APPROACHES TO THE DETECTION OF STEROID ABUSE IN VETERINARY SPECIES

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STEROID ABUSE IN VETERINARY SPECIES

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Abbreviations

AAS	anabolic-androgenic steroid.
API	atmospheric pressure ionization.
BHA	British Horseracing Authority.
C18 steroid	a steroid based on the estrane nucleus.
C19 steroid	a steroid based on the androstane nucleus.
C21 steroid	a steroid based on the pregnane nucleus.
C24 steroid	a steroid based on the cholane nucleus.
C27 steroid	a steroid based on the cholestane nucleus.
CAD	collision activated dissociation.
CCα	decision limit.
CCβ	detection capability.
CRL	European Community Reference Laboratory.
CYP	cytochrome P450.
DHEA	dehydroepiandrosterone.
DMF	dimethylformamide.
DNA	deoxyribonucleic acid.
EI	electron ionisation.
EIA	enzyme immunoassay.
ELISA	enzyme linked immunosorbent assay
EPI	enhanced product ion scan.
ERC	endogenous reference compound.
EU	European Union.
FDA	Food and Drug Administration.
FEI	Federation Equestre Internationale.
FWHM	full width at half maximum height.
GC-C-IRMS	gas chromatography combustion isotope ratio mass spectrometry.
GC-MS	gas chromatography-mass spectrometry.
GC-MS/MS	gas chromatography-tandem mass spectrometry.
GBGB	Greyhound Board of Great Britain.
HCD	higher-energy collision decomposition.
HCl	hydrochloric acid.
HPLC	high performance liquid chromatography.
HR-LC-MS	high resolution-liquid chromatography-mass spectrometry.
IA	Immunoassay.
ICRAV	International Conference of Racing Analysts and Veterinarians.
IFHA	International Federation of Horseracing Authorities.
ILAC	International Laboratory Accreditation Cooperation.
IM	intramuscular.

IUPAC	International Union of Pure and Applied Chemistry.
IV	intravenous.
LC-MS	liquid chromatography-mass spectrometry.
LC-MS/MS	liquid chromatography-tandem mass spectrometry.
LLOQ	lower limit of quantification.
LOD	limit of detection.
LOQ	limit of quantification.
LTQ	linear trap quadrupole.
MeOH	methanol.
MO	methoxyamine.
MO-TMS	methoxyamine-trimethylsilyl.
MRPL	minimum required performance limit.
MSTFA – N-Methyl-n	(trimethylsilyl)-trifluoroacetamide.
MTBSTFA – N	(t-butyldimethylsilyl)-N-methyltrifluoroacetamide.
m/z	mass to charge ratio
NAD$^+$	nicotinamide adenine dinucleotide.
NADPH	nicotinamide adenine dinucleotide phosphate.
NaOH	sodium hydroxide.
NMR	nuclear magnetic resonance.
ND	not detected.
NMP	national monitoring programme.
PTV	programmable temperature vaporiser.
QC	quality control.
qRT-PCR	quantitative real time reverse transcriptase polymerases chain reaction technology
RIA	radio-immunoassay
RSD	relative standard deviation (also known as coefficient of variation).
SCVPH	Scientific Committee on Veterinary Measures relating to Public Health.
SRM	selected reaction monitoring.
SPR	surface plasmon resonance.
THG	tetrahydrogestrinone.
TLC-FL	thin layer chromatography-fluorescence.
TBDMS	tertiary-butyl,dimethyl-silyl
TMS	trimethylsilyl.
TOF	time of flight.
u	atomic mass unit
ULOQ	upper limit of quantification.
UV	ultraviolet.
WADA	World Anti-Doping Agency.

INTRODUCTION

Steroid structure and pharmacology

If one were to ask a member of the public what images the word 'steroid' conjured into their imagination, the use of anabolic-androgenic steroids (AASs) in athletics or bodybuilding would no doubt rank near the top of the list. However, steroids have a range of structures and pharmacological actions that reach far beyond the anabolic effects of AASs. The term 'steroid' itself refers to any compound possessing the basic perhydrocyclopentanophenanthrene nucleus (Figure 1) (Makin, 1995).
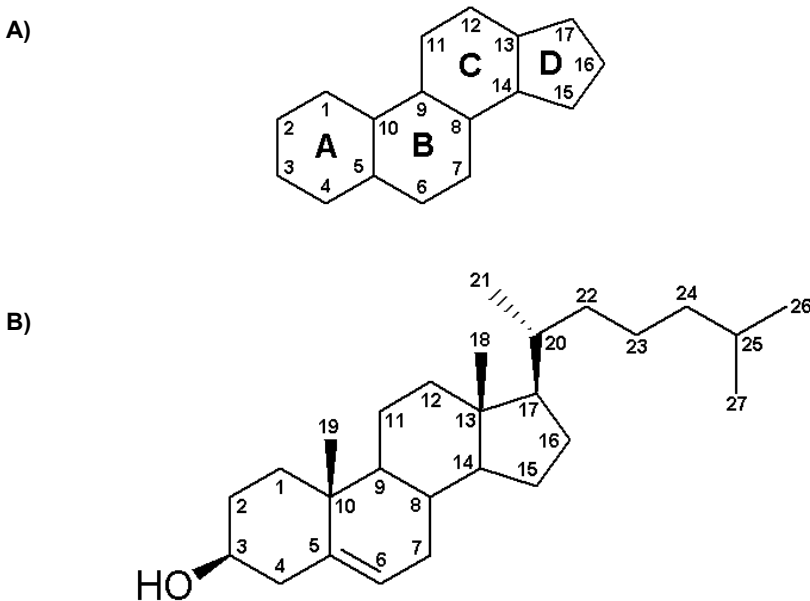
A)

B)

*Figure 1 – **A)** the perhydrocyclopentanophenanthrene nucleus, on which all steroids are based and **B)** cholesterol as an example. Each carbon is assigned a number and the four hydrocarbon rings are numbered A-D, as shown.*

The nomenclature of this class of compounds is complex and large arrays of different systems are used. These include; the official International Union of Pure and Applied Chemistry (IUPAC) recommended systematic nomenclature (IUPAC, 2010), a range of 'trivial' or 'common' names and those of some proprietary preparations. Additionally, many organisations use their own nomenclature (for example the company Steraloids). However, these often deviate from the IUPAC recommendations. The choice of how to

best name a steroid in a particular situation is, therefore, dependent on a number of factors. If one were to always use only the IUPAC systematic name, then this could make the text difficult to read for a non-expert. However, inappropriate over-use of trivial names does not always give enough information in order to inform the reader. Therefore, a combination of systematic and trivial names is often employed as a pragmatic compromise (such as described by Makin et al. 1995) and this will be used in the current text. As an example of the different ways of naming a steroid, some options for testosterone are given below:

Trivial name: testosterone.
**IUPAC systematic name:** 17β-hydroxy-androst-4-en-3-one.
Proprietary example (containing testosterone esters): Sustanon.

When depicted in the orientation shown in Figure 1, substituents on the steroid backbone may protrude below or above the plane of the paper and are drawn as such using either a dashed or solid wedge respectively (indicating the stereochemistry α and β respectively). Hydrogens in positions 8, 9, 10, 13 and 14 (when present) take β, α, β, β, and α orientation respectively in all steroids discussed in this manuscript so their stereochemistry will not be shown in any of the subsequent diagrams. A substituent in position 5 may take either the α or β form, so hydrogens in this position will always be labelled. A wavy line indicates that stereochemistry is unspecified.

In order to aid in the systematic naming of steroids, a number of different hydrocarbon backbones are specified for use by IUPAC. These differ in the number and orientation of carbons, which range from the 17-carbon (C17) gonane nucleus to the 27-carbon (C27) cholestane nucleus (on which cholesterol is based). The range of steroid backbones used in systematic nomenclature is shown in Figure 2. In this text, when describing the trivial name for the oestrogens, the English version will be used (as opposed to the USA use of estrogens). However, when systematically naming steroids that are based on the estrane nucleus, the 'o' will not be used (in accordance with IUPAC guidelines).

No endogenous and very few exogenous steroids are based on the gonane nucleus. The oestrogens and nandrolone (17β-hydroxy-estr-4-en-3-one) are based on the estrane nucleus. The majority of androgens are based on the androstane nucleus and the majority of progestagens and corticosteroids are based on the pregnane nucleus. Most
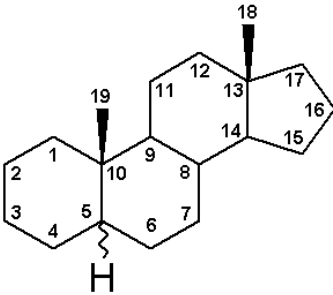
of the bile acids are based on the cholane nucleus and sterols such as cholesterol are based on the cholestane nucleus.
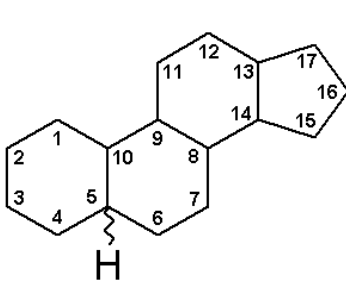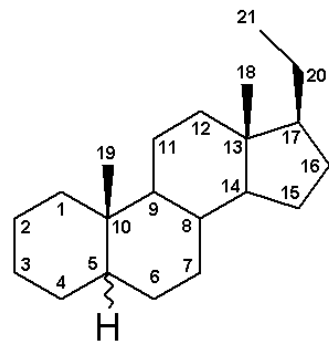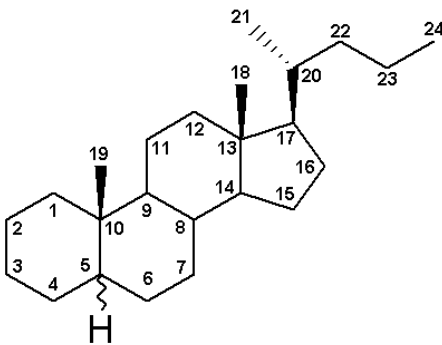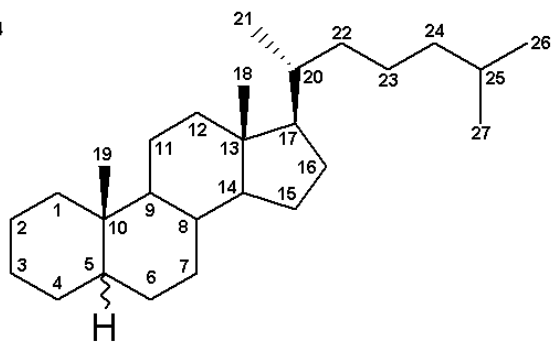

Gonane (C17)
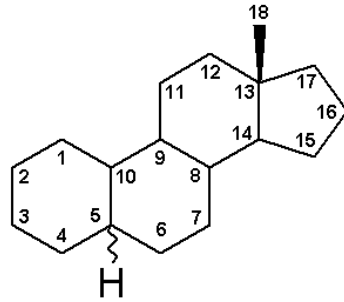
Estrane (C18)

Androstane (C19)

Pregnane (C21)

Cholane (C24)

Cholestane (C27)

Figure 2 – the range of hydrocarbon backbones used in steroid nomenclature.

While many steroids are known to be endogenous (discussed further in chapter 1), a wide range of exogenous steroid structures have been synthesized by chemists over the years in order to optimise their biological properties. Pharmacologically, steroids possess a range of activities far more diverse than their seemingly similar structures may suggest. The following discussion considers the major effects of different steroid classes in mammals. There are some subtle differences between various species, but these will not be considered here since it is only a general overview.

Cholesterol (cholest-5-en-3β-ol – Figure 1) is derived from dietary intake, but is also synthesized in the body. Cholesterol acts to regulate the fluidity of cell membranes and is the precursor to the endogenous androgens, oestrogens, progestagens, corticosteroids, vitamin D, the bile acids and, in certain species, to pheromones such as the 16-androstenes (Hadley and Levine, 2006).

Bile acids such as cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid – Figure 3) are secreted by the gall bladder into the intestine where they aid the absorption of lipids into the body by reducing their surface tension (Hadley and Levine, 2006).
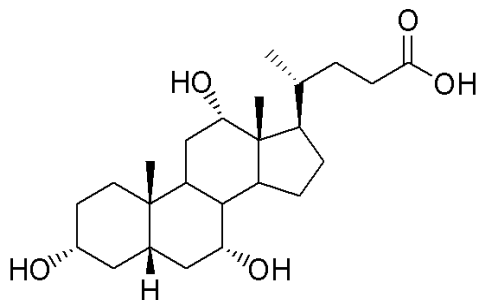
Figure 3 – cholic acid (a bile acid).

Progestagens such as progesterone (pregn-4-ene-3,20-dione – Figure 4) are produced by both males and females in the adrenal glands and gonads. However, they are secreted in much higher concentrations by females during certain stages of the ovulatory cycle (including by the corpus luteum) and during pregnancy (Hadley and Levine, 2006). Progestagens produce the majority of their effects through agonism of the progesterone receptor. This leads to an increased metabolic rate, changes in breast morphology and development/maintenance of the uterus/oviduct before and during pregnancy (Hadley and Levine, 2006). Progestagens (and synthetic progestins) may also be used as

contraceptives in females, which act by suppressing endogenous gonadotrophin release and by inhibiting sperm penetration due to a change in viscosity of the cervical mucous (Westhoff et al. 2010).
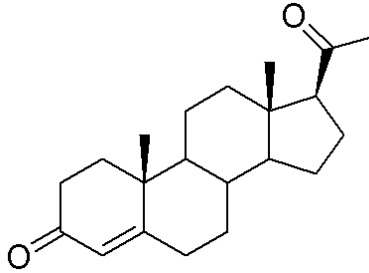
Figure 4 – progesterone (a progestagen).

Corticosteroids are produced by the adrenal cortex and fall into one of two broad classes, depending on their predominant mechanism of action. However, there is some overlap in the effects of the two classes. Glucocorticoids such as cortisol (11β,17α,21-trihydroxy-pregn-4-en-3,20-dione – Figure 5a) agonise the glucocorticoid receptor and act to regulate inflammation and immunity as well as fat, protein and carbohydrate metabolism (Hadley and Levine, 2006). Mineralocorticoids such as aldosterone (11β,21-dihydroxy-3,20-dioxo-pregn-4-en-18-al – Figure 5b) agonise the mineralocorticoid receptor and act to maintain sodium and potassium balance (Hadley and Levine, 2006).

A   **B**
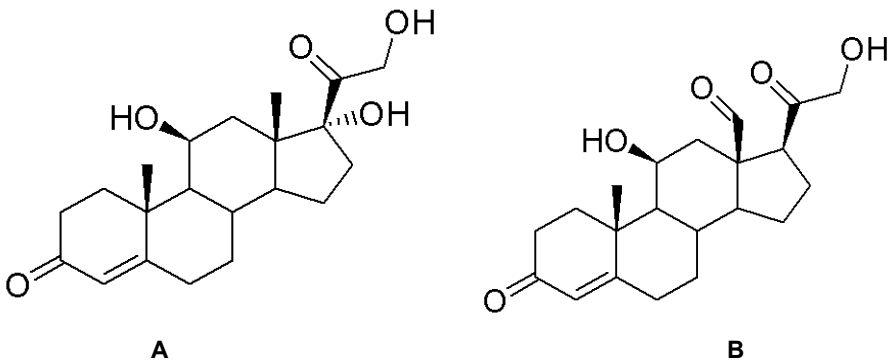
*Figure 5 – **A)** cortisol (a glucocorticoid), **B)** aldosterone (a mineralocorticoid).*

Androgens are produced by both males and females in the adrenal glands and gonads. However, they are secreted in much higher concentrations by male gonads. Testosterone (17β-hydroxy-androst-4-en-3-one – Figure 6) is the most abundant circulating androgen in males, but requires reduction in position 5 to produce the fully active androgen, 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one – Figure 6). The androgenic effects of these steroids are produced by agonising the androgen receptor (AR) (Hadley and Levine, 2006). Androgens produce both androgenic (masculinising) and anabolic (growth promoting) effects to varying degrees. This leads to their more correct classification as anabolic-androgenic steroids (AASs). The androgenic effects are the characteristic male secondary sexual features such as facial/body hair growth and deepening of the voice, while the anabolic effects are predominantly muscle and bone growth (Hadley and Levine, 2006). Whether or not all the anabolic effects of AASs are mediated through the AR is currently unknown. Another possible mechanism of action is antagonism of the glucocorticoid receptor (subject discussed further in Kicman, 2008).
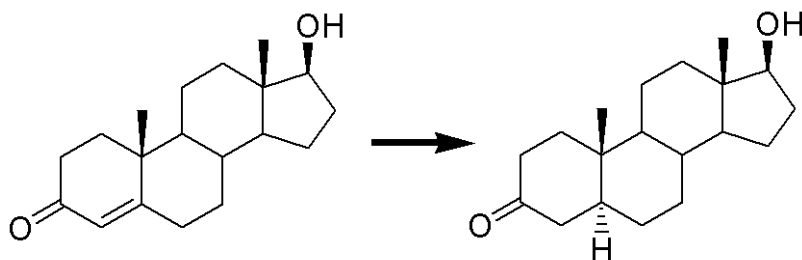
Figure 6 – testosterone and its conversion to the more active androgen 5α-dihydrotestosterone.

Oestrogens such as oestradiol (estra-1,3,5(10)-triene-3,17β-diol – Figure 7) are produced by both males and females in the adrenal glands, gonads and adipose tissue. However, they are secreted in much higher concentrations by females during certain stages of the ovulatory cycle (including by the corpus luteum) and during pregnancy (Hadley and Levine, 2006). Oestrogens produce the majority of their effects through agonism of the oestrogen receptor. This leads to breast growth and redistribution of fat within the body, development/maintenance of the uterus before and during pregnancy, changes to skin morphology and they are also important for bone growth (Hadley and Levine, 2006). Oestrogens are also used as female contraceptives, which act primarily by suppressing endogenous gonadotrophin release (Westhoff et al. 2010).
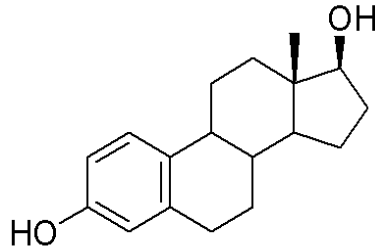
Figure 7 – oestradiol (an oestrogen).

A wide range of synthetic AASs, oestrogens, progestagens and corticosteroids have been produced over the years with the aim of enhancing their pharmaceutical qualities. Modifications that have been applied to the majority of the steroid classes include alkylation (in order to produce more orally active versions), esterification (to prolong duration of action), acetylation (to enhance absorption) and halogenation (to enhance potency) (Kicman, 2008). Specifically relating to AASs, the addition of a double bond at position 1, the attachment of a pyrazole group to the A-ring or the removal of the 19 methyl group have been employed in order to increase the anabolic to androgenic ratio and/or to inhibit their conversion to oestrogens (Kicman, 2008). Similar modifications to glucocorticoids have been engineered in order to try and maximise the glucocorticoid to mineralocorticoid effect ratio. Figure 8 shows a range of different synthetic AASs.
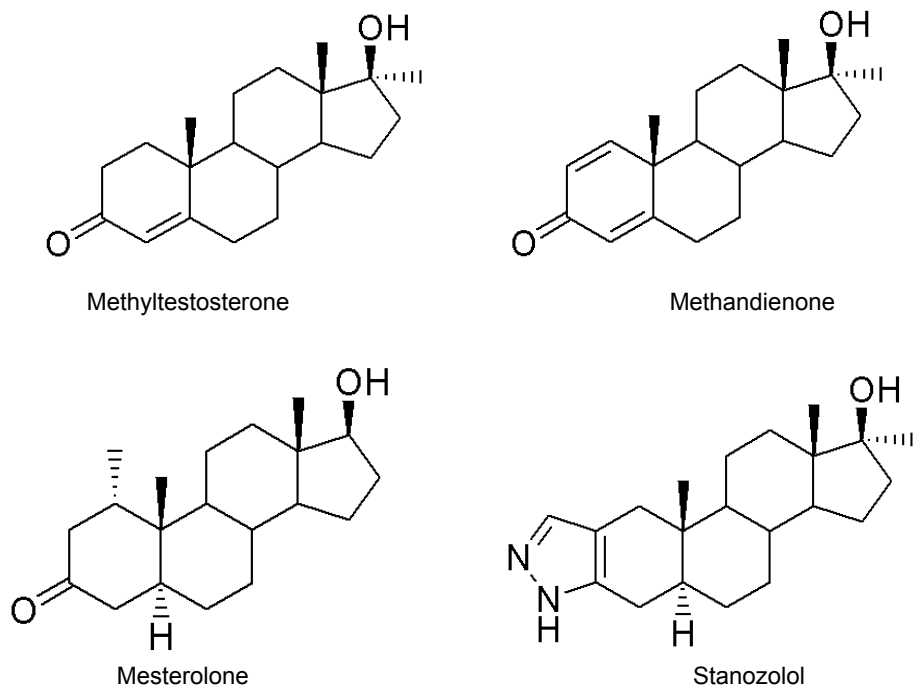
Figure 8 – structures of the synthetic AASs methyltestosterone (17β-hydroxy,17α-methyl-androst-4-en-3-one), methandienone (17β-hydroxy,17α-methyl-androsta-1,4-dien-3-one), mesterolone (17β-hydroxy,1α-methyl,5α-androstan-3-one), stanozolol (17β-hydroxy,17α-methyl-5α-androstano[3,2-c]pyrazole).

Steroid use in food production and competitive sports

The above discussion highlighted the potential anabolic effects of AASs in mammals. In some species, however, oestrogens and progestagens may also produce anabolic effects. In addition to effects on muscle and bone, steroids may also affect the pattern of fat deposition within the body, leading to differential partitioning of muscle and fat; although this depends on the steroid, species and sex of animal in question (Heitzman, 1975, Lone, 1997). Corticosteroids may produce some positive metabolic effects following initial administration, but long-term use of high doses produces a general state of catabolism within the body (Hadley and Levine, 2006).

Because of their potential anabolic effects, some steroids have been used to boost the mass and quality of animal carcasses in food production for economic reasons

(Heitzman, 1975, Lone, 1997, Kay, 2010). Although there are a number of steroid preparations authorised for this purpose in countries such as the USA, the use of growth promoters (also including non-steroidal products such as the oestrogenic compound zeranol, growth hormone and the β_2-agonist class of drugs) is banned within the EU (EU Council Directive 96/22/EC). The reasons for this ban were highlighted in two reports from the European Commission in 1999 and 2002, which concluded that the presence of hormones in meat products may potentially be harmful to human health through endocrine disrupting or carcinogenic mechanisms (SCVPH, 1999, SCVPH, 2002). However, two subsequent opinions published by the UK Veterinary Products Committee failed to agree with the findings of the earlier European Commission's studies (VPC, 1999, VPC, 2006). For example, the latter of these two UK reports estimated that, as a worst case scenario, a postmenopausal woman eating a kilogram of meat (kidney) containing the highest concentration of oestradiol detected (56 ng/kg) following administration of the steroid would experience an increased oestrogen level of only 0.01% of average endogenous production. Indeed, it has also been speculated that the ban may have more to do with regulating trade, leading to official disputes between the EU and USA (Charlier and Rainelli, 2002). Nonetheless, the hormone ban remains and non-EU countries are, therefore, required to provide sufficient animal segregation and residue testing schemes to ensure that treated animals are not sold in the EU.

In addition to their use in food production, steroids may also be used in competitive human and animal sports in order to improve performance. The range of steroids used for this purpose is generally limited to the AASs. These may enhance performance through a number of mechanisms including increased muscle mass, enhanced recovery from training, raised red blood cell count and heightened aggression (Kicman, 2008). Because of their potential to affect performance, the use of AASs in the majority of horseracing, greyhound racing and human sports is prohibited (IFHA, 2008, GBGB, 2009, FEI, 2010, WADA, 2009a). Protection of the welfare of individual competitors is another reason for prohibiting these substances; an aspect that takes increased importance in animal sports where trainers decide on the animal's behalf what substances are administered.

The detection of steroid abuse in food production and animal sports

In order to enforce the ban on hormone use in food production, EU Council Directive 96/23/EC (and EU Commission Decision 2002/657/EC) lay down the requirements for residue testing. Enforcement of the ban on steroid use in competitive sports is not regulated in law in the same way as in food production, but guidelines regarding the analytical methods that must be followed by individual laboratories when confirming cases of steroid abuse have been produced by both the animal (AORC, 2003, ILAC-G7, 2009) and human authorities (WADA, 2009b). A comparison of the regulations used in the food residue and sports doping control arenas can be found in Van Eenoo and Delbeke, 2004.

The type of matrix used for steroid residue analysis in food and sports drug surveillance differs by a number of variables including the country, the individual authority concerned, whether samples are taken from live animals, at slaughter or from a food import programme and whether the analyses for a particular analyte are suited to a specific tissue. Other than food import programmes, where analysis of meat and organs are typically required, urine and blood are the most common matrices for testing in both the food and sports residue arenas (Wynne, 2004, Stolker et al. 2005). However, faeces and hair are also important matrices in some countries. When dealing with blood or hair, detection of unchanged 'parent' drug is often considered suitable for determination of drug abuse. However, when dealing with urine or faeces, a large proportion of the excreted dose can take the form of metabolites. This is a particularly important consideration in the case of steroids, which are typically heavily metabolised (Scarth et al. 2009). It is, therefore, often necessary to conduct metabolism studies in order to determine the appropriate target metabolites for the detection of steroid abuse. The metabolism of the steroid can be broadly categorised into phases 1 and 2. Phase 1 typically involves the modification of existing functional groups within the steroid molecule (namely oxidation, reduction, hydrolysis etc.), whereas phase 2 involves conjugation with, typically, polar moieties such as glucuronic or sulphuric acid in order to increase water solubility and, therefore, aid excretion (see example in Figure 9).
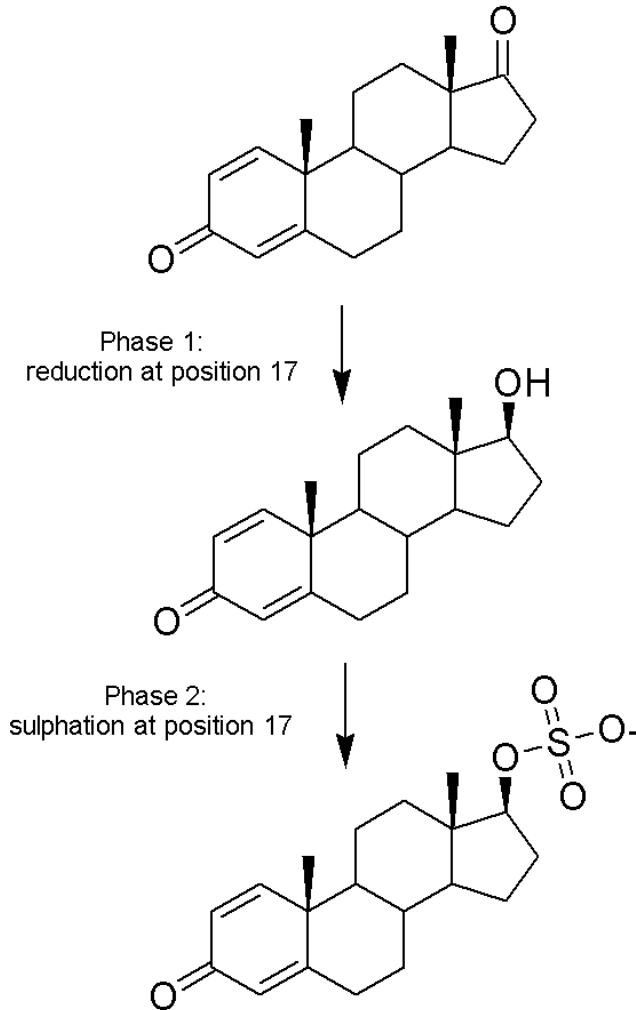
Figure 9 – examples of theoretical phase 1 and 2 metabolic pathways for the boldenone 'pro-drug' boldione (androsta-1,4-diene-3,17-dione - top). A possible pathway of phase 1 metabolism is reduction of the 17-keto group to form boldenone (17β-hydroxy-androsta-1,4-dien-3-one - middle). This may be followed by phase 2 conjugation with sulphate to form boldenone-17-sulphate (17β-hydroxy-androsta-1,4-dien-3-one-17-sulphate - bottom).

Because of the common aims of food residue and sport drug surveillance laboratories, the development and application of analytical techniques for detecting steroid abuse has been broadly similar between the two fields over the years. Indeed, many individual laboratories across the world are involved in residue analysis within both of these fields. Review articles concerning the analytical methods used specifically for veterinary steroid

analysis can be found in Stolker et al. 2005 and De Brabander et al. 2009 (food residue analysis) and McKinney, 2009 (equine sports). Figure 10 and the following discussion serves as a brief overview of the scientific evolution within these two fields.
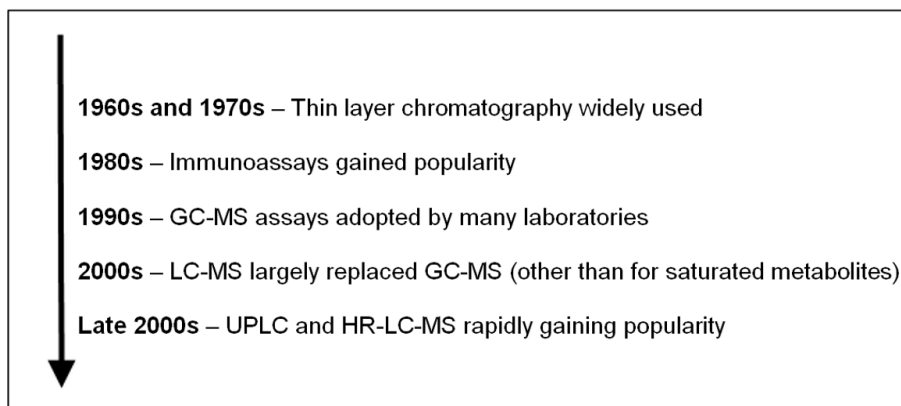
Figure 10 – summary of the evolution of steroid screening techniques used in residue analysis.

Initially, thin layer chromatography-fluorescence detection (TLC-FL) was widely used (Moss and Rylance, 1967, De Brabander and Verbeke, 1975). Immunoassay techniques such as enzyme-linked immunosorbent assays (ELISA) became popular during the 1980s and 1990s, but were largely replaced in the late 1990s and early 2000s by more definitive mass spectrometric-based techniques such as gas- and liquid-chromatography mass spectrometry (GC- and LC-MS respectively) (McKinney, 2009). The ability of many modern GC- and LC-MS instruments to carry out MS^n experiments makes them particularly useful for identifying compounds due to their high selectivity. Also, the recent emergence of higher resolution LC equipment allowing the use of sub-2 μm particle sizes and high flow rates (ultra-pressure liquid chromatography or UPLC) means that metabolites with similar molecular masses and retention times can now be more easily resolved and that analytical run times are shorter (Plumb et al. 2009).

Although there has been a general shift from GC-MS to LC-MS for drug residue analysis during the past decade, GC-MS has remained an important tool for analysing saturated steroid metabolites. This is because saturated steroids generally suffer from poor ionisation properties under the atmospheric pressure ionisation conditions of LC-MS (McKinney et al. 2009, Teale and Houghton, 2010). Although the majority of current

urinary screening procedures are based on detection of the 'free' steroid (liberated from its phase conjugates), it may be possible in the future to design assays based on the analysis of intact conjugates. At present, progress in this area has been limited by a lack of availability of the relevant analytical reference standards for animal specific metabolites. However, the potential of this approach has already been demonstrated in human sports, where the availability of reference material has allowed the development of LC-MS/MS assays for intact steroid conjugates (Kuuranne et al. 2002, Hintikka et al. 2008, Pozo et al. 2008).

Most recently, robust high-resolution-accurate-mass LC-MS (HR-LC-MS) systems operating at an increased level of resolution, typically ranging between 7,500 and 100,000 full width at half maximum height (FWHM) depending on the type of mass analyser employed, have become commercially available and have started gaining popularity for sports drug surveillance screening and research (Virus et al. 2008, Scarth et al. 2010). Because the data acquired are full scan analyses of intact $[M+H]^+$ or $[M-H]^-$ species at very high resolution, a very large number of analytes can be simultaneously monitored. Another advantage of using HR-LC-MS includes the ability to retrospectively analyse data once new drug information comes to light.

The powerful technique of gas chromatography combustion isotope ratio mass spectrometry studies (GC-C-IRMS) has been applied to the confirmation of endogenous steroid abuse (discussed further in chapter 1) in both food and sports drug residue analysis. However, it is not currently suitable as a screening technique due to its low throughput nature (Piper et al. 2010).

In addition to the classical analytical chemistry techniques that are targeted toward the detection of 'parent' steroids or their metabolites, a number of indirect techniques have recently gained attention (discussed further in chapter 1). These include immunoassay and receptor based biosensor assays as well as a range of 'omics' biomarker approaches such as metabolomics, proteomics and transcriptomics (Scarth et al. 2006). Because these techniques are targeted toward pharmacological activity rather than individual drug structure, they produce complementary screening data that can be used to indicate whether steroid abuse may have occurred. However, these techniques have yet to find widespread application in the confirmation of steroid abuse, which is typically still achieved by the direct measurement of a steroid or its metabolite.

While the above discussion served to summarise the range of instrumental techniques that may be used to detect steroid abuse, the ability to detect the abuse of each individual steroid is determined by a number of factors. Firstly, the sample needs to be taken from the animal at a time close enough to the point of steroid administration for the concentrations to be above the limits of instrumental sensitivity. If the sample is taken too long after steroid administration, then traces of the drug may be too low to be detected. Also, each individual steroid can be classified into one of three broad categories, which impacts on the ability to detect the abuse of each:

'Exogenous' steroids are known marketed 'classical' steroids, such as stanozolol. These contain synthetic structures that are thought not to occur naturally. Detection of this class of steroid is relatively straightforward since a purely qualitative demonstration of the presence of these synthetic steroids is all that is required in order to determine abuse

'Endogenous' steroids are also known marketed steroids, such as testosterone, but contain structures that are known to exist naturally. Detection of the abuse of 'endogenous' steroids is more complicated because they are, by definition, 'natural' to some extent and so a simple qualitative demonstration of their presence is insufficient to indicate abuse (discussed further in chapter 1). Some endogenous steroids such as testosterone, progesterone and oestradiol are known to be ubiquitous amongst mammals. However, the classification of a steroid as 'endogenous' is a grey area and there are some steroids that may be considered 'semi'-endogenous. This term signifies that the steroid in question has been suspected to be endogenous, but only in certain situations i.e. in a specific species or at particular time. Analytical sensitivities for detecting steroids have increased significantly over the years, which has resulted in more and more compounds being suspected as 'endogenous' or 'semi-endogenous' at low concentrations.

'Designer' steroids are previously unmarketed steroids that contain synthetic structures that are thought not to occur naturally. The use of a designer steroid first came to the public attention in 2003 when a syringe containing the novel steroid tetrahydrogestrinone (THG) was handed to doping officials. This resulted in the disqualification of several athletes after they were subsequently found to have used the steroid (Catlin et al. 2004). Designer steroids have chemical structures based on previously marketed products, but

with minor modifications which make them undetectable by the majority of current targeted mass spectrometric procedures. In the case of THG for example, the drug's structure is based upon gestrinone, but with the 17-alkyl side chain fully saturated such that the relative molecular mass of THG is 4 atomic mass units (u) higher than gestrinone. Designer steroids are synthesized either to deliberately evade detection, or as appears more common, to enable them to be marketed freely on the Internet to customers in some countries because their structures do not fall within the scope of legal regulations that prevent the sale of defined steroidal products.

Detection of the abuse of the latter two classes of steroids in food production and animal sports is very challenging. The development of analytical approaches to tackle these associated issues forms the basis of the current thesis.

Aims and objectives of the current work

Within the author's laboratory, analytical methods are already available to detect the abuse of the majority of endogenous AASs in horseracing. However, the same is not true for the majority of endogenous steroids in other food producing animals. 'Designer' steroids could in theory be abused in both horseracing and food production, but at present the majority of work on designer steroids has been commissioned by the sports regulatory authorities. One reason that this class of compounds has received more attention in sports doping control is because of the proven use of designer steroids such as THG by a number of athletes. Another factor may relate to the fact that human and animal sports typically involve single individuals looking to gain marginal advantages for significant financial and/or sociological gain, whereas food production involves large herds of animals with smaller financial return relative to the risk. These differences in return relative to risk could, therefore, be considered to make the abuse of relatively expensive 'exotic' treatments such as designer steroids more likely in competitive sports compared to food production. In light of the aforementioned discussion, the following were determined as the overall aims and objectives of the research reported herein:

Overall aims: To develop novel analytical approaches for the detection of 'endogenous' steroid abuse in food-production (part 1) and of 'designer' steroid abuse in animal sports (part 2). The primary focus of this thesis will relate to AASs such as nandrolone, boldenone, testosterone and their synthetic 'designer' analogues. However, chapter 3

will also consider the important natural steroids oestradiol and progesterone in relation to the detection of their abuse in the bovine.

Part 1 objectives:

- To review the literature regarding endogenous steroids and their detection in food production, focussing mainly on endogenous AASs (chapter 1).
- To develop approaches for the detection of nandrolone abuse in the porcine (chapter 2).
- To develop approaches for the detection of androgen, oestrogen and progestagen abuse in the bovine (chapter 3).

Part 2 objectives:

- To review the literature regarding steroid metabolism and detection in the equine (especially in relation to designer steroids) and to compare the trends with those observed in other species (chapter 4).
- To develop and assess the suitability of *in vitro* techniques for conducting equine drug metabolism studies (chapter 5).
- To use the newly developed *in vitro* methods to study the metabolism of a novel 'designer' steroid in the equine (chapter 6).

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PART 1: DETECTION OF 'ENDOGENOUS' STEROID ABUSE IN FOOD-PRODUCTION

Chapter 1: Presence, metabolism and detection of 'endogenous' steroid hormones in food producing animals

After:

Scarth, J., Akre, C., Van Ginkel, L., Le Bizec, B., De Brabander, H., Korth, W., Points, J., Teale, P. and Kay, J. The presence and metabolism of endogenous androgenic-anabolic steroid hormones in meat producing animals. A review. (2009). *Food Additives and Contaminants: part A.* Vol. 26(5), 640-671.

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1.1   Introduction

As discussed in the introductory chapter, EU Council Directive 96/22/EC of 1996 states that "substances having a hormonal action" are prohibited for use in animals intended for meat production. As well as purely novel steroids not existing in nature, the directive also covers synthetically produced versions of steroids that are known to occur naturally in certain species under particular circumstances. However, in some countries, including the USA, Canada and Australia, some (combinations of) steroids and a related synthetic compound Zeranol are officially registered for use as hormonal growth promoting compounds. Due to their anabolic and/or partitioning effect they increase the profit per unit head for the farmer. EU Council Directive 96/23/EC (and EU Commission Decision

2002/657/EC) lays down the requirements for residue testing in order to ensure compliance with the EU prohibition.

The steroid hormones considered in this review chapter are the androgenic-anabolic steroids (AASs) that potentially derive from precursors within the body such as cholesterol and pregnenolone (see figure 1). These include testosterone, androstenedione, nandrolone, boldenone and dehydroepiandrosterone (DHEA), as well as their numerous catabolic products and any precursor compounds that might potentially lead to conversion to these steroids within the body. The major focus of this chapter will relate to phase 1 steroid metabolites because there is much less information relating to phase 2 metabolism. However, details of the phase 2 metabolism of some steroids will be given where they have been shown to usefully distinguish situations of abuse (for example boldenone). The task of detecting the abuse of synthetically produced hormones that are also known to be endogenous under certain conditions, dubbed 'pseudo-endogenous' or 'grey zone substances' due to their dual synthetic/endogenous nature (Van Thuyne Wim 2006), is problematic for many reasons. The most significant challenge arises due to the fact that when they are shown to occur naturally within a particular type of animal, a simple qualitative demonstration of their presence does not necessarily prove abuse. Most, but not all, steroid preparations are ester versions of these potentially endogenous steroids. However, a simple demonstration of the presence of the steroid ester as proof of abuse is not always possible (with the exception of hair and injection/implant sites in some cases) due to a large proportion of the steroid ester being cleaved by the time it reaches the test matrix i.e. plasma or urine. Some type of quantitative uni- or multi-variate threshold approach is therefore usually required in order to confirm abuse. Furthermore, as analytical limits of detection decrease, the list of compounds that are suspected to be endogenous at low concentrations increases. These and some further analytical and physiological considerations are taken up again later in this review.

Analytical methods of various kinds have in the past been employed to identify and quantify endogenous steroids, their metabolites and precursors, but their effectiveness and the harmonisation of their application in different countries and situations is questionable. For example, Van Ginkel et al. 1993 highlight the wide range of different analytical methods and thresholds that have been applied in different EU countries in the past. Since the author was aware of no comprehensive published review on the

concentrations and metabolism of such steroids in food producing animals, the overall aim of the work reported herein was therefore to carry out a survey of the existing literature. This then guided further practical work in order to increase knowledge and to develop more effective testing methods (Chapters 2 and 3).
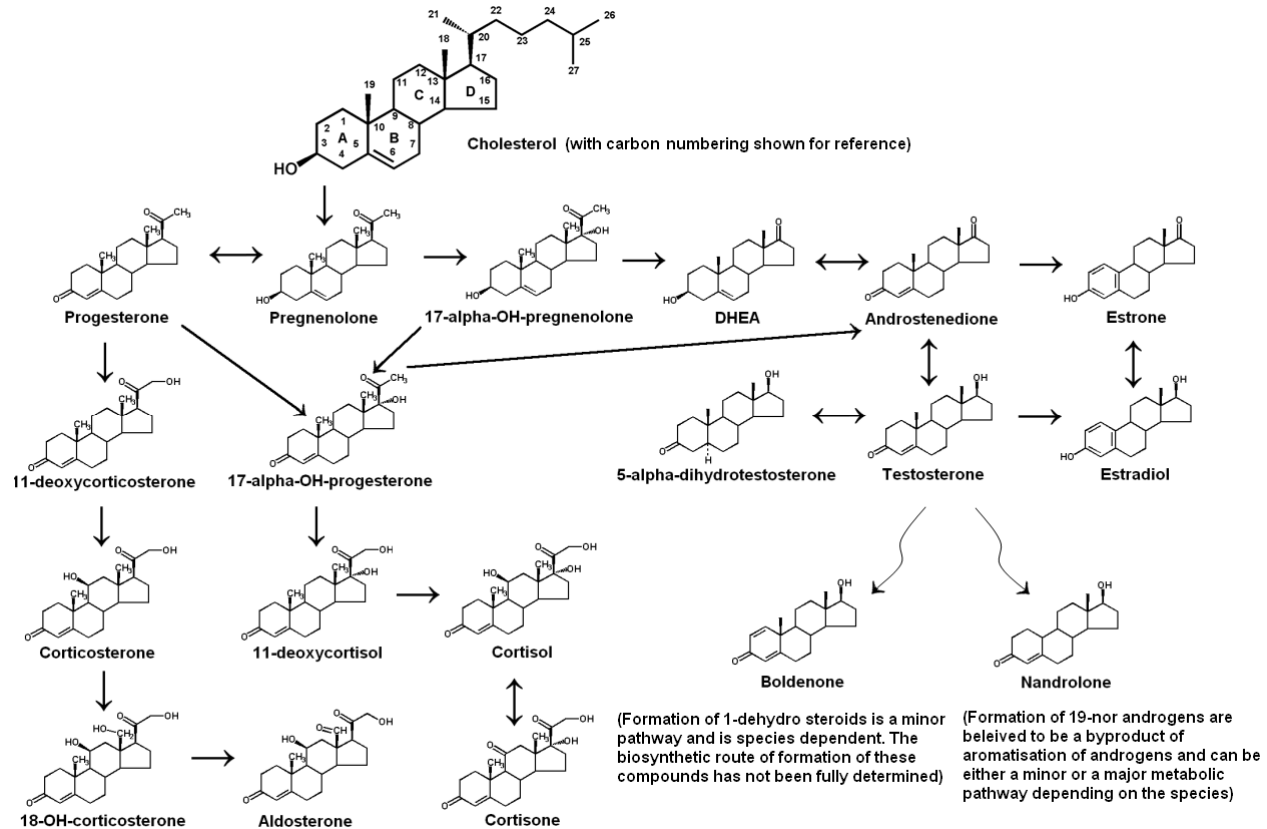
Figure 1 – schematic of the biosynthetic pathways for endogenous steroids in mammalian species. Many of the reactions that involve oxidation or reduction of hydroxyl and ketone groups respectively are reversible. Wavy arrows indicate a putative pathway only.

1.2   Literature survey methods

The overall aim was to collect as much published and unpublished data as possible in order to provide for the most comprehensive evaluation of the field. This review was originally published in the journal *Food Additives and Contaminants* in June 2009. However, studies reported after this date (up to October 2010) have now also been reviewed and are discussed in the text. Literature searches were conducted using the Pubmed facility of the USA National Centre for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/), Scopus (www.scopus.com), the Web of Science (www.scientific.thomson.com/products/wos/) and various 'grey' literature sources. Many researchers were also contacted so that as much data as possible from individual animals and particularly for values below the reported LODs, decision limit (CCα) or detection capability (CCβ) could be obtained. This had the advantage that some appreciation of concentrations could be obtained where analyte concentrations were currently too challenging for fully rigorous quantitative analysis.

In the following sections, the occurrence of precursors and metabolites of testosterone, nandrolone and boldenone in bovine, porcine and ovine matrices is reviewed in narrative fashion. Also reviewed are precursors and metabolites of nandrolone and boldenone in equine, cervine and caprine matrices. Ideally, a statistical analysis of results using a meta-analysis (defined here as "the statistical analysis of a large collection of analysis results for the purpose of integrating the findings" – from Glass, 1976) would be a desirable outcome. However, due to a lack of sufficient data this approach could not be used. Where differences between results in this review are stated to be "statistically significant," this refers to comparisons of controlled populations within a single study and not between results of different studies. Unless otherwise stated, the results reviewed derive from controlled studies where the use of banned steroids can be ruled out.

As many relevant matrices as possible have been considered in this review. However, due to the magnitude of the literature and the overall scope of this review being predominantly targeted at control of abuse rather than the safety implications, there is an inevitable bias in the output toward plasma, urine, bile, faeces and hair over tissues such as muscle and fat. Also, longitudinal studies using solid tissues are not often possible because this usually means slaughter of the animal (hence only one sample can be taken). In addition to the individual studies reviewed herein, several reviews dealing with

the concentrations of steroid hormones in different food products are also available (Velle 1976, Henricks et al. 1976, Hartmann et al. 1998, Arnold 2000, Daxenberger et al. 2001, Stephany et al. 2004, Fritsche et al. 1999 and Mouw et al. 2006).

1.3 Physiological and analytical considerations regarding comparisons of steroid concentrations within and between different species

A basic understanding of the analytical and physiological context of natural steroids is assumed in this review. Nevertheless, some information of specific relevance is given below and the biosynthetic and catabolic pathways of some representative natural steroids are summarised in Figures 1 and 2 and Table 4. Further background information on general analytical aspects can be found in Makin et al. 1995 and Stolker et al. 2005 while further physiological information can be found in Mason et al. 2002 and Hadley and Levine 2006.

Although the background given here is separated into analytical and physiological factors, there are areas of overlap between the two. A critical theme that will become apparent is that the lack of reporting of sufficient method details (at least in a standard format) often means that rigorous quantitative comparisons between different studies are not possible. It was also necessary to limit the number of parameters chosen for study. The remaining analytical parameters subject to full analysis were chosen by consideration of a combination of their impact on any results as well as the frequency and reliability of their reporting.

1.3.1 Analytical factors

Although most published methods rely on direct identification and/or quantification of analytes, indirect approaches utilising biosensors, biomarkers, gas chromatography-combustion-isotope ratio-mass spectrometry (GC-C-IRMS) or the detection of intact steroid esters have also been investigated (discussed further in later sections). For the purposes of the main body of this review chapter however, studies were limited to direct detection/quantification using such techniques as immunoassay (IA), high performance-liquid chromatography with ultraviolet detection (HPLC-UV) and liquid or gas chromatography coupled to mass spectrometry (LC- and GC-MS respectively).

17β-Hydroxy-estr-4-en-3-one
(nandrolone or 19-nortestosterone)

Estr-4-ene-3,17-dione
(19-norandrostenedione)

5α-Estrane-3,17-dione
(5α-dihydro-19-norandrostenedione)

17α-Hydroxy-estr-4-en-3-one
(epinandrolone)
(a major bovine metabolite)

3β-Hydroxy-5α-estran-17-one
(19-norepiandrosterone)
(a major porcine metabolite)

5α-Estrane-3β,17α-diol
(a major equine metabolite)
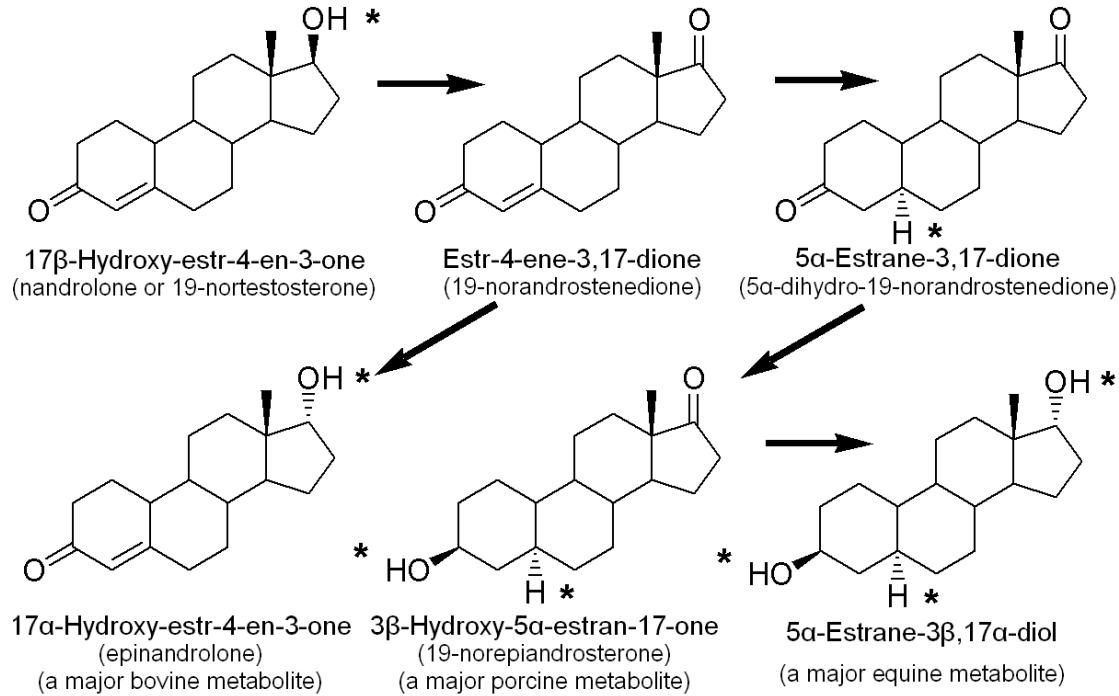
Figure 2 – schematic representation of the phase 1 metabolism of nandrolone in the three species for which most information is available (bovine, porcine and equine). Sites of possible metabolic epimerisation are highlighted (*). For space purposes, and due to the number of potential isomers, it is only possible to depict the major metabolites of nandrolone. For the same reasons it is also not possible to give schematic metabolic pathway representations for all the individual steroids covered in this review. However, it is worth noting that the same functional groups that are subject to metabolism in nandrolone are also liable to metabolism in other androgenic-anabolic steroids.

When comparing data between studies, it becomes apparent that while 'true' differences between data points and populations do exist, that variation can also be caused by biases in sampling designs or the type of analysis used. In many cases, comparison of data is further complicated by the reporting of different types of information i.e. LOD or CCα/β are often not reported. Some examples of analytical aspects that can lead to variation within the data are given below:

● *Qualitative, semi-quantitative and fully quantitative data* – While the ultimate aim of this review was to consider concentrations of natural steroids in a quantitative fashion, it was also recognised that a number of useful studies only reported data in a qualitative or semi-quantitative fashion. While the results of these analyses were not subject to any statistical analysis, they were considered useful in answering certain qualitatively focussed questions e.g. does boldenone occur naturally at any concentration in species X? Where qualitative or semi-quantitative data are analysed, this will be highlighted and any assumptions stated. Even with studies that are reported to be 'quantitative' it is important to understand that all data has a degree of uncertainty attached. However, tor the majority of studies reviewed herein, insufficient validation data were available to fully assess the degree of uncertainty of the results.

● *Method of calibration line construction* – When dealing with endogenous substances, quantification can sometimes be complicated by the difficulty of finding a true blank matrix. In cases where a blank matrix of the same type as the study samples is not available then one can either use standard addition; where known amounts of steroids are added 'on top' of the existing concentrations present, or alternatively a surrogate matrix can be used. If using a surrogate matrix devoid of endogenous steroid, then appropriate measures need to be taken to ensure the chosen matrix behaves in a similar way to the actual sample matrix in order to control for any variation in the analytical procedure. Neither of the two aforementioned measures is perfect and each can lead to different reported concentrations for the same data set due to differential matrix effects or recovery of analyte. In many published reports, the actual calibration range applied was not explicitly given. This made it difficult to evaluate whether individual results fell within a linear range. Due to the need to limit the number of factors that were being taken into account in this review, adjustment of analytical data for recovery and matrix effects was not attempted. In any case, many of the aforementioned parameters were not always reported by authors.

