designer steroids have not been studied in equine systems, despite receiving significant attention in human sports. Studies such as these provide valuable information on the equine metabolism of designer steroid compounds, and as a result this review will hopefully assist future anti-doping research.

One of the primary limitations of current anti-doping research as identified in Chapter One is a lack of reference materials available to anti-doping laboratories for use in the detection and confirmation of designer steroid misuse. In particular, steroid sulfate metabolites have remained relatively understudied, despite the fact that sulfation is known to be a key pathway in the metabolism of steroid compounds in equine systems. Recent advances in the use of LC-MS/MS analysis have also facilitated their direct detection, and as a consequence these metabolites are of growing importance to anti-doping laboratories. Chapter Two presents a new approach for the synthesis of steroid sulfate metabolites, suitable for use by anti-doping laboratories. This approach makes use of SPE, a technique familiar to, and routinely utilised by anti-doping laboratories. A library of sixteen steroid mono-sulfate, and twelve steroid bis-sulfate compounds has been prepared using
this method. A number of these compounds have not been studied previously, and
the characterisation data available in the literature is lacking in most cases. Characterisation including $^1$H NMR, $^{13}$C NMR, and (-ESI) LRMS data has been made available, and these steroid compounds are now able to be prepared for use as reference materials by anti-doping laboratories, without the need for significant investment in chemical synthesis. In addition, the MS behaviour of these compounds has been studied, identifying the key modes of fragmentation. Steroid mono-sulfates were observed to give rise to ions of [M-H], and a fragment ion at $m/z$ 97 (corresponding to [HSO$_4$]). A fragment ion of $m/z$ 80 (corresponding to [SO$_3$]$^-$) was also observed in some instances. Steroid bis-sulfates gave rise to [M-2H]$^{2-}$, and [M-H]$^-$ ions, together with the fragment ions [M-2H-HSO$_4$], $m/z$ 97, $m/z$ 81 (corresponding to loss of [HSO$_3$]$^-$), and $m/z$ 80. The key fragmentation mode appears to be loss of the [HSO$_4$]$^-$ anion to give rise to the fragment ion [M-2H-HSO$_4$], with an increase in $m/z$ of the resulting fragment ion. This unique fragmentation mode formed the basis of a common ion loss scan (CIL) method for the untargeted detection of steroid bis-sulfate compounds. The developed CIL approach allowed for the untargeted detection of endogenous bis-sulfates at the low ng/mL range. This method allowed for the detection of steroid bis-sulfates corresponding to a number of different steroid classes in urine samples obtained from healthy volunteers, and will hopefully be suitable for the detection of other classes of steroid bis-conjugates such as bis-glucuronides, bis-sulfate-glucuronides, or other multiply-charged metabolites. The ability to detect endogenous bis-sulfate metabolites could potentially assist in monitoring for EAAS misuse, or even for the detection of designer steroid misuse, and as such broadens the utility of this method for use in anti-doping laboratories.

The detection of novel anabolic agents intended for doping purposes remains a significant problem in both human and equine sports. The greatest hurdle to responding effectively to this problem is often obtaining intelligence regarding the availability of designer steroids, and therefore identifying the most likely candidates for misuse. Chapter Three presents a study in which an unknown sample (determined to contain 3α/β-chloro-17α-methyl-5α-androsta-17β-ol) was seized by law-enforcement. A workflow was developed which details an effective
response to the threat of new designer steroid compounds, suitable for adoption by anti-doping laboratories. Central to this approach was the use of synthetically-derived reference materials, androgen bioassays, and in vitro metabolism. The availability (or lack thereof) of steroid reference materials is a common theme throughout this work, and this study highlights the utility of high quality chemical synthesis in anti-doping research. Androgen bioassays provide a useful way to gauge the threat a particular compounds poses to anti-doping laboratories, and may eventually have applications in routine screening. The use of in vitro techniques to study the metabolism of designer steroids is highlighted as an alternative to in vivo administration, particularly when it is difficult to justify these on ethical grounds. These studies identified the key metabolites resulting from metabolism of 3α-chloro-17α-methyl-5α-androsta-17β-ol, and in particular, highlighted the need for the study of these compounds specifically in equine systems, as the major metabolite resulting from equine in vitro metabolism (3α-chloro-17α-methyl-5α-androsta-16α,17β-diol) did not match the major metabolite resulting from human in vitro metabolism (17α-methyl-5α-androstan-3α,17β-diol). The data obtained from these studies was then used to develop and evaluate the translation into routine anti-doping screening protocols, which will hopefully allow for detection of these compounds in human and equine sports.