● *Limit of Detection (LOD) or CCα/β* – For some steroids in certain physiological situations, a large number of reported concentrations are 'not detectable' (ND). This causes problems for two main reasons, one statistical and the other regulatory. From a statistical angle, the existence of large numbers of concentrations below the LOD are problematic because these values have to be effectively treated as zero, making it more difficult to define the population distribution and hence set threshold levels. If the LOD is not reported at all then this further complicates meta-analyses. From a regulatory point of view, because sensitivities generally increase as technologies improve, this sometimes means that steroids once thought to be purely synthetic appear to exist naturally at very low levels. However, it is difficult to assess whether new clusters of positive findings at such low levels are due to an increased abuse of steroids or natural occurrence. The issue in this context is therefore not the LOD per se, but the LOD in the physiological, analytical and regulatory context. Where possible, authors of published works were contacted and information on LODs or CCα/βs was requested. Also requested (where relevant) were any results that were quantified, but which were below the LOD or CCα. In these cases, an estimation of the reliability of the additional trace concentration data was also requested.

● *Sample collection and subsequent preparation technique* – Prior to analysis, most techniques require some degree of sample preparation, which typically involves extraction of the analytes of interest from unwanted or interfering matrix components. The treatment of the sample once taken from the animal can influence the analytical results in several ways, all of which highlight the need to stabilise samples appropriately and to take into account any artefactual processes occurring prior to analysis. For example, it is known that a number of meat producing species, e.g. bovine, ovine and equine, but not porcine, have a propensity to convert 17β-hydroxy or ketone functions into 17α-hydroxy compounds. (Gaiani et al. 1984). Bovine plasma in particular is known to be especially active at catalysing this reaction and the addition of methanol to the matrix has been shown to inhibit the activity (Gaiani et al. 1984).

It has also been shown that the new-born of the ovine, caprine and bovine display very high rates of 20α-hydroxysteroid dehydrogenase activity (acting on progestagens and corticosteroids) and that this activity diminishes rapidly with age; possibly due to the replacement of fetal with adult erythrocytes (Nancarrow et al. 1983). Due to a general dearth of knowledge on the metabolism of steroids in caprine, and ovine species, the

significance of this finding for surveillance of steroid abuse is currently difficult to assess. However, for all the species concerned, the data suggests that it is important to choose the correct age of the animals from a reference population that is used for establishing thresholds. This is so that the ages reflect those of the animals likely to be encountered in routine surveillance programmes.

Bovine faeces are known to be capable of producing boldenone and other 1-dehydro steroids as metabolites from some steroidal precursors *ex-vivo* (Pompa et al. 2006). It is therefore recommended that sampling of bovine urine be devoid of faecal contamination in order to avoid boldenone false positives (De Brabander et al. 2004).

It has also been shown that nandrolone related compounds can be formed from testosterone derivatives in human urine. The authors of this work showed that this reaction can be partially stabilised by adding EDTA to the samples (Grosse et al. 2005).

Many steroids can also be conjugated with polar moieties such as sulphuric and/or glucuronic acid. Samples are often hydrolysed prior to analysis in order to produce the 'free' steroid. Hydrolysis can be performed before or after preliminary extraction or group separation and even then can be performed by a variety of methods. *Helix pomatia* digestive juice is the most often applied enzymatic form of deconjugation and this method affords hydrolysis of glucuronic acid conjugates and aryl sulphates at optimum pH. However, it is also known to contain hydroxylase and oxidoreductase enzyme activity that can artefactally oxidise or reduce some steroids (Houghton et al. 1992). Another preparation that is frequently used is the β-glucuronidase enzyme from *E. coli*, which as its name suggests cleaves glucuronic acid conjugates but not sulphate conjugates (Houghton et al. 1992). In a 'two fraction' extraction, glucuronic acid conjugates may be cleaved by enzymes from extracts of *Helix pomatia*, while sulphate conjugates can be cleaved using acidified ethyl acetate:methanol (termed solvolysis; as reported in Teale and Houghton, 1991). An alternative is to cleave both types of conjugates simultaneously using acidified methanol (termed methanolysis; as reported in Tang and Crone, 1989), but this can lead to more complex mixture of components retained within extracts (James Scarth, personal observation). The use of a number of different hydrolysis (or no hydrolysis at all) steps in the literature, all with varying capacities to deconjugate steroids, is another factor that potentially leads to variation in the reported concentrations.

A mixture of purification/concentration approaches were identified in the literature including solid phase extraction, liquid-liquid extraction, protein precipitation, immunoaffinity column chromatography, supercritical fluid extraction, accelerated solvent extraction (ASE) and some very elaborate, but often effective, multi-step HPLC fractionation processes. Results using these methods are generally not compared in this review, unless there was specific relevance to a result.

● *Type of analytical method used* – A major factor leading to variation between reported values lies in the type of end-point detection method used. These included (in approximate descending order of reported use), immunoassay (IA), gas-chromatography-mass spectrometry (GC-MS), liquid-chromatography-mass spectrometry (LC-MS), high-performance-liquid chromatography-ultra-violet detection (HPLC-UV) and thin-layer-chromatography-fluorescence detection (TLC-FL). Immunoassay and mass spectrometry techniques generally afford higher sensitivity over HPLC-UV or and TLC-FL and are also generally more selective. Mass spectrometry is considered to offer more selectivity than IA, predominantly due to variable extents of cross-reactivity of steroids against the IA antibody, although the impact of any cross-reactivity can be reduced by performing HPLC separation of sample extracts prior to analysis. Although generally considered very selective, mass spectrometry is still subject to matrix effects such as ion suppression or enhancement (LC-MS generally more so than GC-MS). However, these can usually be overcome through the use of matrix matched standards (where available). As a general rule, it has been observed that IA tends to overestimate oestrogen levels at low concentrations while underestimating them at high concentrations (Stephany et al. 2004).

● *Statistical analyses used within the studies reviewed* –. Depending on a number of factors, including the steroid, species, matrix and analytical LOD, a Gaussian distribution of steroid concentration population data may or may not be determined. In this respect, a large number of parametric and non-parametric approaches were reported by authors, reflecting the different findings under varying conditions. With such major differences in statistical reporting, such as mean vs median or standard deviation vs inter-quartile range, it is very difficult to make quantitative comparisons between data sets. It is also important to highlight a major difference between a statistical method being able to discriminate a control from a steroid treated population (i.e. a T-test result) and a statistical method that allows a workable threshold to be calculated (i.e. allowing a

degree of certainty that at a particular threshold a false positive will not occur). There can be a significant amount of overlap in individual steroid concentrations from control and treated steroid populations that can be discerned using a T-test, but this does not necessarily mean they are significant enough differences to allow a realistic threshold to be fixed.

It is also important to add that the uncertainty of measurement was very rarely reported in the studies reviewed and was not easily calculated from the data available, further adding to the difficulty in making quantitative comparisons between data sets.

1.3.2 Physiological factors

Some of the physiological considerations regarding steroid concentrations were given in the analytical section above. In addition to inter-individual differences, there are many further factors that lead to variation in the observed concentrations. For example, Challenger (2004) has reviewed the peak ovarian cycle plasma/serum oestradiol and progesterone concentrations in mammalian species. It was found that oestradiol concentrations spanned around four orders of magnitude while those for progesterone spanned three orders of magnitude. Oestradiol concentrations were on average two orders of magnitude lower than progesterone concentrations and there were significant differences between different animal orders. Maximum oestradiol concentrations were more variable in artiodactyls and primates than in carnivores. Absolute oestradiol concentrations were not correlated with dietary niche, but the progesterone to oestradiol ratio was lower in artiodactyls and primates compared with carnivores. Although this study refers to oestrogens and progestagens rather than androgens (a comparable study for androgens could not be found by the author), it highlights the significant differences in steroid concentrations between species and identifies the need to obtain endogenous population data for hormones in each species before detection strategies for regulatory surveillance are devised. As many references in this review will demonstrate, intra- and inter-species genetic variation may be responsible for a large proportion of the observed variation between animals.

● *4- vs 5-ene pathways* – As well as the absolute differences in oestradiol and progesterone described above, species are also known to vary in their utilisation of the 4- and 5-ene pathways for the production of steroids; which can be traced back to

differences in the substrate requirements of the CYP17 enzyme (Mason et al. 2002). This means that some species produce more steroid precursors with a 4-ene group (e.g. androstenedione) whereas others produce more with a 5-ene group (e.g. DHEA [dehydroepiandrosterone]). Of relevance to meat producing animals, 5-ene precursors are relatively high in bovine, porcine, ovine and equine species, while the cervine is lower in 5-ene and higher in 4-ene steroids (Wichman et al. 1984).

● *Pregnancy and pseudopregnancy* – It is well known that pregnancy can lead to extremely high concentrations of certain relevant steroids. Pregnant animals are therefore usually excluded from threshold value calculations. However, a phenomenon termed pseudo-pregnancy (also known as phantom pregnancy or pseudocyesis) also exists. In some species this condition leads to the physiological appearance of a state of pregnancy (including raised steroid concentrations), but without an actual fetus being conceived (Johnson and Everitt 2000). The effect is certainly frequent in rodent and canine species, but some references to its occurrence in the porcine (Pusateri et al. 1996), caprine (Lopes Junior et al. 2002) and ovine-caprine hybrids (Maclaren et al. 1993) were also obtained. While the condition does seem to occur naturally at a high incidence in some caprine species, the porcine reports were of artificially induced pseudo pregnancy by administering oestradiol. No reports of pseudopregnancy in bovine species could be found in the published literature.

● *Oestrous synchronisation* – The effects of oestrous synchronisation devices are not covered in this survey, but the subject has received comprehensive review in Rathbone et al.1998.

● *Route of excretion* – Endogenous and artificially administered steroids are predominantly excreted from the body via the urine and faeces. The excretion of steroids is species and compound dependent, with some species preferentially excreting in faeces and some in urine. Consideration of whether urine, bile or faeces are the most suitable choices for a particular steroid/species combination depends on a number of factors (taken up later in this review), but their relative excretion in the form of recovered radioactivity in urine versus faeces is one consideration. Although an important factor for consideration, a predominance of radioactivity in one or other matrices does not always imply greater suitability for that matrix since a smaller proportion of radioactivity present as one analyte may be more useful than a larger proportion of radioactivity present as

many metabolites. Differences in the total volume of excreted material can influence resulting concentrations. On the whole, urine generally suffers less analytical matrix effects and residual *ex-vivo* metabolism than faeces. Figure 3 exemplifies the range of different excretion patterns that have been observed for some steroids.
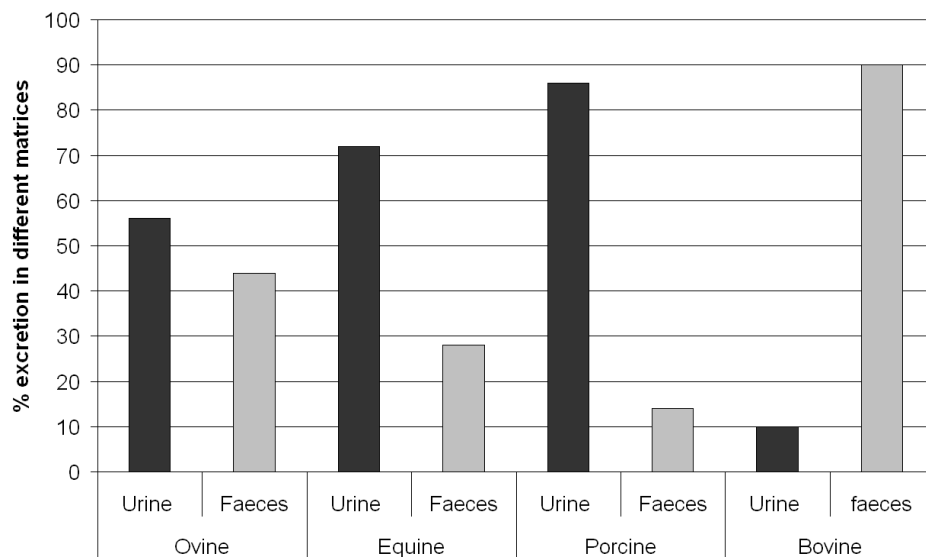
Figure 3 – Percentage excretion of radioactivity in different waste products after intravenous infusion of testosterone into different species (adapted from Martin 1966, Calvert et al. 1975, Velle 1976 and Palme et al. 1996).

● *Hydration status*– The concentrations of steroids in some matrices, especially urine, can be affected by the hydration status of the animal (Wolfgang Korth – personal observation). One could predict that this might be a particularly important factor in countries that have experienced frequent droughts in recent years, for example Australia. The adjustment of urinary steroid concentrations for the hydration status of the animal (often measured as the specific gravity or the creatinine concentration of the urine) therefore has potential to reduce the variation in steroid values among the population. Like many physiological variables, it is also possible that dehydration may be a stressor that affects minor metabolic pathways such as the rate of biosynthesis/catabolism of steroids. However, the author is not aware of any studies that have assessed this particular variable.

● *Other variables* – Many other physiological variables can affect the concentrations of steroids in different animals. Previously proposed regulatory thresholds for natural steroids in meat producing species (i.e Scippo et al. 1993, Arts et al. 1991) have taken into account at least the age and sex of the animal when constructing thresholds. In the current review, some of the factors that were analysed include the steroid in question, matrix, age, sex, herd demographics, gestation and castration status, geographical factors, housing conditions, season and time of day, disease, stress, medication, housing conditions, diet and breed.

1.4 Natural androgenic-anabolic steroid concentrations in the bovine

1.4.1 General trends in the data

Figures 4 and 5 summarise the different analyte/matrix and analytical technique/analyte combinations found for the bovine studies reviewed (as of June 2009 when the original literature review on which this chapter is based was published in *Food Additives and Contaminants*. Studies published after this date are reviewed in a narrative fashion in the text, but do not appear in figures 4 and 5). This analysis was not repeated for other species as it was apparent that a similar analysis of other species would not provide sufficient data for a meaningful comparison.
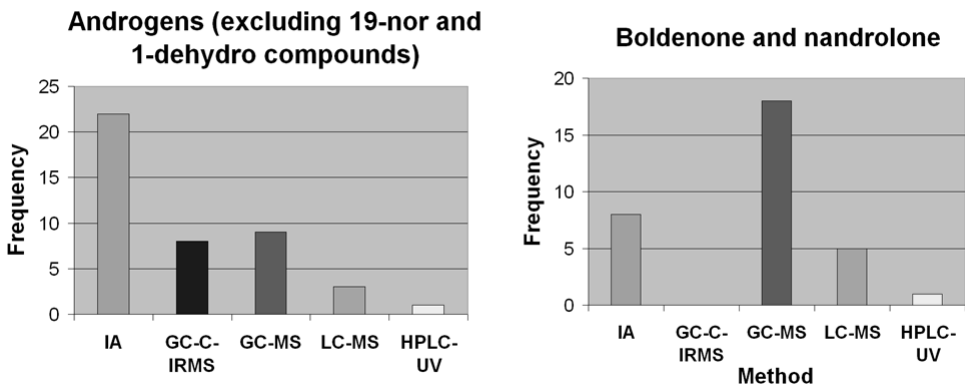
Figure 4 – Summary of the use of different analytical techniques used in the bovine studies reviewed in this report.

As Figure 4 shows, testosterone has most often been analysed using IA, whilst nandrolone and boldenone by GC or LC-MS. The predominant use of IA (most often radioimmunoassay [RIA], followed by enzyme immunoassay [EIA]) is mainly due to its ease of application, its cost effectiveness, the fact that many studies were carried out in research laboratories that do not have mass spectrometry facilities or only use them for confirmatory analysis and because of its high sensitivity in determining analytes present at low concentrations. Nandrolone and boldenone on the other hand are most often analysed by GC-MS or LC-MS. This can be partially explained by the fact that proportionally more research on these analytes is reported by residue screening laboratories; which are more likely to use mass spectrometry than research departments focussing on physiology. However, it may also be because of the ambiguous status of these analytes and their metabolites i.e. are they endogenous or not?
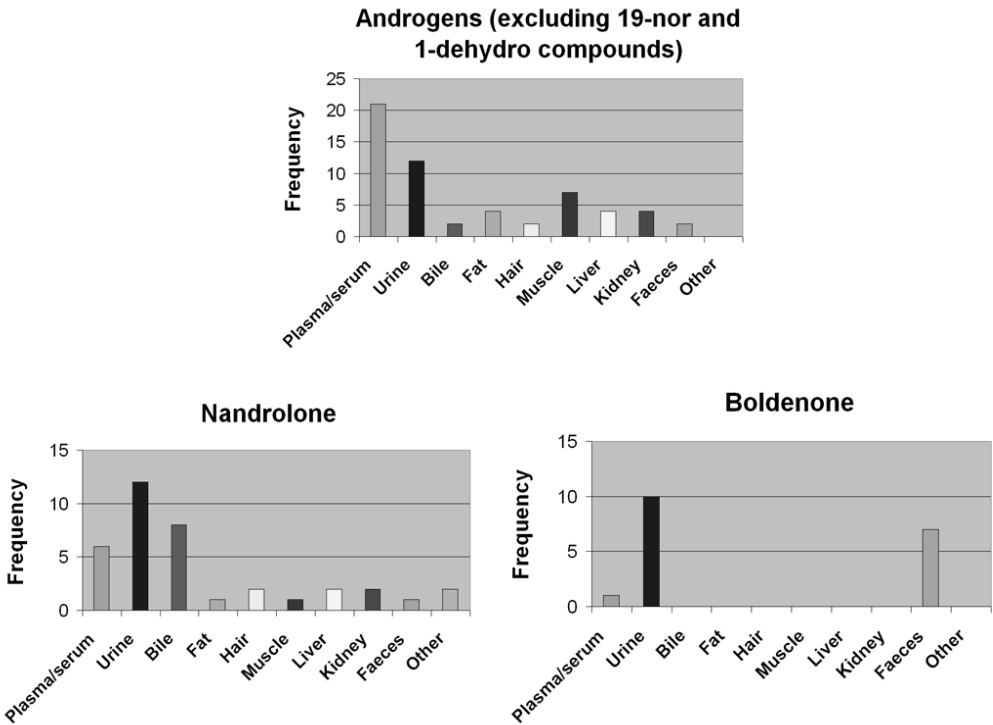

Figure 5 – Summary of the matrices used in the bovine studies reviewed in this report (all methods of analysis included).

As Figure 5 shows, testosterone has most often been analysed in plasma or serum, whereas nandrolone and boldenone have most often been analysed from urine/bile or urine/faeces respectively. As in the case of the explanation for the use of different analytical techniques for different analytes, these differences can be in part explained by quantitative biases in the type of research being carried out: either 1) physiology research looking at matrices indicating relevant circulating concentrations (i.e. plasma) for testosterone and 2) research for residue control in matrices more relevant to the detection of abuse (i.e. concentrated amounts in urine, bile or faeces for nandrolone and boldenone).

Data on the endogenous presence of androgenic-anabolic steroids in the bovine are summarised in Table 3 while details of the major phase 1 metabolic products following exogenous administration are given in Table 4.

1.4.2 Testosterone and related androgens in the bovine

1.4.2.1   Endogenous occurrence

As a general rule for all steroids, circulating plasma and tissues from non-excretory organs contain relatively high concentrations of unchanged 'parent' steroid while excretory products such as urine, bile or faeces contain relatively higher concentrations of metabolites. As well as a relative difference in the proportion of each steroid/metabolite present, excretory products generally contained higher absolute concentrations of total analyte/metabolite due to a concentrating effect.

Testosterone and related steroids such as epitestosterone, androstenedione and DHEA are ubiquitous among male and female animals of all mammalian species, so differences among various groups and times are purely quantitative. When surveying the ranges of mean, minimum and maximum values among the published studies (over 1,000 papers for all species concerned), an approximate overall rank order of absolute concentrations can be constructed. It must be stressed that some positions within this rank may be caused by biases in the amount of information reported for each steroid in different matrices. An approximate rank order for testosterone concentrations in the bovine is hair > urine ~ fat ~ faeces ~ kidney > plasma > liver ~ muscle. An approximate rank order for the significant testosterone metabolite epitestosterone is urine > faeces > plasma >

muscle > hair (no data in fat, liver or kidney). In terms of absolute values, testosterone and epitestosterone were present at similar concentrations in muscle and plasma, testosterone was at least a factor of 10 higher in hair and epitestosterone was around a factor of 10 higher in urine and faeces (no data for fat, liver or kidney). There was more variation among epitestosterone values relative to those for testosterone. As mentioned elsewhere in this review, the majority of plasma results that contributed to the aforementioned results do not use sample hydrolysis. However, Scippo et al. 1993 showed that while the maximum testosterone concentration found in bull plasma were 5.8 and 0.97 ng ml^{-1} for unconjugated and conjugated, respectively; the reverse was seen for epitestosterone with values of 0.97 and 1.8 ng ml^{-1} for unconjugated and conjugated, respectively. This could lead to artificially low reported concentrations of epitestosterone in plasma relative to testosterone.

Several other precursors including DHEA, androstenediol isomers and androstenedione were also occasionally quantified and there may be value in monitoring perturbations of endogenous steroid feedback loops after exogenous steroid administration.

Existing EU guidelines for positive decision limits (as proposed by Heitzman 1994) in the bovine already rely on separation of sex, age and gestation status as summarised in Table 1 below.

Table 1 – EEC decision limits for testosterone in plasma (as proposed by Heitzmann 1994).

Age/sex of animal	EEC decision limit in plasma (ng ml^{-1})
	Testosterone
Female (non-pregnant)	0.5
Male (< 6-months)	10
Male (> 6-months)	30

From the current review, ranges of mean plasma/serum concentrations of testosterone and epitestosterone were found to be approximately 10-fold higher in intact mature males relative to females (no data for steers). One significant finding was of a study that stated that plasma testosterone was exceptionally high for a very brief time during the late luteal phase of the normal female oestrous cycle exceeding 1.8 ng ml^{-1} (Dobson et

al. 1977). All other ranges of testosterone reported by this author were in-line with those of other studies, so if real, this phenomenon could have a serious, negative impact on the validity of the existing EU decision limit for females.

From the current review, ranges of mean urinary concentrations of testosterone and epitestosterone were found to be approximately three-fold higher in mature males relative to females (no data for steers). Ranges of mean muscle concentrations of testosterone were found to be approximately 10-fold higher in mature males relative to females or steers, although epitestosterone was similar between steers and bulls (no data for females). Ranges of mean liver and kidney concentrations of testosterone were found to be approximately 10-fold higher in mature males relative to females (no data for androgens in steers or epitestosterone in any sex). Ranges of mean hair concentrations of testosterone were found to be approximately three-fold higher in mature males relative to females and steers (no data for epitestosterone). There were insufficient data to compare testosterone concentrations by sex in faeces, fat or bile.

Several studies have assessed the effect of age on the plasma/serum concentrations of testosterone in males, although the different ages, matrices and conditions under which the animals were studied and a lack of standardisation in reporting the uncertainty of measurement makes meaningful comparisons difficult. The results from three of the most informative studies are summarised below:

1) Bagu et al. 2006 showed that mean male serum testosterone concentrations at 4-weeks of age were around 0.1 ng ml^{-1}. Concentrations then rose to 1.0 ng ml^{-1} at 20-weeks, then dropped back to 0.4 ng ml^{-1} at 28-weeks and rose again to 1.1 ng ml^{-1} at 32-weeks. The authors of this study also referenced other studies that have shown a trough in testosterone concentrations between 20 and 32 weeks of age.

2) Looking at older animals, Moura et al. 2001 reported that mean male serum testosterone concentrations were 1.8 ng ml^{-1} at 26-weeks of age, rose to 8 ng ml^{-1} at 43 weeks and then dropped to 6.5 ng ml^{-1} at 52-weeks. In the same study, androstenedione was 0.45 ng ml^{-1} at 17-weeks and dropped to 0.25 ng ml^{-1} at 52-weeks.

3) The most informative single study on the effect of age was published by Arts et al. 1990. Median male plasma testosterone concentrations at 15-weeks of age were 0.8 ng ml^{-1} and then rose to 1.3 ng ml^{-1} at 28-weeks. Concentrations of epitestosterone, however, dropped from 7.1 ng ml^{-1} at 15-weeks to 0.8 ng ml^{-1} at 28-weeks. In the same study, median male urinary testosterone concentrations were 1.0 ng ml^{-1} at 15-weeks and rose to 3.7 ng ml^{-1} at 28-weeks. Epitestosterone concentrations did not change with age and values 15- and 28-weeks were 40 and 41 ng ml^{-1} respectively. As a result of the aforementioned testosterone and epitestosterone concentration changes with age, the epitestosterone:testosterone ratio fell significantly from 15- to 28-weeks of age. On this note, the testosterone:epitestosterone ratio has been found to be a good indicator of testosterone abuse in humans and horses (due to selective elevation of testosterone after testosterone doping), but Angeletti et al. 2006 showed it to be of less use in the bovine, probably due to the relatively high 17α-hydroxylase enzyme activity.

Relatively fewer studies have analysed the effect of age on female testosterone concentrations. Nakada et al. 2000 reported that mean female plasma testosterone concentration immediately after birth was 0.075 ng ml^{-1} but then fell, ranging between means of 0.015 and 0.021 ng ml^{-1} between birth and puberty. Mean (and standard error of the mean) age to puberty was 43.3 (1.3) weeks, with a range of 38-55 weeks. The same study by Arts et al. 1990 that reported male testosterone data by age also reported female data. Median female plasma testosterone concentration was less than the LOD at both 15- and 28-weeks, while median plasma epitestosterone was less than the LOD at 15-weeks and then rose to 0.2 ng ml^{-1} at 28-weeks. Median female urinary testosterone concentration at 15-weeks was less than the LOD and then rose to 1.1 ng ml^{-1} at 28-weeks. Median female urinary epitestosterone at 15- and 28-weeks were 6 and 17 ng ml^{-1} respectively. There were insufficient data to compare the effect of age on testosterone or related metabolites/precursors in faeces, liver, kidney, bile, muscle, hair or fat.

Data on the concentration of testosterone in any matrix from pregnant females was not available, but it would be expected to be elevated relative to non-pregnant females in line with other steroids (see later sections). However, mean plasma concentrations of DHEA and androst-5-ene-3β,17β-diol were found to be approximately three-fold higher in pregnant females (Gabai et al. 2004).

In a 2001 study, Plusquellec et al. showed that lactating cows of the Herens breed (artificially selected for fighting ability) had significantly higher (P<0.05) median plasma testosterone concentrations compared to Brune des Alpes animals, with values of 0.21 and 0.11 ng ml^{-1} respectively. This conforms to the observation that aggression correlates with increasing testosterone levels. The biochemical observations were also borne out by secondary sexual characteristics, which were more prominent in the Herens breed.

A study by Moura et al. 2001 showed that bulls suffering spermatic arrest had only slightly lower serum testosterone concentrations than healthy controls. However, serum androstenedione in one diseased animal was > 0.8 ng ml^{-1} at 12-months, relative to a mean of 0.25 ng ml^{-1} in healthy controls. No reports on the effect of other factors known to increase the androgen output in other species were found i.e. stress or congenital adrenal hyperplasia.

No studies were found that directly compared concentrations of testosterone or related precursors/metabolites in similar breeds under different housing conditions or in different countries, nor of diet, time of day or season on testosterone or related precursor/metabolite concentrations.

In most species long-term treatment with gonadotrophin-releasing hormone (GnRH) agonists such as deslorelin decrease luteinzing hormone (LH) output (and therefore testosterone secretion) due to desensitization of the pituitary gland. However, Aspden et al. 1997a reported that testosterone concentrations in mature bulls are increased following deslorelin administration, although another effect of this drug is that LH pulsatility is lost, leading to a flat LH secretion profile. On the other hand, Renaville et al. 1996 showed that administration of GnRH to immature bulls between 70- and-150 days of age delayed puberty relative to controls with mean pubarche ages of 180- and 120- days respectively. No reports of the effects of other non-steroidal medications on androgen concentrations were found, but several types of medication in other species are known to affect increase or decrease in concentrations e.g. cytochrome P450 enzyme inducing inhibiting drugs.

1.4.2.2   Metabolism following administration

Many studies have reported the changes in natural steroid profiles after exogenous steroid administration and some general trends as well as the results of one particularly informative study, whose results are in line with other reports, are summarised below. Testosterone itself was most often reported as being given in esterified form as an implant in the ear or as an intramuscular (IM) injection, either alone or in combination with oestradiol esters. A general trend in the bovine is for animals of both sexes to epimerise 17β-hydroxyl groups to the corresponding 17α isomer. While this epimerisation results in significant amounts of 17α-hydroxy steroids in urine, the enzyme/s responsible for the activity are also present in plasma/serum. This highlights the need to consider steroid stability studies in all matrices in order to ensure that appropriate action to maintain stability is taken.

Biddle et al. 2003 reported the effect of component-EH administration (a testosterone plus oestradiol preparation) to heifers and steers on the concentrations of urinary, serum and bilary androgens and compared the results to 'natural' populations (sampled from the field so not considered a fully 'controlled' population with respect to ruling out steroid abuse). For heifers and steer serum, basal androgen levels were very low (all most all results below the LOD) and no significant changes were noted after administration of component-EH. In heifer and steer urine, epietiocholanolone, 5β-androstane-3α,17β-diol and 5β-androstane-3β,17α-diol were present at similar levels and were around 10 times the concentration of testosterone. No data for epitestosterone were reported. After component-EH administration, the most significantly increased urinary metabolites relative to baseline values were 5β-androstane-3β,17α-diol and 5β-androstane-3α,17β-diol. Testosterone was not detectable in the majority of steer and heifer bile samples collected at slaughter.   Epietiocholanolone and 5β-androstane-3α,17β-diol were detectable somewhat more frequently, with 5β-androstane-3β,17α-diol being present at higher concentrations and therefore detectable in the majority of samples. However, no significant differences were observed in any of these analytes in animals administered Component-EH. Overall, concentrations of testosterone metabolites were present in the following rank order of concentration: bile > urine > serum. It is important to add that the conclusions reached in the above study may be partially dependent on the analytical detection capabilities i.e. if significantly reduced LODs were applied then it might be possible to discern steroid abuse from the natural population.

1.4.3   Nandrolone and related 19-nor androgens in the bovine

1.4.3.1   Endogenous occurrence

Nandrolone was once thought to be a solely synthetic steroid, but in the 1980s it was isolated as a natural hormone in the stallion (Houghton et al. 1984) and boar (Maghuin-Rogister et al. 1988). Since then, nandrolone related compounds have also been detected in matrices originating in the bovine (Vandenbroeck et al. 1991), ovine (Clouet et al. 1997), caprine (Sterk et al. 1998), human (Dehennin et al. 1984) and cervine (Van Hende 1995). Non-phenolic C18 steroids (19-nor androgens) such as nandrolone are likely to be produced predominantly as minor pathways of the normal aromatisation process of androgens that gives rise to the phenolic oestrogens. C18 androgens are therefore most often encountered in situations of high oestrogen out-put (Van Eenoo et al. 2001) such as pregnancy, although other contributory causes such as consumption of contaminated dietary products (Le Bizec et al. 2000), increased physiological stress (De Geus 2004) and *in*-situ formation in stored urine samples (Grosse et al. 2005) (including whilst stored in the bladder prior to sampling), have also been implicated. As the later section on the equine describes, the possibility that 19-nor androgens may arise as artefactual products of 19-carboxy compounds also has to be considered because most methods to date have not taken this possibility into account (Houghton et al. 2007). It is also possible, however, that the production of these 19-carboxy compounds is limited to certain animals such as the equine due to unique genetic sequences in these species. A representative selection of studies reporting endogenous bovine nandrolone are summarised below:

In 1993, Daeseleire et al. reported that traces of 5α-estrane-3β,17α-diol could be detected in urine from a pregnant control cow and that 19-noretiocholanolone was detected in two control steers by GC-MS (the breed not reported). Nandrolone and epinandrolone could not be detected in the cow or steer urine (method not quantitative so no LOD reported). No C18 androgens were present in calf urine.

In 1994, De Brabander et al. reported the results of a multi-laboratory study on the natural occurrence of C18 androgens in the bovine. Although no nandrolone could be detected at an LOD of 0.5 ng ml^{-1}, all four laboratories involved found that urine from pregnant cows (the breed was not reported) contained epinandrolone for up to four-

month pre-partum and two-days post-partum when analysed by GC-MS. Although most laboratories agreed when epinandrolone was or wasn't present in a sample (with some exceptions probably due to concentrations close to LODs), there was significant variation in the quantified concentrations when the compound was found (i.e. between 0.7 and 4.3 ng ml^{-1} for one sample). *This study is seminal in that it highlights the huge uncertainty of measurement that can surround the determination of steroid concentrations. It highlights the need to standardise as many experimental factors as possible and then cross-validate methods before the results of such studies can be compared with confidence. It also serves as caution when trying to make too detailed a comparisons between data already in the published literature.*

In 1998, McEvoy et al. studied the natural occurrence of nandrolone and epinandrolone in the bile of pregnant Friesian cows using GC-MS. Nandrolone itself was not detectable in all samples from all stages of pregnancy (LOD not reported), but epinandrolone was detected from days-120 pre-partum onwards. Concentrations at 120-days pre-partum were around 1 ng ml^{-1}, rising to 37 ng ml^{-1} at parturition and then dropping to not detectable within 1-week post-partum. Cows carrying male fetuses had higher (P<0.001) epinandrolone concentrations than those carrying female fetuses. Cows had lower (P<0.001) epinandrolone concentrations during their second pregnancy compared to during their first pregnancy. In 1999, McEvoy subsequently showed that the bile of steers and bulls derived from an untreated population did not contain nandrolone or epinandrolone above a GC-MS LOD of 0.4 ng ml^{-1}. However, some bile samples from steers (but not from bulls) suspected of nandrolone abuse did contain epinandrolone.

In 2009, Kennedy et al. reported the detection of epinandrolone and, on occasion, nandrolone in the urine of male cattle (bulls and steers) slaughtered on farm because of trauma. Similar results were also found in animals that were found to be injured on anti-mortem inspection. Significant circumstantial evidence was presented that strongly suggested that the occurrence of these steroids was causally linked to the trauma. The authors therefore proposed that the production of these steroids may be related to increased adrenal steroid output during times of extreme stress. This theory was supported by the observation that urinary nandrolone could be detected in the urine of male animals that were administered the adrenal androgen DHEA sulphate.

1.4.3.2 Metabolism following administration

Van Ginkel et al. (1989a) reported that in calves administered nandrolone (animal and administration details not available), that concentrations of epinandrolone were always greater than nandrolone, while concentrations of each were greater in bile than urine.

Samuels et al. 1998 used GC-MS to study plasma, urine and bile steroid levels after IM administration of a 1:1 mixture of nandrolone and a deuterated labelled analogue to cull cows, steers and heifers, with only urinary results being reported in this paper. Between zero and 12-hour after administration, urinary epinandrolone and 5β-estran-3α,17β-diol peaked at around 200 ng ml^{-1} while 5β-estran-3α,17α-diol and 5α-estran-3β,17α-diol were lower at around 160 and 80 ng ml^{-1} respectively. However, between 12 and 24-hours 5β-estran-3α,17α-diol and 5α-estran-3β,17α-diol were predominant with concentrations of around 120 and 90 ng ml^{-1} respectively, followed by epinandrolone and 5β-estran-3α,17β-diol at concentrations of 30 and 40 ng ml^{-1} respectively. The aforementioned compounds were detected for both the deuterium labelled and non-labelled mass transitions, suggesting that they were direct metabolites of the administered nandrolone laurate rather than through latent natural metabolic pathways being activated.

Biddle et al. 2003 reported the effect of nandrolone laurate administration to heifers and steers on the concentrations of urinary, serum and bilary C18 androgens and compared the results to 'natural' populations (sampled from the field so not considered a fully 'controlled' population with respect to ruling out steroid abuse). The majority of steer and heifer plasma samples contained natural concentrations of nandrolone, epinandrolone, 5α-estrane-3β,17α-diol and 5β-estrane-3α,17α-diol that were below the LOQ and there was no significant increase in C18 androgens after administration relative to the 'natural' population. The majority of steer and heifer urine samples contained concentrations of nandrolone, epinandrolone, 5α-estrane-3β,17α-diol, 5β-estrane-3α,17α-diol, 5β-estrane-3α,17β-diol and 19-noretiocholanolone that were below the LOQ. Laurabolin (nandrolone laurate) administered to females produced significant increases in epinandrolone, 5α-estrane-3β,17α-diol, 5β-estrane-3α,17α-diol and 19-noretiocholanolone concentration. 5β-Estrane-3α,17β-diol was also elevated but to a much less marked extent. However, administration of Laurabolin to males only produced a slight rise in epinandrolone and 5α-estrane-3β,17α-diol concentrations. In bile, the majority of steer, heifer and bull

samples contained concentrations of nandrolone, epinandrolone, 5β-estrane-3α,17β-diol, 5α-estrane-3β,17α-diol and 5β-estrane-3α,17α-diol that were below the LOQ. In post-administration samples taken at slaughter from both heifers and steers (no samples for bulls), concentrations of 5β-estrane-3α,17α-diol were the most obviously increased of the steroids relative to samples from the 'natural' population. Nandrolone itself was not detectable in post-administration heifer bile samples, while interpretation of nandrolone data in steers and epinandrolone data in heifers and steers was complicated by the co-elution of a large interfering peak in the relevant mass transition windows.

In 2008, Pinel et al. reported the effect of nandrolone laurate administration to male and female calves (concomitant with oestradiol benzoate administration) on the resulting urinary metabolite profiles. Of the nandrolone metabolites identified following steroid administration, epinandrolone was highest in concentration. Nandrolone, 19-noretiocholanolone and 19-norandrostenedione were identified as secondary metabolites. Overall, nandrolone metabolite profiles were similar between males and females, with the exception that epinandrolone concentrations in females were approximately twice those in males.

Most recently (2010), Pinel et al. have extended the above study protocol to assess the effects of nandrolone laurate administration to male and female calves (concomitant with oestradiol benzoate administration) on the profile of estranediol nandrolone metabolite isomers. Of the isomers identified, 5α-estrane-3β,17α-diol was the most abundant, with less abundant isomers corresponding to 5β-estrane-3α,17α-diol, 5β-estrane-3α,17β-diol and 5α-estrane-3β,17β-diol. Concentrations of 5α-estrane-3β,17α-diol, 5β-estrane-3α,17α-diol and 5α-estrane-3β,17β-diol were found to be an average of 2, 1.5 and 1.5 times greater in females relative to males. Recent evidence (Gaud Pinel, personal communication) has suggested that while the isomer 5α-estrane-3β,17α-diol can occur naturally in the urine of pregnant and injured cattle, the 17β-isomers 5β-estrane-3α,17β-diol and 5α-estrane-3β,17β-diol are only found following nandrolone administration. The 17β-isomers may therefore be appropriate marker metabolites of nandrolone abuse.

1.4.4   Boldenone and related 1-dehydro androgens in the bovine

1.4.4.1   Endogenous occurrence

As in the case of nandrolone, 1-dehydro steroids such as boldenone were once thought to be purely synthetic in origin. Since the 1990s however, boldenone related compounds have been detected in different matrices from several species including microbes (Mahato et al. 1997), maggots (Verheyden et al. 2007), crustaceans (Verslycke et al. 2002), rats (Song et al. 2000), pigs (Poelmans et al. 2005a), horses (Ho et al. 2004) and cattle (veal calves) (Arts et al. 1996). A comprehensive review of the presence and metabolism of boldenone in various animal species was published by De Brabander et al. in 2004. Extra-enteral production of 1-dehydro steroids within the body (i.e. testes) has not been demonstrated in the bovine (De Brabander et al. 2004), but boldenone has been identified in porcine testes (Poelmans et al. 2005a). One of the most likely origins of 1-dehydro compounds in the bovine is currently considered to be through conversion of precursors such as phytosterols or other steroids, possibly by gut microbes (Pompa et al. 2006, Draisci et al. 2007, Verheyden et al. 2007, Verheyden et al. 2010a). The endogenous occurrence of 1-dehydro steroids in the bovine has been the subject of some debate. 1-dehydro compounds appear to be present in some animal populations but not others. The extent to which this is due to real physiological variation versus experimental design is hard to establish, but the general consensus (various personal communications) is that it is likely a mix of both. The following selection of studies highlights many of the issues relating to the possible 'endogenous' nature of boldenone in the bovine:

Draisci et al. (2003) analysed urine samples for boldenone, epiboldenone and androsta-1,4-diene-3,17-dione by LC-MS from 25 untreated animals. Boldenone (LOQ = 0.2 ng ml^{-1}), epiboldenone (LOQ = 0.5 ng ml^{-1}) and androsta-1,4-diene-3,17-dione (LOQ 0.2 ng ml^{-1}) were not detected above the LOQ in any of the urine samples from the untreated animals.

Pompa et al. (2006) studied the concentrations of boldenone, epiboldenone, androsta-1,4-diene-3,17-dione, testosterone and epitestosterone in the urine, skin swabs and faeces of Friesian calves and also assessed the effect of drying the faeces on the resulting faecal steroid concentrations. In urine, LODs for all steroids were 0.1 ng ml^{-1}

and in faeces LODs for all steroids were 0.5 ng g^{-1} (based on S:N>3:1). Boldenone, epiboldenone and androsta-1,4-diene-3,17-dione in urine were not detected in any of the samples from 10 calves. Boldenone was detected in faeces sampled directly from the rectum (rectal faeces) in all the calves at concentrations ranging from 28 to 89 ng g^{-1}. Epiboldenone in rectal faeces was not detectable in six calves and between 2.6 and 5.9 ng g^{-1} in the other four animals. Androsta-1,4-diene-3,17-one was not detected in the rectal faeces from 9 calves while one calf had 21 ng g^{-1}. Results from faeces scraped from the skin, faeces taken from the stall floor and faeces stored for up to 13 days at room temperature in a cowshed showed that the concentrations of all steroids increased significantly (but variably) over time. This is especially true of epiboldenone and androsta-1,4-diene-3,17-dione, which by day-13 of storage are present in high concentrations, while boldenone was reduced to not detectable by day-13. This study exemplifies the need for avoiding faecal contamination of urine during sampling and to ensure swift storage and analysis of any samples taken.

In 2007, Draisci et al. reported the effect of feeding two different types of milk replacers, containing different concentrations of phytosterols, on the urinary excretion of boldenone, epiboldenone and androsta-1,4-diene-3,17-dione in male calves. Boldenone and androsta-1,4-diene-3,17-dione were not detected in any animal during either treatment, but epiboldenone conjugates were present in samples from animals receiving both types of milk replacer. Although concentrations of conjugated epiboldenone were always below 2 ng ml^{-1}, concentrations in urine samples from the animals receiving the milk replacer with the higher phytosterol content were significantly higher. These results support the suggestion that epiboldenone may be formed through conversion of phytosterols and that it may also be present 'naturally' as a conjugate.

In a publication assessing the ability of several invertebrate species to metabolise a number of different steroids, Verheyden et al. 2010a reported that feed-borne fungi were capable of converting phytosterols into 1-dehydro-steroids. Specifically, several unidentified fungal species growing on corn were found to be capable of converting the phytosterol β-sitosterol into androstenedione. On further incubation with androstenedione at higher concentrations, androsta-1,4-diene-3,17-dione was detected. Furthermore, following subsequent incubation with androsta-1,4-diene-3,17-dione at higher concentrations, boldenone was detected. This study demonstrates the usefulness in using *in vitro* models to study the possible pathways of steroid formation in cattle and

also further supports the proposal that phytosterols may be precursors to boldenone related compounds.

Most recently, Verheyden et al. 2010b have demonstrated that some types of wooden crate in which veal calves are housed may contain precursors to boldenone. Specifically, the related compound androsta-1,4-diene-3,17-dione was detected at concentrations ranging from 20 to 34 ng g^{-1} in the wood from calves housing. The wood was also found to contain progesterone, androstenedione and epitestosterone. This raises the possibility that 1-dehydro steroids may be consumed inadvertently by calves due to environmental contamination. In light of these results, some kind of threshold concentration approach might therefore be more appropriate for detecting boldenone abuse compared to a qualitative marker metabolite based method.

1.4.4.2   Metabolism following administration

In a 1998 study, Van Puymbroeck et al. studied the metabolism of boldenone in the bovine using semi-quantitative GC-MS analysis of urine and faecal samples. In urine from animals given various boldenone ester administrations, epiboldenone was the major metabolite in urine, followed by significant amounts of two 5-reduced metabolites with unidentified stereochemistry at positions 3, 5 and 17 with molecular masses two units higher than boldenone. Other urinary metabolites identified were, 6z-hydroxyboldenone, androsta-1,4-diene-3,17-dione and 5β-androst-1-ene-3,17-dione ('z' indicates that the isomer configuration has not been established). In faeces, boldenone, androsta-1,4-diene-3,17-dione and 6z-hydroxyboldenone were absent, while only small amounts of epiboldenone, 5β-androst-1-ene-3,17-dione and some unidentified reduced metabolites were found.

In a 2004 study, Sterk et al. used GC-MS to study the effect on urinary and faecal metabolites after IM boldenone ester administration to veal calves. Not all experimental details or results were reported, but the authors stated that "..both 17α and 17β-boldenone were almost 100% present in urine as glucuronic acid conjugates. The 6z-hydroxy-boldenone metabolite was present as a sulphate conjugate. In faeces boldenone was present in the non-conjugated form."

Biddle et al. reported a quantitative metabolism study of boldenone in the bovine in 2005. Two steers were sequentially treated with A) bolus 400mg boldenone IM injection, B) followed 14-days after the bolus injection by an oral 500 mg androsta-1,4-diene-3,17-dione administration, C) followed 10-days after the oral administration by a 700 mg boldenone undecylenate IM injection. Samples of plasma and urine were analysed as 'free' and 'glucuronic acid conjugated' fractions using differential extraction and E. coli hydrolysis. Analysis was by GC-MS and was qualitative/semi-quantitative only. *Where an analyte is preceded by a question mark (?) in the remainder of this section, it signifies that the structure is putative (although with strong evidence to suggest its structure) as no reference standard was available.* After oral androsta-1,4-diene-3,17-dione administration, the free plasma metabolites were of the order of epiboldenone > ?6β-hydroxyepiboldenone > boldenone ~ 6β-hydroxyboldenone. The plasma glucuronic acid conjugate fraction metabolites were also of the order of epiboldenone > ?6β-hydroxyepiboldenone > boldenone ~ 6β-hydroxyboldenone, but absolute concentrations were approximately 10-times those of the free fraction. Of these, boldenone and 6β-hydroxyboldenone were only transiently visible at low concentrations. The free urinary metabolites were of the order of epiboldenone > ?5β-androst-1-ene-17α-ol-3-one > 6β-hydroxyboldenone ~ 5β-androst-1-ene-17β-ol-3-one. The urinary glucuronic acid conjugate fraction metabolites were of the order of epiboldenone > ?5β-androst-1-ene-17α-ol-3-one > 5β-androst-1-ene-17β-ol-3-one > ?6β-hydroxyepiboldenone > 6β-hydroxyboldenone. After IM boldenone undecylenate administration, the only free plasma analyte present was boldenone, which was detected for the whole 56-days after administration. The glucuronic acid conjugate plasma metabolites were of the order of epiboldenone > ?6β-hydroxyepiboldenone > boldenone. Of the glucuronic acid conjugated metabolites, ?6β-hydroxyepiboldenone had the longest detection time after administration. Boldenone itself was present in greater quantities in the free than glucuronic acid conjugate fraction in plasma. The free urinary metabolites were of the order of epiboldenone > boldenone. The glucuronic acid conjugated urinary metabolites were of the order of ?6β-hydroxyepiboldenone ~ ?5β-androst-1-ene-17α-ol-3-one ~ 5z-androst-1-ene-3z-ol-17-one ~ epiboldenone > 5β-androst-1-ene-17β-ol-3-one. Boldenone and epiboldenone were present at higher concentrations as glucuronic acid conjugates than free steroids.

In a 2006 study, Le Bizec et al. used GC-MS to analyse the urinary metabolite profile after various boldenone administrations in cattle. Treated animals received either a

single oral boldenone + androsta-1,4-diene-3,17-dione dose, a daily oral boldenone ester dose for three-days, a daily oral androsta-1,4-diene-3,17-dione dose for six-days, a daily oral boldenone ester dose for five-days or a single IM injection of boldenone esters. In all treated animal's hydrolysed urine, epiboldenone was by far the most predominant metabolite, while 17α-hydroxy-5β-androst-1-en-3-one, 17β-hydroxy-5β-androst-1-en-3-one and 3α-hydroxy-5β-androst-1-en-17-one were the only other metabolites always present. Analysis with and without hydrolysis suggested that the majority of metabolites were glucuronic acid conjugated; with boldenone having a variable degree of sulphate conjugation. Analysis using LC-MS was also carried out and it was found that urine from treated animals contained boldenone sulphate conjugate whereas urine from untreated animals did not. In a subsequent study from the same laboratory in 2009, Destrez et al. measured intact boldenone sulphate from urine in a larger number of treated and untreated animals using a number of different analytical techniques. This study supported the findings from Le Bizec et al. 2006 and provided further evidence of the suitability of boldenone sulphate as a marker metabolite of boldenone administration. In 2008, Van Poucke et al. presented an alternative method for the analysis of boldenone conjugates based on the separation of free, glucuronide and sulphate fractions prior to hydrolysis of the conjugate fraction and analysis of the resulting 'free' steroids. One of the stated advantages of this method was the lack of requirements for conjugate reference standards.

A recent study by Piper et al. (2010) highlighted the potential use of gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) to differentiate endogenous and exogenous boldenone and its main metabolite in human urine. The potential applications of GC-C-IRMS are taken up further in section 1.11.2.

1.4.4.3   EU recommendations regarding boldenone testing

The outcome of an experts meeting on the control of Boldenone in veal calves in September 2003 (European Commission 2003) recommended the following:

"On the basis of the scientific information available, the experts of the Member States agreed that the presence of boldenone conjugates at any levels in urine from veal calves is proof of illegal treatment. In order for positive results for boldenone to be used as evidence of illegal treatment, the following must be fulfilled:

● *Sampling of urine must be done without faecal contamination of the samples.* The samples should be frozen as soon as possible after collection in order to avoid hydrolysis of the conjugates.

● *Analytical results related to boldenone residues (boldenone or epiboldenone) must always be specified as free or conjugated forms, with the explicit identification of the animal species, including breed, gender and age of the animal.*

There is sufficient scientific knowledge to conclude that the presence of epiboldenone in urine and faeces of bovine animals can come from other sources than illegal treatment. A number of explanations are currently being investigated by the scientific community. If only epiboldenone is found and if the levels are above 2 ng g^{-1} in urine of veal calves, additional investigations would need to be carried out before concluding on illegal use of boldenone.

An MRPL for the analytical methods for the detection of boldenone and epiboldenone in urine of veal calves should be set at 1 ng g^{-1}. Further studies of appropriate marker metabolites of boldenone are encouraged. The member states should transmit existing and future data to the CRL in Bilthoven. This position could be amended in the light of additional data from ongoing and future research."

1.5.1   Testosterone and related androgens in the ovine

Data on the endogenous presence of androgenic-anabolic steroids in the ovine are summarised in Table 3 while details of the major phase 1 metabolic products following exogenous administration are given in Table 4.

1.5.1.1   Endogenous occurrence

In this review, ranges of mean plasma/serum testosterone concentrations were found to be between three- and 100-fold higher in rams relative to ewes (depending on the season), while concentrations in wethers were similar to those of ewes. Concentrations of androstenedione in the only report of concentrations in wethers were similar to the lowest mean concentrations reported in rams, but approximately 60-fold lower than the highest mean concentrations reported in rams.

Plasma/serum concentration levels of testosterone in late pregnant ewes were similar to those of non-pregnant animals, while pregnant ewe DHEA concentrations were similar to concentrations in rams. Androstenedione concentrations in pregnant ewes were similar to the maximum mean concentrations reported in rams and approximately 60-fold higher than the only report of concentrations in wethers.

Several studies have assessed the effect of age on plasma/serum testosterone concentrations in rams. Fahmy et al. 1997 studied the serum concentrations of testosterone from the ages of 10 to 34-weeks in rams of the Romanov breed as well as the Booroola Merina x DLS breed. Testosterone concentrations increased in a linear fashion with age in both breeds (P<0.01). Mean concentrations in the Romanov breed at 10-weeks were 3.8 ng ml^{-1}, rising to 13 ng ml^{-1} at 34 weeks. Mean concentrations in the Booroola Merina x DLS breed were 0.8 ng ml^{-1} at 10-weeks, rising to 8.0 ng ml^{-1} at 34-weeks. Concentrations were significantly higher (P<0.05) in the Romanov breed at all ages other than 14-weeks, which corresponded to a temporary reduction in concentrations in the Romanov breed.

Langford et al. 1998 reported the serum concentrations of testosterone in each of the Canadian Arcott, Outaouais Arcott, Rideau Arcott and Finnish Landrace breeds at six-, eight- and 12-months then at three years. Mean concentrations in the Canadian Arcott, Outaouais Arcott, Rideau Arcott and Finnish Landrace at six-months were 3.3, 4.0, 3.5 and 3.9 ng ml^{-1} respectively, at 8-months were 3.1, 5.0, 5.5 and 8.2 ng ml^{-1} respectively, at 12-months were 3.5, 5.3, 5.4 and 7.8 ng ml^{-1} respectively. Mean concentrations at three-years were 8.0, 7.0, 5.7 and 7.0 ng ml^{-1} respectively. Concentrations were significantly higher (P<0.05) in Finnish relative to Canadian Arcott male lambs at eight and 12-months, but none of the other differences were significant (P<0.05).

There was insufficient data to compare concentrations of testosterone or related precursors/metabolites between sexes, ages, gestation and castration statuses in any other matrix.

No reports on the effect of time of day or geographical factors were found, but two reports on the effect of diet and several reports on the effect of season and housing conditions were retrieved. Schanbacher et al. 1979 reported the effect of transferring animals from a 12-hour light, 12-hour dark photoperiod to either short day photoperiod

(eight hour light, 16 hour dark) or long day photoperiod (16 hour light, eight hour dark) on serum testosterone concentrations in Suffolk-Hampshire rams. The animals exposed to short days had increased testosterone concentrations, heavier testes, larger seminiferous tubules and produced more sperm relative to the long-day rams. This effect of photoperiod has obvious implication for resulting testosterone concentrations, dependent upon the type of artificial light conditions and the hemisphere inhabited. Borque et al. 1999 studied the effect of season on the concentration of plasma testosterone in Manchego rams living in the northern hemisphere (Spain). Concentrations varied with the season, such that mean peak values were in the second week of September at 7.2 ng ml^{-1}, while trough concentrations were 0.40 ng ml^{-1} in the second week of February. The authors also commented that increasing testosterone concentrations were correlated with a decreasing photoperiod.

Rosa et al. 2000 studied the effect of housing males with females on plasma testosterone concentrations in Texel and Suffolk rams. Testosterone concentrations were increased on introduction to ewes ($P<0.05$), but the effect did not depend on whether ewes were in oestrous or not. Mean basal concentrations in different groups varied from 5 to 10 ng ml^{-1}, while after introduction to ewes, concentrations increased by between 3 to 5 ng ml^{-1} to between eight and 15 ng ml^{-1}.

Parkinson et al. 2001 studied the effect of intersex relative to concentrations in 'normal' rams and ewes during the breeding season. Freemartin ewes (XX/XY chimaeras) were classified as either 'male (MF)' type or 'undifferentiated (UF)' depending upon the masculinisation of their genitalia. In one of the experiments (1b) mean basal testosterone concentrations in MF, UF, ewes and rams were 0.79, 0.29, 0.14 and 2.4 ng ml^{-1} respectively. The testosterone concentrations in Freemartins were significantly higher ($P<0.05$) than in ewes, but lower than in rams.

Stellflug et al. 2004 studied the effect of administering the opioid antagonist naloxone on plasma testosterone concentrations in rams with different sexual orientations. Mean basal testosterone concentrations in the first experiment were 2 ng ml^{-1} for all animals. After naloxone administration, mean testosterone concentrations rose significantly ($P<0.01$) after 60-minutes in both sexually active and male oriented rams to 8.0 and 8.2 ng ml^{-1} respectively, but a rise in testosterone concentrations in sexually inactive rams to 3.5 ng ml^{-1} at 60-minutes was not reported as significant. Although this example on it's

own may have little relevance to the regulation of steroid abuse, it highlights the fact that concomitant medication can sometimes have effects on natural steroids and may therefore need to be considered.

1.5.1.2   Metabolism following administration

Only one study analysing androgen profiles after testosterone administration to sheep was found. Yamamoto et al. 1978 reported the effect of infusing a ram with radiolabelled testosterone and androstenedione and a second ram with radiolabelled epitestosterone and testosterone on resulting androgen profiles in bile and urine. Between 80-90% of the doses were excreted as either glucuronic acid or sulphate conjugates. Urine contained mainly glucuronic acid conjugates, while bile mainly sulphate conjugates. Epitestosterone, along with some unidentified polar compounds, was the major metabolite in urine and bile, with androsterone, etiocholanolone, 5β-androstane-3α,17β-diol and 2 other androstanediols also identified. The study was not quantitative and metabolites were identified on the basis of their co-crystallisation with standards using TLC-FL.

1.5.2   19-nor and 1-dehydro steroids in the ovine

Very few reports on the occurrence of 19-nor and 1-dehydro-androgens in the ovine were found.

In a 1991 study by Vandenbroeck et al, the authors reported that the urine of 11 rams/lambs and 10 pregnant/non-pregnant ewes were positive for nandrolone at around 2.5 ng ml^{-1} when analysed by RIA. However, the samples were not confirmed positive by GC-MS (the LOD was not reported).

In 1995, Van Hende analysed the urine of four ewes at different stages of pregnancy and the amniotic fluid of one ewe for the presence of epinandrolone. Animals were sampled between 43 and zero-days prior to parturition, some at multiple times and others only at one time-point. Using semi-quantitative GC-MS analysis, the amniotic fluid did not contain epinandrolone above the LOD of 1 ng ml^{-1}. The urine of the four pregnant animals was found to contain epinandrolone at concentrations ranging from below the LOD to above 2 ng ml^{-1}. There was no clear correlation between the stage of gestation

and the concentration of epinandrolone determined. The ages, parity and fetal number of the animals were not reported.

Clouet et al. 1997 reported the analysis of urine from 30 pregnant and non-pregnant French Vendenne four-year-old ewes for the presence of nandrolone and epinandrolone using GC-MS with an LOD of 0.2 ng ml^{-1}. Nandrolone was not detected in the pregnant ewe's urine and neither analyte was detected in the non-pregnant ewe's urine. However, epinandrolone was detected at somewhere between the LOD and 0.5 ng ml^{-1} (insufficient experimental details available to allow the quantitative significance of this statement to be determined) in pregnant animals at 120 to 39-days before parturition, with concentrations then increasing to 3.4 ng ml^{-1} at seven days before parturition. There was no correlation between the sex or number of fetuses with the epinandrolone concentrations found.

The above results contrast with those of Sterk et al. 1998, who could not detect nandrolone or epinandrolone in the urine of five Flevolander ewes during early or late stages of pregnancy using a GC-MS method with an LOD of 0.5 ng ml^{-1}. The ages, parity and fetal number of the animals were not reported.

Casson et al. 2006 reported on the use of LC-MS to assess whether nandrolone and epinandrolone were natural in a population of 130 male and female sheep in the UK. Although the population could not be guaranteed 'clean', the authors report that no evidence of steroids abuse was found on any of the farms tested. The background to this research was an observation that the incidence of nandrolone positives rose once an LC-MS screening method with an LOD of 0.5 ng ml^{-1} replaced an ELISA method with an LOD of 2 ng ml^{-1} in 2004. The authors commented the following:

"Nortestosterone seems endemic in British sheep; primarily as the 17α-isomer, but also with some 17β- present. There does not seem to be much correlation with age or sex of the animal, although the majority of the population tested was 6-12 months and some of the other categories contained very few samples (e.g. all 5 males of over 12-months contained the 17α-isomer at 0.4ng ml^{-1} or greater). In light of this, it seems unwise to extrapolate the "17α- in male animals indicates abuse" rules from cattle to sheep. An exercise to test the urine of a controlled population will validate these conclusions, and demonstrate any link to other physiological factors such as breed or feedings regime."

Most recently (2009), Rosegger et al. have reported the plasma, urinary and faecal concentrations of nandrolone, epinandrolone and 19-norandrostenedione in pregnant ewes and male/female lambs in Austria before and after nandrolone laurate IM administration. Following extraction of the analytes from the samples, analysis was carried out using both HPLC-EIA and LC-MS. None of the three analytes were detected in the plasma of pregnant ewes. However, epinandrolone and 19-norandrostenedione (but not nandrolone) were detected in urine (concentrations up to 15 and 2.8 ng ml^{-1} respectively) and faeces (concentrations up to 15 and 18 ng g^{-1} respectively) during pregnancy. None of the three nandrolone related analytes were detected in the plasma or faeces of untreated male or female lambs. However, epinandrolone (but not nandrolone or 19-norandrostenedione) was sporadically detectable in the urine from untreated male and female lambs at concentrations up to 3.0 ng ml^{-1} in females and 1.6 in males. Following administration of nandrolone laurate to male and female lambs, nandrolone (but not epinandrolone or 19-norandrostenedione) could be detected in plasma up to 0.9 ng ml^{-1}. In the urine of treated lambs, maximum mean nandrolone concentrations were 17 and 20 ng ml^{-1} for males and females respectively, while the corresponding figures for epinandrolone were 242 and 254 ng ml^{-1} respectively and for 19-norandrostenedione were 12 and 11 ng ml^{-1} respectively. In the faeces of treated lambs, epinandrolone and 19-norandrostenedione were only detected sporadically at concentrations around the LOD. Following the statistical treatment of the endogenous population data, the authors proposed a screening threshold of 2.06 ng ml^{-1} of urinary epinandrolone in sheep (using the 95th percentile from a normal distribution of the population) and then also recommended that confirmation of nandrolone abuse be based on the qualitative detection of nandrolone itself in urine or plasma. The latter suggestion conflicts with the report of Cason et al. 2006, in which the possible endogenous occurrence of nandrolone was raised. However, the population animal population studied by Cason et al. was uncontrolled for steroid use, so further studies are warranted before a strategy for prosecution can be fully ratified.

No reports on the endogenous or post-administration levels of boldenone or related compounds in the ovine were found, but 2 of 961 ovine urine samples tested in an Australian national monitoring programme (not therefore a controlled population with respect to ruling out steroid abuse) were above the 1 ng ml^{-1} LOD for epiboldenone with concentrations of 5.9 and 17 ng ml^{-1} respectively (Wolfgang Korth, personal communication). No samples were above the LOD for boldenone. Clearly, more work is

required to establish the endogenous versus exogenous nature of boldenone in the ovine.

1.6.1 Testosterone and related androgens in the porcine

Data on the endogenous presence of androgenic-anabolic steroids in the porcine are summarised in Table 3 while details of the major phase 1 metabolic products following exogenous administration are given in Table 4.

1.6.1.1 Endogenous occurrence

From the current review, ranges of mean plasma/serum concentrations of testosterone in boars were found to be between seven and 500-fold greater than those observed in barrows (castrated males), while those in gilts/sows were around five-fold lower than those in barrows. There were insufficient data to compare the concentrations of any other steroids by sex as there were few reports of steroid concentrations in barrows and gilts/sows.

The most extensive study of steroid concentrations with age in boar plasma (Yorkshire breed) was carried out by Schwarzenberger et al. in 1993. Before detailing the results, it is important to point out that the concentrations reported seem to be much higher than those reported by other authors. The authors commented that steroids were almost exclusively present in plasma as sulphate conjugates, so unless indicated, the results below are for sulphated steroids. Androstenedione and DHEA-sulphate were measured directly, whereas other steroids were measured after extraction and solvolysis of separated fractions (solvolysis carried out by heating samples in 5 mL of acidified ethyl acetate [6 drops of 6 M sulphuric acid in 100 mL ethyl acetate] overnight at 45°C), thus inferring, but not proving, the status of most conjugates. For testosterone, concentrations after birth were around 1 ng ml^{-1} for both free and sulphate conjugates, rising to around 3 ng ml^{-1} for each at one month. Concentrations of each then dropped back to around 1 ng ml^{-1} at five months where they then increased again, in the case of free testosterone to around 3 ng ml^{-1} at six months and in the case of testosterone-sulphate to around 6 ng ml^{-1} at seven months. Concentrations of free and sulphated testosterone seemed to dip slightly after seven months. For free androstenedione, the pattern was similar to testosterone such that concentrations after birth were around 6 ng ml^{-1}, increasing to

around 24 ng ml^{-1} at one month, then dropping to around 6ng ml^{-1} until five months. Concentrations then increased again to around 18ng ml^{-1} at six months and then dipped to around 9 ng ml^{-1} at eight months. For DHEA-sulphate (authors reported that <10% of the total concentration of this steroid of was present in the unconjugated form), the pattern was again similar to testosterone such that concentrations after birth were around 3 ng ml^{-1}, increasing to around 80 ng ml^{-1} at one month, then dropping to around 15 ng ml^{-1} at five months. Concentrations then increased to around 80 ng ml^{-1} at six months and then dropped to around 60 ng ml^{-1} at eight months. For 5α-androstane-3β,17β-diol-sulphate (authors reported that <10% of the total concentration of this steroid of was present in the unconjugated form), the pattern was again similar to testosterone such that concentrations after birth were around 3 ng ml^{-1}, increasing to around 12 ng ml^{-1} at one month, then dropping to around 3-6 ng ml^{-1} at five months. Concentrations then increased to around 35 ng ml^{-1} at seven months. There was then only a very slight drop between seven and eight months. In summary, sulphate conjugates were (or were inferred to be) the most predominant steroids measured in plasma; with the exception of androstenedione, which was measured in the free fraction only. Concentrations of most steroids peaked first at one month after birth and then dropped until around five months, before increasing again at six-eight months (with some dropping slightly after seven months). The rank order of absolute determined concentrations in plasma was DHEA sulphate > 5α-androstane-3β,17β-diol-sulphate > androstenedione > testosterone sulphate.

McCoard et al. 2003 studied the concentrations of plasma testosterone in Meishan and White Composite fetal and neonatal boars. Fetal testosterone concentrations were not significantly different (P<0.05) in Meishan and White Composite boars, with values ranging from around 1 to 2 ng ml^{-1}. However, testosterone concentrations in both breeds increased neonatally, peaking at 14-days post-partum with concentrations of around 5.5 and 7 ng ml^{-1} in Meishan and White Composites respectively (significantly higher in White Composites at P<0.05). Concentrations then dropped to around 4 ng ml^{-1} in both breeds by 25-days post-partum. Park et al. 2002 reported serum testosterone concentrations in Duroc versus Yorkshire boars living in the northern hemisphere country South Korea during spring, summer, autumn and winter. Mean testosterone concentrations in Duroc boars during spring, summer, autumn and winter were 3.1, 0.73, 1.3 and 1.4 ng ml^{-1} respectively, while in Yorkshire boars were 5.1, 2.6, 2.5 and 2.6 ng ml^{-1} respectively. The higher testosterone concentrations in Spring occur at a time when

photoperiod is increasing in this country, but peak photoperiod (during the summer) does not correlate with peak testosterone concentrations. The testosterone concentrations in Yorkshire boars were significantly higher (P<0.05) than for Duroc boars at all stages of the year. Testosterone concentrations were higher in all breeds in spring compared with the rest of year (P<0.05). Walker et al. 2004 measured plasma testosterone concentrations in Duroc boars that had or had not been subject to selection for high testosterone concentrations over 10 generations. Mean testosterone concentrations in boars selected over 10 generations were 49 ng ml^{-1}, which were significantly higher (P<0.01) than controls with a mean testosterone concentration of 28 ng ml^{-1}.

No reports of the effect of pregnancy, diet, housing conditions or geographical factors on testosterone concentrations were found.

1.6.1.2   Metabolism following administration

No studies assessing the metabolism of testosterone following administration to porcine animals were found.

1.6.2   Nandrolone and related androgens in the porcine

1.6.2.1   Endogenous occurrence

As discussed in the earlier bovine section, nandrolone has been shown to be produced naturally in the porcine (Maghuin-Rogister et al. 1988). The following discussion reviews a selection of studies assessing the concentrations of nandrolone related compounds in different porcine matrices.

Van Ginkel et al. 1989b (article in Dutch but abstract in English) studied the natural concentrations of nandrolone in 25 young boars using RIA for bile and urine and GC-MS for muscle and liver. The mean and maximum urinary nandrolone concentrations were 55 and 130 ng ml^{-1} respectively, biliary concentrations were 88 and 210 ng ml^{-1} respectively, muscle concentrations were 1.1 and 13 ng g^{-1} respectively and liver concentrations were 23 and 200 ng g^{-1} respectively.

Vandenbroeck et al. 1991 studied the concentrations of nandrolone in the urine of a population of boars, cryptorchids, castrated males and pregnant/non-pregnant sows. The limit of detection was not reported. Screening was by RIA and confirmation by GC-MS. A cut-off for reporting positives by RIA was set at 2 ng ml^{-1}. Samples above 3 ng ml^{-1} (or more precisely, the result of a 'blank' cattle urine with 2 ng ml^{-1} nandrolone added externally) were subsequently analysed by GC-MS. For 10 boars, the mean nandrolone concentrations determined by RIA was around 300 ng ml^{-1} and for six cryptorchids was around 120 ng ml^{-1}. The presence of nandrolone in these animals was then confirmed by GC-MS. No nandrolone was detected at the RIA cut-off level for pregnant and non-pregnant sows (n=10). In 12 castrated males (barrows), nandrolone was around 2 ng ml^{-1} by RIA, but the samples were negative when analysed by GC-MS.

In 1993, Rizzo et al. reported the plasma and urinary concentrations of nandrolone in boars, cryptorchids and barrows of the Landrace x Large White breed. Concentrations in the boars and cryptorchids were determined by ELISA and GC-ECD, while those in the barrows were determined by ELISA. LODs of 7 and 5 ng ml^{-1} for plasma and urine respectively were reported for GC-ECD, but the LODs for the ELISA were not reported. Using ELISA, mean (range) urinary nandrolone concentrations in boars (n=20), cryptorchids (n=10) and barrows (n=23) were 25 ng ml^{-1} (10-55 ng ml^{-1}), 25 ng ml^{-1} (10-47 ng ml^{-1}) and 1.0 ng ml^{-1} (0.3-2.1 ng ml^{-1}) respectively, while in plasma concentrations were 4.8 ng ml^{-1} (0.6-17.5 ng ml^{-1}), 5.0 ng ml^{-1} (1.1-15 ng ml^{-1}) and 0.5 ng ml^{-1} (0.1-1.2 ng ml^{-1}) respectively. With GC-ECD, mean (range) urinary nandrolone concentrations in boars (n=10 above LOD and 1 not detected) and cryptorchids (n=6 above the LOD and 1 not detected) were 83 ng ml^{-1} (1-280 ng ml^{-1}) and 93 ng ml^{-1} (10-150 ng ml^{-1}). With GC-ECD, mean (range) plasma nandrolone concentrations in boars (n= six above LOD and one not detected) and cryptorchids (n=3 above LOD and 2 not detected) were 4 ng ml^{-1} (2-8 ng ml^{-1}) and 6 ng ml^{-1} (2-13 ng ml^{-1}) respectively (not including the not detectable samples). The authors recognised the fact that the ELISA and GC-ECD results correlated well, but that urinary results by GC-ECD were much higher than ELISA. The authors suggested that this was due to the increased linear range of GC-ECD relative to the ELISA method.

In 1993, Raeside et al. presented HPLC evidence for the presence of 19-hydroxyandrostenedione and 19-hydroxytestosterone in leydig cell incubates *in-vitro* and testicular venous plasma *in-vivo* from boars of the Yorkshire breed in a semi-qualitative

fashion. 19-hydroxytestosterone was present at apparently higher concentration in plasma than 19-hydroxyandrostenedione. Miyashita et al. 1990 (article in Japanese, but abstract in English) were able to detect 19-hydroxyandrostenedione (a putative precursor to 19-nor-androgens) and 19-norandrostenedione after *in-vitro* incubations of porcine adrenal tissue with androstenedione.

Van Cruchten et al. (2002) reported the presence of 19-nor-androgens in what initially appeared to be a female pig. However, close inspection determined that the animal was in fact a hermaphrodite (intersex animal) due to the presence of both a left ovary and a right un-descended testicle (with functioning leydig cells, but no spermatozoa). Samples of urine, fat, faeces, liver, kidney, muscle and testes were analysed for nandrolone and 19-norandrostenedione using GC-MS for fat, urine and faeces and LC-MS for the remaining matrices. Concentrations of nandrolone in fat, urine, faeces, liver, kidney, muscle and testes were 0.3 ng g^{-1}, 27 ng ml^{-1}, not detectable, not detectable, 1.6 ng g^{-1}, not detectable and 5.3 ng g^{-1} respectively, while concentrations of 19-norandrostenedione in these matrices were not detectable, 0.5 ng ml^{-1}, not detectable, not detectable, 1.1 ng g^{-1}, not detectable and 0.9 ng g^{-1} respectively. The authors proposed visual inspection of pig external sexual organs in female pigs suspected of nandrolone abuse at slaughter in order to discern false positives due to intersex.

Poelmans et al. 2005b reported the results of the most comprehensive analysis of nandrolone and boldenone related compounds in the porcine to date. Samples of muscle, liver, kidney, testicles and urine from boars, cryptorchids, barrows, gilts and sows derived from France, the Netherlands, Belgium and the USA were analysed for boldenone, nandrolone and 19-norandrostenedione using GC-MS and LC-MS (with n = between 5 and 14 for the different sexes). The results from an inter-sex animal were also reported, but the 19-norandrogen results for this animal appear to be the same as those given in Van Cruchten et al. 2002, so will not be reproduced again here. The results of this study are summarised in Table 2. Limits of detection were not reported. Since barrows are castrated males, the major source of steroid production in this animal is likely to be the adrenal gland. While it is theoretically possible that androgens produced by the adrenal gland in the barrow may be converted into 19-nor-androgens and/or their precursors in the systemic circulation, i.e. in adipose tissue, a study by Miyashita et al. 1990 suggests that these compounds may be directly secreted from the adrenal gland.

1.6.2.2   Metabolism following administration

Debruyckere et al. (1991) presented the results of a metabolism study of nandrolone in Gottinger x Vietnamese mini-pigs before and after injection of nandrolone laurate. One boar, one barrow and one sow (all two years old) were used and GC-MS was employed in a qualitative fashion to identify the compound present in urine. Nandrolone was detected in boar urine, but not barrow or sow urine, prior to nandrolone administration and in all animals after administration. No 17-keto, A-ring reduced compounds were detected in any of the animals before nandrolone administration, but after administration 19-noretiocholanolone and 19-norepiandrosterone were detected in all animals. 19-norandrosterone was detected in barrows after nandrolone administration, but not in the boar or sow. 19-Norepietiocholanolone was not detected after nandrolone administration in any animal. No estranediols were detected before nandrolone administration in any animal. Neither 5α-estrane-3α,17β-diol or 5α-estrane-3β,17α-diol were detected after nandrolone administration, but two other estranediols, proposed to be 5β-estrane-3α,17β-diol and 5α-estrane-3β,17β-diol, were found in some of the animals.

McEvoy et al. 1998 studied the bioavailability of nandrolone phenylpropionate in prepubertal Landrace gilts given the drug as either an IV or parenteral dose (in either arachis oil or aqueous solution). The same animals were used for each of the different formulations with suitable washout periods in-between doses and resulting plasma nandrolone levels were analysed by EIA. The bioavailability (and range) was 0.35% (0.25-0.41%) for the aqueous oral dose and 2.25% (0.86-2.85%) for the arachis oil oral dose. Peak plasma concentrations after the IV, arachis oil PO and aqueous PO doses were approximately 200, 36 and 3.2 ng ml^{-1} respectively. The plasma concentrations 24 hours after the IV dose were orders or magnitude lower than those resulting from oral dosing.

In 2007, Claus et al. studied the effect of sublingual nandrolone administration to five groups of five German Landrace barrows. two groups were controls, while the other three groups received either 1 mg testosterone, 1 mg, estradiol or 1mg nandrolone sublingually (under anaesthesia). Mean plasma nandrolone concentrations in pre-treatment animals, as measured by EIA, were 0.45 ng ml^{-1}, whilst mean maximum concentrations 10-minutes after nandrolone application were 4.3 ng ml^{-1}. Concentrations were still significantly higher (P<0.01) after 1-3 hours relative to controls.

Roig et al. 2007 reported the results of a quantitative GC-MS metabolism study following IM nandrolone laurate injection to a ten week old boar. Only post-administration urine results were reported. Following the administration, the boar urine contained nandrolone in predominantly the sulphate fraction (the most abundant analyte in this fraction) with peak concentrations of around 80 ng ml^{-1} at day one dropping to around 10 ng ml^{-1} by day four. Peak concentrations of different analytes in the glucuronic acid conjugate fraction were generally around 20 ng ml^{-1} or lower while in the free fraction, norepiandrosterone, noretiocholanolone and 5β-estrane-3α,17β-diol were most abundant with peak concentrations of around 40, 60 and 100 ng ml^{-1} respectively.

More recently, Ventura et al. 2008 extended the urinary methodology used in Roig et al. 2007 to look at the effect of administering 2 nandrolone laurate IM injections to a larger number of boars. The authors also assessed the endogenous concentrations from 60 boars taken during a routine monitoring programme. The post administration metabolite profiles were similar to those reported in Roig et al. 2008, but nandrolone and 19-norandrostenedione were also found to be present in the urine of untreated animals in the free, sulphate and glucuronide fractions. However, none of the A-ring reduced metabolites were detected in the untreated animals. The authors therefore proposed that noretiocholanolone and norepiandrosterone might be suitable qualitative markers for detecting nandrolone abuse in boars since they were detectable for the longest time period following nandrolone administration.

A major consideration regarding 19-nor-androgens in the porcine is whether or not these compounds are excreted as 19-nor compounds or whether they are, in fact, artefacts of sample preparation produced from an initial 19-carboxy metabolite; analogous to what is experienced in the male horse (Houghton et al. 2007). Several lines of evidence support this hypothesis, including the above detailed observation that no A-ring reduced metabolites are found in untreated boar urine (Debruyckere et al. 1991). These A-ring reduced metabolites would be expected to occur as metabolic products if free nandrolone and/or 19-norandrostenedione were present *in-vivo*. Their absence suggests that nandrolone and/or 19-norandrostenedione are naturally present as carboxylic acid precursors since these compounds would be unlikely to undergo A-ring metabolism directly. However, they may be cleaved to form nandrolone and 19-norandrostenedione during any sample preparation procedures that involve a derivatisation step or even a minor downward pH change. The 19-carboxylic metabolite of androstenedione has also

previously been identified in porcine granulosa cells (Garrett et al. 1991). Lastly, an unusually high ratio of 19-nor-androgens in boar urine, relative to faeces, has been observed (H. De Brabander, unpublished observation). This adds further weight to the theory of 19-nor-androgens in the porcine actually being the 19-carboxylic acid, as these acidic compounds could be substrates for organic anion transport proteins in the liver/gut that actively transport compounds from one area to another and maintain large concentration gradients (something that is less likely to occur for neutral steroids). If the compounds in porcine tissues that are currently thought to be 19-nor-androgens are proven to be predominantly 19-carboxylic acids (similar to the situation in the equine) then this could have major implications for nandrolone residue screening methods in the porcine. For example, Debruyckere et al. 1991 and Ventura et al. 2008 have shown that A-ring reduced metabolites are only detectable after nandrolone administration. It may therefore be feasible to use a threshold of nandrolone sulphate or an A-ring reduced metabolite as an indicator of nandrolone abuse in this species.

1.6.3 Boldenone and related androgens in the porcine

Only one published study relating to the occurrence of boldenone in the porcine was found. In a study on boars, barrows, gilts and sows, Poelmans et al. 2005b reported the concentrations of endogenous boldenone and 19-nor-androgens in muscle, liver, kidney, testicles and urine using GC-MS and LC-MS methods. Calibration ranges and LODs were not reported, but the resulting concentration ranges in the different tissues were reported. The results from this study are presented in Table 2 along with the nandrolone data discussed above. In addition to the data in Table 2, the authors looked at levels of boldenone in an intersex animal, with muscle, liver, kidney, urine and testicles found to contain no boldenone above the LOD. More studies to determine the endogenous occurrence of boldenone in the porcine are clearly warranted.

Table 2 – concentrations of nandrolone, 19-norandrostenedione and boldenone quantified by Poelmans et al. 2005b in different matrices from different sex porcine animals (the number of animals are given in brackets. < refers to concentrations below the LOD).

Nandrolone (ng g^{-1})	Gilt	Sow	Boar	Barrow	Cryptorchid
Meat	< (11)	0.4-0.5 (11)	0.7-13.4 (11)	0.7-11.8 (11)	0.1-2.4 (11)
Liver	0.1-0.9 (11)	< (11)	1-63 (11)	< (11)	0.2-12.3 (14)
Kidney	0.2-0.5 (11)	0.2-1.5 (11)	2.5-232 (11)	0.1 (10)	1.3-78 (14)
Urine (ng ml^{-1})	1.3-2.8 (11)	1.3-1.9 (9)	51-344 (11)	0.5-16.3 (11)	8.6-343 (14)
Testes	-	-	24-144 (5)	-	2.2-101 (11)
19-norandrostenedione (ng g^{-1})	**Gilt**	**Sow**	**Boar**	**Barrow**	**Cryptorchid**
Meat	< (11)	0.04-0.07 (11)	0.1-5.5 (11)	0.05-0.8 (11)	0.04-0.4 (11)
Liver	0.3-1.3 (11)	3.1-8.3 (11)	0.1-24 (11)	1.9-16 (11)	0.4-3.5 (14)
Kidney	8.3-25 (11)	2.7-18 (11)	2.3-535 (11)	0.1-15 (10)	0.2-159 (14)
Urine (ng ml^{-1})	1.8-17 (11)	0.9-18 (8)	5-109 (11)	1.1-16 (11)	9.9-103 (14)
Testes	-	-	6.2-110 (5)	-	1.3-25 (11)
Boldenone (ng g^{-1})	**Gilt**	**Sow**	**Boar**	**Barrow**	**Cryptorchid**
Meat	< (11)	< (11)	0.5-2.5 (11)	< (11)	0.7 (11)
Liver	< (11)	< (11)	1.3-4.9 (11)	< (11)	0.5-2.3 (11)
Kidney	< (11)	< (11)	0.8-9.2 (11)	< (10)	0.3-8.1 (14)
Urine (ng ml^{-1})	0.5-0.6 (11)	< (11)	5.1-120.5 (11)	1.1 (11)	0.9-57.6 (14)
Testes	-	-	2.1-16 (5)	-	0.6-15.1 (11)

1.7 Natural androgenic-anabolic steroid concentrations in the equine

Data on the endogenous presence of androgenic-anabolic steroids in the equine are summarised in Table 3 while details of the major phase 1 metabolic products following exogenous administration are given in Table 4.

While the majority of data reported for androgenic-anabolic steroids in the equine relate to urine (discussed below), interest is beginning to grow regarding their endogenous presence in blood. This is especially true in the US, where a large proportion of testing focuses on this matrix. While it has long been known that testosterone is endogenous in the blood of equine animals of all sexes, recent studies have also shown that nandrolone can be detected in intact males (Soma et al. 2007 and 2008). To date, nandrolone and boldenone related compounds have not been shown to occur naturally in the blood of non-pregnant females or castrated males and boldenone has not been shown to occur in the blood of intact males (McKinney, 2009). In the future, it may be possible to establish thresholds for endogenous steroids in blood, but all the currently internationally thresholds related to this matrix (IFHA, 2008). The following discussion therefore focuses on urine.

1.7.1 Testosterone

Concentrations of testosterone in equine matrices were not fully reviewed as part of the current survey, but testosterone and its precursors/metabolites are known to be endogenous in males and females of this species at varying concentrations. A large amount of data is available on the detection of steroid abuse in horseracing, the results of which are relevant to residue testing in meat production because the strategies for detection apply equally. The approach to controlling testosterone abuse in horseracing varies depending upon the sex and gestation status of the animal (James Scarth, personal experience with the application of these assays). There is no threshold in the intact male due to the high and variable concentrations produced in this animal. In the gelding and filly/mare, population studies have allowed urinary threshold concentrations for testosterone confirmation to be set at 20 and 55 ng ml^{-1} respectively in order to control the abuse of this steroid. A maximum urinary ratio threshold of testosterone:epitestosterone of 12:1 was in the past adopted in the filly/mare (analogous to the 4:1 ratio for either sex in human urine), but an absolute threshold of 55 ng ml^{-1} is

now used (IFHA, 2008). The major phase 1 urinary metabolites of testosterone after exogenous administration have been reported as 5α-androstane-3β,17β-diol, 5α-androstane-3β,17α-diol and 3β-hydroxy-5α-androstan-17-one (Dumasia and Houghton 1981).

1.7.2   Nandrolone

Early nandrolone administration studies on castrated males (geldings) in the 1970s and early 1980s suggested that 19-nor-androgens did not occur naturally in the horse (Houghton 1977, Houghton et al. 1978, Houghton and Dumasia 1980, Houghton and Teale 1981 and Dumasia and Houghton 1984). Indeed, since 19-nor-androgens have not been shown to be endogenous in the gelding or filly/mare, their presence at any concentration is considered evidence of abuse in horseracing (at least with current detection capabilities). Roig et al. 2007 have recently confirmed the findings of some of the earlier studies on nandrolone metabolism following its administration to geldings. While pre-administration samples contained no 19-nor-androgens, the major post-administration metabolites were 5α-estrane-3β,17α-diol in the glucuronic acid conjugate fraction followed by nandrolone in the sulphate fraction. In contrast to the situation in geldings and filly/mare, in 1984 Houghton et al. showed that the urine of untreated intact male horses appeared to contain nandrolone and a range of other 19-norandrogens. A threshold approach utilising a ratio of 5α-estrane-3β,17α-diol:estr-5(10)-ene-3β,17α-diol of greater than 1:1 as indicative of abuse was subsequently introduced for detecting nandrolone abuse in intact male horses (Houghton et al. 1984). However, this has now been replaced with a threshold for the nandrolone metabolite 5α-estrane-3β,17α-diol of 45 ng ml^{-1} in urine (IFHA, 2008).

Despite the lack of data to suggest that 19-norandrogens are endogenous in non-pregnant filly/mares, Sterk et al. 1998 have shown that pregnant mares may excrete the epinandrolone naturally in urine at concentrations ranging from less than their LOD of 1 ng ml^{-1} up to 26 ng ml^{-1}. Nandrolone was not detected at any concentration above the LOD of 1 ng ml^{-1}. This study highlights the need to consider the gestation status in mares.

Although intact male horse urine has for many years been considered to contain 19-nor-androgen sulphates, Houghton et al. 2007 have recently shown that these compounds

are predominantly artefactual in origin. The authors suggested that it is in fact 19-carboxy-nandrolone and 19-carboxyandrostenedione that occur naturally in intact male horse urine and that their decarboxylation under acidic sample preparation procedures leads, respectively, to the artefactual production of nandrolone and 19-norandrostenedione. The finding that nandrolone and 19-norandostenedione may be predominantly de-carboxylation artefacts during sample preparation is significant since nandrolone administration studies in horses have confirmed that, exogenously, nandrolone *is* excreted as a sulphate (Teale et al. 2000). This study also raises the possibility that 19-nor androgens found in other species, i.e. the porcine, may also be artefactual in origin.

In 2008, Grace et al. used LC-MS/MS analysis of intact nandrolone sulphate to demonstrate that while the majority of nandrolone detected in intact male urine may be artefactual in nature, low concentrations of nandrolone sulphate are present. In a post-race samples population of 77 intact males, nandrolone sulphate was present at concentrations ranging from less than the LOQ (1 ng ml^{-1}) up to 21 ng ml^{-1}. Because these concentrations are significantly lower than those typically produced following nandrolone administration, the authors proposed that a threshold of nandrolone sulphate in urine may be suitable alternative to the current 'free and glucoconjugated' 5α-estrane-3β,17α-diol threshold currently in use.

1.7.3   Boldenone

Boldenone has recently been shown to be naturally occurring as a sulphate conjugate in the urine of intact males (Ho et al. 2004). However, to date it has not been reported to be endogenous in geldings or fillies/mares. The major phase 1 urinary metabolite of boldenone after exogenous administration is epiboldenone in the glucuronide fraction, but significant quantities of parent boldenone are excreted as a sulphate conjugate (Houghton and Dumasia 1979). 1-Dehydro-androgens are prohibited at any concentration in the urine of non-pregnant females or castrated males, but a urinary threshold of 15 ng ml^{-1} has been validated internationally for controlling abuse in males (IFHA, 2008).

In 2008, Grace et al. applied LC-MS/MS analysis of intact boldenone sulphate to a post-race urine sample population of 77 intact males, and found that boldenone sulphate was

present at concentrations ranging from less than the LOQ (0.5 ng ml^{-1}) up to 3 ng ml^{-1}. Because these concentrations are significantly lower than those typically produced following boldenone administration, the authors proposed that an intact boldenone sulphate urinary threshold may be a useful alternative to the 15 ng ml^{-1} 'free and conjugated boldenone' threshold currently in use.

1.8   Natural androgenic-anabolic steroid concentrations in the cervine

Data on the endogenous presence of androgenic-anabolic steroids in the cervine are summarised in Table 3. Data on the major phase 1 metabolic products following exogenous administration was not available.

Concentrations of testosterone in cervine matrices were not reviewed as part of the current survey, but testosterone and its precursors/metabolites are known to be endogenous in males and females of this species at varying concentrations (Hamasaki et al. 2000). Only one report on the natural occurrence of 19-nor-androgens in the cervine was found. Van Hende (1995) analysed the urine of pregnant red deer for the presence of epinandrolone. Using semi-quantitative GC-MS analysis, epinandrolone was not detected at an LOD of 1 ng ml^{-1} in two of the animals and was somewhere between 1 and 2 ng ml^{-1} in the third animal. The ages, parity, fetal number and gestation status of the animals was unknown. No reports on the endogenous or post-administration levels of boldenone or related compounds in the cervine were found, but one of 35 cervine urine samples analysed for nandrolone and epinandrolone in an Australian national monitoring programme (not therefore a controlled population with respect to ruling out steroid abuse) was above the 1 ng ml^{-1} LOD for epinandrolone with a concentration of 4.5 ng ml^{-1}. No samples were above the 1 ng ml^{-1} LOD for nandrolone. Clearly, more work is required to establish the endogenous versus exogenous nature of 19-nor and 1-dehydro-androgens in deer of different ages, sexes, gestation/castration status and breeds etc.

1.9   Natural androgenic-anabolic steroid concentrations in the caprine

Data on the endogenous presence of androgenic-anabolic steroids in the caprine are summarised in Table 3. Data on the major phase 1 metabolic products following exogenous administration was not available.

Concentrations of testosterone in caprine matrices were not reviewed as part of the current survey, but testosterone and its precursors/metabolites are known to be endogenous in males and females of this species at varying concentrations (Ahmad et al. 1996, Flint and Burrow 1979). Only two reports on the occurrence of 19-nor-androgens in the caprine were found and both relate to female urine. Van Hende (1995) analysed the urine from two pregnant animals and a pool of urine from a number of pregnant animals for the presence of epinandrolone. Using semi-quantitative GC-MS analysis, the two individual samples were found to contain epinandrolone at a concentration above 2 ng ml^{-1}, while the pooled urine contained concentrations somewhere between 1 and 2 ng ml^{-1}. The ages, parity, fetal number and gestation status of the animals was unknown. In 1998, Sterk et al. analysed the urine from two pregnant animals throughout gestation and four non-pregnant animals for the presence of both nandrolone and epinandrolone using GC-MS (LOD of 1g ml^{-1} for each). Nandrolone was not detected in any of the samples while epinandrolone was only detected in one sample from one animal at 16-weeks into pregnancy with a concentration of 2 ng ml^{-1}. The ages and parity of the animals was not reported. No reports on the endogenous or post-administration concentrations of boldenone or related compounds in the caprine were found. Clearly, more work is required in order to establish the endogenous versus exogenous nature of 19-nor and 1-dehydro-androgens in goats of different ages, sexes, gestation/castration status and breeds etc.

1.10   Summary tables of the occurrence and metabolism of endogenous steroids in meat-producing animals

Data on the endogenous presence of androgenic-anabolic steroids in the species currently reviewed are summarised in Table 3 while details of the major phase 1 metabolic products following the exogenous administration of testosterone, nandrolone and boldenone are given in Table 4.

Table 3 – summary of the endogenous occurrence of androgenic-anabolic steroids in mammalian meat-producing animal species. NMP = National Monitoring Programme, therefore not controlled with respect to steroid abuse.

Details of endogenous presence in different species

Steroid	Bovine	Ovine	Porcine	Equine	Cervine	Caprine
Testosterone	Ubiquitous in males and females at varying concentrations	Ubiquitous in males and females at varying concentrations	Ubiquitous in males and females at varying concentrations	Ubiquitous in males and females at varying concentrations	Ubiquitous in males and females at varying concentrations	Ubiquitous in males and females at varying concentrations
Nandrolone and related 19-nor androgens	Epinandrolone detectable during pregnancy. Most other studies find no nandrolone or related metabolites in males, but some find trace amounts in male and female urine i.e. following casualty (Kennedy et al. 2009)	Epinandrolone detectable during pregnancy. Two reports published on the endogenous presence of urinary nandrolone, epinandrolone and 19-norandrostenedione in male + female UK and Austrian populations	Nandrolone and 19-norandrostenedione detected in urine and some other matrices of animals of all sexes (including intersex animals) at different concentrations	Detected in urine of pregnant mares and at high concentrations in stallions (probably as a byproduct of the high concentration of aromatisation in the testes) but not in geldings or fillies	Urinary epinandrolone detected in a pregnant red deer, but no other animals studied. One of 35 urines from an Australian NMP contained epinandrolone but not nandrolone	Epinandrolone, but not nandrolone, detected in urine during pregnancy, while neither analyte detected in non-pregnant females. Studies on endogenous concentrations in males is lacking
Boldenone and related 1-dehydro androgens	Boldenone and related compounds have been detected in urine and faeces, possibly secondary to their formation by gut bacteria. Some phase 1 + 2 metabolites only detected after boldenone admin. Boldenone precursors also detected in wood crates from calves housing	Insufficient data to draw any conclusions, although 2 of 961 urines from an Australian NMP contained low concentrations of epiboldenone	Boldenone detected in urine and some other matrices of boars, cryptorchids, gilts and barrows at different concentrations, but not above the LOD in sows or an intersex animal	Boldenone detected at low concentrations in the urine of stallions, but not geldings or fillies	Insufficient data to draw any conclusions, although zero of 35 urines from an Australian NMP contained boldenone or epiboldenone	Insufficient data to draw any conclusions

*Table 4 – summary of the major catabolic pathways of endogenous androgenic-anabolic steroids in mammalian meat-producing animal species. * More definitive quantitative studies required in order to confirm results*

Steroid	Details of major phase 1 urinary metabolic products after administration to different species					
	Bovine	**Ovine**	**Porcine**	**Equine**	**Cervine**	**Caprine**
Testosterone	Epitestosterone, epietiocholanolone, 5β-androstane-3α,17β-diol + 5β-androstane-3β,17α-diol	Epitestosterone, androsterone, etiocholanolone + 5β-androstane-3α,17β-diol *	Insufficient data to draw any conclusions	5α-androstane-3β,17β-diol, 5α-androstane-3β,17α-diol + 3β-hydroxy-5α-androstan-17-one	Insufficient data to draw any conclusions	Insufficient data to draw any conclusions
Nandrolone	Epinandrolone, 5α-estrane-3β,17α-diol, 5β-estrane-3α,17β-diol, 5β-estrane-3α,17α-diol, 5α-estrane-3β,17β-diol, 19-noretiocholanolone, 19-norandrostenedione	Epinandrolone (more data required to confirm whether other metabolites also present)	19-norepiandrosterone, 19-noretiocholanolone + 5β-estrane-3α,17β-diol	5α-estrane-3β,17α-diol	Insufficient data to draw any conclusions	Insufficient data to draw any conclusions
Boldenone	Epiboldenone, 17α-hydroxy-5β-androst-1-en-3-one, 6β-hydroxyepiboldenone, 17β-hydroxy-5β-androst-1-en-3-one + 3α-hydroxy-5β-androst-1-en-17-one.	Insufficient data to draw any conclusions	Insufficient data to draw any conclusions	Epiboldenone	Insufficient data to draw any conclusions	Insufficient data to draw any conclusions

1.11   Alternative methods for detecting endogenous steroid abuse

The discussions in this chapter up to now have focussed mainly on direct, univariate steroid measurements. This is because the majority of published methods for detecting their abuse have to date been based on identifying suitable 'qualitative marker metabolite' or 'quantitative threshold' approaches. However, there is increasing interest in the use of alternative approaches to detect endogenous steroid abuse. These include the use of biosensors, biomarkers, gas chromatography-combustion-isotope ratio-mass spectrometry (GC-C-IR-MS) or the detection of intact steroid esters. While none of these techniques form the basis of the experimental side of this thesis, the following discussion serves to provide an overview of this emerging field.

1.11.1   Biosensors and biomarkers

As an alternative to the direct univariate measurement of endogenous steroids in biological matrices, a range of assays based on detecting the biological *effects* of the steroids have been developed. These assays can be broadly split into the areas of 'biosensors' and 'biomarkers'. Biosensors utilise biological techniques to detect the presence of steroidal activity in a sample *ex vivo* (outside of the body), whereas biomarker techniques aim to monitor activity through perturbation of 'normal' *in vivo* physiological parameters. The following discussion introduces some of different approaches that have been investigated according to these two definitions.

1.11.1.1   Biosensors

Biosensors show a great deal of promise for detecting steroid abuse; especially where designer steroids are concerned (discussed further in chapter 4). However, their use is likely to be restricted to screening rather than confirmation of abuse since the structure of the steroid in typically not identified unequivocally. Also, they are less useful for discerning the abuse of endogenous steroids since, by definition, these compounds are natural and some level of biological activity is therefore always present. Given these possible limitations, the following discussion briefly highlights some of the different biosensor approaches available.

In certain respects, biosensors have been applied to residue surveillance for years in the form of immunoassays (Mooney et al. 2009a). Whilst many of the traditional immunoassays have now been replaced with more selective mass spectrometric based methods of detection, an interesting ELISA based assay has recently been reported by Hungerford et al. (2005). This approach utilises an antibody that recognises steroids containing a 17α-methyl,16β,17β-dihydroxy epitope. It therefore produces a suspicious result for any 17α-methyl steroid that undergoes 16β-hydroxylation as part of it's metabolism. Since 16β-hydroxylation is a major pathway of metabolism of such steroids, especially in equine animals (Houghton, 1992), this highlights a suspicious sample for further work to identify the unknown molecules present.

Beyond the established field of immunoassay, several biologically based measurement technologies have recently been developed and are commonly classified as 'biosensor' technologies. Two such techniques are surface plasmon resonance (SPR) and potentiometric detection, where antibody-antigen binding can be measured quickly and assay times therefore reduced. For example, an SPR based immunobiosensor has recently been developed to detect abuse of steroids through a reduced sex hormone-binding globulin binding capacity in bovine plasma (Mooney et al. 2009b). A further application in the biosensor field utilises knowledge of the endogenous biological receptor responsible for the pharmacological effect of the drug. The principle of this approach is that all drugs sharing a common method of action should be detectable through measuring the response of a recombinantly produced version of the relevant receptor. One particularly attractive class of compounds for application of this technology are AASs, since most of these bind to the androgen receptor. However, alternative approaches are also possible for oestrogens, progestagens and glucocorticoids through use of their respective endogenous receptor proteins. A number of radioreceptor assays have been developed, but have the disadvantage of requiring the use of radioactivity (Mooney el al. 2009a). More attractive assays based on reporter gene detection have since been developed. As an example, Peters et al. 2010 have reported the liquid chromatographic separation of extracted urinary steroids prior to analysis using both an androgen receptor reporter gene bioassay and a time-of-flight mass spectrometry (TOF). In addition to identifying steroids such as testosterone with known structures, this approach is also able to identify novel anabolic steroids such as the 'designer' steroid tetrahydrogestrinone (see chapter 4). An extension of the aforementioned method utilises liver S9 bioactivation to convert biologically inactive versions of steroids

(prohormones or metabolites) into active versions that can be detected by the androgen receptor (Rijk et al. 2008).

1.11.1.2 Biomarkers

The rapidly advancing applications of 'omics' related technologies, which allow the simultaneous analysis of a large number of components within a biological system, have huge potential to transform the way drug residue surveillance detection is performed. The idea behind applying the 'omics' approaches are not to detect the presence of a drug directly, but instead to be able to detect its cumulative biological effect (biomarker) within the animal through either targeted (pre-defined profiling) or untargeted (global profiling) approaches. One of the key advantages of the biomarker approach is that no matter what method of doping is used, then a change in the mRNA (transcriptomics), protein (proteomics) or metabolite (metabolomics) profile should be detectable for drugs with common pharmacology. The definition of a 'normal' versus 'suspect' biomarker profile depends on whether 'latitudinal' or longitudinal' comparisons are applied – both of which are currently being investigated. Latitudinal comparisons rely on analysing large populations of individual subjects and defining a 'normal' range, a value outside of which is considered suspect. Longitudinal measurements on the other hand could use a change in an animal's own unique biomarker profile over time to indicate whether external perturbation had occurred.

The following discussion introduces a representative sample of the different 'omics' and other biomarker approaches that have been reported in the veterinary drug residue surveillance area. A full discussion of this topic is beyond the scope of this thesis. For more detailed account the reader is directed to a recent review by Mooney et al. (2009a).

As an example of a recent transcriptomics approach, Riedmaier et al. (2009) have reported the effect of trenbolone acetate plus oestradiol administration on the profile of 38 pre-defined candidate genes in bovine calf blood. The authors determined gene expression in blood cells by quantifying concentrations of mRNA using quantitative real time reverse transcriptase polymerases chain reaction technology (qRT-PCR) and found that 11 of the 38 candidate genes were influenced by treatment. These included the oestrogen receptor-α, the glucocorticoid receptor-α, the apoptosis regulator Fas, the proinflammatory interleukins IL-1α, IL-1β and IL-6 as well as the major histocompatibility

complex II, creatine kinase, myotropin, RNA binding protein 5 and actin-β. Principal component analysis was then applied and was able to discern untreated animals from those treated at days 2 and 16 post-administration.

As an example of a recent proteomic approach, Cacciatore et al. (2009) have reported the effect of concomitant oestradiol and nandrolone on the plasma concentrations of a range of pre-defined candidate proteins using a number of different detection techniques for the various analytes. Concentrations of immunoreactive inhibin were found to be decreased following administration of the steroids to male calves for up to 38 days, but not in females. Concentrations of the remainder of the targeted proteins including insulin-like growth factor-1, insulin-like growth factor-binding proteins-2 and 3, luteinising hormone, follicle-stimulating hormone and prolactin were not affected by steroid treatment.

As an example of a recent metabolomics approach, Di Nardo et al. (2010) have reported the effect of androstenedione administration on the profile of phase 2 steroid conjugate metabolites in bovine urine. The authors used a combination of targeted (selected reaction monitoring) and semi-targeted (precursor ion scanning) LC-MS/MS approaches to identify altered metabolite profiles. Using the targeted approach, concentrations of epiandrosterone-3-sulphate were found to be unregulated in treated animals, while some unidentified metabolites were found to be unregulated when applying the semi-targeted approach.

As an example of an alternative type of biomarker approach, the 1992 PhD thesis of Maria Groot describes and interesting study based on 'Histological Screening for illegal administration of growth promoting agents in veal calves.' This technique showed some potential for using the effects of androgens and oestrogens on male prostate or female clitoris/Bartholin gland as biomarkers of abuse and has recently been followed up by looking at the range of 'normal' histological findings in large numbers of untreated calves (Groot et al. 2007). However, the technique can only be applied post-mortem and, to date, has only been adopted for routine screening in a small number of countries (Maria Groot, personal communication).

Whether biomarker approaches are suitable as confirmatory techniques or just as screening tools remains to be determined. However, further studies are clearly warranted in order to investigate their great potential.

1.11.2   Combustion isotope ratio mass spectrometry studies

Results of gas chromatography combustion isotope ratio mass spectrometry studies (GC-C-IRMS) are considered separately from those quantifying concentrations in bovine tissues because GC-C-IRMS measures the relative composition of ^{12}C and ^{13}C atoms of steroids rather than their absolute concentrations. However, since the aim of using GC-C-IRMS is to be able to distinguish natural from exogenous steroids, a consideration of the results of studies using this technique is relevant to the current review.

The majority of published studies relate to the bovine, but one study looked at porcine tissues. The following overview of the potential use of this technique was based on results of published studies as well as an executive summary from the EU supported ISOSTER project GRD1-2001-40085 (which coordinated several of the published studies).

The technique of GC-C-IRMS relies on detecting differences in the relative ^{12}C and ^{13}C composition of steroids between the natural and synthetic states. Synthetic steroids are typically synthesized from a single C3 plant (often soy), while the natural diet of the bovine is usually a mixture of both C3 and C4 plants (Balizs et al. 2005). The terms C3 and C4 refer to the type of metabolic pathway used by the plant in synthesising organic compounds during photosynthesis, utilising either 3 or 4 carbon-chain metabolites respectively. The significance of this lies in the fact that the two types of pathway display differing degrees of discrimination against ^{13}C and thus result in different ^{13}C to ^{12}C ratios. C4 plants have lower discrimination against ^{13}C than C3 plants, resulting in higher ^{13}C to ^{12}C ratios in C4 plant material (Balizs et al. 2005). Since steroids produced within the body will derive carbon from both C3 and C4 plant material of dietary sources, the resulting ^{13}C to ^{12}C ratio will be lower after exogenous steroid administration (mainly C3 plant material derived) relative to the endogenous state.

The method itself requires substantial sample preparation prior to analysis including hydrolysis, solid phase extraction, liquid-liquid extraction and HPLC fractionation steps,

as the influence of matrix interferences need to be minimised. Suitable derivatisation of the extracts followed by separation using gas chromatography further purifies the extract before introduction into a furnace. The furnace then combusts the introduced sample, which is then analysed alongside a reference gas by mass spectrometry in order to determine the relative levels of ^{13}C and ^{12}C (Prevost et al. 2004).

The resulting ^{13}C to ^{12}C ratio determined by GC-C-IRMS is usually expressed as a $\delta^{13}C$ value, calculated as follows:

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

The range of $\delta^{13}C$ values for C3 plants (hence also abused steroids) is around -25 to -35$^o/_{oo}$, whereas for C4 plants is around -11.9 to -15.2$^o/_{oo}$ (Mason et al. 1998). With diets usually containing both C3 and C4 plants, the range of $\delta^{13}C$ values for endogenous steroids is usually somewhere between these two ranges. However, as will become apparent later in this section, the exact location on this continuum will depend on the type of diet fed to the animal (Buisson et al. 2005). The $\delta^{13}C$ values of the steroid or metabolite being measured is usually referenced to an endogenous reference compound (ERC) such as DHEA that is not affected by exogenous steroid administration (Ferchaud et al. 1998). This is to ensure that the endogenous make up of the animal has not been affected in some way (i.e. diet) so as to give low $\delta^{13}C$ values for all steroids. A high relative difference between the $\delta^{13}C$ values of the steroid or metabolite and the ERC can therefore be used as an indicator of abuse. The absolute sensitivity of the method depends to some extent on the analyte and matrix in question, but 10 ng ml^{-1} is typically required, meaning that only some analyte matrix combinations are currently suitable (ISOSTER project [GRD1-2001-40085], publishable executive summary 2006 and personal observation of Bruno Le Bizec).