The work presented in Chapter Four builds on the work presented in Chapter Two and Chapter Three, and presents two separate studies detailing the metabolism of the designer steroids furazadrol ([1′,2′]isoxazolo[4′,5′:2,3]-5α-androstan-17β-ol), and superdrol (17β-hydroxy-2α,17α-dimethyl-5α-androstan-3-one). In the first study, the in vivo equine metabolism of furazadrol is presented alongside a comparative in vitro phase I metabolism study. Following the controlled administration of furazadrol to a thoroughbred racehorse, the key in vivo metabolites furazadrol 17-sulfate, furazadrol 17-glucuronide, and epifurazadrol 17-glucuronide were identified and matched to synthetically derived reference materials. Additionally, a number of minor metabolites were observed resulting from hydroxylation, and oxidation and hydroxylation, together with sulfate or glucuronide conjugation. Enzymatic hydrolysis of the phase II sulfate and glucuronide metabolites by Pseudomonas aeruginosa arylsulfatase and Escherichia
coli β-glucuronidase provided additional evidence for the identity of these metabolites. The key metabolites, furazadrol 17-sulfate and furazadrol 17-glucuronide, were quantified in equine urine to established an excretion profile, and suitable limits of detection. The comparative *in vitro* phase I study was also undertaken and identified all but two of the minor *in vivo* phase I metabolites observed after hydrolysis of the urine samples. These investigations identified the key urinary metabolites of furazadrol following oral administration, which can be incorporated into anti-doping screening and confirmation procedures. This study highlights the value of synthetically derived reference materials, and *in vitro* techniques in studying the metabolism of designer steroids.

The second study described in Chapter Four presents an investigation into the *in vitro* study of phase II steroid metabolism, which is currently under-studied in anti-doping research. Phase II sulfation was undertaken through the addition of UDPGA and PAPS to the *in vitro* reaction employed in Chapter 4.2. Alternative conditions for phase II sulfation were explored in which ATP, and Na$_2$SO$_4$ were used in place of PAPS. Employing UDPGA, ATP, and Na$_2$SO$_4$ for the metabolism of superdrol identified 3β-RSS, and a reduced superdrol glucuronide G2 as the major metabolites, alongside minor metabolites including two hydroxylated superdrol sulfates, a reduced and hydroxylated superdrol sulfate, a superdrol glucuronide, 3β-RSG, and two reduced and hydroxylated superdrol glucuronides. The major 3β-RSS, and minor 3β-RSG metabolites were confirmed against 2α,17α-dimethyl-5α-androstan-3β,17β-diol 3-sulfate, and 2α,17α-dimethyl-5α-androstan-3β,17β-diol 3-glucuronide reference materials respectively. The phase II conjugates observed appeared to correlate well with the phase I metabolites identified in previously reported equine and human *in vitro* studies. The *in vitro* metabolism of furazadrol was also explored using both phase II systems, with comparison to the *in vivo* study reported in Chapter 4.2. Following phase II metabolism with UDPGA and PAPS, the major *in vivo* metabolites FS, IFS, EFS, FG, IFG, and EFG were all confirmed by matching to reference materials. In addition, a number of minor metabolites were observed, including six hydroxylated furazadrol sulfate metabolites, one oxidised and hydroxylated furazadrol sulfate metabolite, five hydroxylated furazadrol glucuronide metabolites, and two oxidised and
hydroxylated furazadrol glucuronide metabolites. Only one of the minor sulfate metabolites observed using these conditions was observed to match the in vivo profile. The use of UDPGA, ATP, and Na₂SO₄ generated a comparable in vitro profile, with identification of the same major in vivo metabolites, as well as a number of minor metabolites including six hydroxylated furazadrol sulfate metabolites, one oxidised and hydroxylated furazadrol sulfate metabolite, two hydroxylated furazadrol glucuronide metabolites, and three oxidised and hydroxylated furazadrol glucuronide metabolites. Only one of the minor sulfate metabolites observed using these conditions was observed to match the in vivo profile, suggesting that these conditions may be a better choice for generating some in vivo metabolites. Although some of the minor in vivo designer metabolites were also identified in this work, the ability of these systems to fully predict in vivo metabolism is currently limited, and it is hoped that future work will improve the ability to accurately predict the in vivo metabolism of steroid compounds solely from in vitro results.