As mentioned elsewhere in this review, diet is a major factor influencing the $\delta^{13}C$ value of endogenous steroids. In the UK for example, animals are typically fed a much higher base of C3 plants than in Europe, leading to a lower difference in the $\delta^{13}C$ values

between administered steroids and the ERC (Mason et al. 1998). However, as studies by Buisson et al. 2005 and Hebestreit et al. 2006 have shown, the difference after steroid administration to animals consuming a predominantly C3 diet is still usually sufficient to discern testosterone abuse. The Hebestreit et al. 2006 study showed that after testosterone administration, the difference in $\delta^{13}C$ between the testosterone metabolite etiocholanolone and the ERC DHEA for C4 plant fed animals (maize) was typically around $10^o/_{oo}$, whereas the difference for C3 fed animals (grasses) was typically around $4^o/_{oo}$. In the endogenous state, the difference in $\delta^{13}C$ between the testosterone metabolite etiocholanolone and the ERC DHEA was a mean of $0.9^o/_{oo}$ with a standard deviation of $0.7^o/_{oo}$. Applying a confidence interval of three standard deviations to either side of the mean, endogenous $\delta^{13}C$ difference between etiocholanolone and the ERC DHEA allowed a 100% discrimination of samples as either positive or negative.

The ISOSTER project successfully validated the use of this technique for detecting testosterone abuse in urine via monitoring of etiocholanolone values in relation to DHEA as the ERC. The method was also applied to steroids in other tissues, but in most cases the steroid or ERC contents were too low to be of use in the technique. In addition to testosterone, Buisson et al. 2005 showed that detection of oestradiol abuse in the bovine was possible via monitoring the $\delta^{13}C$ value of the urinary oestradiol metabolite epioestradiol (17α-oestradiol) relative to DHEA. GC-C-IRMS has also been used to analyse porcine nandrolone and 19-norandrostenedione concentrations in testicles, liver and kidney, although post administration samples were not included (Prevost et al. 2004).

One question that still remains to be answered (several researchers, personal communications) is what the final statistical approach to determining the threshold for $\delta^{13}C$ value differences between target steroids and the ERC will be. If the use of an ERC is adopted as standard, then it is theoretically possible that some positive samples would be called negative due to a high proportion of C3 plant in their diet, but the converse situation of calling false positives is less likely than using the technique without an ERC.

The laborious nature of the sample preparation technique currently makes the technique unsuitable for use as a screening tool (various researchers, personal communications), but it has already found use as a confirmatory technique for determining testosterone abuse in human sports (Saudan et al. 2006). The executive summary of the ISOSTER

project GRD1-2001-40085 in 2006 showed that the GC-C-IRMS method for detecting testosterone abuse in bovine urine was successfully validated in several European laboratories. *It is therefore recommended that the use of this technique be further explored in order to act as a confirmatory method following screening using a uni- or multi-variate threshold of some kind.*

1.11.13 The detection of intact steroid esters in hair and plasma

The majority of injectable steroid preparations contain steroids in esterified forms. The direct detection of steroid esters in matrices from an animal may therefore be indicative of steroid abuse. The only exception to this would be if the ester is also known to occur naturally. For example, oestradiol and testosterone have been shown to be present in enzymatically hydrolysed fractions, but not unhydrolysed fractions, of human high density lipoprotein and rat adipose tissue respectively (Hockerstedt et al. 2006 and Borg et al. 1995). While the exact nature of steroid esters were not unequivocally identified in either of these studies, the oestradiol related compound identified by Hockerstedt et al. 2006 co-crystallised with an oestradiol stearate reference standard. However, in all studies that have looked at the detection of steroid esters in animal tissues following administration of commercially available steroid ester preparations, no endogenous concentrations of the esters were detected in the pre-dose or untreated samples. Depending on the matrix in question, contamination issues also need to be considered carefully in order to eliminate environmental contamination as a possible cause of false non-compliant results.

The detection of intact steroid esters in hair has attracted the most attention in the literature for this purpose and has already found use in some European labs as a confirmatory technique for detecting natural steroid abuse (see for example Marcos et al. 2004, Nielen et al. 2006, Boyer et al. 2007, Anielski, 2008, Stolker et al. 2009 and Duffy et al. 2010). Until recently, the detection of intact steroid esters in plasma was hampered by their typically low concentrations in this matrix relative to analytical limits of detection (Kim et al. 2000, Hooijerink et al. 1994). However, analytical methods based on the detection of intact testosterone, nandrolone and boldenone esters below 0.01 ng ml^{-1} in equine plasma have recently been developed (Gray et al. 2010). When a testosterone ester is administered as a depot injection, these sensitive assays allow detection of testosterone abuse in female and castrated male equine animals for several weeks

longer than the existing (2010) international urinary testosterone concentration thresholds (IFHA, 2008). They also allow the detection of testosterone abuse in intact males for the first time. However, these assays are not suitable for detecting the abuse of steroids that are administered in a non-esterified form. Also, it is likely that the detection time for the administration of an oral preparation of steroid esters would be reduced compared to that of a depot injection due to the lack of sustained release. Despite these potential limitations, the detection of steroid esters shows great promise for detecting the abuse of endogenous steroids (at least when they are administered as esters), more studies in other species and matrices are clearly warranted.

1.12   Summary and consideration of areas for future research

In this chapter, the occurrence of steroids related to testosterone, nandrolone and boldenone in bovine, ovine and porcine matrices, as well as the occurrence of steroids related to nandrolone and boldenone in equine, caprine and cervine matrices have been reviewed.

Several specific questions have been raised during this review, including some unusually high steroid concentrations under specific physiological conditions and a relevant dearth of information regarding some steroids in different species (i.e. boldenone/ nandrolone). Furthermore, continuously improving standards of analytical detection, as well as artificial selection within certain animal populations suggests that constant re-evaluation of detection approaches is necessary. An ideal situation might therefore be analogous to human sports: the use of a uni/multivariate concentration threshold or biomarker approach as a screening strategy (as are currently in use for some steroid/matrix/species combinations already) and the use of more laborious but definitive techniques such as carbon isotope ratio mass spectrometry, intact steroid ester analysis or more conservative concentration thresholds for confirmation.

A major deficiency in much of the existing published literature is the lack of standardisation and formal validation of experimental approach. Key articles are cited that highlight the huge variation in reported steroid concentrations that can result when samples are analysed by different laboratories under different conditions. These deficiencies are in most cases so fundamental that it is difficult to make reliable comparisons between data sets and hence it is currently impossible to recommend

definitive detection strategies. Standardisation of experimental approach would need, most importantly, to involve communication among researchers before experiments are conducted and methods cross-validated. As far as cross-validation is concerned, it is also important to ensure access to the relevant reference standards and/or incurred sample material.

In order to standardize data amongst researchers aiming to producing thresholds for controlling steroid abuse, the following specific recommendations are made:

Physiologically based recommendations:

1) Adequate consideration should be given to the demographic of the animal population used for the study. Specifically, the population needs to be representative of that which control of abuse is aimed at i.e. it must take into account sex, age, gestation and castration status, breed (genetic history), housing conditions, geographical region of origin, disease, medication, hydration and stress status, diet, housing, time of sampling (clock hour and sun hour) and season (and particularly the terminology surrounding season i.e. the word 'September' may relate to a different photoperiod in one hemisphere compared to the other hemisphere so could lead to confusion when considering seasonal effects). When delineating the resulting data for the control of endogenous steroid abuse, it would seem necessary to separate animals based upon, at the very least, their age, sex, castration, gestation, disease (particularly casualty animals) and medication status otherwise the variation under these different conditions may lead to irrelevantly high thresholds for the control of steroid abuse. However, there would appear to be balance between a physiological need to separate animals and a statistical requirement for a large enough population on which to base threshold calculations.

2) When considering sample collection strategies, it is very difficult to produce unilateral recommendations because different matrices are likely to be suitable in different locations i.e. many labs will have access to urine or plasma, but export testing programmes may need to analyse meat. Factors that apply to all situations include the need to avoid contamination of one matrix with that from another e.g. no faecal contamination of urine and the need for samples to be frozen as quickly as possible after collection in order to reduce degradation or the artefactual production of certain

steroids. It may be necessary to consider data derived from live animals differently to dead animals, not necessarily because the physiology of a live animal would be different to a dead animal, but because 'on-farm' testing of live animals compared to 'slaughter-house' testing of dead animals may have implications for type of matrix used, the age of the animal, stress levels etc. When sampling from dead animals, the time from slaughter to sample collection should be minimised.

3) Care should be taken to distinguish between the usefulness of longitudinal and latitudinal studies. Latitudinal studies may be of most use when applying unilateral thresholds to whole populations, while longitudinal studies may be of more use in probing the individual variables that may need to be taken into account when designing the demographic of animal populations used in latitudinal studies.

Analytically based recommendations:

1) All methods should be subject to a full quantitative validation prior to use. As a minimum, the following factors should be covered and reported:

   - Intra- and inter-batch precision and accuracy.
   - The ability to dilute samples from outside the calibration range.
   - Method selectivity.
   - Method sensitivity (defined here using the common [non-IUPAC] usage of this term to describe the ability of the method to determine low concentrations of sample). The LOD or CCα/β and LOQ should be reported where appropriate.
   - Recovery.
   - Linearity.
   - Stability (of analyte solutions, extracted samples and samples stored in matrix under the appropriate conditions).

2) Following validation of any new method, the laboratory should attempt, where possible, to cross-validate their method with those from other laboratories and participate in the inter-laboratory exchange of QC samples.

3) Data should only be considered fully quantitative if a certified reference standard is available.

4) Mass spectrometry (preferably MS/MS) following chromatographic separation should be the method of choice for definitive quantitative studies.

5) Measures should be taken to ensure suitable chromatographic reproducibility and resolution; especially where isomers of the target analyte are suspected to be present.

6) Sample preparation procedures should be designed such that it can be stated whether data are derived from an analysis of the 'total' concentration of steroid, or the concentration from a particular fraction i.e. glucuronic acid conjugates. When hydrolysing glucuronide steroid conjugates, recombinant versions of the glucuronidase enzymes should be used. This is because heterogeneous preparations such as that from *Helix pomatia* are known to produce artefacts of certain steroids.

7) Given the above quantitative validation requirements, consideration needs to be given as to whether calibration lines and QCs are best prepared in matrix i.e. standard addition, or through alternative means i.e. using isotope dilution or surrogate matrices. The most suitable option will depend on the steroid, matrix and species in question.

8) Where possible, attempts should be made to monitor various precursors, metabolites and other compounds related to the steroids in question so that multi-variate approaches based on the perturbation of 'normal' profiles can be investigated.

9) Once published, the raw data from all studies should be made available to the EU Community Reference Laboratory (CRL) so that independent statisticians are able to combine data from a number of studies and produce more workable unilateral thresholds. The current approach of reporting certain statistical parameters, but not others, makes the amalgamation of data-sets difficult.

General recommendations:

When publishing the results of studies, as many details as possible regarding the aforementioned factors (including, critically, the analytical method validation results) should be reported so that comparisons can be made between different studies.

Further work:

The results of this literature review (originally completed in February 2007 but since updated for publication in *Food Additives and Contaminants* in June 2009 and then again for the current thesis in October 2010) were discussed with Sponsors at the UK Government Department of Food, Environment and Rural Affairs (DEFRA) in 2007 and used to inform an appropriate strategy for the remainder of part 1 of this thesis. As discussed in the introduction, this involved developing approaches for the detection of nandrolone abuse in the porcine (chapter 2) and for the detection of androgen, oestrogen and progestagen abuse in the bovine (chapter 3).

1.14   References

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Chapter 2: Validation and application of an analytical biomarker approach for the detection of nandrolone abuse in the porcine

After:

2.1   Introduction

As discussed in the introductory chapter, the use of anabolic steroids as growth promoting agents in food production is prohibited under European Union legislation (European Union, 1996). However, some androgenic-anabolic steroids, such as testosterone (17β-hydroxy-androst-4-en-3-one), nandrolone (17β-hydroxy-estr-4-en-3-one), and boldenone (17β-hydroxy-androsta-1,4-dien-3-one), are known to be endogenous in certain species (Scarth et al. 2009), making a simple qualitative determination of their presence insufficient for proving abuse (see chapter 1 for further details). The detection of intact steroid esters or the use of combustion isotope ratio mass spectrometry can be useful for the confirmation of certain endogenous steroids (Scarth et al. 2009), but these methods are not suitable for all steroids or are too laborious to be used as screening approaches. Therefore, a quantitative threshold or biomarker approach is usually required to regulate the use of endogenous steroids.

For many years, nandrolone was considered to be a purely synthetic steroid, but in the 1980s it was isolated as a natural hormone in the stallion (Houghton et al. 1984) and

boar (Maghuin-Rogister et al. 1988). Since then, nandrolone related compounds have also been detected in the bovine (Vandenbroecke et al. 1991), ovine (Clouet et al. 1997), caprine (Sterk et al. 1998), human (Dehenin et al. 1984) and cervine (Van Hende, 1995). The presence of nandrolone in boar meat also has potential consequences for consumers, since it has been shown that following consumption of boar meat, human urine contains increased concentrations of nandrolone metabolites (Le Bizec et al. 2000).

Studies on the metabolism of nandrolone following administration to the pig (Roig et al. 2007, Ventura et al. 2008) have shown that nandrolone itself is excreted in urine predominantly as a sulphate, while a number of A-ring reduced metabolite isomers are present in the free, glucuronide and sulphate fractions. Although nandrolone has been found to be present in the urine and several other matrices of untreated porcine animals (Debruyckere et al. 1990, De Brabander et al. 1994, Poelmans et al. 2005), these studies have reported that the A-ring reduced metabolites were not detected in porcine urine.

The absence of A-ring reduced metabolite in the 'natural' state is hard to rationalise if nandrolone is truly produced by the porcine, since nandrolone administration studies have clearly demonstrated them to be major metabolites. A clue to the real source of nandrolone in porcine urine may lie in studies carried out in the equine, where similarly to the porcine, nandrolone is detected in the urine of intact males, but the A-ring reduced metabolites are much less abundant than would be expected if nandrolone itself were present (Houghton et al. 2007). Nandrolone in the equine is now known to be predominantly an analytical *artefact* of the breakdown of a 19-carboxylic acid precursor that is endogenous in this species (Houghton et al. 2007). It has therefore recently been suggested (Scarth et al. 2009) that such 19-carboxylic acids may also be the precursors to urinary nandrolone in the porcine (Fig. 1). This theory is supported by studies in porcine granulosa cells, where the existence of a 19-carboxylic acid derivative of androstenedione has been demonstrated (Garrett et al. 1991).
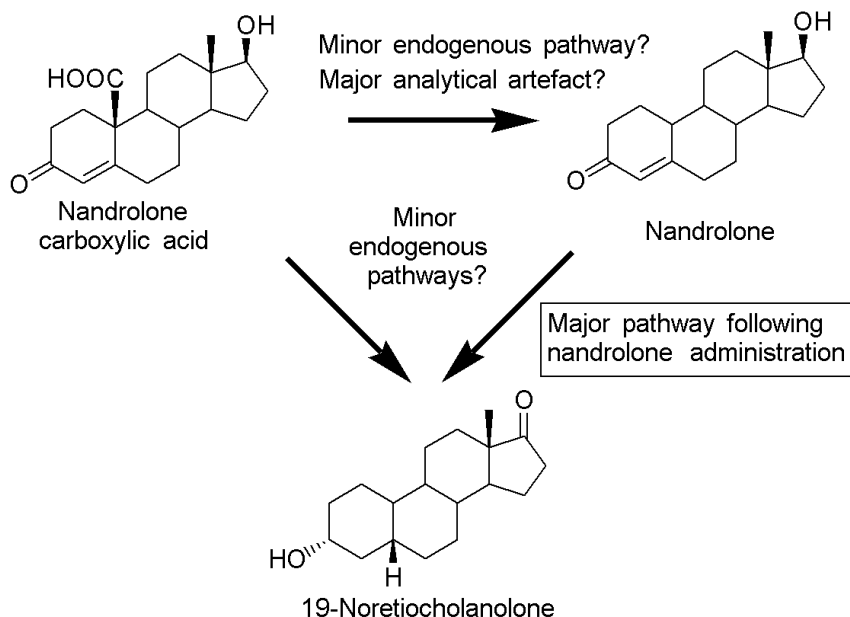
Figure 1 – diagram depicting some of the possible metabolic pathways leading to the presence of 19-noretiocholanolone in porcine urine. A similar pathway could also operate starting with androstenedione carboxylic acid in place of nandrolone carboxylic acid, leading to either 19-noretiocholanolone or 19-norandrostenedione as metabolites.

Since the A-ring reduced nandrolone metabolites are present in large quantities in the urine of treated, but not untreated porcine animals, a quantitative method for 19-noretiocholanolone (3α-hydroxy-5β-androstan-17-one) (Fig. 1) in the urinary free fraction was therefore validated and applied to real samples. The ultimate aim of this work was to use this data to produce thresholds for screening and/or confirmation of nandrolone abuse in the porcine. The reason for choosing 19-noretiocholanolone in particular is because it has been shown to be one of the most abundant urinary metabolites following nandrolone administration and was therefore predicted to be a good biomarker of nandrolone abuse (Roig et al. 2007). Analysis of the free fraction was preferred over the glucuronic or sulphate fractions because the free fraction contains the highest concentrations of 19-noretiocholanolone (Roig et al. 2007) and also because the other fractions contain significant quantities of other materials that interfere in GC-MS based analytical assays (James Scarth, personal observation).

2.2 Experimental

2.2.1 Chemicals and Reagents

Deionized water was prepared using an SG Ultrachem TWF UV system (Barsbuttel, Germany). All organic solvents, buffers and bases were analytical grade and were purchased from Fisher Scientific (Loughborough, UK). Ammonium iodide, N-(t-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA), dimethylformamide (DMF) and ethane thiol were obtained from Sigma-Aldrich (Dorset, UK). C18, 6 cc, 500 mg and silica, 6 cc, 500 mg solid phase extraction cartridges were supplied by Waters (Wexford, Ireland). 19-noretiocholanolone was supplied by Steraloids (Rhode Island, USA) and D_4-19-noretiocholanolone was provided by LGC Promochem (Teddington, UK).

2.2.2 Collection of Samples from Untreated Animals

Urine samples were obtained in the UK from both live animals (various university and research institute owned herds; from animals housed in metabolic cages and collecting urine using a floor-based drainage system) and from animals going for slaughter (organically farmed animals slaughtered for food production; collecting urine directly from the bladder post-mortem). A total of 205 gilt urine samples (none from pregnant animals) were obtained from animals ranging from 10 to 39 weeks of age and a total of 263 boar urine samples were obtained from animals ranging from 10 to 28 weeks of age. Whilst future European legislation may lead to a more widespread banning of porcine castration, many countries in Europe currently prefer to rear castrated males because of the potentially unsavoury 'boar taint' odour of meat from intact males (Font i Furnols et al. 2008). Boar taint is believed to result from the increased concentrations of skatole and androstenone produced by intact boars (Font i Furnols et al. 2008). However, the rearing of intact males is currently preferred in the UK because these animals are generally slaughtered earlier than in Europe, with the result that the animals are less likely to reach sexual maturity and hence produce lower concentrations of skatole and androstenone. Also, reducing profit margins in the UK pork industry have meant that the economic benefits to rearing intact males are also factors in refraining from castration (various researchers and organic farming consultants – personal communications 2008 and 2009). Because of the current UK practice to rear intact males, no urine samples from castrated males were available for study.

Samples were collected and stored chilled on ice within 1-hour. Samples on ice were then delivered by courier within 48-hours before being frozen at -80°C until the time of analysis.

2.2.3   Preparation of Calibration Lines and QCs

Calibration lines were constructed using pooled urine from castrated male bovine animals as a surrogate matrix. Bovine calibrant urine was augmented with 19-noretiocholanolone at concentrations of 0, 25 (the lower limit of quantification or LLOQ), 50, 100, 250, 500, 1,000, 1,500, 2,000 and 3,000 (the upper limit of quantification or ULOQ) pg ml^{-1}. This calibration range was chosen in order to provide maximal sensitivity at the low concentrations (instrumental responses saturated at concentrations above the upper limit of the calibration range due to the high sensitivity of the method). The reason for not using pooled porcine urine for constructing calibration lines was two-fold; firstly because it contained measurable endogenous concentrations of 19-noretiocholanolone (bovine urine did not) and secondly because the volumes needed to produce enough of the pool for both the validation and sample analysis studies were not available.

In order to validate the use of bovine urine as a surrogate calibration line matrix, pooled porcine boar urine was used as the QC matrix. Porcine QC concentrations were; the endogenous concentration only (E), endogenous augmented with 25 pg ml^{-1} (E+25), 100 pg ml^{-1} (E+100), 1,000 pg ml^{-1} (E+1,000) and 2,000 pg ml^{-1} (E+2,000).

2.2.4   Extraction Method

Samples were prepared and extracted according to the scheme shown in Fig. 2. The D$_4$-19-noretiocholanolone internal standard was added at a concentration of 800 pg ml^{-1} to all samples (other than an extracted blank bovine urine control sample).
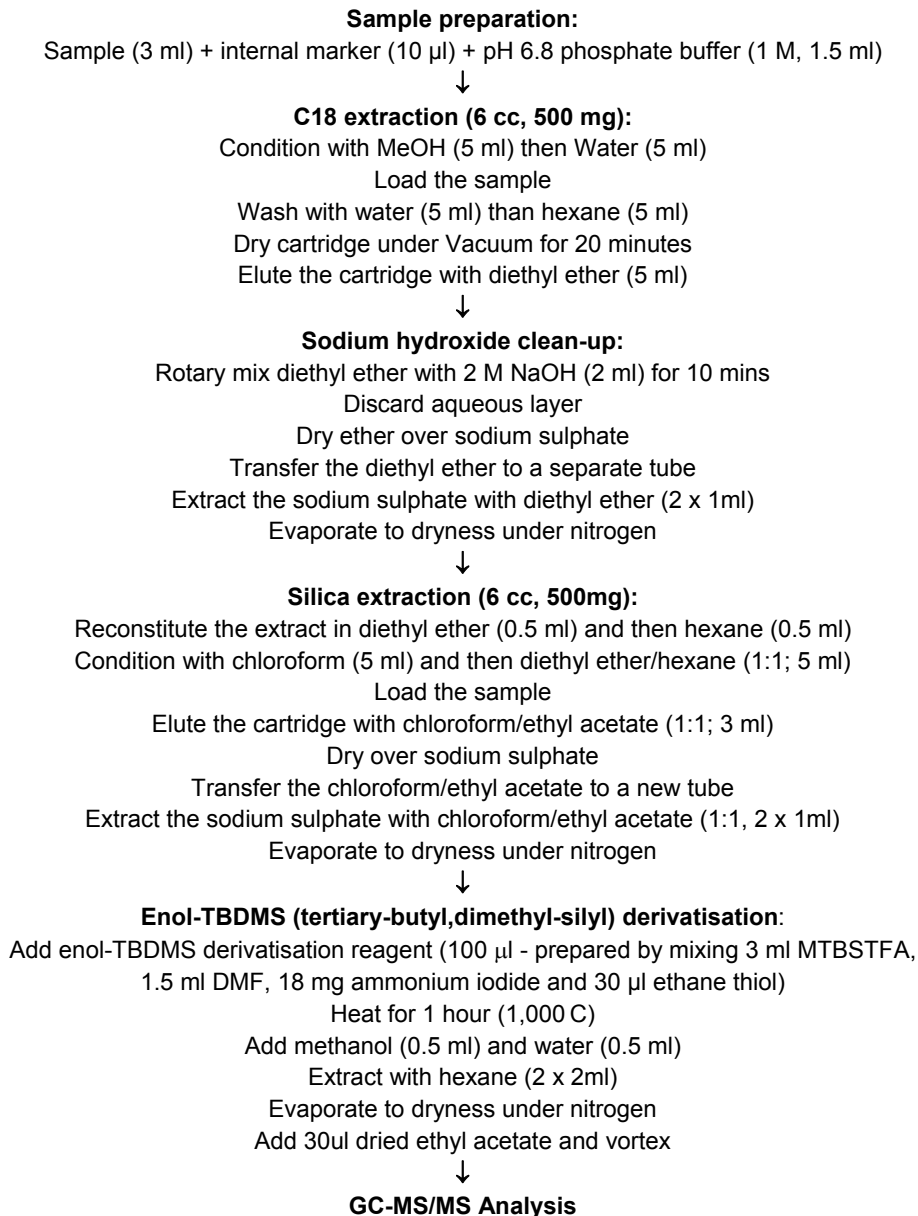
Sample preparation:
Sample (3 ml) + internal marker (10 µl) + pH 6.8 phosphate buffer (1 M, 1.5 ml)
↓
C18 extraction (6 cc, 500 mg):
Condition with MeOH (5 ml) then Water (5 ml)
Load the sample
Wash with water (5 ml) than hexane (5 ml)
Dry cartridge under Vacuum for 20 minutes
Elute the cartridge with diethyl ether (5 ml)
↓
Sodium hydroxide clean-up:
Rotary mix diethyl ether with 2 M NaOH (2 ml) for 10 mins
Discard aqueous layer
Dry ether over sodium sulphate
Transfer the diethyl ether to a separate tube
Extract the sodium sulphate with diethyl ether (2 x 1ml)
Evaporate to dryness under nitrogen
↓
Silica extraction (6 cc, 500mg):
Reconstitute the extract in diethyl ether (0.5 ml) and then hexane (0.5 ml)
Condition with chloroform (5 ml) and then diethyl ether/hexane (1:1; 5 ml)
Load the sample
Elute the cartridge with chloroform/ethyl acetate (1:1; 3 ml)
Dry over sodium sulphate
Transfer the chloroform/ethyl acetate to a new tube
Extract the sodium sulphate with chloroform/ethyl acetate (1:1, 2 x 1ml)
Evaporate to dryness under nitrogen
↓
Enol-TBDMS (tertiary-butyl,dimethyl-silyl) derivatisation:
Add enol-TBDMS derivatisation reagent (100 µl - prepared by mixing 3 ml MTBSTFA,
1.5 ml DMF, 18 mg ammonium iodide and 30 µl ethane thiol)
Heat for 1 hour (1,000 C)
Add methanol (0.5 ml) and water (0.5 ml)
Extract with hexane (2 x 2ml)
Evaporate to dryness under nitrogen
Add 30ul dried ethyl acetate and vortex
↓
GC-MS/MS Analysis

Figure 2 – schematic of the extraction method.

2.2.5   Method Validation Protocol

The validation protocol was based on the USA Food and Drug Administration (FDA) Centre for Drug Evaluation and Research guidelines for bioanalytical method validation (FDA, 2001), with accuracy defined as the degree of bias (% bias) and precision as the relative standard deviation (% RSD). In terms of % bias and % RSD acceptance limits, the FDA recommended +/- 20% limit for the lower limit of quantification (LLOQ) and +/- 15% limit for all other concentrations were relaxed to 25 and 20 % respectively. This was because the assay was considerably more complex than the majority of small molecule applications that the FDA guidelines are designed for. These somewhat relaxed limits were considered 'fit-for-purpose' according to the recommendations for biomarkers of Lee et al. (2006), the results of which are taken into account through the application of uncertainty of measurement (see later section on statistical analysis). Three separate precision and accuracy batches were extracted and analyzed in order to determine the inter-batch precision and accuracy, chromatographic separation, selectivity, linearity, lower and upper limits of quantification (LLOQ and ULOQ respectively) and the limit of detection (LOD). Each precision and accuracy batch contained a blank sample (extracted bovine urine with no internal standard or analyte added), a bovine urine calibration line (as described in the above section) and 5 replicates of each of the porcine urine QC concentrations (E, E+25, E+100, E+1,000 and E+2,000 pg ml^{-1}).

Stability of the analytes in the porcine E+100 and E+1,000 QCs was assessed by measuring 19-noretiocholanolone concentrations at 3-, 6-, 9-, 12 and 13.5-months (the longest period for which 'real' samples were stored) and comparing the results to those determined at time-zero.

Recovery was assessed by comparing porcine urine spiked with 19-noretiocholanolone at 1,000 pg ml^{-1} before (matrix fortified) and after (matrix matched) extraction (n=5).

The ability to dilute from outside the calibration range was assessed by spiking pooled porcine urine with 16,667 pg ml^{-1} 19-noretiocholanolone and then performing a 1 in 10 dilution with pooled porcine urine in order to bring the resulting concentration of analyte into the workable calibration range.

2.2.6   Analysis of Samples from Untreated Animals

Following validation of the method, samples from untreated animals were analyzed alongside a bovine urine spiked calibration line (25 to 3,000 pg ml^{-1}, made up of 9 separate calibrant concentrations) and 6 porcine urine spiked QCs (2 at each of the E+100, E+1,000 and E+2,000 pg ml^{-1} concentrations) interspersed with the samples throughout the batch.

2.2.7   GC-MS/MS Method

Nine microlitres of sample was introduced into a Varian 1079 programmable temperature vaporiser (PTV) injector held at 125°C for 1 min and then ramped to 280°C at a rate of 150°C min^{-1} before being held at 280°C for 13 mins. The injector split/column flow ratio was 20:1 until 1 min, before being reduced to 0:1 until 3 mins, before being increased to 50:1 for the remainder of the run-time. Chromatography was carried out using an Varian CP-3800 gas chromatograph with helium as the carrier gas (1.0 mlmin^{-1}) and a 30m x 250 um x 0.25 um Varian VF-17MS column initially held at 120°C for 2.00 mins and then ramped by 50°C min^{-1} to 220°C at 4.00 mins, ramped by a further 4.5^{o}C min^{-1} to 291°C at 19.78 mins, held for 2.00 mins, ramped by a further 4.5^{o}C min^{-1} to 300°C at 23.78 and then ramped at 50°C min^{-1} to 320°C at 28.18 mins.

Mass spectrometry was carried out using a Varian 320-MS triple quadrupole mass spectrometer operated in the electron ionization (EI) mode. Transfer line temperature was 280°C, ion source temperature was 220°C, electron energy was 20 eV and electron multiplier voltage was typically around 1,800 V. Positive ion MS/MS data was acquired in the selected reaction monitoring (SRM) mode using argon as the collision gas at a pressure of 1.5 m Torr. Precursor and parent *m/z* transitions monitored were 451.3 to 375.2 for D$_4$-19noretiocholanolone (collision energy 7 V), 447.3 to 371.2 for 19-noretiocholanolone transition 1 (used for quantification) (collision energy 7 V) and 447.3 to 239.1 for 19-noretiocholanolone transition 2 (used to provide additional support for the peak of interest relating to 19-noretiocholanolone) (collision energy 10 V) (Figs. 3 and 4). Dwell times were 0.167 seconds for each transition. Data were acquired and processed using the Varian Workstation version 6.9 software.
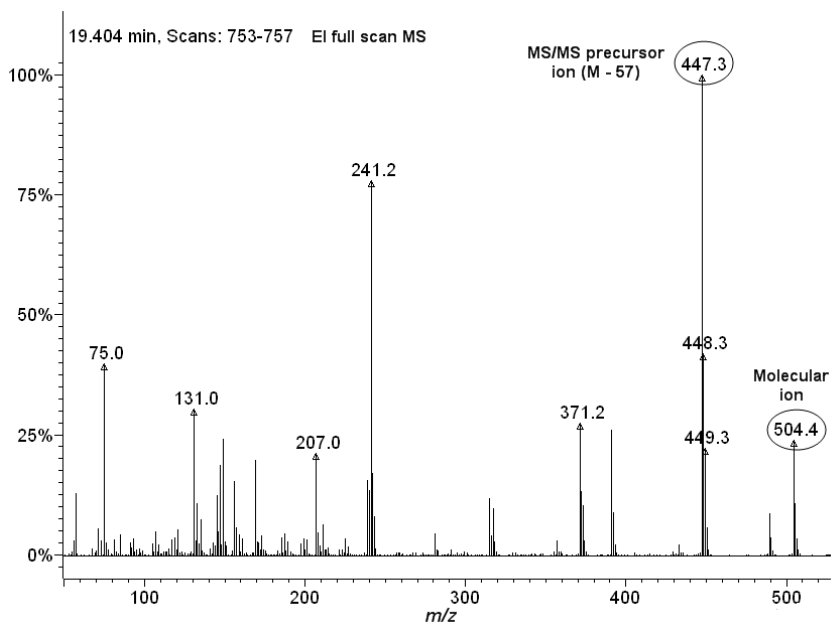
Figure 3 – full scan EI-MS spectrum of 19-noretiocholanolone-bis-TBDMS (tertiary-butyl,dimethyl-silyl) at 20 eV, highlighting the molecular ion and the fragment chosen as the MS/MS precursor ion.
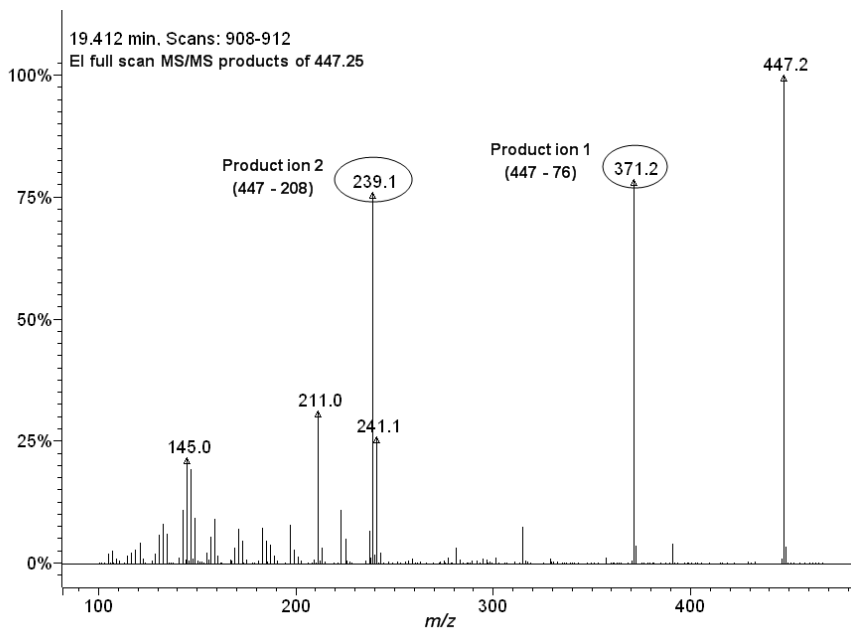

Figure 4 – full scan EI-MS/MS spectrum of 19-noretiocholanolone-bis-TBDMS at a collision energy of 7 eV, highlighting the MS/MS product ions.

2.2.8   Statistical Analysis of the Untreated Animal Population Data

In order to control the abuse of nandrolone in the porcine, the statistical analysis set out to suggest 19-noretiocholanolone thresholds with various probabilities (1 in 20, 1 in 100, 1 in 1,000 and 1 in 10,000) of finding a larger value by chance in a natural population. The one-tailed version of the Chebyshev inequality was used. This method, described by Estler (1997), is based on probability theory and makes minimal assumptions about the distribution of the data. Chebyshev confidence intervals are as far as possible from a mean for a given standard deviation; they are distribution-independent confidence intervals and are ideally suited to dealing with non-normally distributed data such as those resulting from the current study.   A one-tailed Chebyshev confidence interval is given by

$$p \leq \frac{1}{1 + t^2/V}$$

Hence

$$t \leq \sqrt{\frac{V}{p} - V}$$

Where:

t is the difference between the mean and concentration at the upper confidence interval

V is the variance (square of the standard deviation),

p is the probability that difference between the mean concentration and the concentration of a sample taken at random is greater than t.

To assess the extent to which concentration differed between gilts and boars, a Monte Carlo permutation test was implemented (Manly, 2007). Here, gender was permuted amongst individuals and the mean difference in concentration of 19-noretiocholanolone between gilts and boar was calculated for each permutation. The observed difference

was then compared to the distribution of mean differences for 1,000 permutation and significance assessed on the basis of the position of the observed mean in the distribution.

Uncertainty of measurement was calculated using a Monte Carlo sample (Manly, 2007) from the distribution consistent with the observed result along with the analytical performance observed at validation. 1,000 samples were taken from these distributions and 1,000 Chebyshev threshold estimates were generated. The different probabilities of finding a value by chance in a 'natural' population are then reported at the upper 95% quantile results after factoring in the determined method uncertainty. Values for *CCα* and *CCβ* (Decision limit and detection capability respectively) on each Chebyshev threshold associated with these probabilities (European Union, 2002), again at the 95% confidence interval, were also calculated for each analytical method used.

2.3   Results

In the following section all raw data and calculated threshold concentrations are uncensored and are given to one decimal place. All other summary statistics are presented to three significant figures.

2.3.1   Chromatography

Complete chromatographic separation for 19-noretiocholanolone in bovine urine was achieved (Fig. 5). In some porcine urine samples there was a small peak close after 19-noretiocholanolone (Fig. 5), but this did not reach significant enough a size to interfere with the integration of 19-noretiocholanolone.

2.3.2   Selectivity

In addition to the chromatographic separation, selectivity was assessed through monitoring the ratio of two separate MS/MS transitions for 19-noretiocholanolone, which support the suggestion that the peaks found in the porcine samples relate to 19-noretiocholanolone (Figs. 4 and 5).

2.3.3   Linearity

The calibration curves (weighted 1/x) were linear over the range studied (25 to 3,000 pg ml^{-1} – see chromatograms in Fig. 5). The mean r^2, slope, intercept and response factor % RSD values were 0.995, 0.00143, 0.0150 and 14.8 respectively (n=15 over the validation and sample analysis phases).
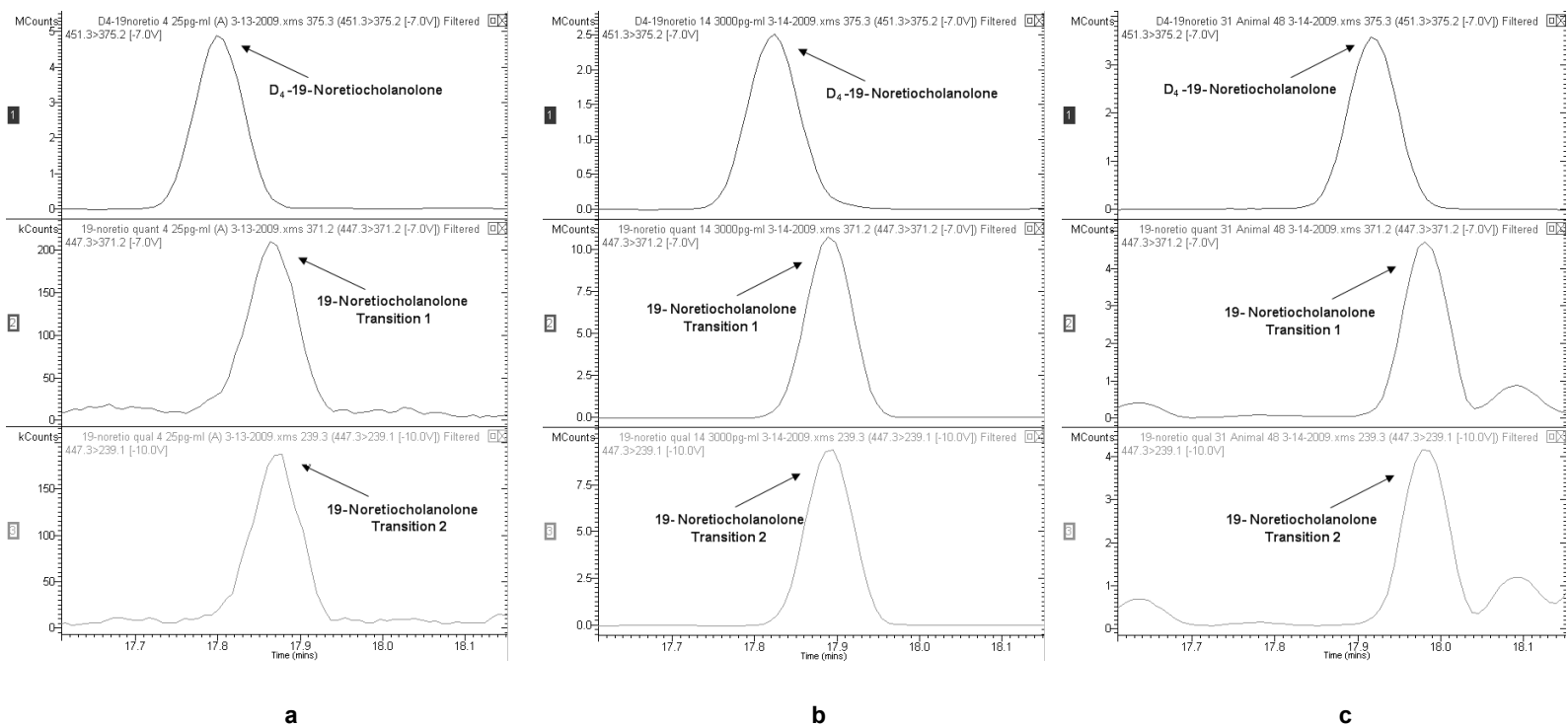
*Figure 5 – chromatograms obtained for samples at **a)** the lower limit of quantification (a 25 pg ml^{-1} spiked calibration line sample. Note: this peak was absent in unspiked bovine urine), **b)** the upper limit of quantification (a 3,000 pg ml^{-1} spiked calibration line sample) and **c)** a porcine urine from the sampled population with a determined concentration of 896.5 pg ml^{-1}. The top trace refers to the D4-19-noretiocholanolone internal standard (m/z 451 to 375), the middle trace refers to 19-noretiocholanolone transition 1 (used for quantification) (m/z 447 to 371) and the bottom trace refers to 19-noretiocholanolone transition 2 (used to provide additional support for the peak of interest relating to 19-noretiocholanolone) (m/z 447 to 239).*

2.3.4   Lower Limits of Quantification (LLOQ) and Detection (LOD)

The limit of quantification (25 pg ml^{-1}) was defined by the lowest point on the bovine urine calibration curve that could be quantified with a % bias and % RSD of less than 25% (based on the FDA guidelines, as discussed earlier). The LOD was calculated using the endogenous porcine QCs analyzed during the validation. The mean S:N of the 19-noretiocholanolone peaks in these samples was 8.15 at a mean calculated concentration of 35.9 pg ml^{-1}. When defining the LOD as the concentration that leads to a S:N of 3:1, extrapolation of the endogenous porcine QC data leads to a predicted LOD of 13.2 pg ml^{-1}.

2.3.4   Precision and Accuracy

Inter-batch precision and accuracy during both the validation and sample analysis stages were good, with % bias and % RSD within 15.0% throughout (Tables 1 and 2).

2.3.6   Linearity of Dilution

Results of QC samples diluted 1 in 10 from above the calibration range were acceptable, with the % bias and % RSD being less than 2% in each case (Table 3).

2.3.7   Recovery

The mean recovery of 19-noretiocholanolone spiked at 1,000 pg ml^{-1} (n=5 for each of the matrix fortified and matrix matched spikes) was 87.4%.

2.3.8   Matrix Stability

19-noretiocholanolone was found to be stable in urine at -80°C during the 13.5-months period studied (within +/- 20.0% of time-zero values at all subsequent time-points studied).

*Table 1 – inter-batch precision and accuracy results for 19-noretiocholanolone concentrations in porcine QCs during the validation (n=15 at each concentration). * The theoretical concentration for the endogenous QCs was the mean of the determined concentrations in the endogenous QCs in the first of the three precision and accuracy validation batches. The theoretical concentration for the remainder of the QCs was this endogenous concentration plus the known spiked amount on top of this number i.e. endogenous + 100 pg ml^{-1} for LOW QCs.*

	Endogenous QCs pg ml^{-1} (E)	Endogenous + 25 pg ml^{-1} QCs (E+25)	Endogenous + 100 pg ml^{-1} QCs (E+100)	Endogenous + 1,000 pg ml^{-1} QCs (E+1,000)	Endogenous + 2,000 pg ml^{-1} QCs (E+2,000)
Theoretical conc.*	36.1	61.1	136.1	1,036.1	2,036.1
Measured mean conc.	35.9	68.6	136.4	1,049.8	1,945.9
SD	5.22	10.1	16.1	56.6	147
% RSD	14.5	14.7	11.8	5.39	7.55
% Bias	-0.391	12.3	0.237	1.32	-4.43

Table 2 – inter-batch precision and accuracy results for 19-noretiocholanolone concentrations in porcine QCs during the sample analysis phase of the study (n=24 at each concentration). * See footnote to Table 1.

	Endogenous + 100 pg ml⁻¹ QCs (E+100)	Endogenous + 1,000 pg ml⁻¹ QCs (E+1,000)	Endogenous + 2,000 pg ml⁻¹ QCs (E+2,000)
Theoretical conc.*	136.1	1,036.1	2,036.1
Measured mean conc.	141.0	1,036.3	2,021.0
SD	15.0	134	320
% RSD	10.6	12.9	15.8
% Bias	3.61	0.0186	-0.743

Table 3 – results of the 1 in 10 dilution QCs (note: no samples needed to be diluted during the population sample analysis phase). * Because of the endogenous contribution in the porcine urine used for dilution, in order to compare the % bias of the measured concentrations in the diluted samples compared to the theoretically spiked concentration, it was necessary to adjust the measured values for the endogenous contribution. This was achieved by subtracting the endogenous concentration of the diluting matrix (as measured by the mean of five endogenous QCs run in this batch) from the dilution QC measured value and then multiplying the result by the dilution factor of 10.

	Measured concentration (pg ml⁻¹) after 1 in 10 dilution	Adjusted concentration (relative to the theoretically spiked concentration of 16,667 pg ml⁻¹)*
Measured mean conc.	1,679.4	16,399.4
SD	28.9	289
% RSD	1.72	1.76
% Bias	N/A	-1.61

2.3.9   Application of the Method to a Population of Samples from Untreated Animals

The descriptive statistics of the population data from untreated animals are shown in Table 4. 19-noretiocholanolone distributions in boars and gilt were bimodal, with a small number of concentrations in each sex at around the 1,000 pg ml^{-1} region and the majority of concentrations closer to the lower end of the calibration range. The ratio of the two MS/MS transitions in these samples is consistent with them relating to 19-noretiocholanolone (Fig. 5).

Table 4 – summary statistics for determined 19-noretiocholanolone concentrations in the free fraction of porcine urine.

	Boars	Gilts
Mean concentration (pg ml^{-1})	12.2	40.5
SD	69.7	183
% RSD	572	452
Minimum determined concentration (pg ml^{-1})	< LOD	< LOD
Maximum determined concentration (pg ml^{-1})	937.2	1,181.8
n	263	205

2.3.10   Statistical Analyses of the Population Data

19-noretiocholanolone concentrations in gilt and boar urines were determined to be statistically significantly different from each other (Monte Carlo permutation test $p < 0.025$, 1,000 permutations) and hence threshold calculations were performed separately for each sex. The estimated thresholds of finding a value by chance in a 'natural' population at different probabilities are given in Table 5 and are reported as the '95% confidence interval,' signifying that they are the upper estimates for the thresholds after factoring in the method's uncertainty of measurement. Table 5 also lists the *CCα* and *CCβ* values relating to these thresholds when using the analytical methodology presented herein.

The application of the resulting thresholds for screening and confirmation of nandrolone abuse will be discussed in the following section.

Table 5 – the 95% quantile thresholds (the upper threshold estimate after factoring in uncertainty of measurement) and associated CCα/β concentrations (p=0.05) for free fraction 19-noretiocholanolone in porcine urine at different false non-compliance probability rates.

		Chebyshev thresholds (pg ml^{-1}) at different false non-compliance probability rates			
		1 in 20	1 in 100	1 in 1,000	1 in 10,000
Boar	**95% quantile**	345.4	763.8	2,384.3	7,501.6
	CCα	396.4	876.4	2,735.8	8,607.5
	CCβ	464.9	1,027.9	3,208.9	10,096.0
Gilt	**95% quantile**	885.1	1,955.8	6,103.4	19,200.4
	CCα	1,015.6	2,244.2	7,003.2	22,031.1
	CCβ	1,191.2	2,632.2	8,214.2	25,840.8

2.4   Discussion

Previous studies have suggested the existence of endogenous nandrolone in porcine urine and at the same time the absence of the A-ring reduced metabolites in untreated animals (Roig et al. 2007, Ventura et al. 2008, Debruyckere et al. 1990, De Brabander et al. 1994, Poelmans et al. 2005). Several authors have therefore suggested that 19-noretiocholanolone could be used as a biomarker for nandrolone abuse (Scarth et al. 2009, Roig et al. 2007, Ventura et al. 2008). The results of the current study have shown that while concentrations of 19-noretiocholanolone are relatively low (maximum of 0.937 and 1.182 ng ml^{-1} in boars and gilts respectively), it has been demonstrated for the first time that 19-noretiocholanolone is endogenous in this species. Since the limit of detection (13.2 pg ml^{-1}) was much lower than previously (Roig et al. 2007, Ventura et al.

2008) reported methods (due to the analysis of the urinary 'free' fraction, the use of a PTV injector and analysis by GC-MS/MS), it is not surprising that a small amount of 19-noretiocholanolone can be detected in porcine urine, since it might be expected that a small proportion of the suggested nandrolone carboxylic acid precursors might be converted into nandrolone metabolites in vivo.

The endogenous nature of 19-noretiocholanolone, albeit at low concentrations, means that a threshold approach rather than a simple qualitative demonstration of it's presence is required in order to be able to use this compound as a biomarker of nandrolone abuse. The principle of adopting thresholds to control the abuse of endogenous compounds has long been an accepted approach in both food production (Heitzman, 1994) and animal sports (Houghton and Crone, 2000). If used to formally confirm the abuse of an endogenous substance, the standard approach is to set the threshold at a statistical probability of finding a false non-compliance at a rate of 1 in 10,000 in a natural population (Houghton and Crone, 2000), since this is considered to offer a sufficiently large safety margin to prevent the occurrence of false non-compliances.

Thresholds for screening may be set at a lower probability, but there then needs to be a secondary mechanism for confirmation if the confirmatory threshold is not also breeched. Typically, this may include follow-up analyses using gas chromatography carbon isotope mass spectrometry (GC-C-IRMS) (Prévost et al. 2004), detection of an intact steroid ester (Boyer et al. 2007) or an on-farm inspection (Jack Kay, personal communication). In an ideal world, a confirmatory threshold would also be suitable as a screening threshold, but this requires that the threshold is able to produce both low rates of false compliance and non-compliance; an ideal that is seldom achieved.

The analytical method presented herein was validated to a high standard and therefore considered suitable for application to a population of animals for use in establishing urinary 19-noretiocholanolone confirmatory thresholds. Because of the non-normal distribution of the data, it was necessary to use a non-parametric method of statistical analysis and the Chebyshev was considered the most suitable as it makes minimal assumptions about the population distribution and produces conservative thresholds relative to methods based on normally distributed data (Roy Macarthur, personal communication).

As shown in Table 5, the suggested confirmatory thresholds for free fraction 19-noretiocholanolone concentrations at a false non-compliance probability rate of 1 in 10,000 are 7.502 and 19.200 ng ml^{-1} for boars and gilts respectively. To put these thresholds into context, in a recent study administering 2 mg/kg nandrolone laurate via intra-muscular injection to six boars aged 8-10 weeks, the mean free fraction 19-noretiocholanolone concentration at the last time-point of the study (15 days following administration) was 28.4 ng ml^{-1}, with a range of 9.6 to 53.6 ng ml^{-1} (Ventura et al. 2008). The suggested threshold of 7.502 ng ml^{-1} in boar urine should therefore be able to detect the abuse of nandrolone for a significant time period in most treated animals and lead to rates of both low false compliance and non-compliance.

Quantitative data on 19-noretiochlanolone concentrations following nandrolone administration to gilts are lacking, but if the results are similar to boars, then the suggested threshold of 19.200 ng ml^{-1} in gilt urine should still be able to detect abuse in a significant number of cases in this sex also. Since no castrated males were available within the current study, it was not possible to suggest thresholds for these animals, but if the current (2009) UK trend to leave animals intact continues, then a threshold for castrated animals would not be required.

Although the current study has focussed on male and female porcine animals, recent studies have also suggested that inter-sex porcine animals may occasionally be encountered (Van Cruchten et al. 2002, Cornillie et al. 2009). Therefore, it is important that these animals be identified during regulatory surveillance procedures in order that they can either be excluded from measurement against threshold concentrations or be subject to further investigations to rule out nandrolone abuse should a breech of one of the threshold concentrations be observed.

Threshold concentrations at false non-compliance probabilities of lower than 1 in 10,000 are given in Table 5 in order that these concentrations could also be used in screening programmes prior to confirmatory analysis using one or more of the alternative follow-up techniques described earlier, should this be required. For example, should screening thresholds be set at a false non-compliance probability of 1 in 1,000 instead of the 1 in 10,000 used for confirmation, then the resulting threshold concentrations for boars and gilts are reduced by a factor of approximately three-fold to 2.384 and 6.103 ng ml^{-1} respectively. With reference to the 19-noretiocholanolone concentrations reported in the

Ventura et al. 2008 nandrolone administration study, these threshold concentrations would be able to detect 100% of the instances of nandrolone use in boars at the last time point studied (15 days), while also leading to a low rate of follow-up work (1 in 1,000 samples tested). Ultimately, the decision to establish a particular screening threshold is at the discretion of the individual regulatory authority and will no doubt reflect the striking of a balance between the amount of unnecessary follow-up work generate and the ability to minimise false non-compliances at the different probabilities. Although the CCα and CCβ values for the thresholds using the method above are also given in Table 5, these are unique to this laboratory. If other laboratories adopt these thresholds, then CCα and CCβ values must be independently determined in these laboratories before applying the method to 'real-life' samples.

In conclusion, the analytical method presented above has been validated and applied to a population of boars and gilts in order to suggest urinary 19-noretiocholanolone screening and confirmatory biomarker thresholds for the detection of nandrolone abuse. The establishment of these thresholds is significant since there is currently no internationally accepted method for detecting nandrolone abuse in this species.

2.5   Acknowledgements

2.6   References

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Chapter 3: Validation of analytical biomarker approaches for the detection of androgen, oestrogen and progestagen abuse in the bovine

After:

3.1   Introduction

As discussed in the introductory chapter, the use of steroids as growth promoting agents in food production is prohibited under European Union legislation (European Union, 1996). However, some steroids are known to be endogenous in certain species (Scarth et al. 2009), making a simple qualitative determination of their presence insufficient for proving abuse (see chapter 1 for further details). The detection of intact steroids esters or the use of combustion isotope ratio mass spectrometry can be useful for the confirmation of certain endogenous steroids (Scarth et al. 2009), but these methods are not suitable for all steroids or are too laborious to be used as screening approaches. As discussed in chapter 2 in relation to the porcine, some sort of quantitative threshold or biomarker approach is therefore usually required to regulate the use of endogenous steroids

In the work reported in the current chapter, a multi-residue analytical method was developed and validated in order to quantify urinary target analytes indicative of the

abuse of testosterone, nandrolone, boldenone, progesterone and oestradiol in the bovine. Once validated, the aim was then to apply this method to a large number of samples from natural populations of animals in order to determine baseline steroid concentrations and establish thresholds for detecting steroid abuse. Urine was the matrix of choice as it is available at both slaughter and from live animals and is known to contain higher concentrations of most steroids relative to blood (Scarth et al. 2009).

In human sports, an increased ratio of testosterone to epitestosterone is used as a marker of testosterone abuse, but since epitestosterone is a major metabolite of testosterone in the bovine (Angeletti et al. 2006), the ratio has little use in this species. However, testosterone is also metabolised to a number of androstanediol isomers in the bovine (Scarth et al. 2009). 5β-androstane-3α,17β-diol (BAB-androstanediol - Fig. 1) was chosen as the testosterone target analyte for the current study as this analyte has been shown to be increased in the urine of testosterone treated animals (Scarth et al. 2009) and a deuterated version of this analogue is commercially available.

Analogous to testosterone metabolism, nandrolone is converted to epinandrolone, 19-noretiocholanolone and a mixture of estranediol isomers in the bovine. Since it is currently unknown which of these analytes will produce the most useful threshold for detection of nandrolone abuse, epinandrolone, 19-noretiocholanolone and 5α-estrane-3β,17α-diol (ABA-estranediol) were all targeted with the current method (Fig. 1).

Boldenone is metabolized by the bovine to epiboldenone and a number of isomers reduced in the A- and/or D-ring of the steroid molecule (Scarth et al. 2009). Current EU guidelines for detecting boldenone abuse in the bovine (European Commission, 2003) target boldenone conjugates as indicative of abuse, but these guidelines also recommend that further work to identify more appropriate metabolites be encouraged. Therefore, the current study focused on the A-ring reduced boldenone metabolite 17β-hydroxy-5β-androst-1-ene-3-one (Fig. 1), as this compound has been detected in the urine of animals administered boldenone, but not unequivocally in untreated animals (Scarth et al. 2009). It has also been observed that boldenone related compounds are present in some animal sub-populations and absent in others (De Brabander et al. 2004). Therefore, in order to assess whether any absence of detectable 17β-hydroxy-5β-androst-1-ene-3-one in bovine urine was due to it's true absence (even in the presence of endogenous epiboldenone) or instead only absent because there was no endogenous

epiboldenone that could be metabolized through to 17β-hydroxy-5β-androst-1-ene-3-one, epiboldenone was also included in the assay.

In contrast to the aforementioned androgenic-anabolic steroids, data on the metabolism of progesterone following exogenous administration is lacking. Therefore, the target analyte that was chosen for the current study, 5α-pregnane-3β,20α-diol (ABA-pregnanediol – Fig. 1), was one that was shown by preliminary analyses to be detected in the urine of bovine steers at a concentration within the same calibration range used for the other androgenic-anabolic steroids.
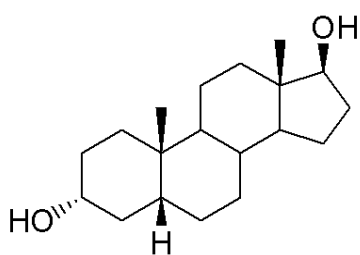
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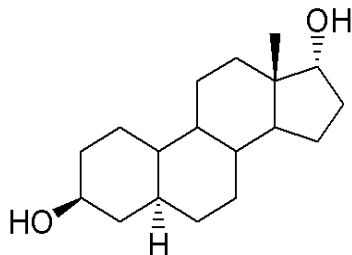
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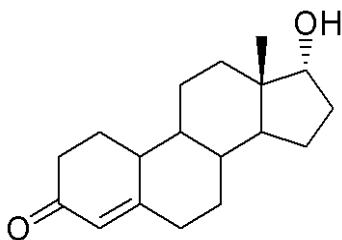
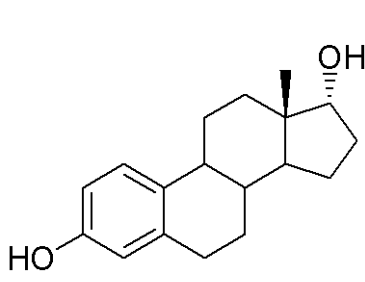**Epinandrolone**
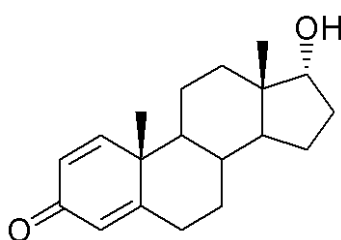
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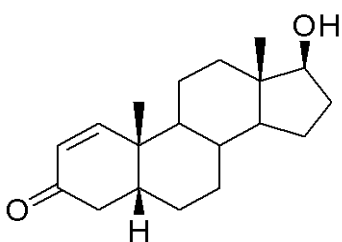
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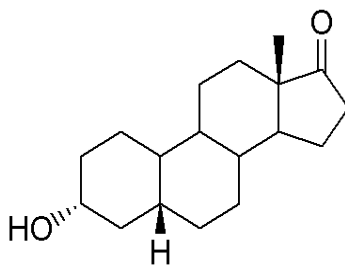
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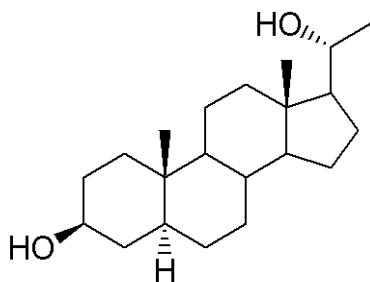
ABA-Pregnanediol

Epioestradiol

Fig. 1 – structures of the analytes studied.

Lastly, the target analyte chosen for detecting the abuse of oestradiol was epioestradiol (Fig. 1) because concentrations of this steroid in urine are much higher than oestradiol (Biddle et al. 2007) and hence are more readily quantified. Additionally, there is an overlap in the concentrations of epioestradiol between treated and untreated animals, so it is anticipated that the control of oestradiol abuse will necessitate a provisional urine screen using epioestradiol followed by confirmation using an auxiliary techniques such as gas-combustion-isotope-ratio-mass-spectrometry (GC-C-IRMS). The higher concentrations of epioestradiol are more amenable to confirmatory analysis using GC-C-IRMS compared to oestradiol.

Due to the high sensitivity and selectivity obtained with GC- or LC-MS/MS, these techniques are the most frequently applied in steroid residue analysis (Stolker et al. 2005). The chemically reduced nature of many of the androgen and progestagen urinary metabolites means that these compounds do not ionize well under atmospheric pressure ionization (API), hence GC-MS/MS was chosen for these compounds. For oestradiol however, its fractionation from the androgens and progestagens using an extractive derivatisation procedure allowed LC-MS/MS analysis to be used. This was desirable since the LC-MS/MS analysis of the dansyl derivative of ethinyloestradiol has been demonstrated to provide high sensitivity by virtue of introducing a functional group with high proton affinity into the molecule (Anari et al. 2002).

3.2 Experimental Data

3.2.1 Chemicals and Reagents

Deionized water was prepared using an SG Ultrachem TWF UV system (Barsbuttel, Germany). All organic solvents, buffers and bases were analytical grade and were purchased from Fisher Scientific (Loughborough, UK). Ammonium iodide, N-(t-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA), di-methyl-formamide (DMF), D_3-testosterone, oestradiol, dansyl chloride, β-glucuronidase from *e. coli* and ethane thiol were obtained from Sigma-Aldrich (Dorset, UK). C18, 6 cc, 500 mg and silica, 6 cc, 500 mg solid phase extraction cartridges were supplied by Waters (Wexford, Ireland). 19-noretiocholanolone, 5α-pregnane-3β,20α-diol, 5α-estrane-3β,17α-diol and epinandrolone were supplied by Steraloids (Rhode Island, USA). D_4-19-noretiocholanolone, D_3-boldenone, epiboldenone, 5β-androstane-3α,17β-diol, D_5-5β-androstane-3α,17β-diol and

17β-hydroxy-5β-androst-1-ene-3-one were provided by LGC Promochem (Teddington, UK). D_2-epioestradiol was obtained from CDN Isotopes (Quebec, Canada). D_3-androstanediol (isomer nature unknown) and D_4-5α-pregnane-3β,20α-diol were synthesized in our laboratories. Urine samples from castrated male bovine animals not treated with steroids were obtained in the UK from both live animals (various university and research institute owned herds) and from animals going for slaughter (organically farmed animals slaughtered for food production).
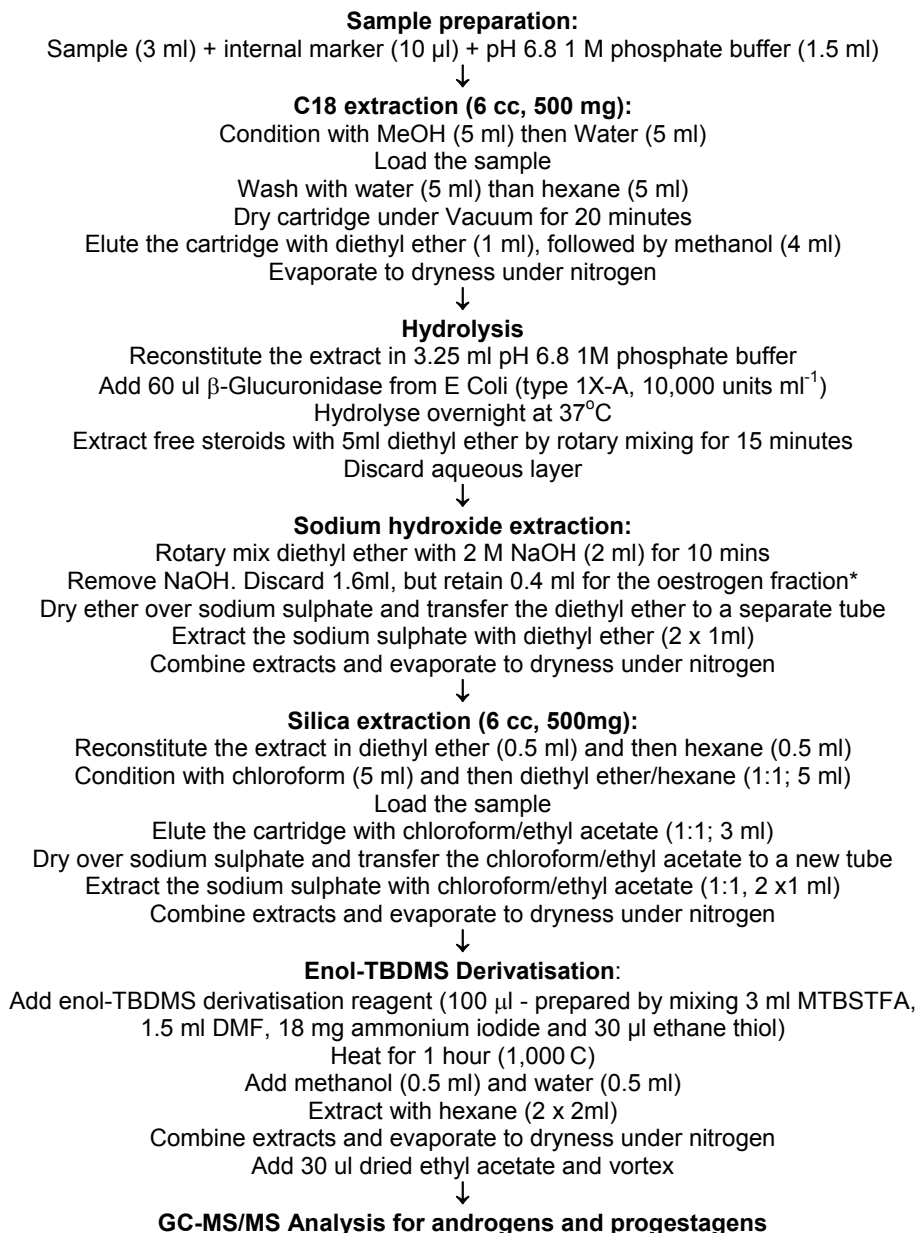
3.2.2   Preparation of Calibration Lines and QCs

Calibration lines were constructed using pooled urine from castrated male bovine animals using a 'standard addition' approach. By spiking known quantities of analyte onto existing endogenous concentrations, the slope and intercept of the calibration line was used to calculate the endogenous concentrations of analyte present. The resulting calibrant concentrations were then adjusted to account for the endogenous plus known spiked amount at each point. Bovine calibrant urine was augmented with steroid at concentrations of 0, 25, 50, 100, 250, 500, 1,000, 1,500, 2,000 and 3,000 pg ml^{-1} for androgens/progestagens and at 0, 20, 40, 80, 200, 400, 800, 1,200, 1,600 and 2,400 pg ml^{-1} for oestrogens. These calibration ranges were chosen in order to provide maximal sensitivity at the low concentrations (instrumental responses saturated at concentrations above the upper limit of the calibration range due to the high sensitivity of the method). QCs were also prepared in pooled urine from castrated males. QC concentrations for androgens/progestagens were: the endogenous concentration only (E), endogenous augmented with 25 pg ml^{-1} (E+25), endogenous augmented 100 pg ml^{-1} (E+100), endogenous augmented with 1,000 pg ml^{-1} (E+1,000) and endogenous augmented with 2,000 pg ml^{-1} (E+2,000). QC concentrations for oestrogens were: the endogenous concentration only (E), endogenous augmented with 20 pg ml^{-1} (E+20), endogenous augmented with 80 pg ml^{-1} (E+80), endogenous augmented with 800 pg ml^{-1} (E+800) and endogenous augmented with 1,600 pg ml^{-1} (E+1,600).

3.2.3   Extraction Method

Samples were prepared and extracted according to the scheme shown in Fig. 2a (androgens and progestagens) and 2b (oestrogens). The internal standards were added to all samples (other than an extracted blank bovine urine control sample) at

concentrations of 800 pg ml^{-1} (androgens) and 400 pg ml^{-1} (oestrogens). A chromatogram for the related internal standard is shown for comparison in Fig. 3 to 10.

Sample preparation:
Sample (3 ml) + internal marker (10 µl) + pH 6.8 1 M phosphate buffer (1.5 ml)
↓
C18 extraction (6 cc, 500 mg):
Condition with MeOH (5 ml) then Water (5 ml)
Load the sample
Wash with water (5 ml) than hexane (5 ml)
Dry cartridge under Vacuum for 20 minutes
Elute the cartridge with diethyl ether (1 ml), followed by methanol (4 ml)
Evaporate to dryness under nitrogen
↓
Hydrolysis
Reconstitute the extract in 3.25 ml pH 6.8 1M phosphate buffer
Add 60 ul β-Glucuronidase from E Coli (type 1X-A, 10,000 units ml^{-1})
Hydrolyse overnight at 37°C
Extract free steroids with 5ml diethyl ether by rotary mixing for 15 minutes
Discard aqueous layer
↓
Sodium hydroxide extraction:
Rotary mix diethyl ether with 2 M NaOH (2 ml) for 10 mins
Remove NaOH. Discard 1.6ml, but retain 0.4 ml for the oestrogen fraction*
Dry ether over sodium sulphate and transfer the diethyl ether to a separate tube
Extract the sodium sulphate with diethyl ether (2 x 1ml)
Combine extracts and evaporate to dryness under nitrogen
↓
Silica extraction (6 cc, 500mg):
Reconstitute the extract in diethyl ether (0.5 ml) and then hexane (0.5 ml)
Condition with chloroform (5 ml) and then diethyl ether/hexane (1:1; 5 ml)
Load the sample
Elute the cartridge with chloroform/ethyl acetate (1:1; 3 ml)
Dry over sodium sulphate and transfer the chloroform/ethyl acetate to a new tube
Extract the sodium sulphate with chloroform/ethyl acetate (1:1, 2 x1 ml)
Combine extracts and evaporate to dryness under nitrogen
↓
Enol-TBDMS Derivatisation:
Add enol-TBDMS derivatisation reagent (100 µl - prepared by mixing 3 ml MTBSTFA,
1.5 ml DMF, 18 mg ammonium iodide and 30 µl ethane thiol)
Heat for 1 hour (1,000 C)
Add methanol (0.5 ml) and water (0.5 ml)
Extract with hexane (2 x 2ml)
Combine extracts and evaporate to dryness under nitrogen
Add 30 ul dried ethyl acetate and vortex
↓
GC-MS/MS Analysis for androgens and progestagens

Fig. 2a – schematic of the androgen/progestagen extraction method.
* See Fig. 2b for the details of the oestrogen extraction from this point.

Extractive derivatisation:
To the 0.4 ml of 2 M NaOH derived from the androgen/progestagen extraction, add a
further 1.6 ml of 2 M NaOH
Rotary mix for 30 mins with 4 ml of 0.175 mg ml^{-1} dansyl chloride in hexane
(in darkness)
Remove the hexane layer and evaporate it to dryness under nitrogen
Reconstitute in 45 ul of 75:25 acetonitrile:0.1% formic acid (aq)
↓
LC-MS/MS Analysis for oestrogens

*Fig. 2b – schematic of the oestrogen extraction method that follows from the
androgen/progestagen method in Fig. 2a.*

3.2.4   *Method Validation Protocol*

The ultimate application of the validated method presented herein will be for analysis of
steroid concentrations in a large population of animals in order to produce quantitative
thresholds for detecting steroid abuse. The establishment of each threshold will be
based on the probability of finding a value above a specified concentration by chance in
the 'natural' population. The statistical model that will be used to produce these threshold
concentrations is known as the Chebyshev inequality and has been described by Estler
(1997). Further details of the Chebyshev inequality and its application will be published
once the population data have been generated.

For the purposes of the current validation in the context of the Chebyshev inequality, the
most critical aspect is to ensure that the analytical methods are validated to a high level
of precision and accuracy across a wide range of concentrations, not just at the lower
limits of detection. Based on previous experience with the use of the Chebyshev
inequality, it has been observed that the population concentrations toward the upper end
of the calibration range are the most important parameters affecting the resulting
thresholds (Roy Macarthur, personal communication). Concentrations around the lower-
limits of detection or quantification have little or no effect on these thresholds (Roy
Macarthur, personal observation). Therefore, the current method validation protocol was
designed to fully characterise the performance of the analytical method over a wide
range of concentrations, rather than focussing on the CCα and CCß at the lower limits of
detection. However, it will be necessary to calculate the CCα and CCß values at the
thresholds produced by the Chebyshev method. Because these calculations can only be

carried out once these threshold concentrations have been determined, they will be published separately once the population studies are complete.

The calculated thresholds are likely to be in significant excess of the upper calibration ranges used in the current study. Therefore, although individual laboratories are welcome to use the current analytical method to regulate each threshold, they will most likely not need to use methods as sensitive or time-consuming as these (which were required here in order to define the population values over the whole range of observed concentrations). However, it will be essential for them validate their own CCα and CCß values around the defined thresholds using whatever analytical methods they choose to adopt.

The validation protocol was based on the USA Food and Drug Administration (FDA) Centre for Drug Evaluation and Research guidelines for bioanalytical method validation (FDA, 2001), with accuracy defined as the degree of bias (% bias) and precision as the relative standard deviation (% RSD). In terms of % bias and % RSD acceptance limits, the FDA recommended +/- 20% limit for the lower limit of quantification (LLOQ) and +/- 15% limit for all other concentrations were relaxed to 20 and 25% respectively. This was because the assay was considerably more complex than the majority of small molecule applications that the FDA guidelines are designed for. These somewhat relaxed limits were considered 'fit-for-purpose' according to the recommendations for biomarkers of Lee et al. (2006), the results of which are taken into account through the application of uncertainty of measurement (see later section on statistical analysis). Three separate precision and accuracy batches were extracted and analyzed for oestrogens and four batches for androgens/progestagens (an additional batch relative to the oestrogens due to the increased complexity of the androgen multi-residue GC-MS/MS method) in order to determine the inter-batch precision and accuracy, chromatographic separation, selectivity, linearity, lower and upper limits of quantification (LLOQ and ULOQ respectively) and the limit of detection. Each precision and accuracy batch contained a blank sample (extracted bovine urine with no internal standard or analyte added), a bovine urine calibration line (as described in the above section) and 5 replicates of each of the bovine urine QC concentrations (E, E+25, E+100, E+1,000 and E+2,000 pg ml^{-1} for androgens/progestagens and E, E+20, E+80, E+800 and E+1,600 pg ml^{-1} for oestrogens).

Stability of the analytes in bovine urine at the E + 1,000 QC (androgens and progestagens) and E + 1,600 QC (oestrogens) points was assessed by measuring concentrations at 3- and 6-months and comparing the results to those determined at time-zero. The ability to dilute samples was assessed by spiking pooled bovine urine with concentrations of each steroid at approximately 10 times the middle of the calibration range and then performing a 1 in 10 dilution with pooled bovine urine in order to bring the resulting concentration of analyte into the workable range.

3.2.5   GC-MS/MS Androgen/Progestagen Method

Nine microlitres of sample was introduced into a Varian 1079 programmable temperature vaporiser (PTV) injector held at 125°C for 1.00 min and then ramped to 280°C at a rate of 150°C min^{-1} before being held at 280°C for 13.00 min. The injector split/column flow ratio was 20:1 until 1.00 min, when it was immediately reduced to 0:1 and held at this ratio until 3.00 min. It was then immediately increased to 50:1 for the remainder of the run-time. Chromatography was carried out using a Varian CP-3800 gas chromatograph with helium as the carrier gas (1.0 ml min^{-1}) and a 30m x 250 um x 0.25 um Varian VF-17MS column initially held at 120°C for 2.00 min and then ramped by 50°C min^{-1} to 220°C at 4.00 min, a further 4.5^{o}C min^{-1} to 291°C at 19.78 min, held for 2.00 min, ramped by a further 4.5^{o}C min^{-1} to 300°C at 23.78 and then by 50°C min^{-1} to 320°C at 28.18 min. Mass spectrometry was carried out using a Varian 320-MS triple quadrupole mass spectrometer operated in the electron ionization (EI) mode. Transfer line temperature was 280°C, ion source temperature was 220°C, electron energy was 20 eV and electron multiplier voltage was typically 1800 V.   Positive ion MS/MS data was acquired in the selected reaction monitoring (SRM) mode using argon as the collision gas at a pressure of 1.5 m Torr. The SRM conditions for each compound are given in Table 1.

3.2.6   LC-MS/MS Oestrogen Method

15 µl of sample was introduced into an Applied Biosystems Sciex 5000 instrument using a Waters Acquity autosampler/HPLC. Chromatography was carried out using a Waters 100 mm x 2.1 ID 1.8 µm HSS T3 column held at 37°C. Flow rate was 650 µl min^{-1} and mobile phase A was acetonitrile and mobile phase B was 0.1% formic acid in water. Mobile phase A was at 76.5% from 0 to 2.90 min, rising to 99% at 2.95 min, held at 99%

until 4.60 min, before being reduced back to 76.5% at 4.65 min and then being held at 76.5% until 5.10 min.

Sample ionisation was carried out in the positive mode using the Turbo Ionspray source at a source temperature of 750°C. Ion spray voltage was 4000 V, gas one had a flow of 30 units, gas two a flow of 70 units, the curtain gas had a flow of 10 units, the CAD gas setting was 7 units, declustering potential was 175 V, collision cell exit potential was 22 V and entrance potential 10 V. The MS/MS transitions and collision energies are given in Table 1. Data was processed using the Analyst version 1.4.2 software.

LC-MS/MS matrix effects were studied by spiking 5 individual bovine urine samples (from different animals with varying endogenous epioestradiol concentrations) with 1200 pg ml^{-1} epioestradiol and measuring the determined concentration of the analyte after subtracting the known endogenous concentration for each sample (determined by analysing the urines unspiked alongside the spiked samples). Since the method uses a standard addition approach with a deuterated epioestradiol internal standard, the key parameter to measure the matrix effect was considered to be a sufficiently low relative standard deviation between the individual replicates to ensure that there were no differential matrix effects that were not corrected for by the deuterated internal standard.

Table 1 – GC/LC-MS/MS conditions for the compounds studied. N.B. qualifier transitions were not required for internal standards

* No qualifier ions could be monitored for epioestradiol as no alternative diagnostic fragments were formed during collision induced dissociation of this compound.

Steroid (and derivative)	Typical retention time (min)	MS/MS quantifier precursor ion m/z	MS/MS quantifier product ion m/z (collision energy in brackets)	MS/MS qualifier precursor ion m/z	MS/MS qualifier product ion m/z (collision energy in brackets)
Androgens and progestagens by GC-MS/MS:					
5β-Androstane-3α,17β-diol (bis-TBDMS)	16.8	463.3 (M – 57)	255.1 (10)	463.3 (M – 57)	387.2 (10)
5α-Estrane-3β,17α-diol (bis-TBDMS)	16.0	449.4 (M - 57)	241.1 (10)	241.2 (M – 265)	145.0 (10)
Epinandrolone (bis-TBDMS)	18.6	502.4 (M)	445.2 (10)	502.4 (M)	369.2 (13)
19-Noretiocholanolone (bis-TBDMS)	16.4	447.3 (M – 57)	371.2 (7)	447.3 (M – 57)	239.1 (10)
17β-Hydroxy-5β-androst-1-ene-3-one (bis-TBDMS)	16.3	516.5 (M)	501.3 (10)	501.5 (M – 15)	369.2 (10)
Epiboldenone (bis-TBDMS)	16.7	514.5 (M)	248.1 (10)	367.1 (M – 147)	271.0 (10)
5α-Pregnane-3β,20α-diol (bis-TBDMS)	22.0	491.5 (M – 57)	159.0 (10)	491.5 (M – 57)	283.2 (10)
D_3-Testosterone (bis-TBDMS)	20.0	519.3 (M)	462.4 (10)	N/A	N/A
D_3-Boldenone (bis-TBDMS)	20.0	517.5 (M)	248.1 (10)	N/A	N/A
D_3-Androstanediol (bis-TBDMS)	16.5	466.3 (M – 57)	258.2 (10)	N/A	N/A
D_5-5β-Androstane-3α,17β-diol (bis-TBDMS)	16.7	468.3 (M – 57)	260.2 (10)	N/A	N/A
D_4-19-Noretiocholanolone (bis-TBDMS)	16.3	451.3 (M – 57)	243.2 (10)	N/A	N/A
D_4-5α-Pregnane-3β,20α-diol (bis-TBDMS)	21.5	495.4 (M – 57)	162.1 (10)	N/A	N/A
Epioestradiol by LC-MS/MS:					
*Epioestradiol (dansyl)	2.7	506.2 (M+H)	427.3 (45)	*N/A	*N/A
D_2-Epioestradiol (dansyl)	2.7	510.2 (M+H of S_{34} isotope)	431.3 (45)	N/A	N/A

3.2.7   Recovery

Since the current method quantifies total 'free and glucuronide conjugated' steroid in bovine urine and since glucuronide derivatives of the majority of steroids were not available as reference standards at the time the work was conducted, it was not possible to determine meaningful absolute recoveries for the different steroids. However, increasing the amount of β-glucuronidase enzyme above that used in the final extraction scheme (Fig. 2) did not increase the yield of metabolites (data not shown), so the extraction was considered optimised with respect to glucuronide conjugate hydrolysis.

3.3   Results

All raw data in the following section are uncensored and hence calculated concentrations are given to one decimal place. All summary statistics are presented to three significant figures.

3.3.1   Chromatography

Complete chromatographic separation was achieved for ABA-estranediol, 19-noretiocholanolone, epinandrolone, epiboldenone, 17β-hydroxy-androst-1-en-3-one, ABA-pregnanediol and epioestradiol in bovine urine (Fig. 4 to 10). In the chromatogram for BAB-androstanediol in bovine urine samples, there were a series of three peaks close before BAB-androstanediol (Fig. 3). However, these peaks did not reach significant enough a size to interfere with the integration of BAB-androstanediol and in all cases the 'cleaner' qualifier ion transition assured the identity of BAB-androstanediol. Interestingly, these three earlier running peaks increased and decreased in proportion with the endogenous BAB-androstanediol peaks in different urines (but did not increase when BAB-androstanediol was artificially spiked into samples), suggesting that these peaks are related androstane-metabolite isomers that are produced in synchrony with BAB-androstanediol.
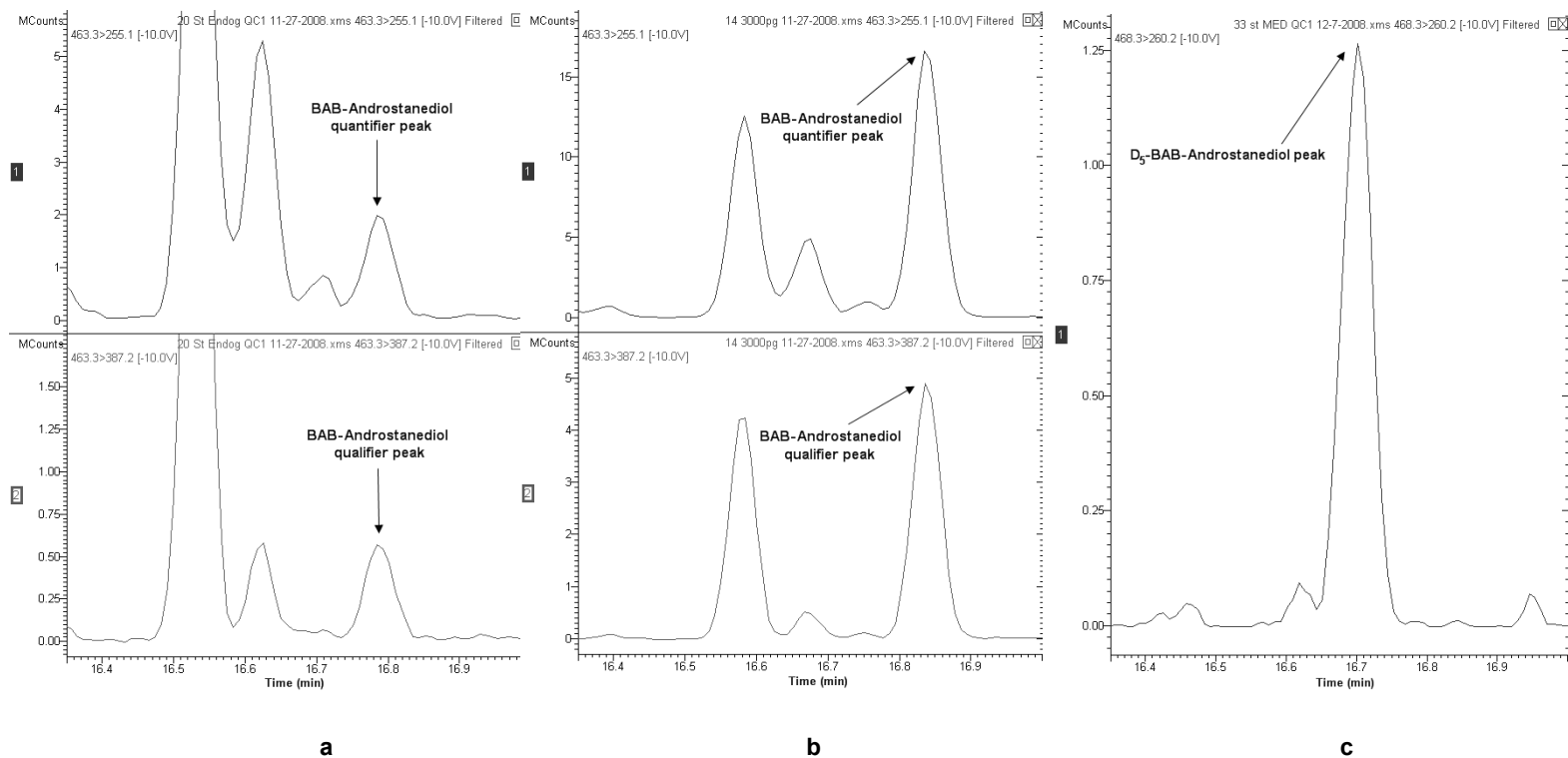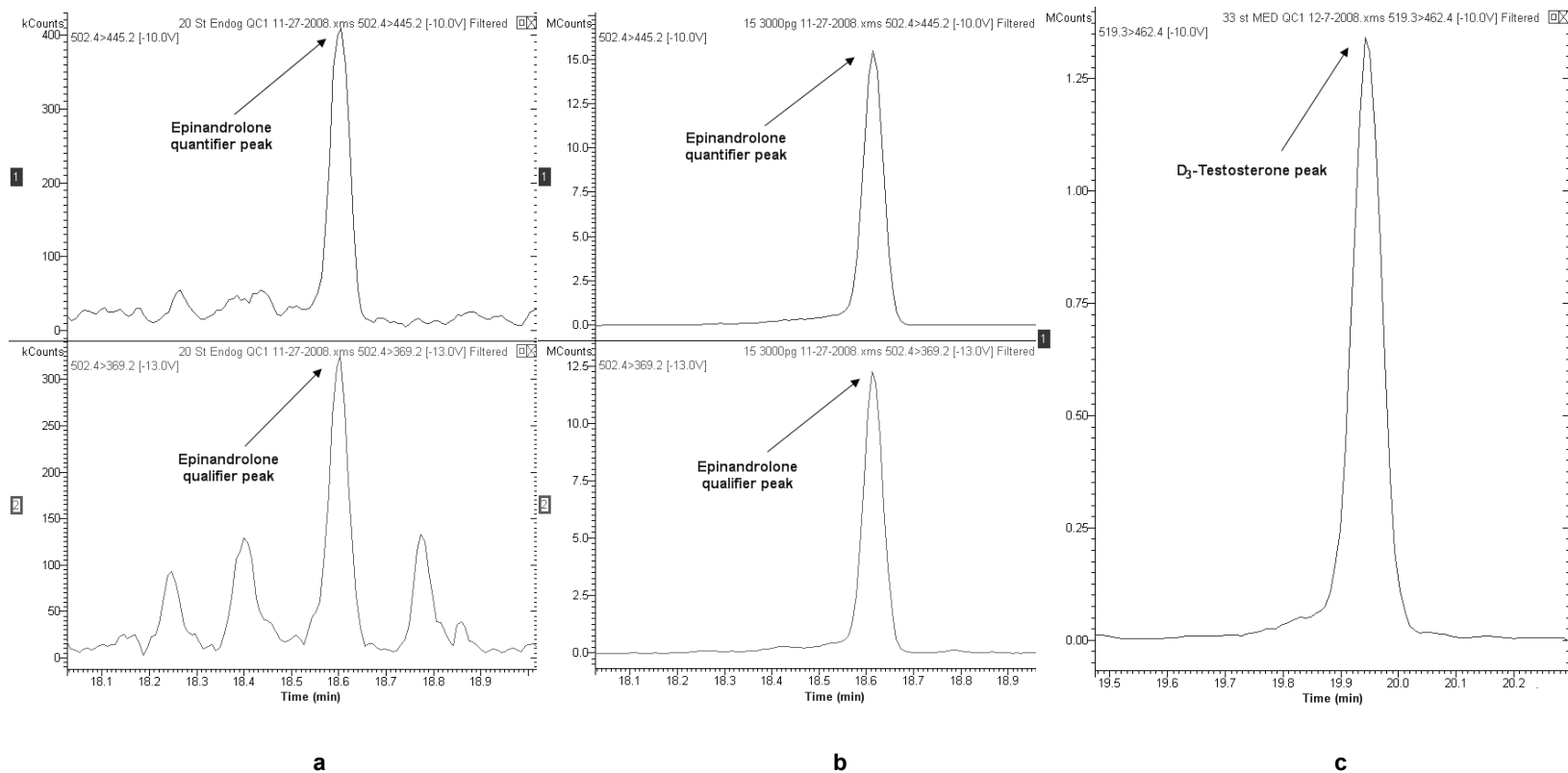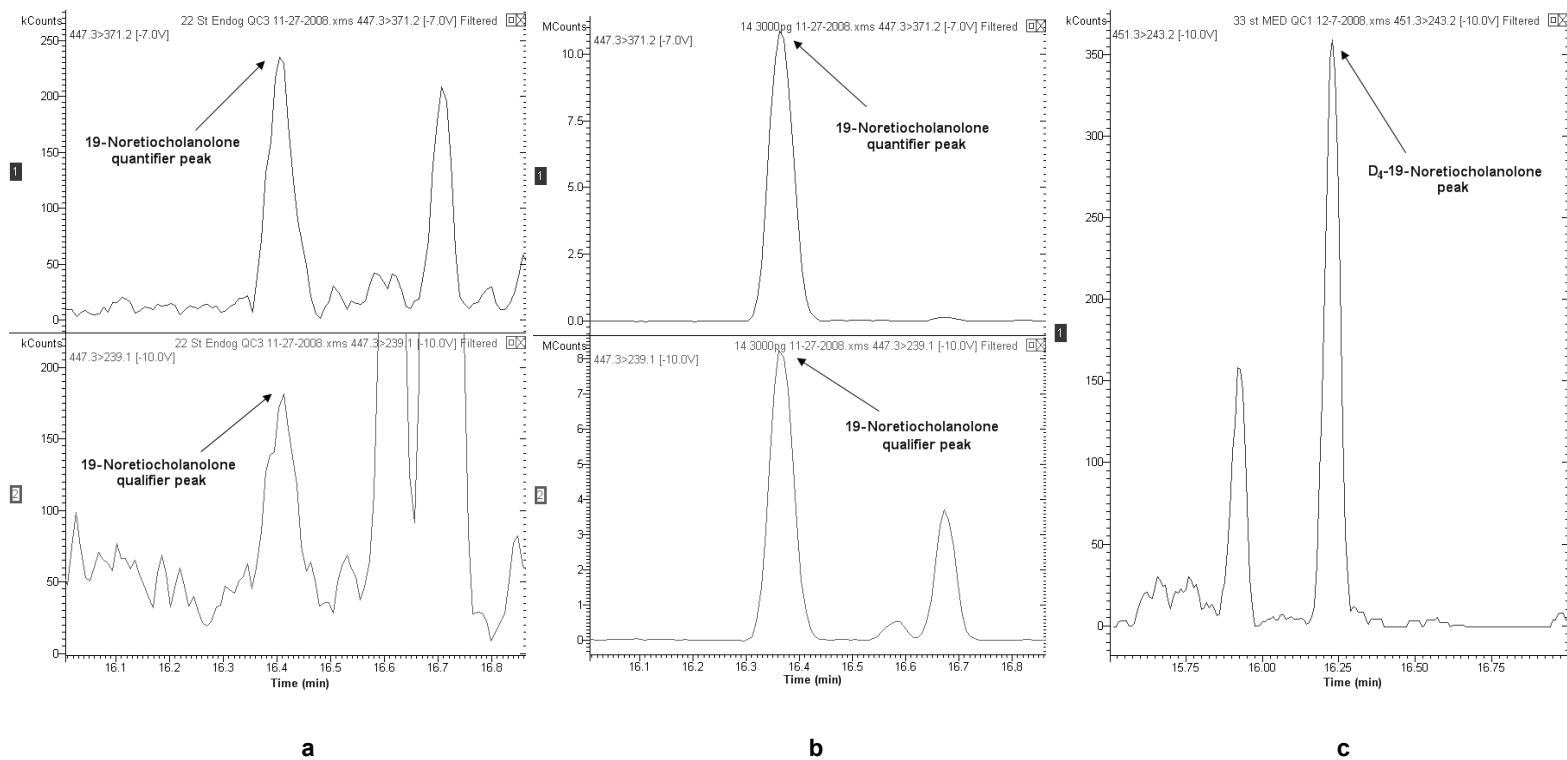
Fig. 3 – chromatograms obtained for BAB-androstanediol at **a)** the bovine urine pool endogenous concentration (measured at 449.3 pg ml^{-1}) **b)** the upper limit of quantification (an E + 3,000 pg ml^{-1} spiked calibration line sample) and **c)** the D$_5$-BAB-androstanediol internal standard used for this analyte (m/z 468.3 to 260.2). The top BAB-androstanediol trace refers to the quantifier ion (m/z 463.3 to 255.1) and the bottom trace refers to the qualifier ion (m/z 463.3 to 387.2).
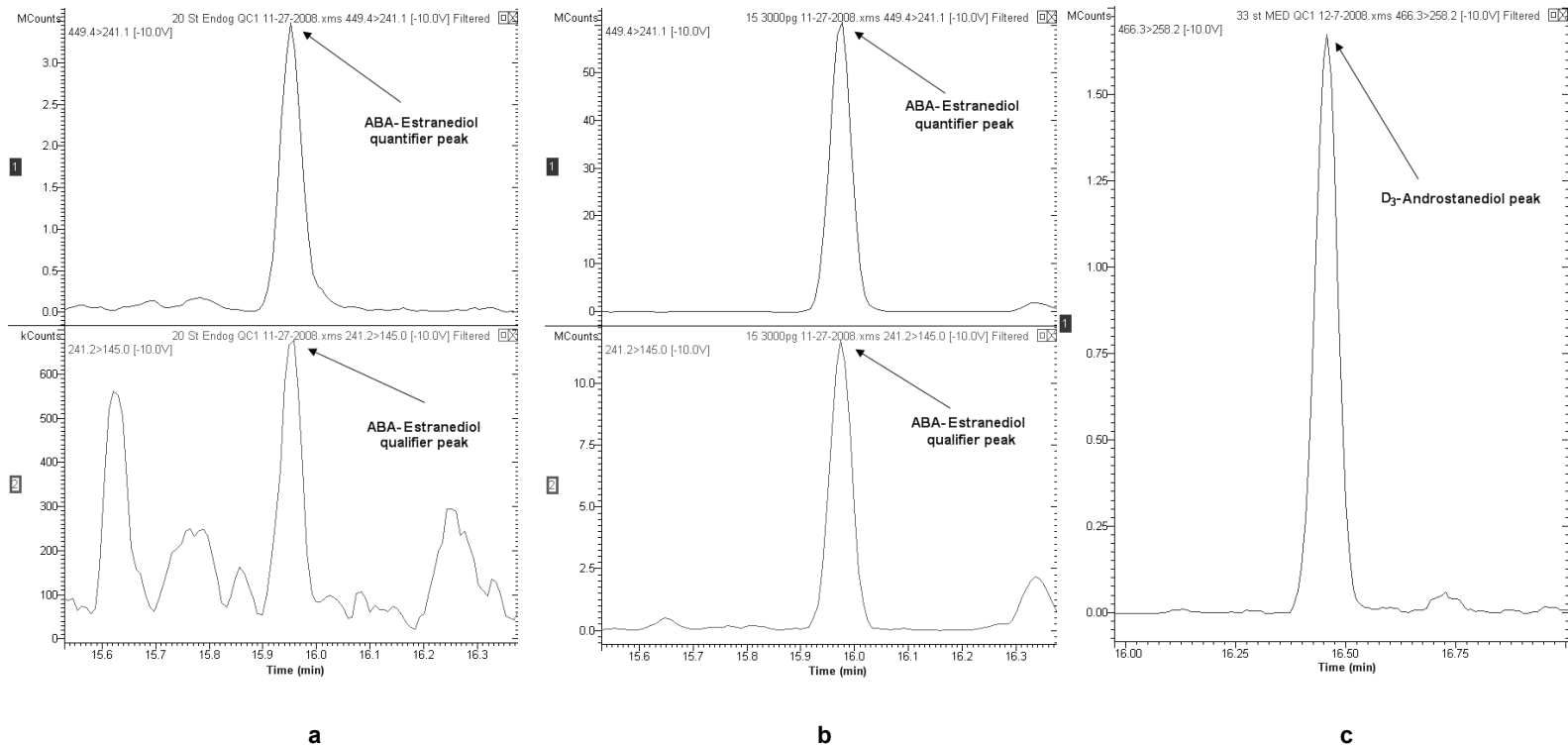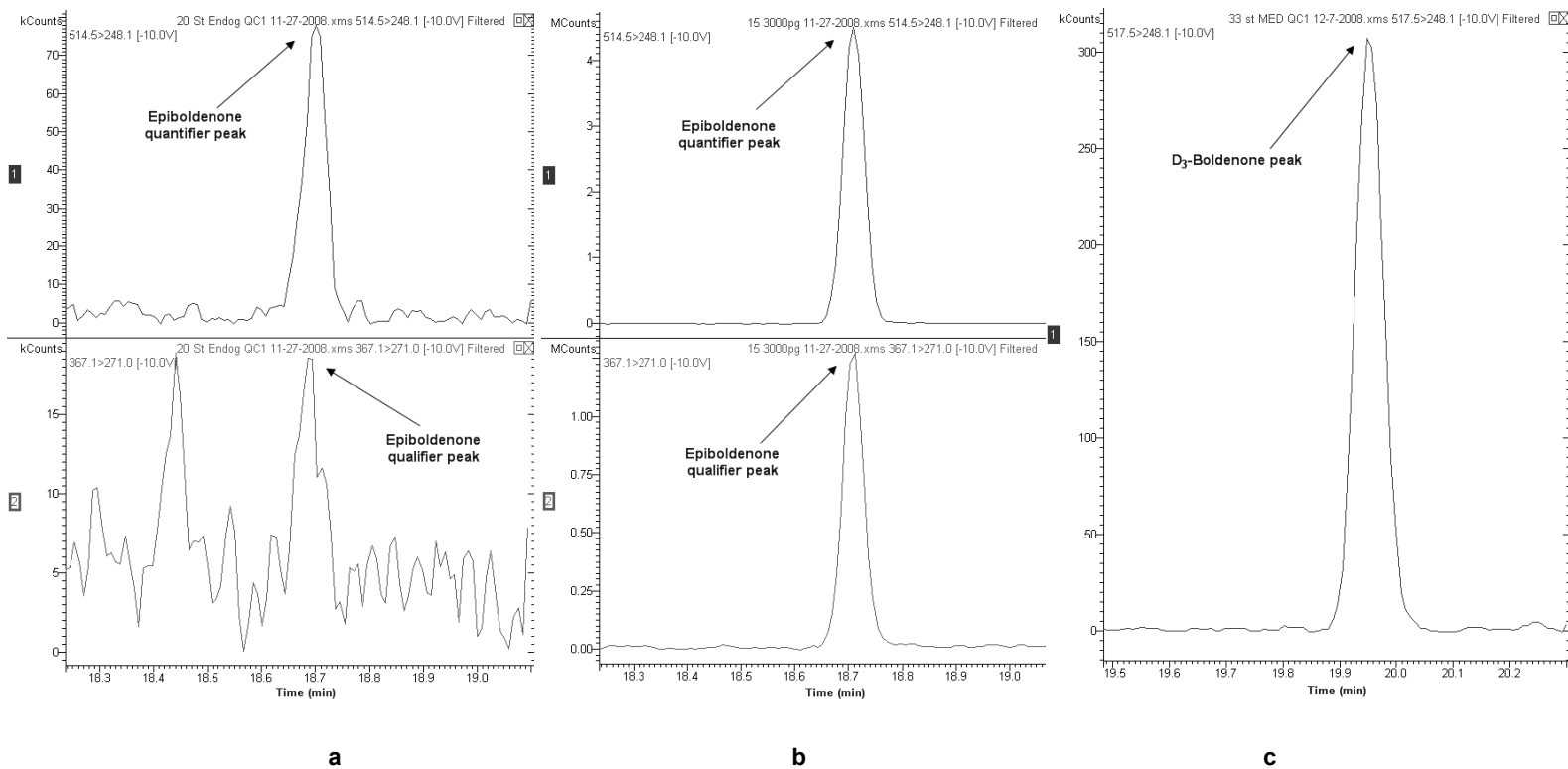
*Fig. 4 – chromatograms obtained for ABA-estranediol at **a)** the bovine urine pool endogenous concentration (measured at 178.7 pg ml^{-1}) **b)** the upper limit of quantification (an E + 3,000 pg ml^{-1} spiked calibration line sample) and **c)** the D$_3$-androstanediol internal standard used for this analyte (m/z 466.3 to 258.2). The top ABA-estranediol trace refers to the quantifier ion (m/z 449.4 to 241.1) and the bottom trace refers to the qualifier ion (m/z 241.2 to 145.0).*