Chapter Five draws on the lessons learnt throughout the previous chapters and details a study of the designer steroid hemapolin (2α,3β-epithio-17α-methyl-5α-androstan-17β-ol). Synthetic hemapolin was prepared through a novel synthetic strategy, and was used in an equine in vivo administration study. Following controlled administration, the key in vivo enone metabolites 17β-hydroxy-17α-methyl-5α-androst-3-en-2-one E₂, and 17β-hydroxy-17α-methyl-5α-androst-2-en-4-one E₄, were identified in equine blood and urine, and were matched to synthetically derived reference materials. Additional minor metabolites were observed, resulting from hydroxylation, dihydroxylation, dihydroxylation and reduction, and trihydroxylation, with and without phase II sulfation or glucuronylation. A number of these were tentatively assigned by comparison to reference materials, which were considered essential to assign the identity of the key enone, and diol metabolites. The excretion profiles of these metabolites were established, with the major enone, and diol metabolites E₄, E₂, M₂, and M₃ observed up to 72 h, 8 h, 48 h, and 48 h post-administration respectively. A number of aspects of this work currently remain incomplete, however this study has identified the key metabolites resulting from hemapolin administration which
can be incorporated into routine anti-doping screening and confirmation protocols in the future.

Overall, the work presented in this thesis contributes to a number of important areas of anti-doping research. First and foremost, this work addresses the issue of access to reference materials, which is a major limitation in current anti-doping laboratories. Through the development of simple methodology for the in-house synthesis of steroid sulfates, as well as the synthesis of the designer steroids 3α-chloro-17α-methyl-5α-androstan-17β-ol, furazadrol, hemapolin, and their respective phase I and phase II metabolites, we have expanded the range of reference materials available to anti-doping laboratories for use in routine screening and confirmatory analysis. Secondly, this work addresses some of the current limitations of in vitro technologies for the study of steroid metabolism. A common criticism of in vitro methods is their suggested inability to replicate in vivo results, and this work addresses these concerns though the development of alternative in vitro methodologies which have been observed to generate in vivo metabolites, including phase II sulfate and glucuronide metabolites. Finally, this work helps to translate these results into meaningful screening protocols which can be adopted by anti-doping laboratories. This has been achieved through the identification of the key metabolites resulting from steroid administration, the determination of their respective excretion profiles, determination of suitable limits of detection and quantification, and the provision of reference materials to anti-doping laboratories. It is hoped that this work can be used by anti-doping laboratories to detect instances of misuse of designer steroids, and as a platform for future research to develop new strategies for the detection of designer steroids.

6.2 Future work
The work presented throughout this thesis has identified a number of aspects which could be the target of future research. As previously discussed, the review presented in Chapter One has identified a number of limitations of current anti-doping analysis which could become the subject of future research. A number of designer steroids which are currently available have not been previously studied
in equine systems, although they have gained attention in human sports\textsuperscript{1–3}. Additionally, new supplements are constantly coming onto the market which will require study in the future. The work presented throughout this thesis will hopefully prove a suitable template upon which to undertake future research. Additionally, the review has highlighted the growing importance of LC-MS analysis in anti-doping laboratories, which is facilitating the direct detection of phase II metabolites. These metabolites are likely to become important markers of drug detection in the future, and developing methods which can utilise these markers would likely benefit anti-doping laboratories greatly. It would also be prudent to review the scientific literature on the application of phase II conjugates in screening and confirmation, which would hopefully provide useful data to anti-doping laboratories, and provide further direction to future research.