*Fig. 5 – chromatograms obtained for epinandrolone at **a)** the bovine urine pool endogenous concentration (measured at 81.7 pg ml^{-1}) **b)** the upper limit of quantification (an E + 3,000 pg ml^{-1} spiked calibration line sample) and **c)** the D$_3$-testosterone internal standard used for this analyte (m/z 519.3 to 462.4). The top epinandrolone trace refers to the quantifier ion (m/z 502.4 to 445.2) and the bottom trace refers to the qualifier ion (m/z 502.4 to 369.2).*
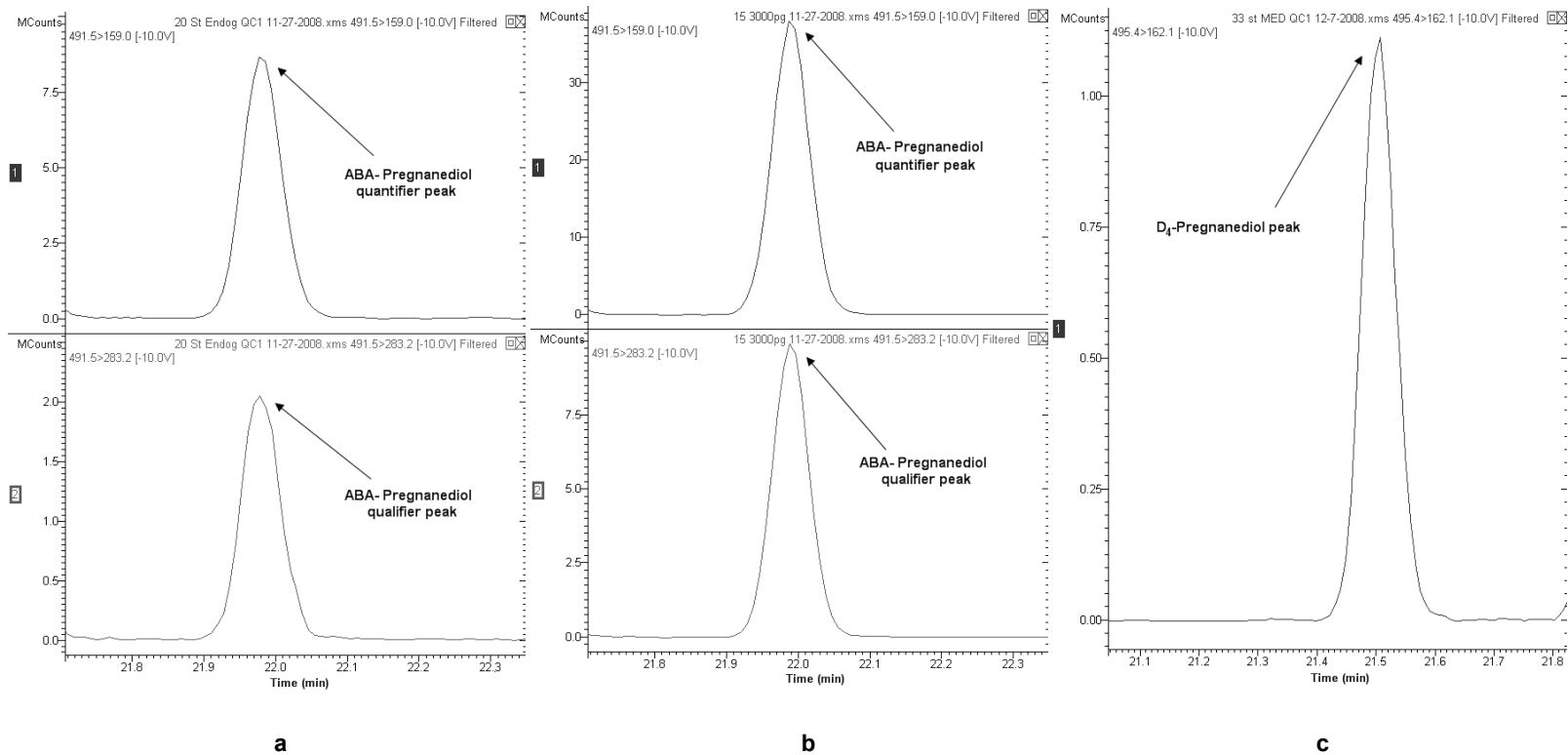
Fig. 6 – chromatograms obtained for 19-noretiocholanolone at **a)** the bovine urine pool endogenous concentration (measured at 82.7 pg ml^{-1}) **b)** the upper limit of quantification (an E + 3,000 pg ml^{-1} spiked calibration line sample) and **c)** the D$_4$-19-noretiocholanolone internal standard used for this analyte (m/z 451.3 to 243.2). The top 19-noretiocholanolone trace refers to the quantifier ion (m/z 447.3 to 371.2) and the bottom trace refers to the qualifier ion (m/z 447.3 to 239.1).
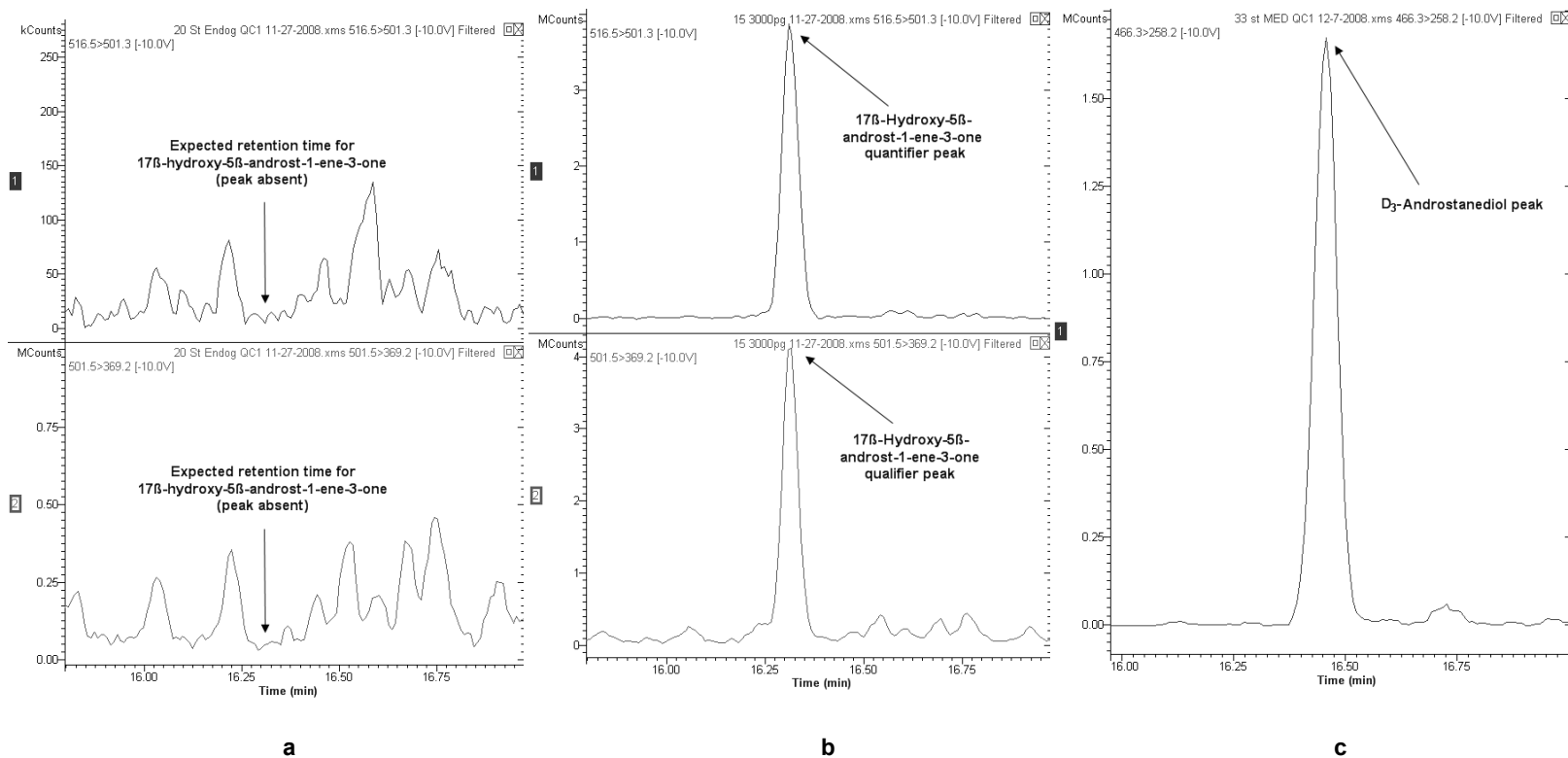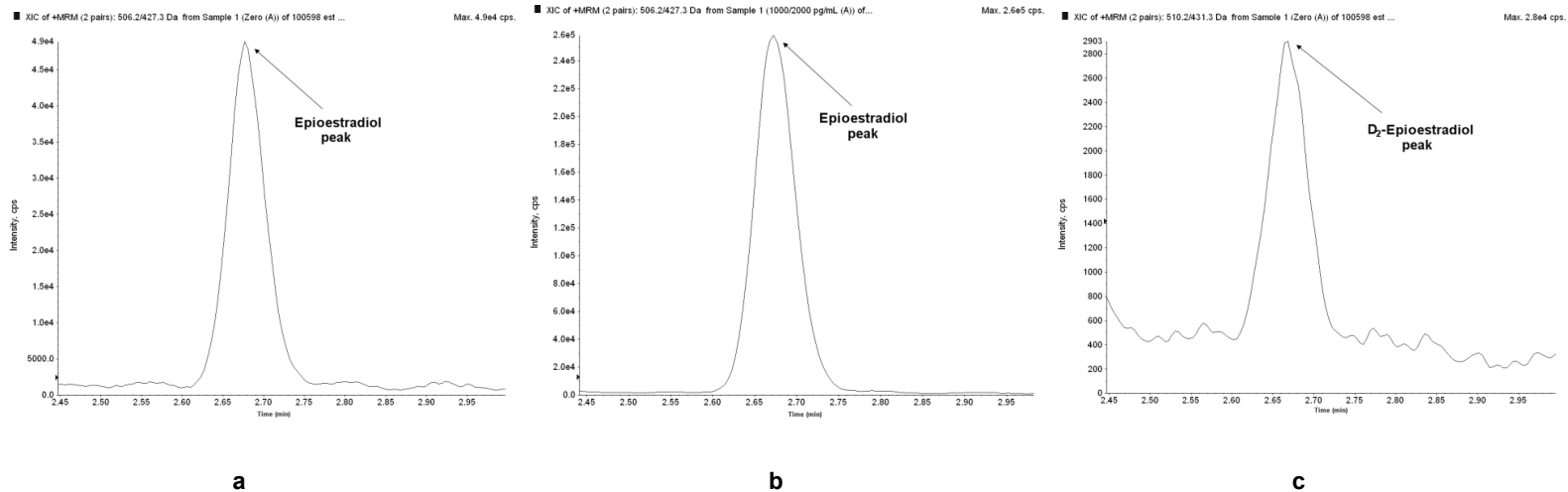
*Fig. 7 – chromatograms obtained for 17β-hydroxy-5β-androst-1-en-3-one at **a)** the bovine urine pool endogenous concentration (no peak) **b)** the upper limit of quantification (an E + 3,000 pg ml[1] spiked calibration line sample) and **c)** the D₃-androstanediol internal standard used for this analyte (m/z 466.3 to 258.2). The top 17β-hydroxy-5β-androst-1-ene-3-one trace refers to the quantifier ion (m/z 516.5 to 501.3) and the bottom trace refers to the qualifier ion (m/z 501.5 to 369.2).*

a **b** **c**

Fig. 8 – chromatograms obtained for epiboldenone at a) the bovine urine pool endogenous concentration (measured at 55.9 pg ml^{-1}) b) the upper limit of quantification (an E + 3,000 pg ml^{-1} spiked calibration line sample) and c) the D$_3$-boldenone internal standard used for this analyte (m/z 517.5 to 248.1). The top epiboldenone trace refers to the quantifier ion (m/z 514.5 to 248.1) and the bottom trace refers to the qualifier ion (m/z 367.1 to 271.0).

*Fig. 9 – chromatograms obtained for ABA-pregnanediol at **a)** the bovine urine pool endogenous concentration (measured at 1168.5 pg ml^{-1}) **b)** the upper limit of quantification (an E + 3,000 pg ml^{-1} spiked calibration line sample) and **c)** the D$_4$-ABA-pregnanediol internal standard used for this analyte (m/z 495.4 to 162.1). The top ABA-pregnanediol trace refers to the quantifier ion (m/z 491.5 to 159.0) and the bottom trace refers to the qualifier ion (m/z 491.5 to 283.2).*

Fig. 10 – chromatograms obtained for epioestradiol (m/z 506.2 to 427.3) at a) the bovine urine pool endogenous concentration (measured at 475.1 pg ml^{-1}) b) the upper limit of quantification (an E + 2,000 pg ml^{-1} spiked calibration line sample) and c) the D$_2$-epioestradiol internal standard used for this analyte (m/z 510.2 to 431.3 – monitoring the S$_{34}$ isotope in order to avoid the interference caused by the S$_{34}$ isotope of epioestradiol-dansyl). No qualifier ions could be monitored for epioestradiol as no alternative diagnostic fragments were formed during collision induced dissociation of this compound.

3.3.2 Selectivity

In addition to the chromatographic separation, selectivity was assessed through monitoring the ratio of two separate MS/MS transitions for each steroid (with the exception of epioestradiol, for which a second selective transition could not be found). The matching transition ratios for these steroids support the suggestion that the endogenous peaks found in the bovine samples relate to the analytes in question (Fig. 3 to 10). The only exception was for 17β-hydroxy-androst-1-en-3-one, where no endogenous peaks were detected.

3.3.3 LC-MS/MS Matrix Effects

After correcting for the known endogenous concentration for each of the 5 individual bovine urines spiked with epioestradiol at 1200 pg ml^{-1}, the % bias and % relative standard deviations were 0.948 and 10.3% respectively, indicating that there were no significant matrix effects that the deuterated internal standard did not correct for.

3.3.4 Calibration Line Linearity

The calibration curves (weighted 1/x) were linear over the range studied (from E to E + 3,000 pg ml^{-1} for all steroids other than 17β-hydroxy-androst-1-en-3-one, which was linear from 250 to 3,000 pg ml^{-1}). R^2 values were greater than 0.99, intercepts were minimal relative to the y axes scale and all back-calculated concentrations were within +/- 20.0% of the nominally spiked concentrations (+/- 25.0% at the LLOQ; based on the FDA guidelines, as discussed earlier) (Table 2) .

Table 2 –calibration line results (mean values of n=4 for androgens/progestagens and n=3 for oestrogens).

Analyte (all weighted 1/x)	R^2	Slope	Intercept	Response factor RSD (%)
BAB-Androstanediol	0.994	1.97×10^{-3}	-8.00×10^{-4}	5.98
ABA-Estranediol	0.998	3.67×10^{-3}	6.27×10^{-3}	5.78
Epinandrolone	0.997	1.20×10^{-3}	-8.03×10^{-3}	6.84
19-Noretiocholanolone	0.994	2.93×10^{-3}	1.04×10^{-2}	8.31
17β-Hydroxy-5β-androst-1-ene-3-one	0.994	1.61×10^{-4}	1.23×10^{-3}	11.9
Epiboldenone	0.998	1.50×10^{-3}	5.60×10^{-3}	7.41
ABA-Pregnanediol	0.994	2.70×10^{-3}	0.125	5.53
Epioestradiol	0.996	3.82×10^{-2}	1.50	5.92

3.3.5   Precision and Accuracy

Inter-batch precision and accuracy were good, with bias (% bias) and relative standard deviations (% RSD) generally within 20% (Table 3). The only instances where values fell outside of +/- 20% were for 19-noretiocholanolone, where the % bias of the endogenous (E) QCs was -20.9% and the RSD of the E+25 QCs was 20.5%, and for epiboldenone where the RSD of the E QCs was 27.4%. In each of these cases, the reason that these values were outside the desired 20.0% (based on the FDA guidelines, as discussed earlier) was because of the limiting sensitivity at these low concentrations. Since uncertainty of measurement will be factored into any real-life utilization of the methods presented, the only practical significance of these low-concentration deviations from the desired 20.0% bracket will be an increase in the uncertainty associated with any results generated. Since the difference between the concentrations of 19-noretiocholanolone and epiboldenone in the urine from untreated and steroid treated animals is expected to be several orders of magnitude, the slight increase in uncertainty of measurement that will result for these analytes is not expected to have a significant impact on any resulting concentration thresholds for detecting steroid abuse once these methods are applied.

3.3.6   Linearity of Dilution

Results of QC samples diluted 1 in 10 from above the calibration range were acceptable, with % bias being less than 15.0% in each case (Table 4).

3.3.7   Limits of Quantification (LOQ) and Detection (LOD)

The limit of quantification was defined by the lowest point on the bovine urine calibration curve that could be quantified with a % bias and % RSD of less than 25.0% (based on the FDA guidelines, as discussed earlier) (Table 5). The limits of detection were calculated using the endogenous bovine QCs analyzed during the validation. The mean S:N of the peaks in these samples were used to extrapolate to the limits of detection at a S:N of 3:1 (Table 5).

3.3.8   Matrix Stability

All analytes were found to be stable in urine at -80°C during the 6-months period studied (within +/- 20.0% of time-zero values at all subsequent time-points studied).

Table 3 – inter-batch precision and accuracy QC results (n=20 at each level for androgens/progestagens and n=15 at each level for epioestradiol).
* The nominal value for the endogenous QCs was the mean of the determined concentrations in the endogenous QCs in the first of the precision and accuracy validation batches. The nominal value for the remainder of the QCs was this endogenous concentration plus the known spiked amount on top of this number i.e. endogenous + 100 pg ml^{-1} for LOW QCs.

All concentrations are in pg ml^{-1}	Endogenous QCs (E)	Endogenous + 25 pg ml^{-1} QCs (E+25)	Endogenous + + 100 pg ml^{-1} QCs (E+100)	Endogenous + 1,000 pg ml^{-1} QCs (E+1,000)	Endogenous + 2,000 pg ml^{-1} QCs (E+2,000)
BAB-Androstanediol					
Nominal conc.*	449.3	474.3	549.3	1,449.3	2,449.3
Measured mean	461.2	505.7	559.7	1,433.9	2,340.6
% RSD	9.56	12.6	9.85	6.22	6.17
% Bias	2.66	6.63	1.90	-1.06	-4.44
ABA-Estranediol					
Nominal conc.*	178.7	203.7	278.7	1,178.7	2,178.7
Measured mean	176.4	202.3	275.0	1,245.3	2,179.2
% RSD	9.06	8.34	6.41	3.15	5.63
% Bias	-1.26	-0.651	-1.32	5.65	0.026
Epinandrolone					
Nominal conc.*	81.7	106.7	181.7	1,081.7	2,081.7
Measured mean	87.6	109.3	186.6	1,154.0	2,118.5
% RSD	15.3	19.0	15.3	6.11	6.25
% Bias	7.21	2.47	2.68	6.68	1.77
19-Noretiocholanolone					
Nominal conc.*	82.7	107.7	182.7	1,082.7	2,082.7
Measured mean	65.4	92.2	163.1	1,109.7	2,042.2
% RSD	19.1	20.5	11.5	8.76	11.3
% Bias	-20.9	-14.4	-10.7	2.49	-1.95
17β-Hydroxy-5β-androst-1-en-3-one					
Nominal conc.*	< LOD	< LOD	< LOD	1,000	2,000
Measured mean	< LOD	< LOD	< LOD	1,042.9	1,994.6
% RSD	< LOD	< LOD	< LOD	10.1	8.84
% Bias	< LOD	< LOD	< LOD	4.29	-0.268
Epiboldenone					
Nominal conc.*	55.9	80.9	155.9	1,055.9	2,055.9
Measured mean	65.0	93.8	166.3	1,142.2	2,087.8
% RSD	27.4	13.9	17.1	8.19	5.34
% Bias	16.4	16.0	6.71	8.17	1.55
ABA-Pregnanediol					
Nominal conc.*	1,168.5	1,193.5	1,268.5	2,168.5	3,168.5
Measured mean	991.2	1,011.7	1,022.9	1,935.1	2,830.6
% RSD	16.1	18.8	11.0	10.1	8.54
% Bias	-15.2	-15.2	-19.4	-10.8	-10.7
Epioestradiol	**(E)**	**(E+20)**	**(E+80)**	**(E+800)**	**(E+1,600)**
Nominal conc.*	475.1	495.1	555.1	1,275.1	2,075.1
Measured mean	444.1	469.8	528.2	1,274.0	2,108.0
% RSD	7.06	8.17	6.62	7.03	7.55
% Bias	-6.54	-5.11	-4.85	-0.088	1.58

Table 4 – results of the 1 in 10 dilution QCs.

* Because of the endogenous contribution in the urine used for dilution, in order to compare the % bias of the measured concentrations in the diluted samples compared to the nominally spiked concentration, it was necessary to adjust the measured values for the endogenous contribution. This was achieved by subtracting the endogenous concentration of the diluting matrix (as measured by the mean of five endogenous QCs run in this batch) from the dilution QC measured value and then multiplying the result by the dilution factor of 10.

All concentrations are in pg ml^{-1}	Measured concentration after 1 in 10 dilution	*Adjusted concentration relative to the nominally spiked concentration of 16667 pg ml^{-1} (12,000 for epioestradiol)
BAB-Androstanediol		
Measured mean	1,828.4	14,185.0
% RSD	5.07	6.54
% Bias	N/A	-14.9
ABA-Estranediol		
Measured mean	1,902.4	17,258.7
% RSD	4.23	4.67
% Bias	N/A	3.55
Epinandrolone		
Measured mean	1,769.0	17,145.5
% RSD	4.84	5.00
% Bias	N/A	2.87
19-Noretiocholanolone		
Measured mean	1,652.4	15,949.7
% RSD	10.1	10.4
% Bias	N/A	-4.30
17β-Hydroxy-5β-androst-1-ene-3-one		
Measured mean	1,418.4	14,184.0
% RSD	11.5	11.5
% Bias	N/A	-14.9
Epiboldenone		
Measured mean	1,811.6	17,388.5
% RSD	7.50	7.82
% Bias	N/A	4.33
ABA-Pregnanediol		
Measured mean	2,227.2	14,691.7
% RSD	3.62	5.49
% Bias	N/A	-11.9
Epioestradiol		
Measured mean	1,824.0	13,400.0
% RSD	7.85	10.7
% Bias	N/A	11.7

Table 5 – calculated limits of detection and limits of quantification for the different analytes.

* The limit of detection was calculated by taking the mean signal to noise ratio of the endogenous peaks determined during the validation and extrapolating these ratios to give the concentration that would lead to a signal to noise ratio of 3:1. In the case of 17β-hydroxy-5β-androst-1-ene-3-one, the calculation was based on the 250 pg ml^{-1} calibrants since no endogenous peaks were present.

** The limit of quantification is given as the endogenous concentration calculated during batch 1 of the validation. In the case of 17β-hydroxy-5β-androst-1-ene-3-one, the limit of quantification is given as the lowest calibrant that can be quantified with a % bias of <25.0%. Although these values are the formal limits of quantification, because this method uses a standard addition approach, the true limits of quantification may in some cases be much lower than those given above.

Steroid	Calculated Limit of Detection (pg ml^{-1})*	Limit of Quantification (pg ml^{-1})**
BAB-Androstanediol	94.1	449.3 (endogenous)
ABA-Estranediol	10.9	178.7 (endogenous)
Epinandrolone	19.8	81.7 (endogenous)
19-Noretiocholanolone	32.4	82.7 (endogenous)
17β-Hydroxy-5β-androst-1-ene-3-one	160.7	250 (not detected endogenously)
Epiboldenone	10.5	55.9 (endogenous)
ABA-Pregnanediol	12.2	1,168.5 (endogenous)
Epioestradiol	14.8	475.1 (endogenous)

3.4   Discussion

The present study provides a convenient method of quantifying 'endogenous' urinary steroids of a wide range of structures using techniques that are available in most drug residue surveillance laboratories. The extractive dansylation of oestradiol away from the androgens/progestagens in particular is useful since it prevents the need for a second aliquot of urine and also enhanced sensitivity. The use of an enol-TBDMS derivatisation approach prior to the GC-MS/MS analysis of the androgens and progestagens was found to be useful for analyzing reduced steroids, since they tend to fragment more extensively when analysed as enol-TMS derivatives; hence limiting the sensitivity compared to the enol-TBDMS version (James Scarth, personal observation).

The methods presented above are, for some steroids, more sensitive than previous studies (discussed further in chapter 7), allowing the demonstration that all of the targeted analytes, with the exception of 17β-hydroxy-5β-androst-1-ene-3-one, were endogenous in the pool of bovine steer urine (Table 5). The fact that epioestradiol, ABA-pregnanediol and BAB-androstanediol were detected endogenously is not surprising since oestradiol, testosterone and progesterone are generally regarded as ubiquitous among male and female mammals; being produced by intact animal's gonads and in castrated animals through adrenal biosynthesis (Hadley and Levine, 2006). Somewhat lower in concentration were the nandrolone metabolites epinandrolone, ABA-estranediol and 19-noretiocholanolone (Table 5). Epinandrolone is known to be produced by pregnant bovine animals at the end of gestation (De Brabander et al. 1994) and, as discussed in chapter 1, by injured animals. However, in contrast to previous published studies (McEvoy et al. 1999), the current research has demonstrated that epinandrolone, 19-noretiocholanolone and ABA-estranediol are also endogenous in uninjured castrated male animals, albeit at relatively low concentrations. Both boldenone and epiboldenone have been shown previously to be endogenous in certain sub-populations of animals (De Brabander et al. 2004), although it is not certain exactly what the source of these 1-dehydro-steroids are and what conditions lead to their presence (Scarth et al. 2009). As discussed further in chapter 1, one theory is that they are produced by microbial transformation of other steroids present in faeces, which is why the EU guidelines require that sampling be performed without faecal contamination (European Commission, 2003). Results from the current study show that while epiboldenone itself was present at low concentrations in a pool of bovine steer urines, the reduced metabolite 17β-hydroxy-5β-androst-1-ene-3-one was not detectable above the limit of detection and may therefore be useful in detecting boldenone abuse (Table 5).

Since it is known that the majority of endogenous steroids in the bovine are excreted predominantly as glucuronide conjugates (Scarth et al. 2009), the current method used selective glucuronide conjugate hydrolysis using recombinantly produced β-glucuronidase enzyme to measure the concentrations of combined free and glucuronide versions of all steroids. This is an important point of clarification, since the use of alternative methods that hydrolyse all steroid conjugates present (therefore including sulphates) may in theory lead to higher determined concentrations than those reported herein. In the future, when applying any thresholds determined using this currently reported method, it will be necessary for laboratories to determine 'free and glucuronide'

concentrations in order to ensure that their results are applicable to the method used to set the thresholds. The methods above have since been applied to urine samples from a large population of untreated male and female animals, the results of which will be published separately in due course.

3.5   Acknowledgements

3.6   References

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PART 2: DETECTION OF 'DESIGNER' STEROID ABUSE IN ANIMAL SPORTS

Chapter 4: Steroid metabolism and detection in the equine

After:

Scarth, J., Teale, P. and Kuuranne, T. Drug metabolism in the horse: a review. (2011). *Drug Testing and Analysis*. 3, 19-53.

4.1   Drug metabolism in the equine

An appreciation of the pathways of drug metabolism and the underlying enzymology is important in the veterinary industry in predicting the toxicology or pharmacology of new chemical entities. It is also important in the horseracing and food production industries in order to target the relevant metabolites for the detection of drugs of abuse. The majority of the published literature on metabolism in the equine derives from studies looking at the detection of drug abuse in horseracing, with a much smaller proportion originating from studies aimed at targeting molecular enzymology aspects for veterinary drug development purposes. However, horses are also considered food-producing animals in some countries, which impacts on therapeutic drug usage patterns (and hence metabolism research) due to legislations regarding the residues of veterinary drugs entering the food chain (EU Council Directive 96/23/EC). Much of the published equine drug metabolism literature is unavailable to the general scientific public because it is has largely been published in the proceedings of the International Conference of Racing Analysts and Veterinarians (ICRAV), which is not searchable on sites such as Scopus or Medline. The following chapter will serve to introduce the topic of equine drug

metabolism, before highlighting issues surrounding the detection of 'designer' steroids (with particular reference to detection of steroid abuse within horseracing). In order to put the following equine information into context, some comparisons of metabolism with the human, bovine and porcine will also be given. These species were chosen because they represent animals of particular interest within competitive sport and food production.

4.1.1   The equidae family

The term equine itself encompasses all species of animal within the equidae family. The major extant species within the equidae family fall into one genera (Equus). Most members of the equidae family are able to interbreed and produce viable offspring, although these are almost always sterile (Skelton, 2000). The horse (Equus caballus) is now virtually extinct naturally in the wild but survives in large numbers in human captivity and in some feral populations that have developed on several continents. Due to the selective breeding of horses by humans that has been occurring for thousands of years, distinct breeds have become apparent. For example Shire horses have been selected for their strength and ability to do work, while Thoroughbreds have been bred for their speed (Budiansky, 1998).

The Thoroughbred is one of the oldest pedigrees with records spanning three centuries and is of particular importance in horseracing. A feature of this breed is the narrow genetic variation with ten founder females accounting for 72% of maternal lineages and one founder stallion being responsible for 95% of paternal lineages (Cunningham et al. 2001). Genetic variability, measured as average heterozygosity, is significantly lower in Thoroughbred horses (H=0.353 +/- 0.065) than in Argentine Creole horses (H=0.585 +/- 0.131) (Diaz et al. 2002). This narrow genetic stock of thoroughbreds may therefore be expected to lead to reduced polymorphism in many characters when compared to other breeds.

The differences that exist in overall morphology between horse breeds may also be relevant to aspects of drug metabolism. As an example, it has been reported that significant differences in the pH of Standardbred and Thoroughbred horse urine exist and that this could be expected to lead to the differential excretion of some drugs (Stanley et al. 1996). Thoroughbred urine showed a bimodal distribution with a major

peak at pH 5.5 and a minor peak at pH 8.0 while Standardbreds had a single peak at pH 8.0.

Donkeys are closely related to the horse (a domestic breed of the species Equus asinus) and a notable difference between the two species is the increased ability of donkeys to survive large losses in body water (Lizarraga et al. 2004). Only a very small amount of qualitative literature is available for the donkey and comparisons are mainly based on pharmacokinetics of the parent drug. Although the clearance of some drugs such as caffeine show similar excretion profiles between the two species, donkeys generally have a much greater capacity for drug metabolism. The clearance of phenylbutazone for example has been shown to be between 5 and 15 times higher in donkeys than in horses (Lizarraga et al. 2004).

Several gross physiological factors may lead to differences in drug metabolism between the equine, bovine, porcine and human. The diet of the four species is different; humans and porcine animals are typically omnivores, while equine and bovine species are herbivores (Kararli, 1995, Budiansky, 1998). Bovine animals are foregut fermenters and possess a large rumen, while equine animals are hindgut fermenters and have a more developed caecum with a rich flora of microorganisms that potentially contribute to the metabolism of a drug (Bailey et al. 2002). A particular feature of the equine caecal flora is that they are known to produce relatively large quantities of monoamines, which are absorbed into the bloodstream and have the potential to influence a number of physiological variables (Bailey et al. 2002 and Elliot et al. 2003). The porcine caecum is reduced in size compared to that of the equine, while it is almost completely regressed in humans (Kararli, 1995). Another feature of the equine is the capacity of its spleen to sequester red blood cells in times of inactivity or, conversely, to expel red blood cells into the circulation during periods of stress or exercise. The equine's huge spleen can alter the red blood cell content of blood by up to 50%, which may have evolved as an adaptation to exercise in this species (Stewart and McKenzie, 2002). The spleens of many other species, however, are relatively much smaller and have a significantly reduced capacity for altering blood volume. The potential for massive changes in blood volume in the equine depending on the exercise and/or excitation status of the animal has obvious implications for variations in the pharmacokinetics of drugs.

4.1.2   Methods used for studying drug metabolism in the equine

Studies on drug metabolism in equine species have been confined to *Equus caballus* (horses) and *Equus asinus* (asses and domestic donkeys). Within these studies, there exists a large body of *in-vivo* and a very much smaller body of *in-vitro* drug metabolism literature. Unless specified otherwise, all studies reviewed from this point onwards were carried out using *Equus caballus* species. A specific breed will only be referred to when there exists a point of comparison with another breed or sub-species.

A large proportion of the published data on drug metabolism in the horse relates to the detection of drug abuse. Although an understanding of enzymology would help in analysing data, it has is not been the primary aim of these studies, which therefore leaves many questions about the underlying biology unanswered.

4.1.2.1   Analytical techniques for studying equine drug metabolism

In general, unless an authentic commercial reference standard is available or is easily synthesized, the structures of equine metabolites in surveillance research studies are generally inferred from their analytical behaviour or through comparison with known metabolic information from other species. The analytical techniques used for the actual metabolite identification have evolved significantly over the last 50 years. Initially, thin layer chromatography-fluorescence detection (TLC-FL), often using radio-labelled drug as tracers or to provide quantitative data, were very popular techniques (Moss and Rylance, 1967). While TLC-FL based detection has now largely been superseded, a minority of laboratories still use some TLC-FL for screening or confirmation of specific substances. High-performance liquid-chromatography linked to ultraviolet detection (HPLC-UV) was very popular until the 1980s/1990s (Limbrey and Chapman, 1983), after which gas chromatography and then liquid-chromatography linked to mass spectrometry (GC- and LC-MS respectively) became the techniques of choice (McKinney 2009, Teale and Houghton, 2010, Scarth et al. 2010a). While immunoassay techniques can be very useful for drug screening, they are generally of less use when it comes to performing metabolism studies as they provide little information on the structure of the metabolites. Nuclear magnetic resonance (NMR) analysis has occasionally been used to definitively identify metabolites (Pearce and Lushnikova, 2006), but this typically requires extensive

purification of large sample volumes and thus takes a significant amount of time and money and is not always feasible.

Although there has been a major shift from GC-MS to LC-MS in the past decade, GC-MS has remained an important tool because certain compounds, especially saturated steroid metabolites, suffer from poor ionisation properties under the atmospheric pressure ionisation conditions of LC-MS (McKinney et al. 2009, Teale and Houghton, 2010). The ability of many modern instruments to carry out MS^n experiments makes them particularly useful for identifying metabolites. Especially feasible are triple quadrupole instruments useful since they allow a combination of full scan MS, selected reaction monitoring (SRM), product ion scanning and more generic neutral loss or precursor ion scans to be performed; each with their own benefits depending on the type of study involved. For both GC- and LC-MS, the availability of deuterated or other labelled analogues of the drug can significantly aid the metabolite identification process, both in terms of the 'shift' technique (Scarth et al. 2010b) and the observation of peak doublets if a mixture of labelled/unlabelled versions of the drug is administered (Samuels et al. 1998). While selective derivatisation procedures prior to GC-MS analysis have been used as aid in the structural elucidation process for several decades, the application of derivatisation to LC-MS structural elucidation has been more recent. Also, the recent emergence of higher resolution LC equipment allowing the use of sub-2 μm particle sizes and high flow rates means that metabolites with similar molecular masses and retention times can now be more easily resolved and that analytical run times are shorter (Plumb et al. 2009).

Most recently, robust high-resolution-accurate-mass LC-MS (HR-LC-MS) systems operating at an increased level of resolution, typically ranging between 7,500 and 100,000 full width at half maximum height (FWHM) depending on the type of mass analyser employed, and have started gaining popularity for sports drug surveillance screening and research (Virus et al. 2008, Scarth et al. 2010a). Because the data acquired are full scan analyses of intact $[M+H]^+$ or $[M-H]^-$ species at very high resolution, the results can be analysed using statistical packages designed to pick out subtle differences between pre and post-administration samples. Some systems also allow in-source or collision activated dissociation to be performed; with the result that the accurate mass assignments of the fragment ions can significantly aid the structural elucidation process. Further advantages of using HR-LC-MS include the ability to

retrospectively analyse data once new information comes to light and the extended analyte coverage through the use of full scan MS.

In light of the fact that equine drug metabolism studies rarely utilize radiolabelled dosing (discussed further below), it is important to design sample preparation, extraction and analysis techniques that are not optimized too specifically on the 'parent' drug as metabolites often differ significantly in their behaviour. For these reasons, the best designed equine studies typically involve the use of sequential or parallel sample preparation, extraction, and analysis steps in order to encompass e.g. the extremes of polarity, ionic nature, volatility, phase two conjugation, metabolite lability to temperature and pH (Wynne et al. 2004). A particular feature of equine urine, which impacts on the choice of sample preparation or extraction technique, is its wide range of pH, which is affected by factors such as diet and exercise (Woods et al. 1990). Also, equine plasma contains relatively high esterase activity, so it is important to consider whether any metabolites detected in the matrix are authentic or artefacts (Scarth et al. 2010a).

A particularly important consideration regarding sample preparation relates to the hydrolysis of urine samples prior to extraction to cleave the phase two conjugates. While this is generally beneficial in the extraction and analysis of the drugs, it means that the mode of conjugation is often undetermined. This is not always the case, however, and selective hydrolytic procedures or LC-MS techniques have sometimes been able to determine the nature of a conjugate. The conjugation pathways for endogenous and exogenous steroids in the horse, for example, are particularly well characterised (Houghton, 1992). The particular method used for hydrolysing phase two conjugates prior to analysis is of critical importance in measuring the metabolite profile. For example, recombinantly produced β-glucuronidase from *E. coli* is relatively selective for hydrolysing glucuronides, whereas β-glucuronidase from *Helix pomatia* also contains aryl sulphatase and other enzymes leading to hydrolysis of some aromatic sulphates and various phase one reactions (Houghton et al. 1992). Simultaneous hydrolysis of glucuronide and sulphate conjugates is possible using strongly acidified methanol (methanolysis), although the harsh conditions may lead to losses or by-products (Gomes et al. 2009). At present, there are very few drug screening assays based on the analysis of intact phase two conjugates (Lampinen-Salomonsson et al. 2006, Grace et al. 2008). This may be partly due to a lack of reference standards for equine phase two metabolites, but also because they are not generally suitable for analysis by GC-MS.

However, the combination of LC-MS analysis with *in vitro* production of conjugated metabolites may now allow the development of intact phase two conjugate screening assays on a larger scale. This would be advantageous since it could in theory significantly reduce the amount of sample preparation required and, in the case of urinary steroid metabolites, mean that they could be detected efficiently using electrospray ionization.

4.1.2.2   In vivo equine metabolism studies

The most comprehensive *in vivo* metabolism studies involve the administration of a radiolabelled analogue of the drug in order to trace its fate with maximum certainty. This offers several benefits including; the ability to study the mass balance of the drug in different excreted products, the ability to focus the analysis on sample fractions containing increased levels of radioactivity and to ensure the suitability and high recovery of sample preparation and extraction techniques as an analytical aid (Seymour et al. 1990). The drawbacks of radiolabelled studies are the cost of preparing the material, the ethical considerations regarding the administration of radioactivity, the requirements for specialized analytical equipment and the precautionary measures in handling and disposing of the samples (especially when dealing with large animals such as horses). In drug surveillance laboratories the number of radiolabelled *in vivo* equine metabolism studies is limited and reducing in frequency, whereas they appear to have been carried out during veterinary drug development as a convenient way to study the mass balance and pharmacokinetics.

With regard to *in vivo* drug administration studies in drug surveillance research, the range of studied matrices generally mirrors the most suitable ones for routine testing. Equine urine has been the most extensively studied matrix to date; partly because of its applicability to post-race testing protocols, but also because it typically contains a much higher proportion of metabolites than plasma. Other matrices that have been studied include faeces (Popot et al. 2004, Popot et al. 2006), hair (Dunnett et al. 2005) and saliva (Horner, 1976). Although not typically containing as large a proportion of metabolites as urine or faeces, blood analysis is gaining importance due to its ease and speed of collection during training or pre-race, a general requirement for less sample purification compared to urine and a greater relevance of the observed parent drug concentrations to evaluation of their pharmacological effect (Kwok et al. 2010). There are

also regional differences, such that testing protocols in the USA place greater emphasis on blood analysis compared to some other parts of the world (Arthur, 2008).

4.1.2.3   In vitro equine metabolism studies

While *in vivo* metabolism studies are the mainstay of equine metabolism research, the use of *in vitro* techniques utilising *ex vivo* liver or lung preparation is gaining popularity (Scarth et al. 2010a). *In vivo* experiments have the advantage that the whole plethora of possible transformation can be considered and allows the most representative picture of the situation for real-life samples. However, *in vivo* experiments require animal experimentation and hence there are additional ethical considerations and significant resource requirements. Also, the identification of drug metabolites in urine is often complicated by the presence of interferences, the timescales of the experiments are relatively long, compounds without previously defined toxicological profiles such as 'designer' drugs cannot be easily studied for ethical reasons and it is difficult to carry out mechanistic studies such as the identification of the enzymes responsible for metabolism. By comparison, *in vitro* methods do not require animal experimentation, although there can be ethical issues related to the supply of tissues, can be carried out quickly, produce a 'cleaner' extract for analysis, can be used to study 'designer' drugs (discussed further later in this chapter) and can be more easily tailored to study mechanistic aspects (Scarth et al. 2010a). Some of the disadvantages of using *in vitro* methods include the lack of an intact biological system and the inability to generate quantitative *in vivo / in vitro* correlation. It is therefore important to recognize these limitations as well as the advantages.

In vitro studies are used in equine surveillance laboratories to complement the *in vivo* studies (Ho et al. 2005, Ho et al. 2007a and b, Kwok et al. 2006, Scarth et al. 2010a), but proportionally more *in vitro* studies have been conducted in the veterinary arena, where they are useful e.g. in determining possible routes of metabolism. Of the published equine drug surveillance studies, *in vitro* techniques have been used to study the biosynthetic pathways of C18 androgens in testicular tissue [Smith et al. 1987] and the B-ring unsaturated oestrogens in placental tissue [Marshall et al. 1989]. More recently, equine liver microsomes have been used to study the phase one metabolism of the anabolic-androgenic steroids clostebol acetate and mesterolone [Ho et al. 2007b], methenolone acetate [Ho et al. 2005], turinabol [Ho et al. 2007a], a range of designer

steroids [Kwok et al. 2006] and the non-steroidal anti-inflammatory drug phenylbutazone [Stanley and Whitley 2006]. Most recently, two equine cytochrome P450 (CYP) enzyme isoforms, named CYP2D50 and CYP2C92, have for the first time been sequenced and their activity compared to human CYP2D and CYP2C isoforms (DiMaio Knych et al. 2008, DiMaio Knych et al. 2009).

In vitro technologies have to date been somewhat more widely applied in the human sports field (for example Levesque et al. 2005 and Gauthier et al. 2009). Several authors have also reported the scaling up of *in vitro* conditions to allow the production and purification of mg quantities of human phase one [Lehnmen et al. 1981, Vail et al. 2005] and phase two (Kuuranne et al. 2002, Kuuranne et al. 2003 Jäntti et al. 2007, Hintikka et al. 2008) drug metabolites, thus enabling characterisation using techniques such as NMR. These approaches often require the use of recombinantly expressed enzymes or tissue from animals that have been administered enzyme inducing chemicals. Since there are currently no recombinantly expressed equine drug metabolizing enzymes available and because there are ethical issues surrounding the administration of enzyme inducing chemicals to horses, these approaches are less viable in the horseracing industry. Other factors that have limited the use of *in vitro* technologies in equine drug surveillance include the availability of equine tissues and a limited knowledge of the application of *in vitro* techniques. However, equine tissues are now becoming more readily available from specialist suppliers and there is increasing demand from many angles to reduce the use of animals in experimentation. The increased usage of HR-LC-MS also provides a strong incentive to utilize *in vitro* methods for studying drug metabolism. The technique not only provides a powerful tool for the rapid interpretation of drug metabolism but is also increasingly used as a screening technique within drug control laboratories. Where only knowledge of the qualitative profile of metabolites is required it is relatively straight forward to insert the relevant masses and retention times into a detection database, in-vitro techniques are ideally suited to this application (Scarth et al. 2010a).

In order to confirm the presence of a metabolite in the case of a positive drug finding it is necessary to compare the mass spectral and chromatographic performance of the analyte against a standard material. Currently in this situation, International Laboratory Accreditation Cooperation-G7 guidelines on the *'Accreditation Requirements and Operating Criteria for Horseracing Laboratories'* [ILAC-G7, 2009] allow the use of *in vivo*

urine/plasma samples resulting from a drug administration as qualitative reference material. However, this is not ideal as it requires animal experimentation. In 2009, a new revision of the ILAC-G7 guidelines (ILAC-G7, 2009) stated for the first time (article 16.4) that *in vitro* incubations can now be used in place of *in vivo* post-administration samples. It is important to stress that under the ILAC-G7 guidelines, it is not necessary to produce large quantities of *in vitro* metabolite that can be isolated and subject to NMR. As with the existing *in vivo* post-administration paradigm, it is simply necessary to demonstrate that the analytical data are sufficient to fully justify the compound's identity as a metabolite by demonstrating the presence of a diagnostic analyte spectrum in post-administration samples but not in pre-dose samples or blanks. It is therefore envisaged that a major benefit of *in-vitro* methods will be to provide reference materials where no 'parent' drug can be detected and for which no chemically synthesized metabolite reference standard is available.

4.2   Metabolism of anabolic-androgenic steroids in the equine

Because of their importance as ergogenic drugs of abuse within the horseracing industry and due to their involvement in equine reproduction, steroid metabolism has received more focus in the equine than any other class of compounds. Anabolic-androgenic steroids are banned under Federation Equestre Internationale (FEI, 2010) rules and also by horseracing authorities that are signatories to Article 6 of the International Agreement on Breeding, Racing and Wagering (IFHA, 2008). Most recently, the use of anabolic-androgenic steroids (AASs) has also become controlled in the majority of US horseracing events (Teale and Houghton, 2010).

In-vivo and in-vitro studies of both endogenous and exogenous steroid metabolism in the horse have been conducted and there is increasing interest within the veterinary field to understand the molecular biology of their action as well as measuring their concentrations in biological fluids. The presence and metabolism of endogenous AASs in meat producing animals, including the horse, has been reviewed in chapter 1 (and published in Scarth el al. 2009) and a review article addressing the metabolism of both endogenous and exogenous AASs in the horse has recently been produced (Teale and Houghton 2010). Also, a review of the analytical methodologies used to detect steroid abuse has also recently been published (McKinney 2009). Because these aforementioned articles already provide a thorough coverage of the field, the current

section of this review will serve to summarize the overall trends within the equine and to provide some further information on the enzymology of steroid metabolism within this species.

All the major mammalian steroid biosynthetic pathways have been demonstrated to be present in the horse: equine follicular cDNAs from CYP11A1, 3β-hydroxysteroid dehydrogenase/Δ5-/Δ4-isomerase (Boerboom and Sirois 2001) and 17β-hydroxysteroid dehydrogenase have been cloned (Brown et al. 2004); equine steroid acute regulatory protein (STAR) and CYP17 mRNA have been detected in theca cells (Watson et al. 2004); CYP17 has been immunolocalised in the mare corpus luteum (Rodger et al 1998); equine CYP19 cDNA has been cloned (Seralini et al 2003) and also immunolocalised in testicular tissue (Almadhidi, 1995). The existence of many of the remaining steroidal CYPs and oxidoreductases is based on the detection of their metabolites in biological fluids (Makin, 1985).

Again, in order to provide a frame of reference, the following section will compare some aspects of steroid metabolism in the equine to the porcine, bovine and human.

A major species difference in steroid biosynthesis involves CYP17, which catalyses both the 17α-hydroxylation and 17,20-lyase of pregnenolone and progesterone in two distinct steps (Mason, 2002). The 17α-hydroxylation step is efficient for both pregnenolone and progesterone in all species but the 17,20 lyase is often selective for one particular substrate. In the equine and porcine, both 17α-hydroxyprogesterone and 17α-hydroxypregnenolone can be substrates, but human and bovine activity is selective for 17α-hydroxypregnenolone. This leads to the human and bovine using predominantly the 5-ene pathway and the equine and porcine both the 4-ene and 5-ene pathways for the biosynthesis of the androgens and oestrogens (Mason, 2002). Although these enzymatic preferences for different precursors exist, circulating 5-ene concentrations are generally high in human, bovine, porcine and equine species relative to those such as the cervine in which 4-ene steroids predominate (Wichman et al. 1984). Males and females of all four species are able to produce the oestrone and oestradiol, but the concentrations produced by intact male equine and porcine animals are far greater than those of the human or bovine (McKinnon and Voss, 1993). Intact male porcine animals also produce relatively large quantities of 16-androstene steroids (Hadley and Levine, 2006), while

pregnant equine animals secrete large amounts of the B-ring unsaturated steroids equilin and equilenin (McKinnon and Voss, 1993).

Differences in the presence and phase 1 catabolism of endogenous AASs and their routes of excretion (urine vs faeces etc.) were discussed at length in chapter 1, so the details will not be repeated here. However, it is pertinent to note that that while $3\alpha/3\beta/5\alpha/5\beta$-reduction and 17-oxidation are important catabolic pathways in each species, equine animals also tend to secondarily reduce 17-keto groups to from a mixture of 17α- and 17β-hydroxy isomers and bovine animals predominantly reduce 17-keto groups to from 17α-hydroxy isomers. For synthetic AAS analogues, in which many of the classical oxidative and reductive routes of metabolism are hindered, multiple carbon skeleton hydroxylations are common and the only major differences between the species are the positions of hydroxylation. Phase two conjugations of AASs involve glucuronidation and sulphation in all four species, but there is a trend for sulphation to predominate in the equine and glucuronidation in the bovine and human. More data would be required before a trend in the porcine can be stated, but endogenous AAS data suggests that sulphation is prominent (Schwarzenberger et al. 1993). In the horse, there is also a preference for steroids with a 17β-hydroxy group to form sulphates and those with a 17α-hydroxy group to form glucuronides (Teale and Houghton et al. 2010).

Table 1 summarizes the metabolism of individual AASs in the equine while Figure 1 summarizes the major routes of anabolic-androgenic steroid catabolism in this species.

Oxidation, leading to possible aromatisation

Reduction if R$_1$ = ketone, but if R$_1$ = hydroxy, then oxidation with variable levels of subsequent reduction

If R$_2$ = alkyl group, then varying levels of hydroxylation

Hydroxylation may occur

If R$_2$ = H, then oxidation with varying levels of subsequent reduction

If R$_2$ = alkyl group, then varying levels of hydroxylation

If R$_2$ = alkyl group, then hydroxylation a major pathway, but if R$_2$ = H, then only minor

Reduction common, but is inhibited when extended conjugation through the a-b ring is present

If R$_2$ = alkyl group, then hydroxylation can be major pathway, although variable. If R$_2$ = H, then only minor

If R$_2$ = alkyl group, then hydroxylation can be a major pathway, although variable

Fig 1 – summary of some of the common pathways of AAS metabolism in the horse. See table 1 for a more extensive summary for individual steroids (including those with various substitutions not shown above).

Table 1a – summary of the phase 1 and 2 metabolism of the androgenic-anabolic steroids studied in the horse.

Steroid	References	Phase 1 metabolites	Phase 2 metabolism	Comments
Boldenone	Dumasia et al. 1983, Ho et al. 2004	A range of metabolites result from modifications including oxidation and subsequent reduction at C17, reduction at $\Delta 1$ and $\Delta 4$ and hydroxylation at C6 and C16.	Boldenone predominantly as sulphate. Minor metabolites predominantly as glucuronides.	Boldenone and boldenone sulphate endogenous in intact males at low levels.
Clostebol	Leung et al. 2005	Major = 4-chloroandrost-4-ene-3α,17β-diol and 4-chloroandrostane-3α,17β-diol. Minor = various metabolites resulting from reduction at C3 and $\Delta 4$, oxidation at C17 and hydroxylation at C6.	The majority of metabolites were detected in the sulphate fraction.	
Danazol	Tang et al. 2000	Major = ethisterone, 6-hydroxyethisterone and 2-(hydroxymethyl)ethisterone. Minor metabolites include a number of other oxidised metabolites following isoxazole ring cleavage.	Mixture of free, glucuronide and sulphate	Cleavage of the isoxazole ring a major feature of danazol metabolism; something that is not observed for the pyrazole ring in stanozolol
Desoxyvinyltestosterone analogues (designer steroid analogues)	Kwok et al. 2006	A mixture of metabolites resulting from the addition of a ketone or hydroxy at C2 and/or C3, reduction or shifting of the double bond within the A-ring and reduction and hydroxylation of the vinyl group	Predominantly in the glucuronide fraction.	A range of other analogues with various substitutions at position 17 were also studied *in vitro*, but not *in vivo*
Ethylestrenol	Gourdie et al. 1994, Kim et al. 1996.	Major = reduction at C17. A range of minor metabolites result from modifications including reduction at C3 and hydroxylation at various sites, including C15/16.	Ethylestrenol in the free fraction, with the other metabolites predominantly in the glucuronide fraction, but with some sulphation	

Table 1b – summary of the phase 1 and 2 metabolism of the androgenic-anabolic steroids studied in the horse.

Steroid	References	Phase 1 metabolites	Phase 2 metabolism	Comments
Fluoxymesterone	Stanley el al. 1997a, Yamada et al. 2008a	Major = 9α-fluoro-11β-hydroxy-17,17-dimethyl-18-norandrosta-4-13-dien-3-one. Minor = 17-epifluoxymesterone, 16- and 20-hydroxyfluoxymesterone	Predominantly in the free fraction for all metabolites, with a smaller proportion in the glucuronide fraction	Loss of the 17-hydroxy group with subsequent migration of the C18 methyl group to the C17-position is an interesting feature of fluoxymesterone metabolism.
Furazabol	Yamada et al. 2008b	Major = 16α-hydroxyfurazabol.	Data not available.	
Mestanolone	Yamada et al. 2007	Forms the A-ring reduced metabolites of methyltestosterone.	Forms the A-ring reduced metabolites of methyltestosterone.	Mestanolone a metabolite of methyltestosterone and oxymetholone.
Mesterolone	Ho et al. 2007b	Major = 1α-methyl, 3,16-dihydroxy-5α-androstan-17-one and 1α-methyl, 3,18-dihydroxy-5α-androstan-17-one. Minor = a range of metabolites resulting from reduction at C3, epimerisation at C17 and hydroxylation at C16.	Split between the glucuronide and sulphate fractions, with 17α-hydroxy isomers predominantly as glucuronides and 17β-hydroxy isomers predominantly as sulphates.	
Methandienone	Edlund et al. 1989, Hagedorn et al. 1992, McKinney et al. 2001b, Kurosawa et al. 2006	Major = 16-hydroxymethandieneone, Δ4-reduced + 6 and 16-hydroxylated as well as Δ4-reduced and 6,16-dihydroxylated methandienone. Minor = a number of metabolites, including epimethandienone, and a range of A-ring fully reduced metabolites (being the longest detected metabolites).	16-hydroxymethandieneone predominantly as sulphate, epimethandienone predominantly as free and the remainder of metabolites split between the glucuronide and sulphate fractions.	Some sulphate conjugates unstable in urine, leading to 'apparent' unconjugated metabolites if storage conditions not adequate
Methandriol	Yamada et al. 2008b, Kurosawa et al. 2006	As the A-ring reduced metabolites of methyltestosterone	A mixture of glucuronide and sulphate for parent methandriol. As methyltestosterone for the A-ring reduced metabolites.	

Table 1c – summary of the phase 1 and 2 metabolism of the androgenic-anabolic steroids studied in the horse.

Steroid	References	Phase 1 metabolites	Phase 2 metabolism	Comments
Methenolone	Ho et al. 2005	Mixture of epimethenolone, 17-keto-16-hydroxymethenolone, 16-hydroxymethenolone and 2,6-dihydroxy-methenolone (this metabolite was only observed *in vitro*)	Metabolites split between both the glucuronide and sulphate fractions.	
Methyltestosterone	Schoene et al. 1994, Stanley et al. 1997b, McKinney et al. 2007	Hydroxylation at C6, C15, C16 and C20, epimerization at C17 as well as a range of metabolites resulting from A-ring reduction, including mestanolone	Predominantly as sulphates.	
Nandrolone	Houghton 1977, Houghton et al. 1984, Roig et al. 2007, Houghton et al. 2007	Major = 5α-estrane-3β,17α-diol. Minor = a number of metabolites, including epinandrolone, norepiandrosterone, 5α-estrane-3β,17β-diol and various C16-hydroxylated metabolites.	5α-estrane-3β,17α-diol predominantly as glucuronide, while nandrolone predominantly as sulphate. Other metabolites split between both fractions.	Some C18 androgens endogenous in intact males and pregnant females. Now thought to be predominantly analytical artefacts caused by degradation of 19-oic acids.
Norethandrolone	McKinney et al. 2001a	Mixture of metabolites resulting from reduction of the A-ring and hydroxylation at C16, C20 + C21 (also forming a carboxylic acid at C21)	Carboxylic acids in free fraction. The remainder of metabolites in either the sulphate, glucuronide or a mixture of both fractions.	Norethandrolone also a metabolite of ethylestrenol
Normethandrolone (nomethandrone)	Fox et al. 2000	Mixture of metabolites including, epinormethandrolone, A-ring reduced metabolites, 6- and 16-hydroxynormethandrolone and A-ring reduced 16-hydroxynormethandrolone	C6- and C16-hydroxylated metabolites predominantly in the glucuronide fraction, with the remainder split between the glucuronide and sulphate fraction	71% of the overall radioactive dose excreted in glucuronide fraction
Oxymetholone	Yamada et al. 2008c, Tang et al. 1998	Mixture of mestanolone, C3-reduced mestanolone isomers, 2-hydroxymethyl-17α-methyl-5α-androstan-3,17β-diol and 2,17α-di(hydroxymethyl)-5α-androstan-3,17β-diol.	Metabolites split between both the glucuronide and sulphate fractions.	

Table 1d – summary of the phase 1 and 2 metabolism of the androgenic-anabolic steroids studied in the horse

Steroid	References	Phase 1 metabolites	Phase 2 metabolism	Comments
6-Oxo (androst-4-ene-3,6,17-trione)	Leung et al. 2010	An extensive array of metabolites resulting from reduction at C3, Δ4, C6, and C17.	Some detected in both free and glucuronide fractions, while others in glucuronide only. Sulphation not assessed due to the acid lability of metabolites.	
Stanozolol	Muck and Henion, 1990, McKinney et al. 2004, Scarth et al. 2010b	Major = 15-, 16α- and 16β-hydroxystanozolol. Minor = 16-ketostanozolol and 3', 4α, 4β and 6α-hydroxystanozolol.	Stanozolol and 15-hydroxystanozolol predominantly as glucuronide, while 16α- and 16β-hydroxystanozolol predominantly as sulphates.	16α- and 16β-hydroxylation proposed to be mediated by an enzyme related to human CYP2C8s
Testosterone	Houghton and Dumasia 1979, Dumaisa and Houghton 1981	Major = 5α-androstane-3β,17α-diol. Minor = a number of metabolites, including epitestosterone, epiandrosterone, 5α-androstane-3β,17β-diol and various C16-hydroxylated metabolites	5α-androstane-3β,17α-diol predominantly as glucuronide, while testosterone predominantly as sulphate. Other metabolites split between both fractions	Testosterone endogenous in intact males, geldings and females.
Testosterone precursors (DHEA or androst-4-ene-3,17-dione)	Dehennin et al. 2001	Increased excretion of testosterone metabolites for both DHEA and androst-4-ene-3,17-dione administration. DHEA also increased levels of androst-5-ene-3,17-diols.	Concentrations given as 'total' only, but is stated that DHEA sulphate 'attained high levels after DHEA administration.'	
Trenbolone	Houghton et al. 1992, Suann et al. 2000	Major = epitrenbolone. Minor = various 16-hydroxylated metabolites.	Mixture of glucuronide and sulphate.	A-ring metabolism inhibited by the extended A-B-C-ring conjugation.
Turinabol	Ho et al. 2007a	Major = epiturinabol, 20-hydroxyturinabol, 6β,20-dihydroxyturinabol and a range of A-ring reduced metabolites. Minor (*in vitro* only) = 6βhydroxy and 6β, 16-dihydroxyturinabol.	Excreted as a mixture of sulphated and free steroids	

4.3 The detection of steroid abuse in horseracing

4.3.1 Detection of the classical 'synthetic' and 'endogenous' steroids

The range of analytical techniques used for detecting steroid abuse in equine sports was discussed in the introduction to this thesis, so this section will serve to discuss highlight issues that are relevant to specific AASs.

In the majority of instances, detection of the abuse of classical 'synthetic' steroids in equine sports relies on a simple qualitative demonstration of their presence. Urine and plasma are the two most popular matrices used for testing and require somewhat different analytical approaches. Abuse of the majority of AASs in plasma can be detected by monitoring 'parent' steroid, whereas in urine it is typically necessary to monitor the deconjugated metabolites listed in table 1 as well (Teale and Houghton, 2010). Steroids such as trenbolone containing a high degree of chemical conjugation are generally more suitable for LC-MS analysis, whereas highly saturated steroids such as methandriol are more suited to GC-MS analysis. Many laboratories therefore operate both LC-MS and GC-MS screens for steroids in order to obtain the highest degree of analyte coverage (McKinney, 2009).

In contrast to the situation for many of the other food producing species, methods for detecting the abuse of the majority of 'endogenous' AASs in equine sports are already available:

Since 19-nor-androgens have not been shown to be endogenous in the gelding or filly/mare, their presence at any concentration is considered evidence of nandrolone abuse (with the exception of the pregnant mare, which is known to produce 19-nor-androgens during gestation). A threshold utilising a ratio of 5α-estrane-3β,17α-diol:estr-5(10)-ene-3β,17α-diol of greater than 1:1 was previously used to indicative nandrolone abuse in male horses (Houghton et al. 1984) since nandrolone is considered endogenous in this sex. However, this biomarker ratio approach has recently been replaced with a 45 ng ml^{-1} threshold for the nandrolone metabolite 5α-estrane-3β,17α-diol (IFHA, 2008).

Since 1-dehydro-androgens have not been shown to be endogenous in the gelding or filly/mare, their presence at any concentration is considered evidence of boldenone abuse. However, boldenone has recently been shown to be naturally occurring as a sulphate conjugate in the urine of intact males (Ho et al. 2004) and a urinary threshold of 15 ng ml^{-1} boldenone has been introduced in order to regulate abuse (IFHA, 2008).

In the gelding and filly/mare, urinary threshold concentrations for testosterone have been set at 20 and 55 ng ml^{-1} respectively in order to control the abuse of this steroid (IFHA, 2008). There is no urinary testosterone threshold in the intact male due to the high and variable concentrations produced in this animal.

Unlike urine, definitive data on the plasma concentrations of 'endogenous' steroids in large populations of animals across different geographical locations are not currently available, so there are no internationally accepted thresholds in this matrix (McKinney 2009). However, analytical methods based on the detection of intact testosterone, nandrolone and boldenone esters in equine plasma have recently been developed by our laboratory; which now provides a means of detecting abuse of these preparations (Gray et al. 2010). In the future, it is anticipated that the detection of intact steroids esters in hair may further enhance detection periods for these compounds.

4.3.2   The threat of 'designer' steroids

Designer steroids first came to the public attention in 2003 when a syringe containing the novel steroid tetrahydrogestrinone (THG) was handed to doping officials, which resulted in the disqualification of several athletes after they were subsequently found to have used the steroid (Catlin et al. 2004). Designer steroids have chemical structures based on previously marketed products, but with minor modifications which make them undetectable by the majority of current targeted mass spectrometric procedures. In the case of THG for example, the drug's structure is based upon gestrinone, but with the 17-alkyl side chain fully saturated such that the relative molecular mass of THG is 4 u higher than gestrinone. Designer steroids are synthesized either to deliberately evade detection, or as appears more common, to enable them to be marketed freely on the Internet to customers in some countries because their structures do not fall within the scope of legal regulations that prevent the sale of defined steroidal products. Although THG was the first such steroid to reach public attention, it is now known that the steroid

turinabol (4-chloro,17β-hydroxy,17α-methyl-androsta-1,4-dien-3-one) was distributed amongst East German athletes in a state sponsored doping programme in order to evade detection procedures in place at the time (Ungerleider, 2001). It is now also known that the company that produced THG, namely the Bay Area Laboratory Cooperative), also supplied the designer steroids norbolethone (17β-hydroxy-13β,17α-diethylgon-4-en-3-one) and desoxymethyltestosterone (17α-methyl-5α-androst-2-en-17β-ol) to athletes (Kicman, 2008, McKinney 2009).

As with any AAS, if testing is based on blood or hair then detection of the 'parent' steroid may be appropriate. However, if detection is to be based on excreted matrices such as urine and faeces, knowledge of the metabolism is typically required in order to direct detection protocols toward the appropriate target analyte. With this in mind, several strategies have been proposed to detect the abuse of designer steroids in biological samples taken from humans and animals.

One approach that has been proposed by several authors is based on predicting the pattern of fragmentation under EI-GC-MS or LC-MS/MS conditions (Thevis et al. 2005, Fragkaki et al. 2009). Using this approach, a variety of scan functions can be used to screen for the presence of common fragments or neutral losses from a range of possible steroid structures in biological samples. If these initial screening analyses produce a suspicious result, then this may trigger a more in depth analysis of the sample in order to determine the possible structure of any unknown components.

Another approach is to use bioassay guided fractionation of samples, followed up by in depth analysis of any fractions that produce positive bioassay results. One such approach has been published by Peters et al. (2010) uses a yeast expressed androgen receptor assay to highlight sample fractions that produce androgenic pharmacological effects. If the bioassay produces a suspicious result, then this may trigger a more in depth analysis of the sample fraction in order to determine the possible structure of any unknown components. An alternative bioassay based on ELISA has been reported by Hungerford et al. (2005). This approach utilises an antibody that recognises steroids that contain a 17α-methyl,16β,17β-dihydroxy epitope, therefore theoretically producing a suspicious result for any 17α-methyl steroid that undergoes 16β-hydroxylation as part of it's metabolism. Since 16β-hydroxylation is a major pathway of metabolism of such

steroids, especially in equine animals (Houghton, 1992), this again highlights a suspicious sample for further work to identify any unknown molecules.

While the above approaches are based on the detection of designer steroids through screening for the presence of the steroid or metabolite itself, an alternative method that is currently being investigated is the use of endogenous 'biomarkers' (Scarth et al. 2006, Mooney et al. 2009). The theory of the biomarker approach is that regardless of structure, the majority of AASs are likely to produce their pharmacological actions through similar means such as through binding to the androgen receptor or antagonising the cortisol receptor. Therefore, as long as they produce similar pharmacological effects, designer steroids may be just as likely as classical steroids to induce a change in endogenous biomarker profile and hence for the suspicious sample to undergo further investigation.

The above approaches are considered most suitable for identifying new designer steroids that regulatory authorities are not aware previously existed, such as THG. However, the extent of abuse of this type of designer steroid is hard to predict and it is anticipated that it would be limited to a small group of elite individuals who are able to obtain access to a small number of rogue chemists. The designer steroids that appear to be the most widely available are those marketed on the Internet. Although these steroids contain novel structures in order to enable them to be marketed freely on the Internet to customers in some countries (because their structures do not fall within the scope of legal regulations that prevent the sale of defined steroidal products), the regulatory authorities are able to maintain an awareness of their emergence through Internet searches. For these steroids, it is therefore possible to purchase materials that can then be used for analytical method development purposes.

While it is theoretically possible to conduct *in vivo* metabolism studies using Internet available designer steroids, the fact that these compounds typically lack toxicological characterisation makes this approach difficult to justify on ethical grounds. Several other approaches have therefore been reported for producing metabolite information where reference standards of designer steroids are available. Peters et al. (2009) proposed an *in silico* approach to identifying the presence of designer steroids and/or their metabolites in samples using a range of possible transformations of existing steroid structures. Lootens et al. (2009) and Pozo et al. (2009) have recently developed a rodent

model transplanted with human hepatocytes. Using this approach, human type metabolism can be investigated without the need for experimenting on people. In the future, it may be possible to adapt this approach to utilise equine hepatocytes. However, this technique still requires the use of animal experimentation. Therefore, attractive alternatives that have shown excellent qualitative *in vivo* correlation are the use of different *in vitro* models, involving incubation of steroids with either *ex vivo* mammalian liver preparations (Ho et al. 2005, Ho et al. 2007a and 2007b, Leung et al. 2004) or surrogate invertebrate models (De Wasch et al. 2002, Verheyden et al. 2007); hence negating the requirement for mammalian *in vivo* experimentation.

Several designer steroid studies have been carried out in the human. Levesque et al. (2005) and Gauthier et al. in (2009) used human hepatocytes to study the *in vitro* metabolism of THG and 17α-methyl-drostanolone respectively, while Rodchenkov et al. in (2006) studied the *in* vivo human metabolism of a number of capsulated products purported to contain different steroidal products. In the latter study, however, reference standards for the compounds in question were not available to confirm the structures of the steroids contained in the capsules obtained on the Internet.

To date, no studies have reported the metabolism of designer steroids in the canine and only one study has been reported in the equine. In the equine study, Kwok et al. (2006) reported the *in vivo* and *in vitro* metabolism of desoxy-vinyltestosterone and some of its analogues. The objective of the remainder of part 2 of this thesis was therefore to further develop and assess the suitability of *in vitro* techniques for conducting equine drug metabolism studies (chapter 5) and to use the developed *in vitro* methods to study the metabolism of a novel 'designer' steroid in the equine (chapter 6).

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Chapter 5: Assessment of the applicability of in vitro technologies to study drug metabolism in the equine

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After:

Scarth, J., Spencer, H., Hudson, S., Gray, B., Teale, P. and Hillyer, L. The application of in vitro technologies to study the metabolism of the androgenic/anabolic steroid stanozolol in the equine. (2010). Steroids. 75, 57-69.

5.1 Introduction

During the past decade, *in vitro* methods for studying drug metabolism have become widespread in the drug development industry (Yuan et al. 2002). In contrast, while a small number of authors have reported the use of *in vitro* studies in sports drug surveillance research, their application has to date not been widespread (see chapter 4 for a more detailed discussion). Although there are many scientific advantages to the use of *in vitro* techniques, the reduced need for animal experimentation is a significant benefit to animal welfare. In the equine, *in vitro* techniques have been used to study the biosynthetic pathways of C18 androgens in testicular tissue (Smith et al. 1987) and the B-ring unsaturated oestrogens in placental tissue (Marshall et al. 1989). More recently, equine liver microsomes have been used to study the phase 1 metabolism of the androgenic-anabolic steroids clostebol acetate and mesterolone (Leung et al. 2004), methenolone acetate (Ho et al. 2005), turinabol (Ho et al. 2007), a range of designer steroids (Kwok et al. 2006) and the non-steroidal anti-inflammatory drug phenylbutazone (Stanley et al. 2006). In general, the *in vitro* studies reported to date have produced comparable qualitative metabolite profiles to those obtained in urine following *in vivo*

administrations, but some quantitative differences have been observed. Most recently, two equine cytochrome P450 (CYP) enzyme isoforms, named CYP2D50 and CYP2C92, have for the first time been sequenced and their activity compared to human CYP2C and D isoforms (DiMaio Knych et al. 2008 and 2009). While the equine and human isoforms shared some substrate specificity, there were significant differences in the enzyme kinetics and the range of metabolites produced in the different species. *In vitro* techniques have also been applied in the human sports field. For example, hepatocyte preparations have been used to study the phase 1 and 2 metabolism of the designer steroid tetrahydrogestrinone (THG) (Lévesque et al. 2009). The authors were able to isolate enough material to perform NMR analysis of some of the resulting metabolites. Although the majority of research has focussed on harvesting mammalian tissues since these are generally considered to be the most representative of the *in vivo* mammalian situation, invertebrate models such as those involving maggots and crustaceans have also shown promise (Verheyden et al. 2007, De Wasch et al. 2002).

In the work reported in the current chapter, the use of equine liver microsomes, and for the first time liver and lung S9 tissue fractions, were used to study the metabolism of the androgenic/anabolic steroid stanozolol in the equine. The overall aim of these experiments was to assess the suitability of *in vitro* techniques for studying steroid metabolism in this species. The major objectives were a) to compare the *in vitro* phase 1 metabolite profile observed here with urinary profiles previously reported *in vivo* in the equine, b) to further characterise some of the metabolites observed and c) to identify some of the enzymatic mechanisms that may be responsible for the metabolism.

Stanozolol (17β-hydroxy,17α-methyl-5α-androstano[3,2-c]pyrazole - see Fig 1) is a synthetic testosterone derivative that first came to public attention following the positive drugs test of Ben Johnson at the 1988 Olympics. The main reason for selecting stanozolol in the current study was because significant data regarding it's *in vivo* metabolism in the equine is available.
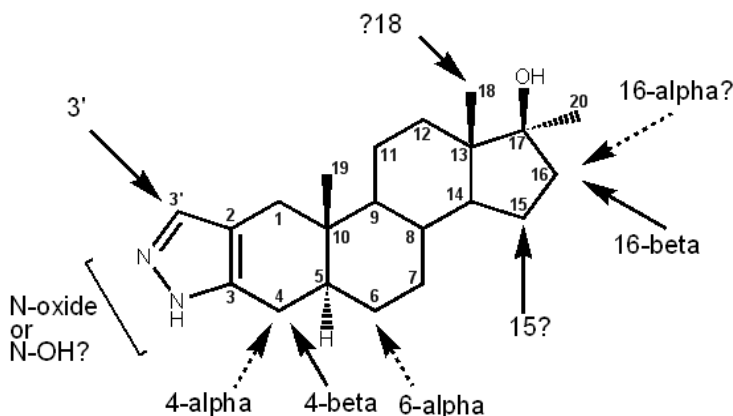
Fig 1 – structure of stanozolol with carbon numbering indicated. Arrows point to the sites of mono-hydroxylation produced in the equine liver microsomal fractions that could either be confirmed by reference standards or suggested (shown by a ?) based on pattern of fragmentation. See details in the text for a full description of these equine metabolites.

Although the early studies in the 1980s and 1990s most often used GC-MS to study stanozolol metabolism, differences in the ease of derivatisation of the different hydroxy products made it difficult to be certain of the relative abundance of the different metabolites. For example, 3'-hydroxy-stanozolol derivatises to the TMS-ether relatively easily while the 16β-hydroxy-isomers do not (Poelmans et al. 2002). The adoption of LC-MS in the 1990s and 2000s was a significant advantage as this technique does not require derivatisation of stanozolol and it is therefore easier to compare the relative abundance of the different hydroxy products (Poelmans et al. 2002, Van De Wiele, 2000). In humans, the major metabolites of stanozolol following oral administration have been shown to be 3'-hydroxy-stanozolol, 4β-hydroxy-stanozolol and 16β-hydroxy-stanozolol, but several di-hydroxy- and 17-epi- metabolites have also been detected (Schanzer et al. 1990, Masse et al. 1989). In cattle, the major metabolite of stanozolol is 16β-hydroxy-stanozolol (Van De Wiele et al. Ferchaud et al. 1997, De Brabander et al. 1998, Delahaut et al. 1998), but depending on the route of administration additional dihydroxy metabolites have also been detected (Ferchaud et al. 1997). An equine study using triple-quadrupole LC-MS/MS analysis of urinary extracts following an oral dose of stanozolol reported the detection of several mono-hydroxy stanozolol metabolites, but in most cases the stereochemistry could not be confirmed (Muck and Henion, 1990). A later equine study using ion trap LC-MS/MS analysis of urinary extracts following an

intra-muscular dose of stanozolol was able to confirm the presence of 16β-hydroxy-stanozolol as a major phase 1 metabolite and postulate two further mono-hydroxy-metabolites as 15-hydroxy- and a 16α-hydroxy-stanozolol based on their pattern of mass spectral fragmentation (McKinney et al. 2004). Phase 2 metabolism of these metabolites was a mixture of sulfation and glucuronidation. Neither 3'-hydroxy-stanozolol, 4α/4β-hydroxy-stanozolol or di-hydroxy-stanozolol isomers could be found. Most recently, a rigorous study of the mass spectrometric behaviour of stanozolol and several mono-hydroxy-metabolites has been conducted using both a quadrupole-linear ion trap and a high-resolution/accurate-mass linear ion trap-orbitrap LC-MS/MS analyzer (Thevis et al. 2005).

5.2   Experimental

5.2.1   Chemicals and reagents

Deionized water was prepared using an SG Ultrachem TWF UV system (Barsbuttel, Germany). All organic solvents, acids and bases were analytical grade and were purchased from Fisher Scientific (Loughborough, UK). Trizma base and HCl, NADPH (variant N-1630), N-Methyl-n-(trimethylsilyl)-trifluoroacetamide, ethane thiol, ammonium iodide, β-glucuronidase from *Helix pomatia* (type HP-2), pancreatin (8xUSP – product P7545) and Stanozolol were obtained from Sigma-Aldrich (Dorset, UK). D_3-stanozolol and 16β-hydroxy-stanozolol were supplied by Cerilliant (Texas, USA). 3'-hydroxy-stanozolol was purchased from the National Measurement institute of Australia (Sydney, Australia). 4α- and 4β-hydroxy-stanozolol were gifts from the Institut für Biochemie, der Deutschen Sporthochschule Köln, (Köln, Germany) and 6α-hydroxy-stanozolol was purchased from the Australian Racing Forensic Laboratory (Kensington, Australia). C18, 6 cc, 500 mg Sep-pak Vac solid phase extraction cartridges and a 100 mm x 2.1ID 3 μm Atlantis T3 column were supplied by Waters (Wexford, Ireland). Strata XC 3ml 60mg solid phase extraction cartridges were obtained from Phenomenex (Macclesfield, UK). Equine liver microsomes and S9 (both at 20 mg ml^{-1} protein and from a Quarter horse) were purchased from Xenotech Llc. (Lenexa, USA), while equine lung S9 (at 20 mg ml^{-1} and from a thoroughbred horse) was prepared by Asterand (Royston, UK). The horses used were euthanized by ethically approved means for reasons not related to the current study.

5.2.2   In vitro experiments

Incubations of stanozolol, D_3-stanozolol, 6α-hydroxy-stanozolol or 16β-hydroxy-stanozolol were performed with equine liver microsomes, liver S9 or lung S9. Reactions volumes were 0.33 ml and contained drug (25 µM), microsome or S9 (at 0.5 mg ml^{-1} protein), NADPH (0.63 mM) and pH 7.4 TRIS buffer (50 mM). Samples were incubated in a water bath at 37°C for 2 hours and 50 µl aliquots were quenched at 0 and 120 mins by the addition of 75 µl of ice-cold acetonitrile. Control experiments with no added cofactor were also performed in order to check that metabolism was responsible for producing any metabolites observed.

Stanozolol was also incubated with equine liver microsomes under the above conditions, but with the addition of a range of chemicals that are known to inhibit individual human cytochrome P450 isoforms (selectively in most cases) in order to try and provide some preliminary information on the enzymes that might be responsible for the metabolism of stanozolol in the equine. Chemical inhibitors were added in excess of their published human IC50 values in order to allow for any reduction in affinity that may occur between species and were; α-naphthoflavone (at 12 µM to inhibit CYP1A2), 8-methoxypsoralen (at 12 µM to inhibit CYP2A6), setraline (at 12 µM to inhibit CYP2B6), quercetin (at 12 µM to inhibit CYP2C8), sulfaphenazole (at 6 µM to inhibit CYP2C9), ticlopidine (at 12 µM to inhibit CYP2C19), quinidine (at 6 µM to inhibit CYP2D6), clomethiazole (at 12 µM to inhibit CYP2E1) and ketoconazole (at 0.6 µM to inhibit CYP3A4). Chemical inhibitors that resulted in inhibition of stanozolol metabolism in this initial screen were then subject to full IC50 analysis using a full range of inhibitor concentrations.

For samples that were analysed by LC/MS/MS, the quenched aliquots were then centrifuged for 5 mins at 11,000 rpm, the supernatant transferred to a separate vial and blown down to dryness and reconstituted in 5 µl propan-2-ol followed by 95 µl of water, before being submitted to LC-MS analysis on the Thermo LTQ-Orbitrap or Sciex 5500 Q Trap.

For samples that were analysed by GC-MS, the quenched aliquots were diluted to 2 ml with water and then added to 6 ml, 500 mg C18 solid phase extraction cartridges that had previously been primed with 5 ml methanol and 5 ml water. The cartridges were then washed with 5 ml water and 5 ml hexane before being dried under vacuum for 20 mins

and then eluted with 5 ml diethyl ether. Samples were then blown down to dryness, reconstituted in 30 µl of an enol-trimethylsilyl derivatising reagent (made by adding 60 µl of ethane thiol and 30 mg ammonium iodide to 10 ml N-Methyl-n-(trimethylsilyl)-trifluoroacetamide) and heated at 80^0c for 2.5 hours before being transferred to tapered vials and submitted to GCMS analysis on the Agilent 7000A.

5.2.3   In vivo experiments

An oral dose of 140 mg Stanozolol (preparation Stromba) was administered to a thoroughbred gelding following an ethically approved protocol. Urine samples were collected from the animal and pooled over the following three time periods; 0-24, 24-28 and 48-72-hours.

Urine samples were prepared for analysis by mixing 2 ml of urine sample with 1 ml of 1 M pH 4.7 acetate buffer, 100 µl of a β-glucuronidase from *Helix pomatia* solution (40,000 units ml^{-1} in water) and 100 µl of a pancreatin solution (made by dissolving 1.25 g of Sigma product P7545 in 50 ml of 0.05 M pH 6.6 acetate buffer) and then hydrolysing overnight at 40^oC. Samples were then extracted the following day by conditioning a Phenomenex Strata XC 3 ml 60 mg solid phase extraction cartridge with 3 ml methanol and 3 ml water, loading the samples, washing with 3 ml 0.1M pH 9.0 acetate buffer, 3 ml water, 3 ml 0.1 M HCl, 3 ml methanol, 3 ml diethyl ether and then eluting with 2 x 1 ml of ethyl acetate:propan-2-ol:ammonia: 80:17:3. Samples were then evaporated to dryness and reconstituted in 5 µl propan-2-ol followed by 95 µl of water before being submitted to LC-MS analysis on the Thermo LTQ-Orbitrap.

5.2.4   Thermo LTQ-Orbitrap high-resolution/accurate mass LC-MS analyses

Urine samples from the stanozolol *in vivo* administrations and tissue incubates from the *in vitro* incubations were analysed by a full scan, high resolution/accurate mass LC-MS screen.

10 µl of sample was introduced onto a Thermo Accela autosampler/HPLC linked to a Thermo Discovery LTQ-Orbitrap. Chromatography was carried out using a Waters 100 mm x 2.1 ID 3 µm Atlantis T3 column held at 40^OC. Flow rate was 400 µl min^{-1} and

mobile phase A was 0.1% acetic acid with 300 µg l⁻¹ uracil (external calibrant) and mobile phase B was 0.1% acetic acid in acetonitrile with 300 µg l⁻¹ uracil (external calibrant). Mobile phase B was at 0% at 0 mins, rising to 10% at 1.2 mins, 35% at 2.0 mins, 65% at 3.0 mins, 98% at 3.5 mins, held at 98% until 4.5 mins before being reduced back to 0% at 4.51 mins.

Sample ionisation was carried out in the positive mode using the electrospray source at a capillary temperature of 200$^{\mathrm{o}}$C, a sheath gas flow of 30 units, an auxiliary gas flow of 10 units and an ionspray voltage of 4.5 kV. Full scan centroid data over a range from 90 to 650 u was then acquired by the LTQ Orbitrap using a resolution of 30,000 at FWHM. Data was acquired and processed using the Xcalibur version 2.0.7 software.

5.2.5   Applied Biosystems Sciex 5500 Q Trap LC-MS/MS analyses

Since the equine liver microsomal incubations produced the most concentrated and cleanest extracts, a selection of these samples were analysed using product ion scanning LC-MS/MS techniques in order to structurally elucidate some of the metabolites produced. In addition to the *in vitro* samples, 10 ng injections of all available reference standards were also performed in order to match chromatographic retention time and mass spectra to those observed in the samples.

10 µl of sample was introduced into an Applied Biosystems Sciex 5500 Q Trap using a Waters Acquity autosampler/HPLC. Chromatography was carried out using a Waters 100 mm x 2.1 ID 3 µm Atlantis T3 column held at 40$^{\mathrm{o}}$C and using an elongated HPLC gradient relative to the Orbitrap analyses in order to provide some addition separation of the metabolites. Flow rate was 400 µl min⁻¹ and mobile phase A was 0.1% formic acid in water and mobile phase B was acetonitrile. Mobile phase B was at 2% at 0 mins, rising to 7% at 0.2 mins, 10% at 1.2 mins, 50% at 5.0 mins, 100% at 5.2 mins, held at 100% until 6.2 mins before being reduced back to 2% at 6.25 mins and then being held at 2% until 7.0 mins.

Structural information regarding the metabolites was achieved using the 'enhanced product ion scan (EPI)' mode (uses collision induced dissociation of precursor ions in the collision cell followed by product ion scanning using the linear ion trap function). Sample ionisation was carried out in the positive mode using the Turbo Ionspray source at a

source temperature of 600°C. Ion spray voltage was 5500 V, gas one had a flow of 50 units, gas two a flow of 50 units, the curtain gas had a flow of 10 units, the CAD gas setting was 'low', declustering potential was 120 V and entrance potential 10 V. Scan ranges were from 50 u up to 5 u above the chosen precursor ion. The Q1 resolution was 'unit', the Q3 entry barrier was 8 V, the scan rate was 10,000 u s^{-1}, the dynamic fill time setting on the linear ion trap was used and the step size was 0.12 u. Collision energies ranged between 45 and 65 eV, with exact details being given in the relevant results section. Data was acquired in profile mode and processed using the Analyst version 1.5 software.

5.2.6   Agilent 7000A GC-MS analyses

A selection of extracts from the equine liver microsome incubations were analysed using EI-GC-MS in order to ascertain whether a 15-hydroxy-stanozolol metabolite had been produced, as previous *in vivo* studies in our laboratories (Phil Teale, personal communication) had suggested that GC-MS was able to identify this metabolite in samples resulting from an *in vivo* stanozolol administration.

1 μl of sample was introduced into an Agilent 7683B injector held at 260°C in a pulsed splitless mode (at 1 psi with a 0.5 min pressure pulse at 25 psi). The split valve was opened at 1 min with a flow of 50 ml min^{-1}. chromatography was carried out using an Agilent 7890A gas chromatograph with helium as the carrier gas (1.5 ml min^{-1}) and a 3 0m x 250 um x 0.25 um Thermo TR-50MS column initially held at 150°C for 1 min and then ramped by 15°C min^{-1} to 320°C before being held for 6 mins (total run-time 18.33 mins). Mass spectrometry was carried out using an Agilent 7000A triple quadrupole mass spectrometer operated in the electron ionization (EI) mode. Source temperature was 230°C, electron energy was 70 eV, electron multiplier voltage was 500 V and the scan range was 50 to 700 u over 400 ms. Data was acquired and processed using the Agilent Masshunter version B.04.00 software.

5.3   Results and Discussion

5.3.1   *Comparison of in vivo urine and in vitro incubate results*

The results of the *in vivo* urine and *in vitro* tissue samples that were run by high resolution/accurate mass on the Orbitrap are shown in Fig 2. In the analysed *in vivo* post-dose urine samples, no stanozolol related compounds could be detected in the 24-48 and 48-72 hour post-dose urine pools. In the 0-24 hour post-dose urine pool, only a small amount of stanozolol parent drug was observed (not shown). Significant instrument responses in the current study were, however, seen for ions relating to hydroxy and keto stanozolol metabolites. For *m/z* 345.2537 relating to hydroxy-stanozolol, four major peaks at 3.71, 3.84, 4.06 and 4.16 mins were observed. Additionally, a number of very much smaller chromatographically unresolved peaks appeared to be present for this *m/z*. The peak at 4.16 mins correlated in retention time with 16β-hydroxy-stanozolol, but the other peaks in the chromatogram could not be assigned stereochemistry based on their mono-isotopic masses alone. A peak with *m/z* 343.2380 relating to a keto-stanozolol metabolite was also observed in the 0-24 hour *in vivo* post-dose urine sample with a signal intensity approximately 10% of the largest hydroxy-metabolite response.

In the equine liver microsome and S9 *in vitro* post-dose incubations, the same four major hydroxy-stanozolol and the one keto-stanozolol peaks were present as in the 0-24 hour *in vivo* post-dose urine sample. The relative intensities of the overall metabolite profiles were approximately three-fold higher in the liver microsome relative to S9 incubations, consistent with a more concentrated source of CYP mediated activity toward stanozolol being present in the microsomal fraction. The relative profile of the different metabolites between the microsomal and S9 fractions was similar, with the exception that the intensity of the peak relating to 16β-hydroxy-stanozolol varied between incubations when using microsomes (cause unknown). In the equine lung S9 incubations, the yield of metabolites was very much lower and only one of the hydroxy-stanozolol metabolites (peak at 4.05 mins) could be detected in this fraction. This is consistent with the reportedly lower level of certain drug metabolising enzyme activities in the lung of most species (Gibson and Skeet, 2001).
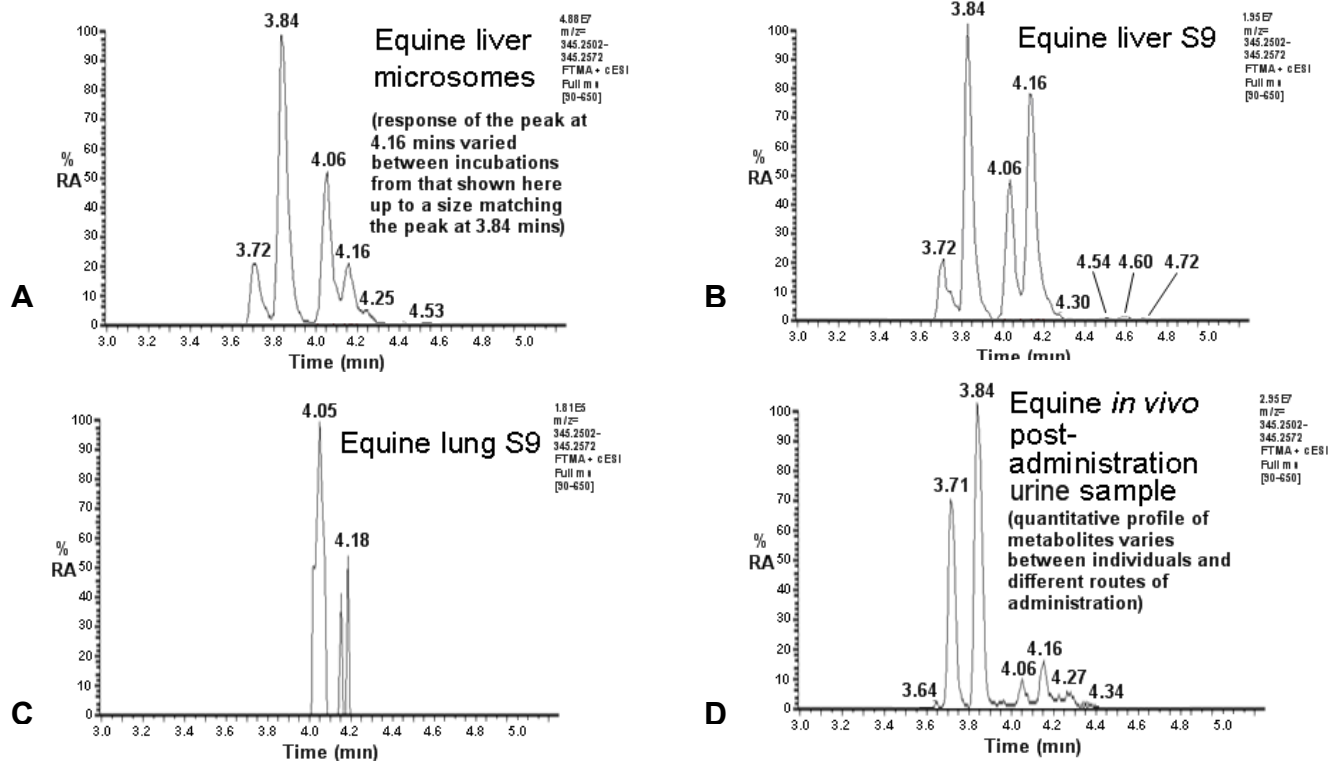
Fig 2 – comparison of hydroxy-stanozolol (m/z 345.2537 at a mass tolerance of 10 ppm) chromatograms on the Orbitrap for; **A)** equine liver microsomes, **B)** equine liver S9 and **C)** equine lung S9 and **D)** equine in vivo post-administration urine sample (see text for details of sample origin). Note: a minor metabolite at 4.14 mins, assigned as a keto-stanozolol (m/z 343.25), was also observed in the liver microsome and in vivo administrations, but is not shown above (see later results from the 5500 Q trap that was able to assign a structure to this keto-metabolite).

The *in vitro* incubation conditions used in the current study were such that only phase 1 enzymatic pathways were activated. This means that metabolites resulting from phase 2 conjugations with moieties such as glucuronic or sulphuric acid would not be produced. However, in theory the relevant cofactors necessary for activating phase 2 metabolism could be added to the incubation and is therefore an area for future research. One result of this selective phase 1 enzyme pathway activation is that sulfates would not be formed in the 17 position of stanozolol or its metabolites. The significance of this lies in that the spontaneous decomposition of the 17-sulfates of stanozolol and its metabolites has been proposed as a mechanism for the formation of 17-epimers of some 17α-methylated steroids (Schanzer et al. 1992). Therefore, the absence of any additional hydroxy-stanozolol peaks in the *in vivo* samples compared to the *in vitro* samples suggests that either the spontaneous decomposition of the 17-sulfates of stanozolol and its metabolites is not the mechanism by which 17-epimers are formed, or that none of the four major hydroxy-stanozolol metabolites observed *in vivo* take the form of 17-epimers. Since the evidence for the spontaneous decomposition of the 17-sulfates of 17α-methylated steroids being the pathway to form 17-epimers is strong, the view of the authors is that the latter of the two explanations is more likely.

5.3.2   Structural characterisation of the equine metabolites produced in vitro

The stereochemistry of the different metabolites produced *in vitro* was analysed in more detail on the Sciex 5500 Q Trap using product ion scanning experiments. The reason that the LTQ-Orbitrap was not used for this purpose was that the low-mass cut-off on the LTQ component of this instrument meant that many of the metabolically diagnostic low mass ions could not be observed. This situation could theoretically be circumvented by the use of a similar LTQ-Orbitrap that makes use of a higher-energy collision decomposition (HCD) chamber.

The product ion spectra acquired at 55 eV for reference standards of stanozolol, D_3-stanozolol, 3'-hydroxy-stanozolol, 6-hydroxy-stanozolol, 4α-hydroxy-stanozolol, 4β-hydroxy-stanozolol and 16β-hydroxy-stanozolol are shown in Fig 3. As reported previously (Muck and Henion, 1990, McKinney et al. 2004, Thevis et al. 2005), the LC-MS/MS spectra of stanozolol and it's analogues are highly complex. Considerable energy needs to be imparted into the molecules before they fragment, and once fragmentation is achieved the molecules typically fragment through a range of pathways