Access to steroid-sulfate metabolites has been provided through the methodology presented in Chapter Two. The use of these compounds as reference materials will facilitate the future development of LC-based analyses, and potentially provide additional tools to assist in confirmatory analysis. Whilst sulfate metabolites are straightforward to prepare, another class of metabolites, steroid glucuronides, are currently lacking reliable and simple methodology for their synthesis. The synthesis of steroid mono-glucuronides, bis-glucuronides, and mixed bis-sulfate-glucuronides would benefit from the development of simple methodology for the synthesis of these materials, and these compounds could be subjected to a systematic study similar to the one presented in Chapter 2.3. Recent work within the McLeod group has developed an enzymatic β-glucuronylsynthase method for the synthesis of steroid glucuronides\textsuperscript{4}, however it is currently limited by a small substrate scope and poor reaction yields. It is hoped that improvements to this enzyme may provide future access to these classes of compounds, and facilitate their study and incorporation into routine anti-doping screening. Additionally, the constant ion loss method developed in Chapter 2.3 should be employed in anti-doping laboratories for the detection of steroid bis-sulfate metabolites, potentially allowing for the detection of new steroid metabolites. This methodology could also be applied to other bis-metabolites, such as bis-glucuronides, and mixed bis-
sulfate-glucuronides, allowing for the untargeted detection of new classes of steroid metabolites.

The identification of new supplements containing unknown steroid compounds is a growing concern, and the workflow developed in Chapter Three should allow anti-doping laboratories to respond more effectively to the threat of designer steroids in future. The use of androgen bioassays is gaining importance for screening, as these assays can rapidly detect samples containing anabolic agents which can then be flagged for further testing. Future testing of new supplements using these methods could provide valuable intelligence to anti-doping laboratories and law enforcement, providing direction to future research.

*In vivo* studies have been a mainstay of steroid metabolism; however, the use of animal studies is not without its risks to the health and well-being of the animal researcher. The use of *in vitro* techniques to replicate steroid metabolism would reduce the need for *in vivo* studies, but cannot be used routinely until reliable protocols are developed which can replicate *in vivo* metabolism. Methodology has been developed in Chapter Four which attempts to address these concerns, and has shown that the incorporation of phase II co-factors into *in vitro* systems can successfully generate phase II metabolites. The use of PAPS to generate phase II metabolites has been successfully replaced with cheaper reagents, and although UDPGA is much cheaper to purchase compared to PAPS, it can still be prohibitively expensive, preventing its use in some instances. The development of alternative conditions for phase II glucuronylation could facilitate their incorporation into *in vitro* techniques, and the incorporation of phase II metabolism is likely to more accurately reflect the metabolism observed *in vivo*. The phase II co-factor UDPGA (AU$100 for 25 mg, Sigma Aldrich) is required for glucuronylation *in vivo*, and is known to be generated from uridine 5'-diphosphogluucose (UDP-glucose, AU$87 per 100 mg, Sigma Aldrich) in a NADH-dependant process. The incorporation of UDP-glucose and NADH could be a viable substitute for UDPGA, as the enzymes required for the biosynthesis of UDPGA should likely be contained within the liver S9 fraction. By making the study of phase II metabolism affordable, through the use
of affordable co-factor systems, it is hoped that it will be used more routinely to study phase II metabolism; eventually resulting in in vitro systems capable of fully replicating in vivo metabolism.

A number of aspects of the work presented in Chapter Five require future work, and have been discussed in detail previously. Briefly, the identification of a number of hemapolin metabolites requires additional work to confirm the identity of these metabolites against reference materials. Additionally, the excretion of the key enone and diol metabolites will require further work to complete. Ultimately, it is hoped that completing this work will provide valuable information about this intriguing designer steroid for use in anti-doping screening

6.3 References
APPENDIX A

Publications
In the period between when this thesis was submitted (September 2016) and the corrections to this thesis were made (May 2017), this review was accepted for publication in the journal *Drug Testing and Analysis*. The full citation for this work is as follows:


A copy of the full text article has been reproduced in Appendix A, with permission of John Wiley and Sons via Rightslink (License Number: 3994540395974).
Appendix A.1 A review of designer anabolic steroids in equine sports
Due to copyright restrictions from the publisher, the journal article presented in section A.1 has been removed from the online version of this thesis. The full text article can be obtained via the ANU Library, or directly from the publisher using the link below:

In the period between when this thesis was submitted (September 2016) and the corrections to this thesis were made (May 2017), this work was accepted for publication in the journal *Analytical Chemistry*. The full citation for this work is as follows:


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Appendix A.2 Constant Ion Loss Method for the Untargeted Detection of Bis-sulfate Metabolites.
Due to copyright restrictions from the publisher, the journal article presented in section A.2 has been removed from the online version of this thesis. The full text article can be obtained via the ANU Library, or directly from the publisher using the link below:

http://pubs.acs.org/doi/abs/10.1021/acs.analchem.6b03671