to a large number of different products. Fragmentation can either proceed through a charge-driven route leading to moieties retaining the protonated pyrazole structure or alternatively through charge-remote fragmentations leading to a range of moieties, including some that retain the D-ring portion of the molecule.
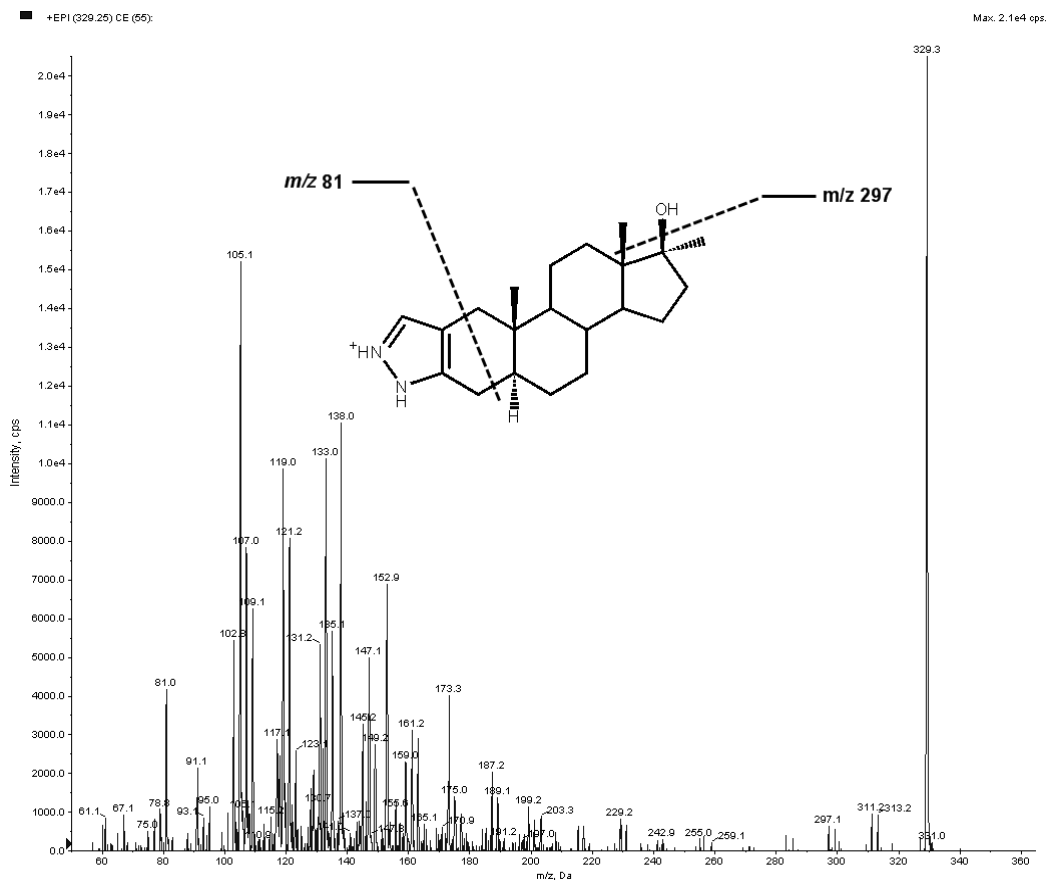
Fig 3a – reference standard product ion spectrum (5.89 mins) on the 5500 Q trap at a collision energy of 55 eV for stanozolol (precursor ion m/z 329.25).
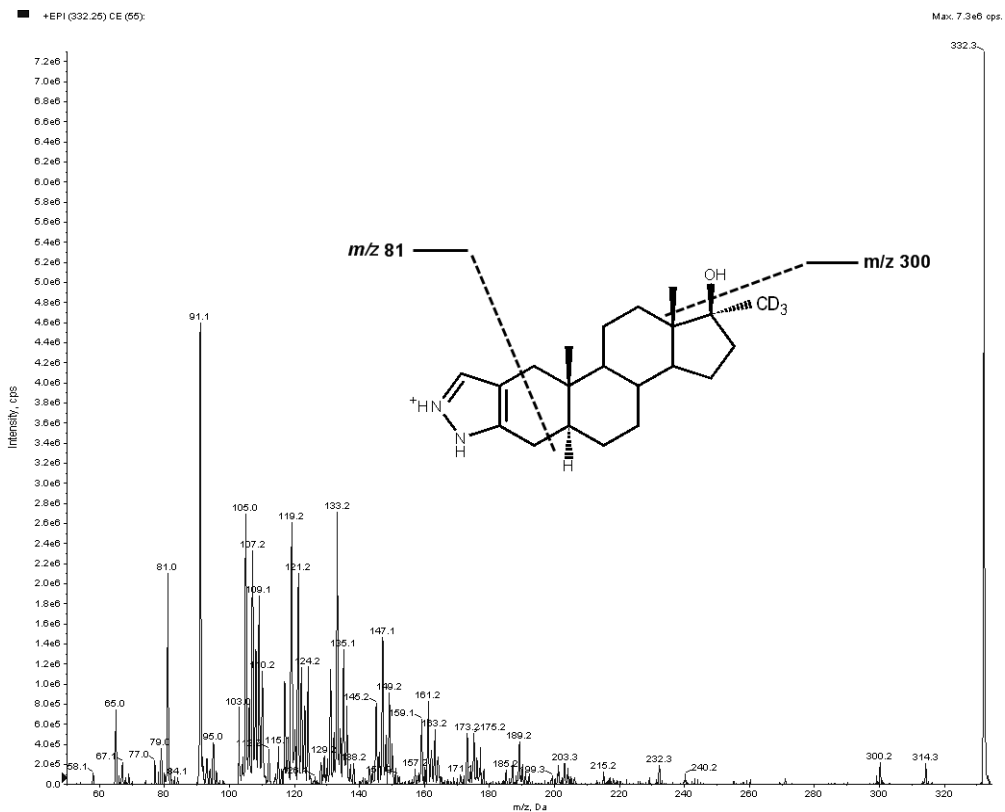
Fig 3b – reference standard product ion spectrum (5.89 mins) on the 5500 Q trap at a collision energy of 55 eV for D₃-stanozolol (precursor ion m/z 332.25).
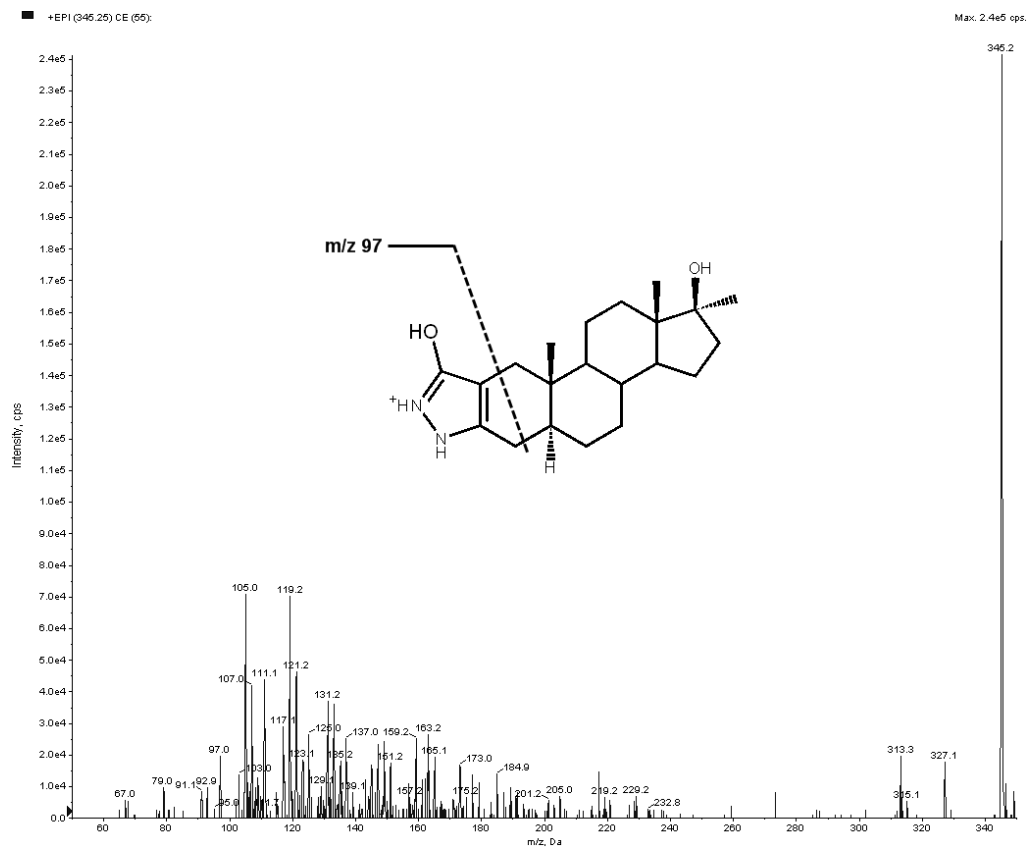
Fig 3c – reference standard product ion spectrum (4.98 mins) on the 5500 Q trap at a collision energy of 55 eV for 3'-hydroxy-stanozolol (precursor ion m/z 345.25).
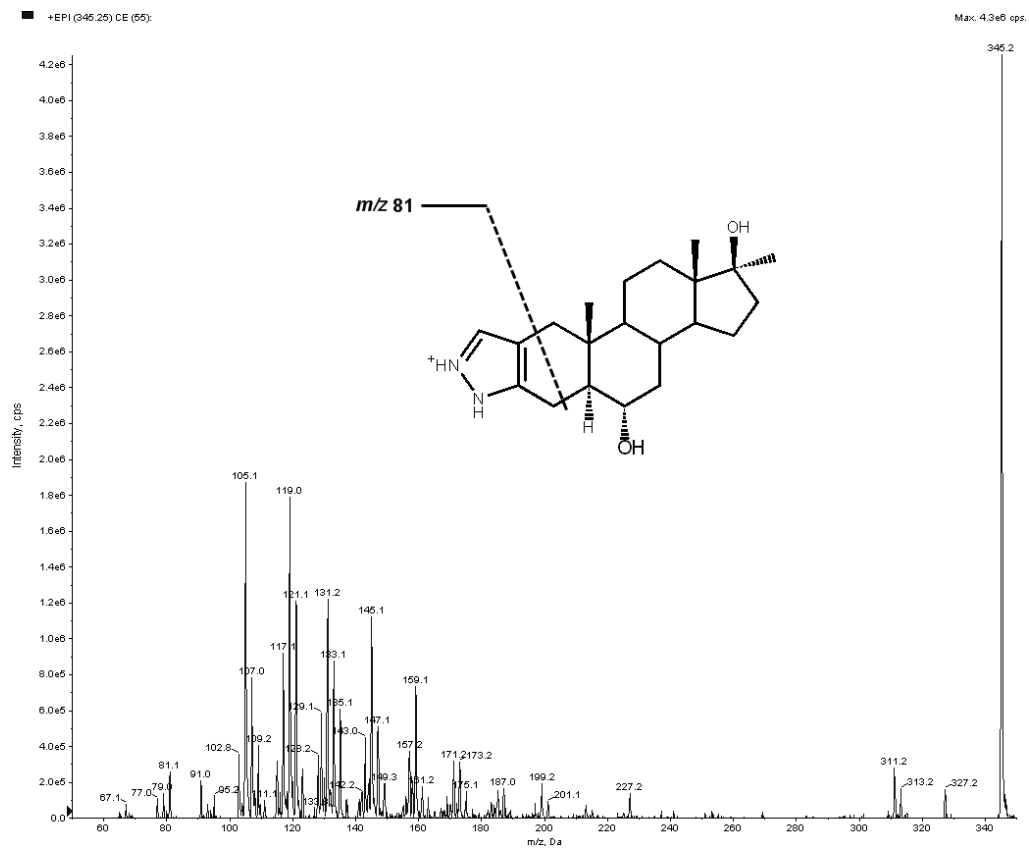
Fig 3d – reference standard product ion spectrum (4.91 mins) on the 5500 Q trap at a collision energy of 55 eV for 6α-hydroxy-stanozolol (precursor ion m/z 345.25).
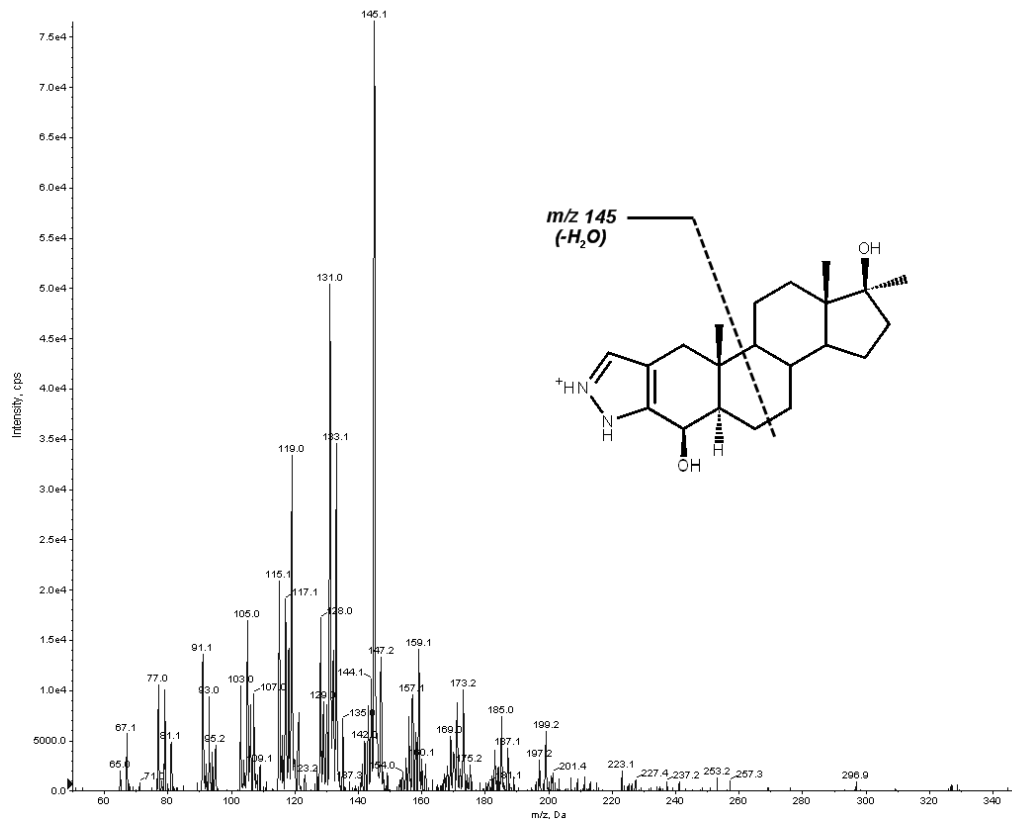
Fig 3e – reference standard product ion spectrum (4.91 mins) on the 5500 Q trap at a collision energy of 55 eV for 4α-hydroxy-stanozolol (precursor ion m/z 345.25).
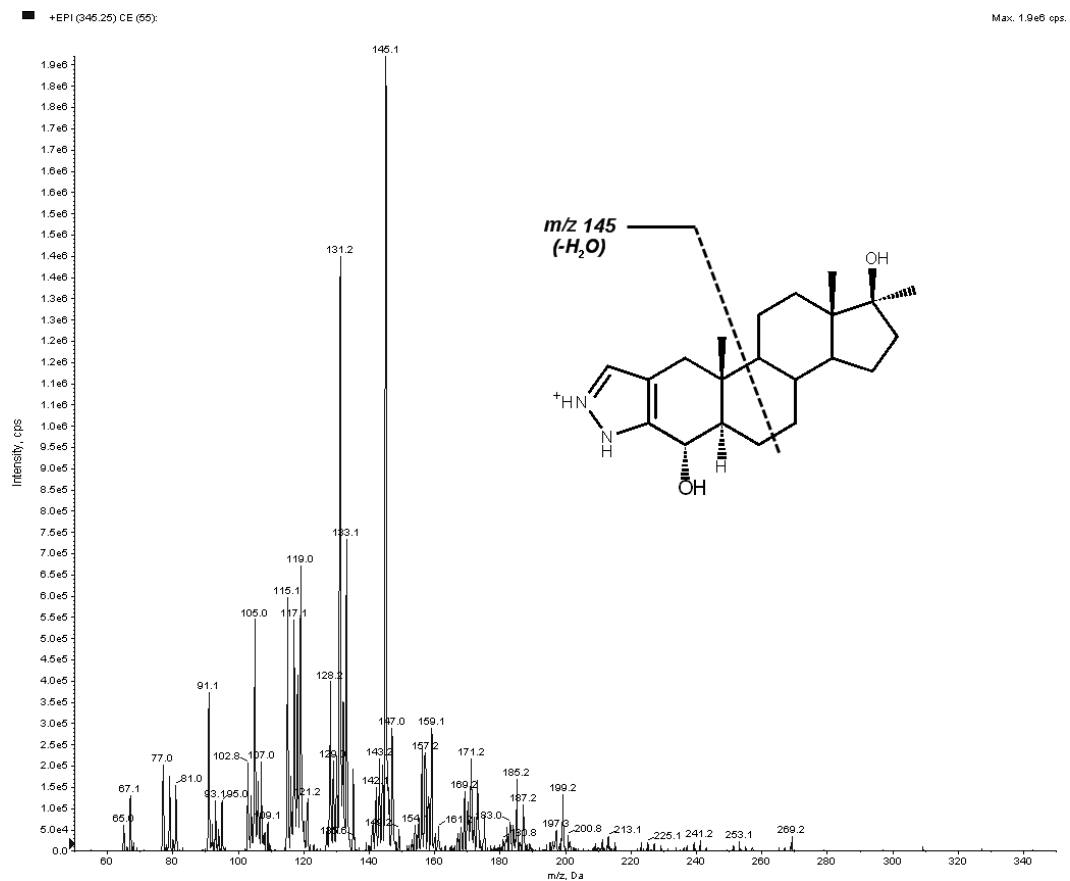
Fig 3f – reference standard product ion spectrum (5.25 mins) on the 5500 Q trap at a collision energy of 55 eV for 4β-hydroxy-stanozolol (precursor ion m/z 345.25).
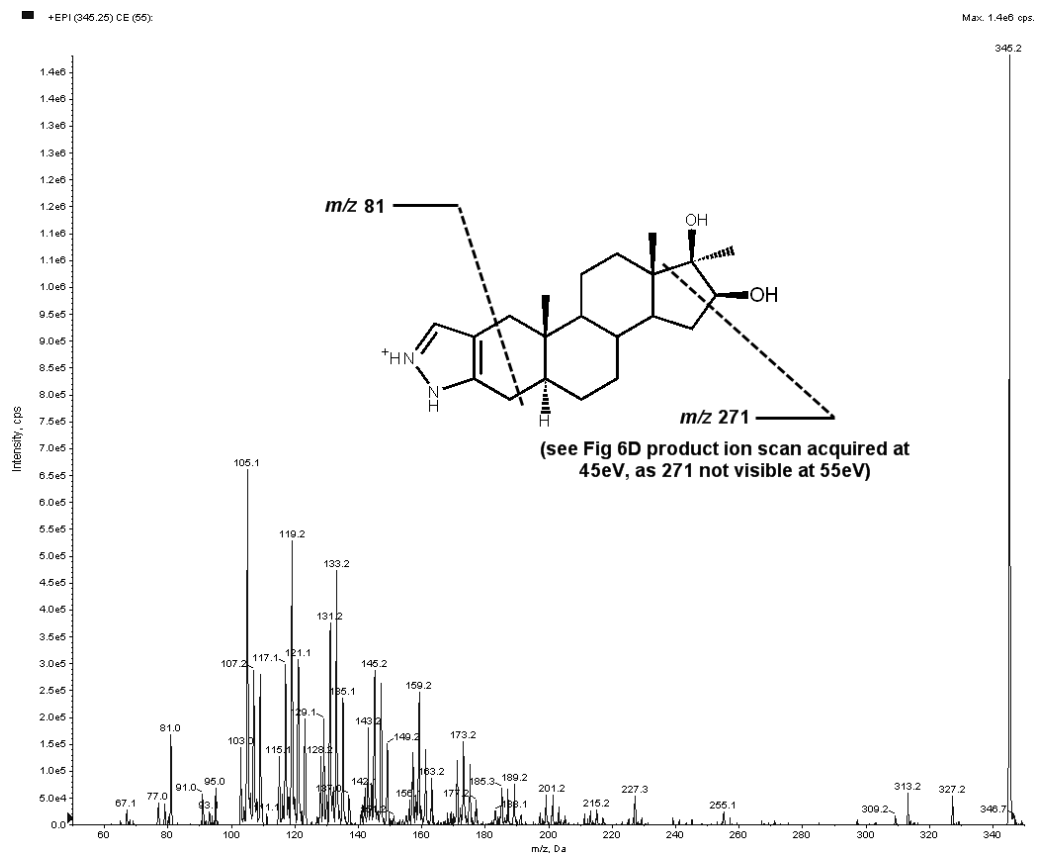
Fig 3g – reference standard product ion spectrum (4.82 mins) on the 5500 Q trap at a collision energy of 55 eV for 16β-hydroxy-stanozolol (precursor ion m/z 345.25).
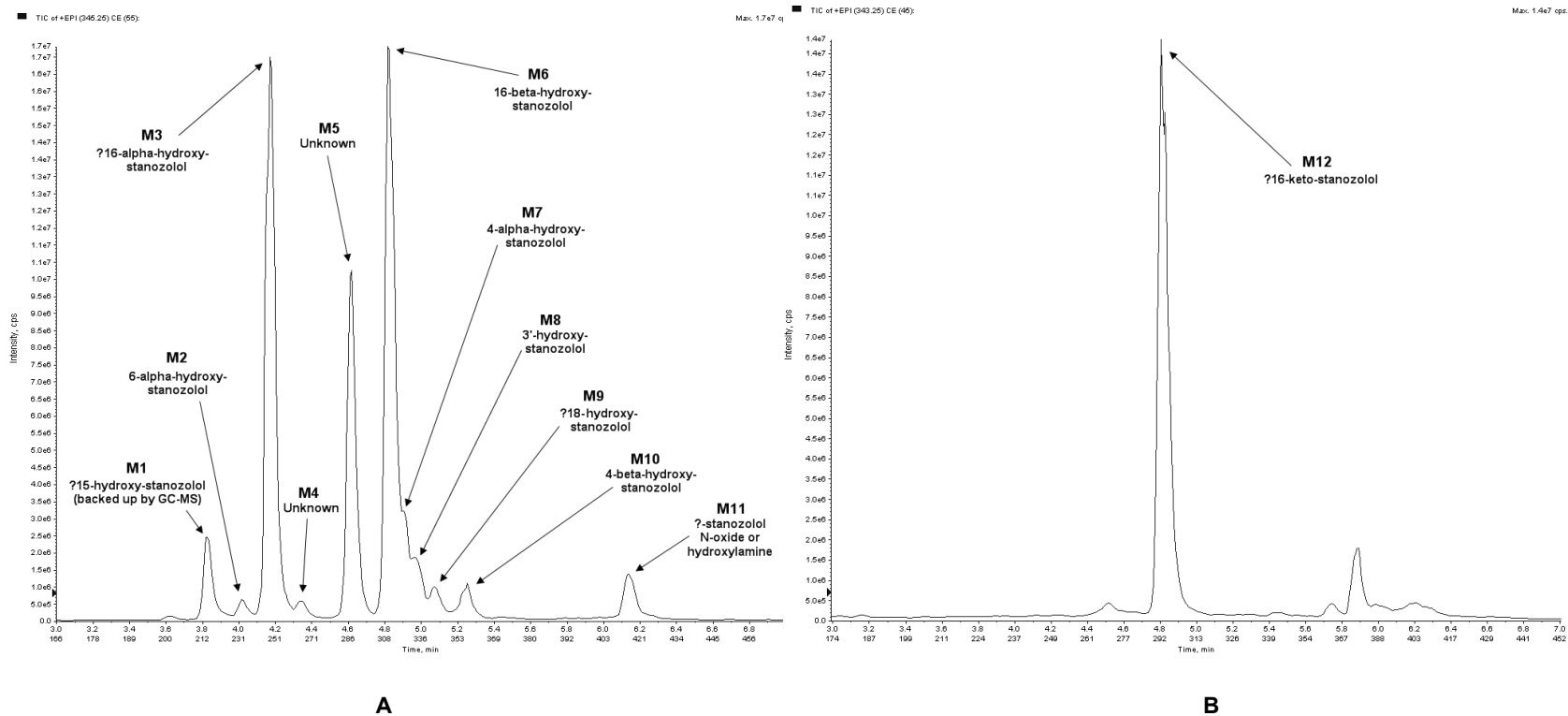
Fig 4 – total ion chromatogram of **A)** a product ion scan at a collision energy of 55 eV of m/z 345.25 (hydroxy-stanozolol) and **B)** a product ion scan at a collision energy of 45 eV of m/z 343.25 (keto-stanozolol) ran on the 5500 Q Trap following a 25 μM incubation of stanozolol with equine liver microsomes. Metabolite peaks that could be confirmed alongside a reference standard are labelled, while metabolites that could be postulated based on pattern of fragmentation are labelled with a question mark (see text for details).
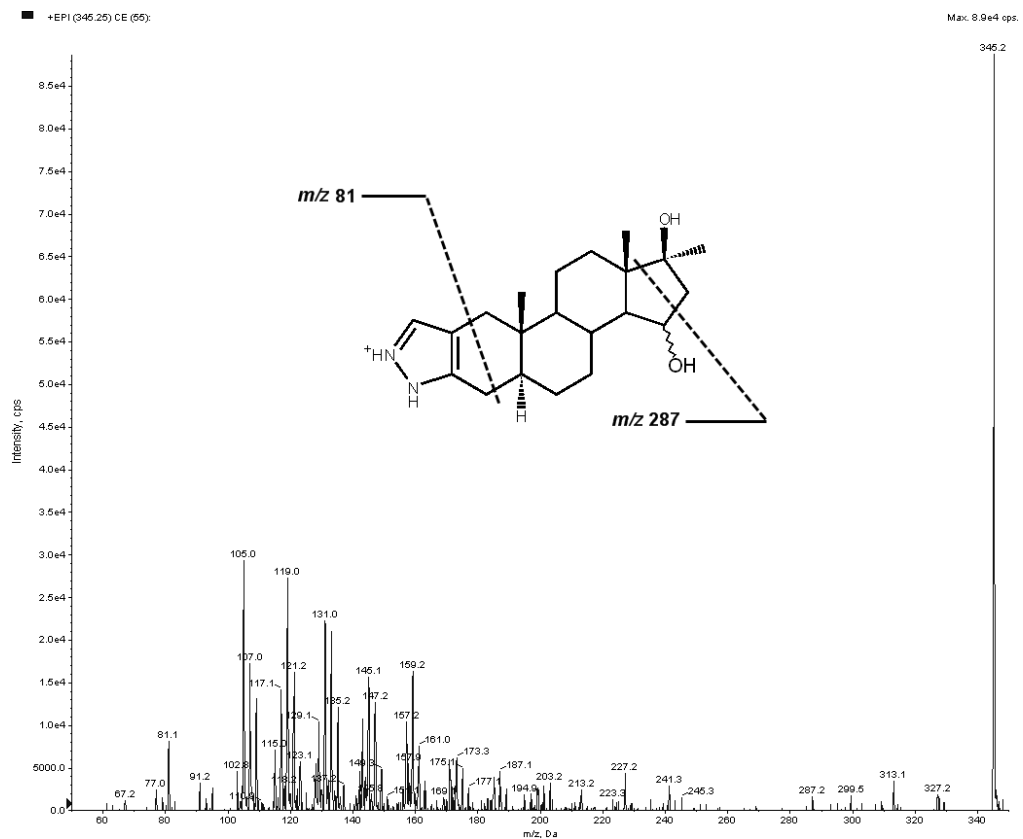
Fig 5a – product ion spectrum obtained on the 5500 Q Trap at a collision energy of 55 eV for the proposed 15-hydroxy-stanozolol metabolite M1 (3.83 mins) obtained following incubation of stanozolol (precursor ion m/z 345.25).
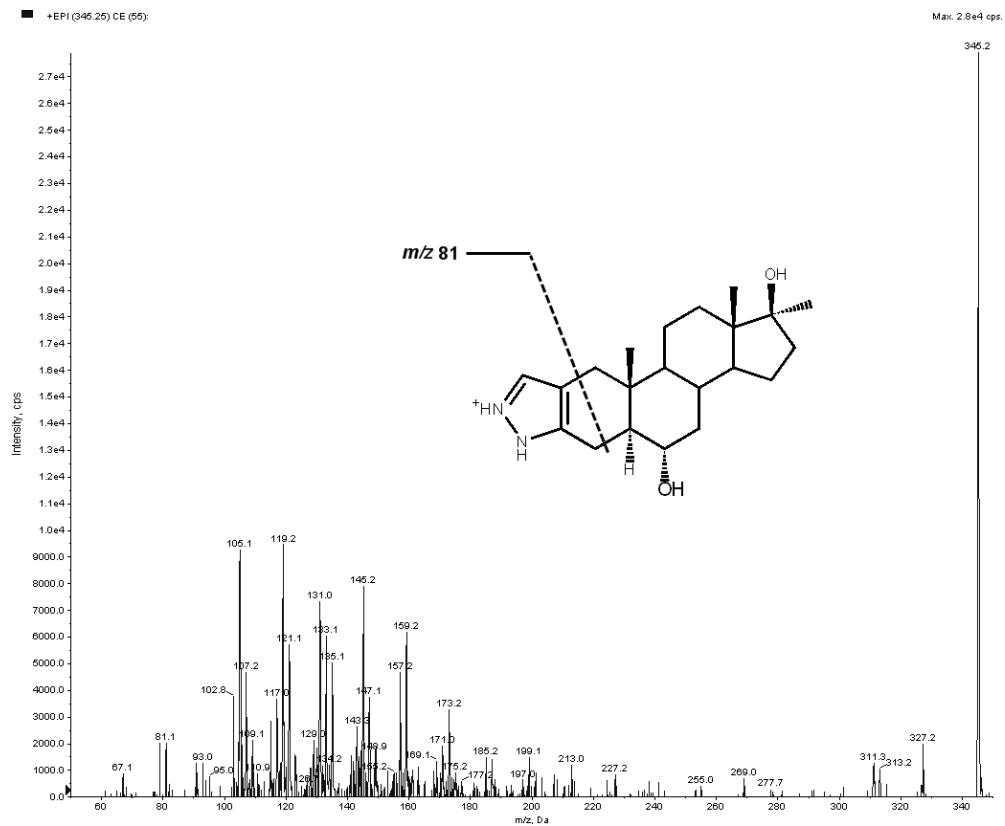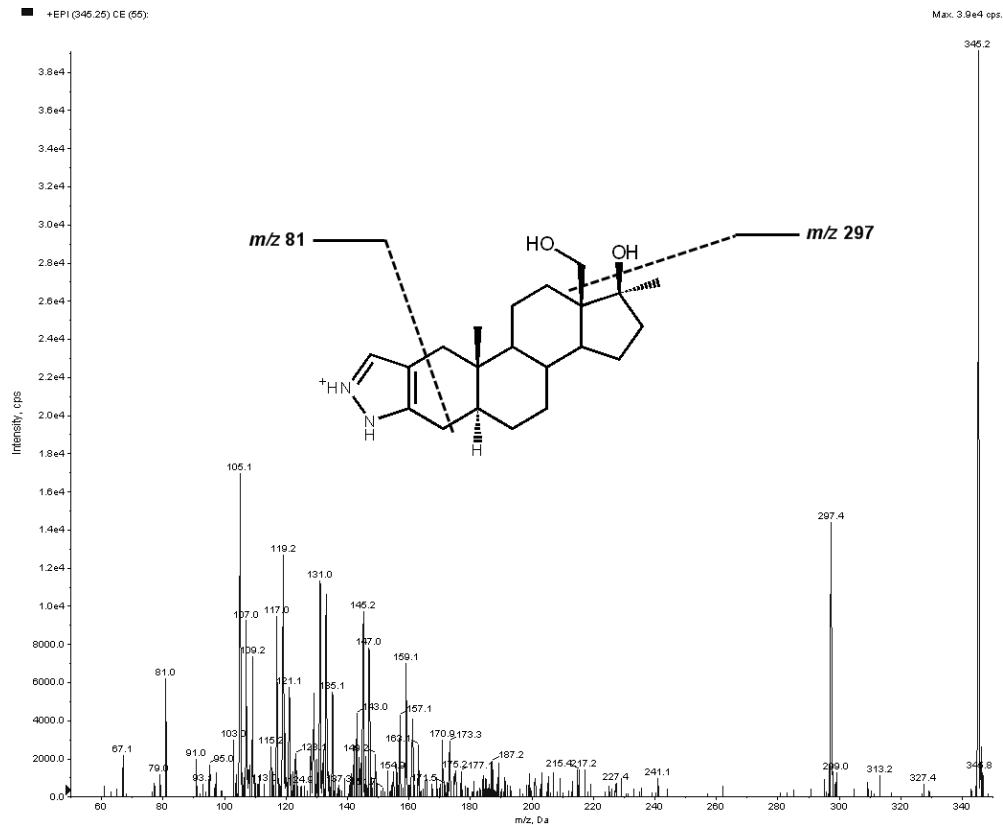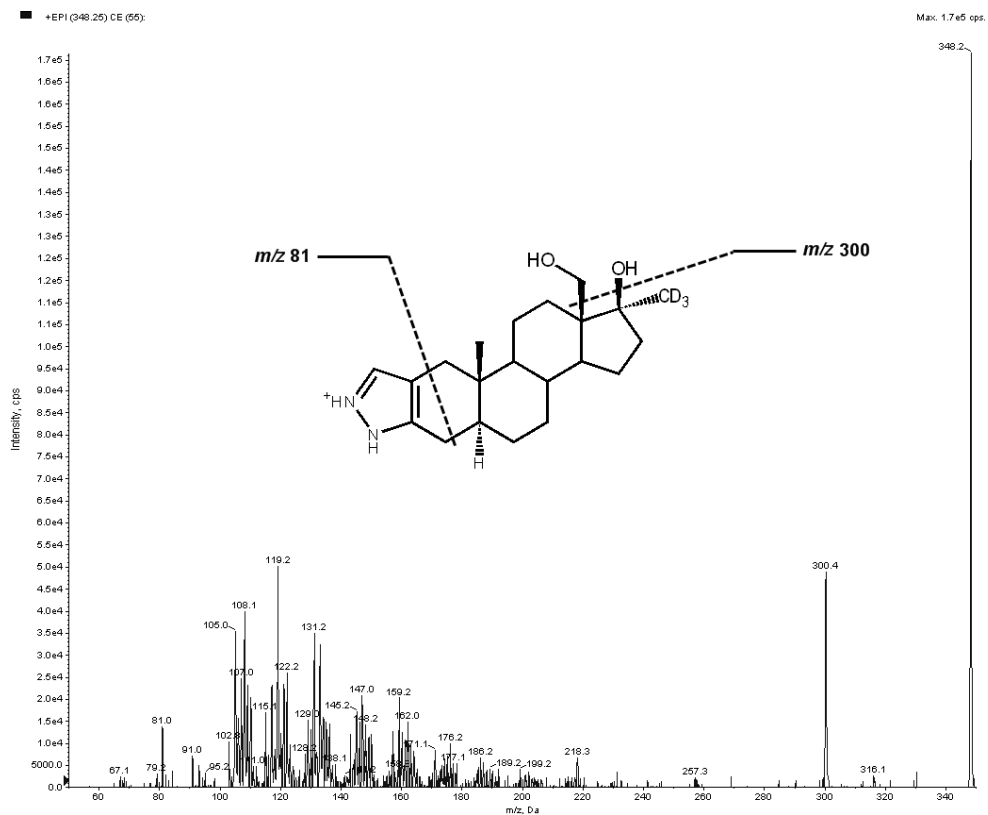
Fig 5b – product ion spectrum obtained on the 5500 Q Trap at a collision energy of 55 eV for the proposed 6α-hydroxy-stanozolol metabolite M2 (4.02 mins) obtained following incubation of stanozolol (precursor ion m/z 345.25).
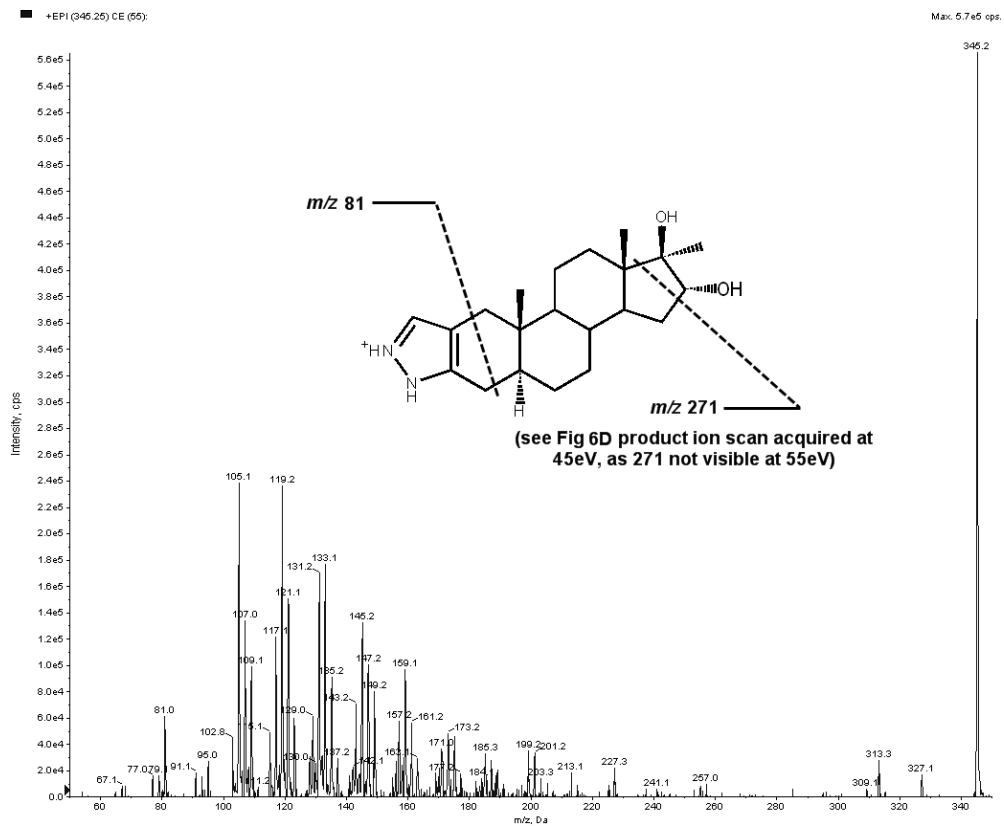
Fig 5c – product ion spectrum obtained on the 5500 Q Trap at a collision energy of 55 eV for the proposed 16α-hydroxy-stanozolol metabolite M3 (4.17 mins) obtained following incubation of stanozolol (precursor ion m/z 345.25).
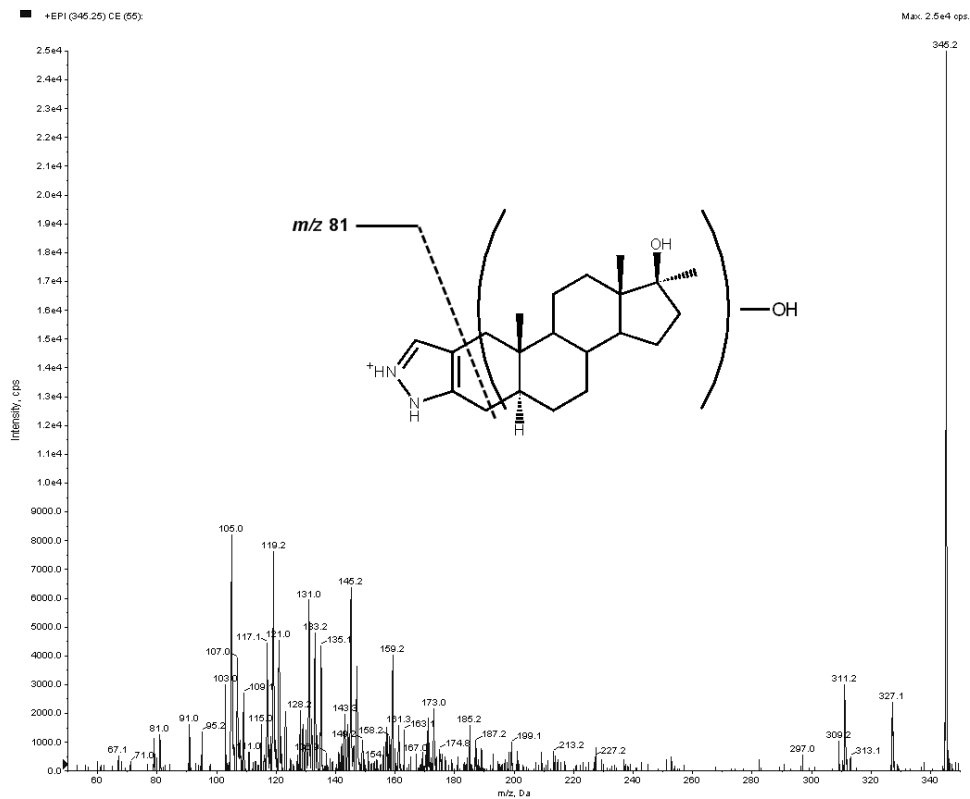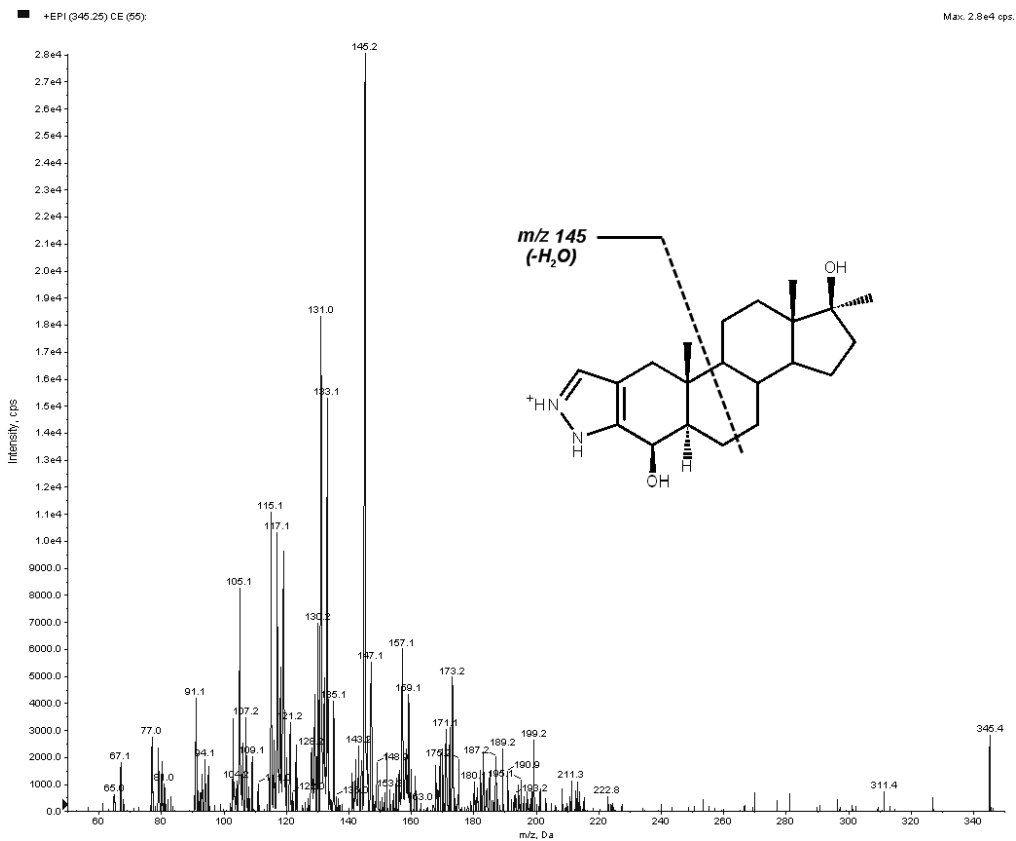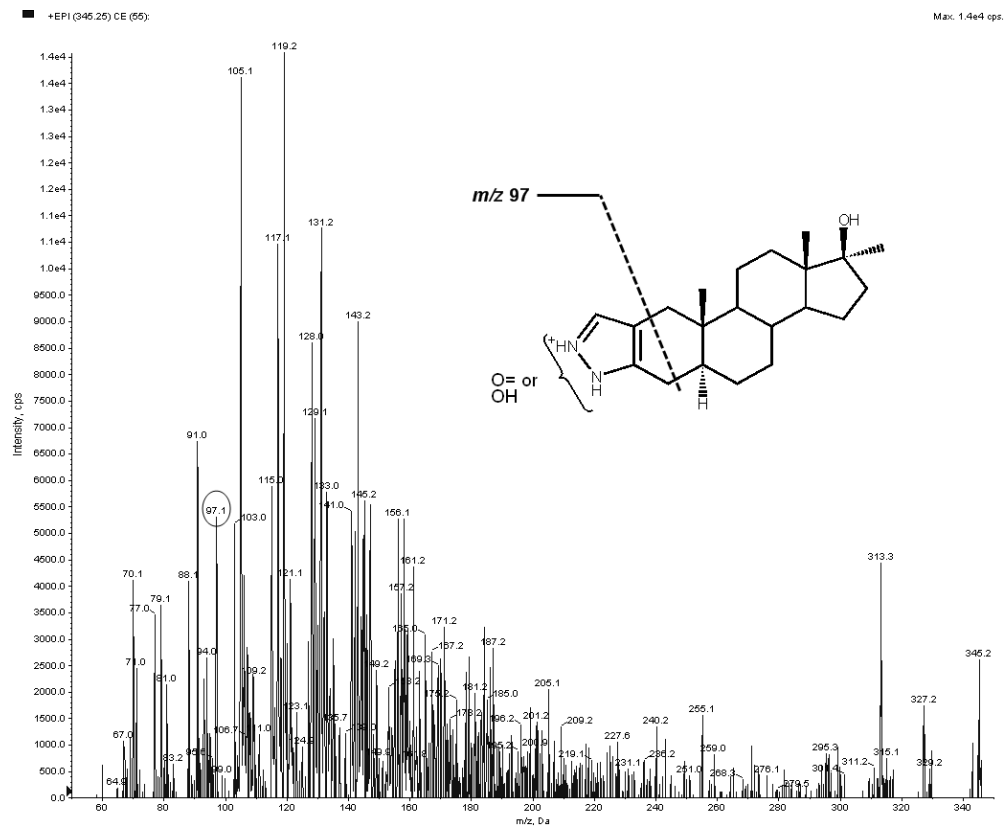
Fig 5d – product ion spectrum obtained on the 5500 Q Trap at a collision energy of 55 eV for one of the unknown hydroxy-stanozolol metabolites M4 (4.34 mins) obtained following incubation of stanozolol (precursor ion m/z 345.25).
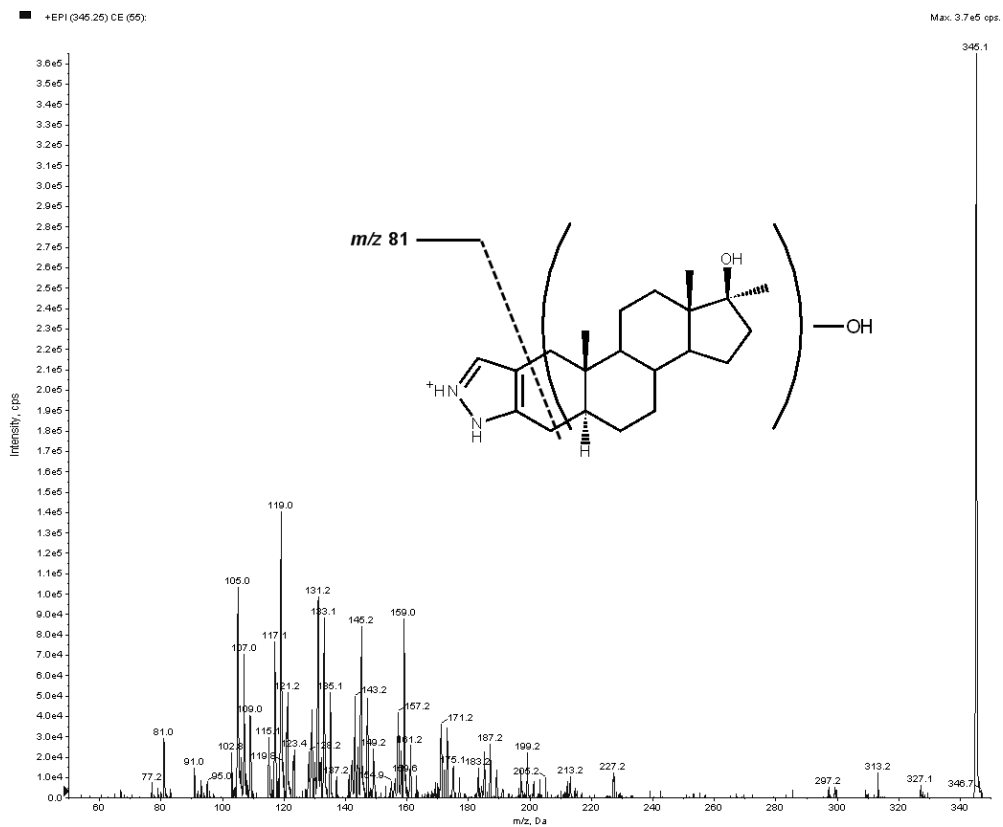
Fig 5e – product ion spectrum obtained on the 5500 Q Trap at a collision energy of 55 eV for one of the unknown hydroxy-stanozolol metabolites M5 (4.61 mins) obtained following incubation of stanozolol (precursor ion m/z 345.25).
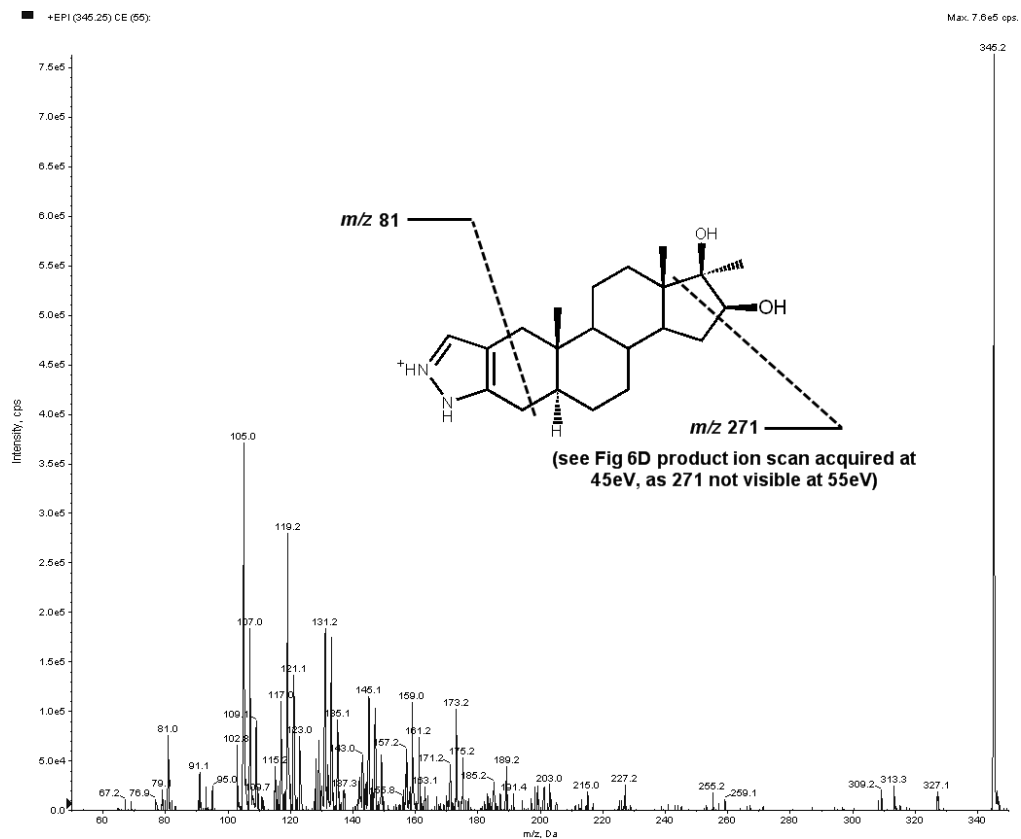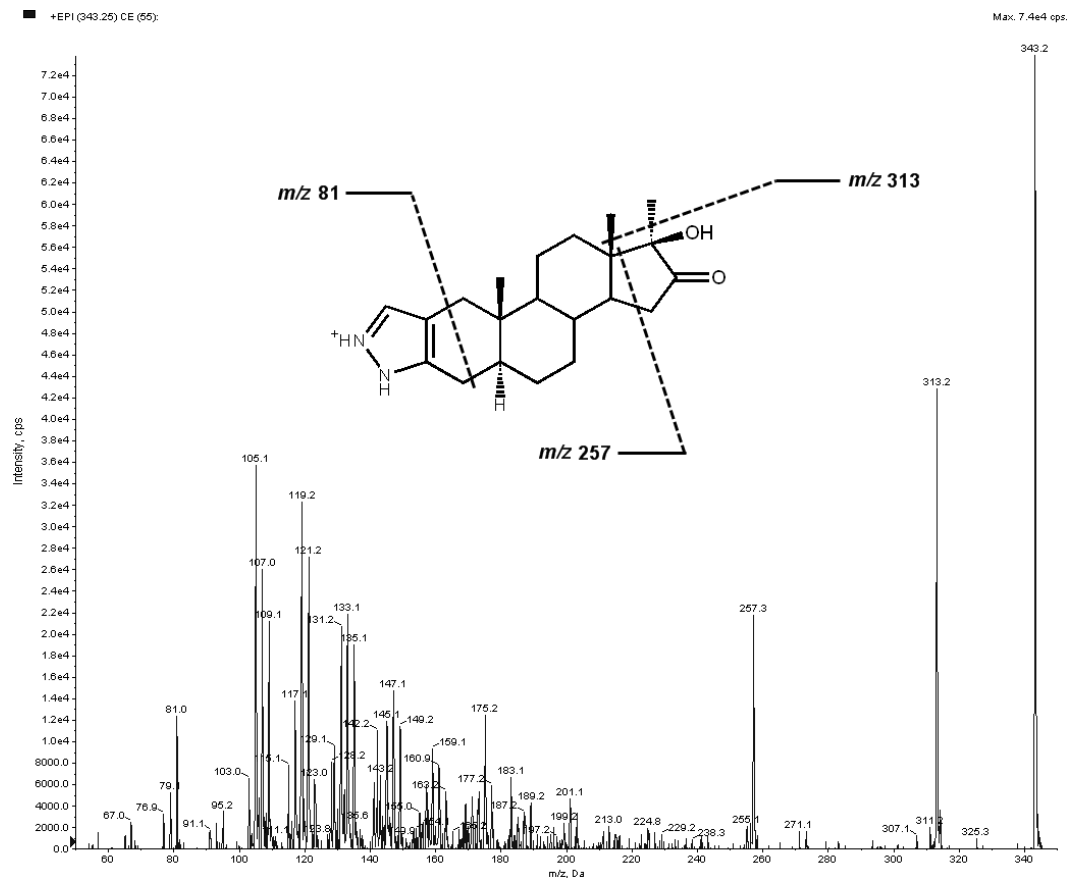
Fig 5f – product ion spectrum obtained on the 5500 Q Trap at a collision energy of 55 eV for the proposed 16β-hydroxy-stanozolol metabolite M6 (4.82 mins) obtained following incubation of stanozolol (precursor ion m/z 345.25).
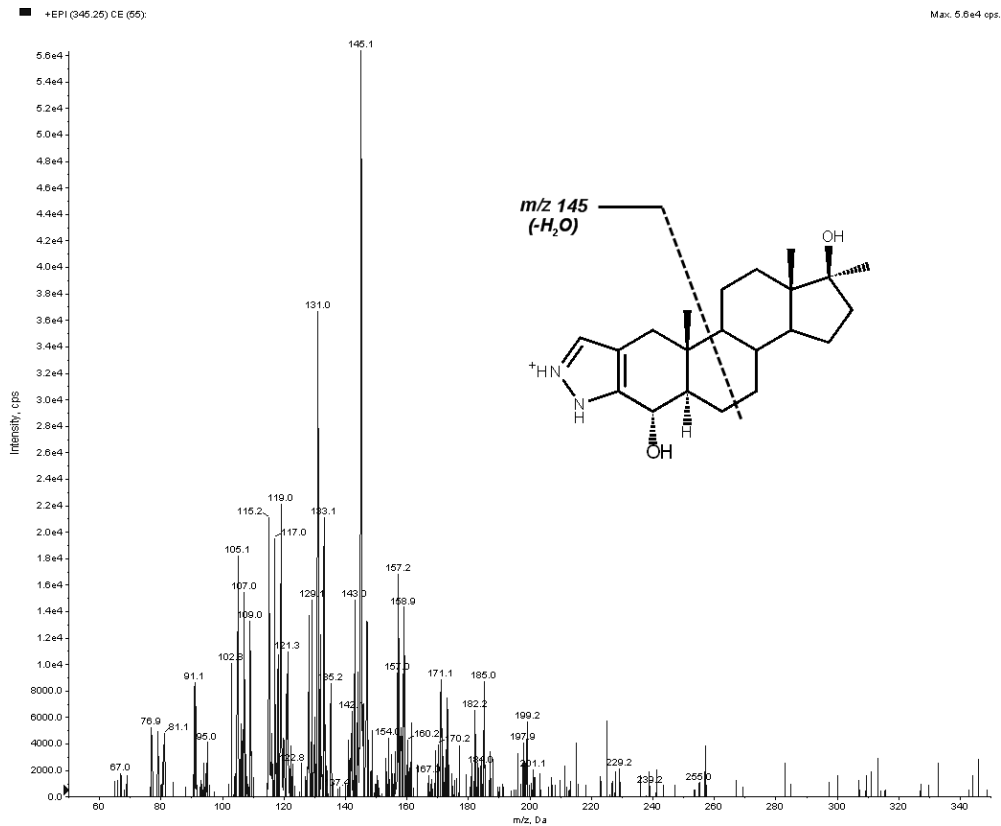
Fig 5g – product ion spectrum obtained on the 5500 Q Trap at a collision energy of 55 eV for the proposed 4α-hydroxy-stanozolol metabolite M7 (4.91 mins) obtained following incubation of stanozolol (precursor ion m/z 345.25).
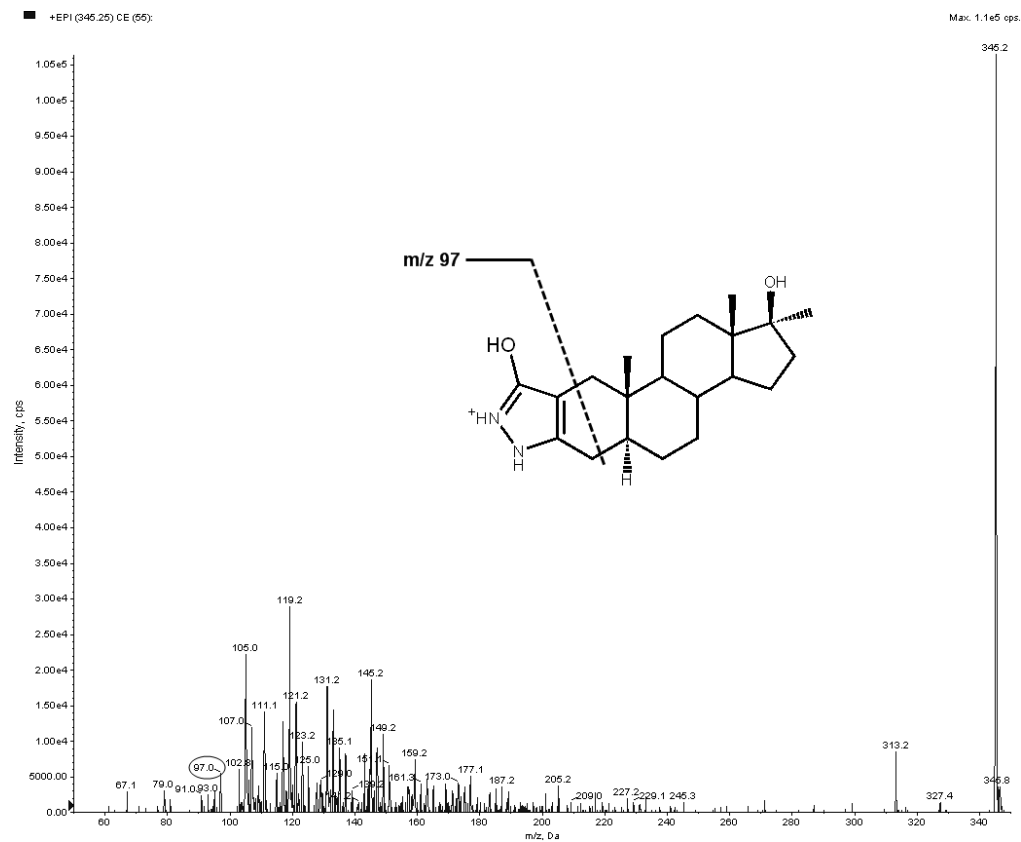
Fig 5h – product ion spectrum obtained on the 5500 Q Trap at a collision energy of 55 eV for the proposed 3'-hydroxy-stanozolol metabolite M8 (4.98 mins) obtained following incubation of stanozolol (precursor ion m/z 345.25).
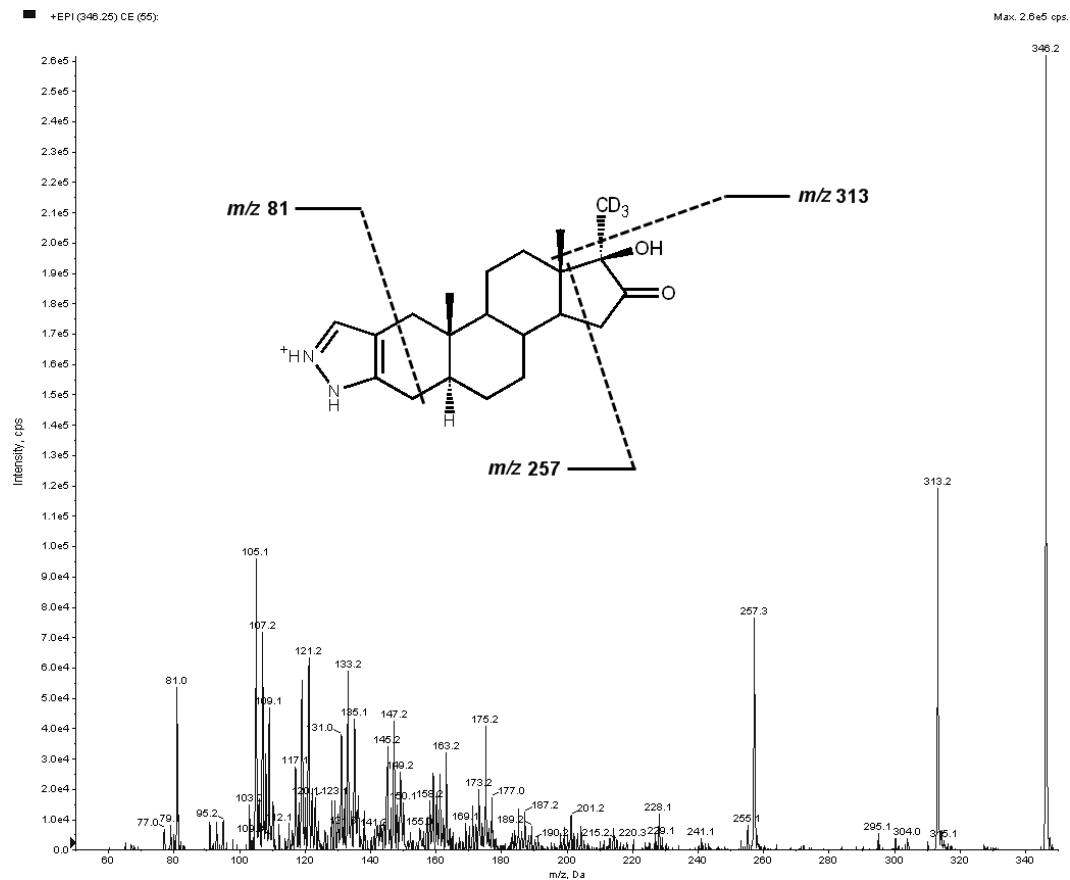
Fig 5i – product ion spectrum obtained on the 5500 Q Trap at a collision energy of 55 eV for the proposed 18-hydroxy-stanozolol metabolite M9 (5.07 mins) obtained following incubation of stanozolol (precursor ion m/z 345.25).
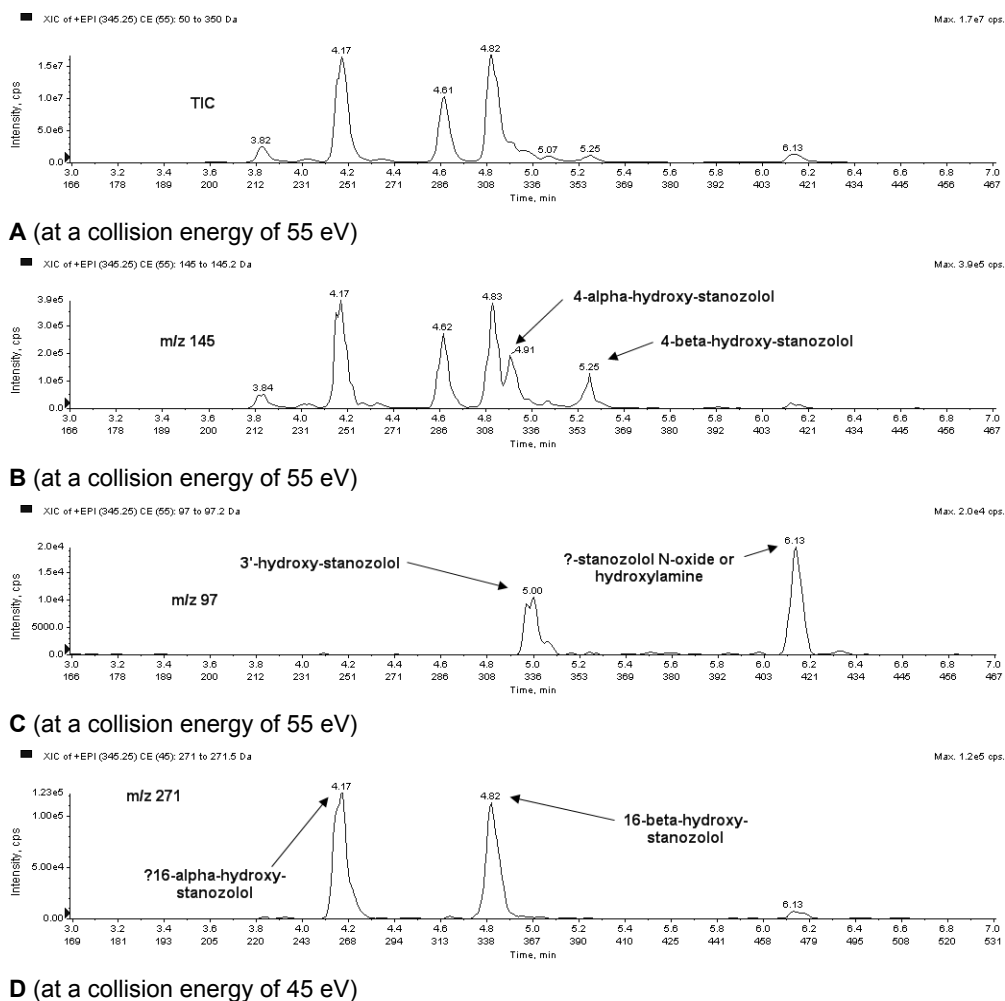
Fig 5j – product ion spectrum obtained on the 5500 Q Trap at a collision energy of 55 eV for the proposed 18-hydroxy-stanozolol metabolite M9 (5.07 mins) obtained following incubation of D3-stanozolol (precursor ion m/z 348.25).

Fig 5k – product ion spectrum obtained on the 5500 Q Trap at a collision energy of 55 eV for the proposed 4β-hydroxy-stanozolol metabolite M10 (5.25 mins) obtained following incubation of stanozolol (precursor ion m/z 345.25).

Fig 5l – product ion spectrum obtained on the 5500 Q Trap at a collision energy of 55 eV for the proposed stanozolol N-oxide/hydroxylamine metabolite M11 (6.13 mins) obtained following incubation of stanozolol (precursor ion m/z 345.25).

Fig 5m – product ion spectrum obtained on the 5500 Q Trap at a collision energy of 55 eV for the proposed keto-stanozolol metabolite M12 (4.86 mins) obtained following incubation of stanozolol (precursor ion m/z 343.25).

Fig 5n – product ion spectrum obtained on the 5500 Q Trap at a collision energy of 55 eV for the proposed keto-stanozolol metabolite M12 (4.86 mins) obtained following incubation of D_3-stanozolol (precursor ion m/z 346.25).

A (at a collision energy of 55 eV)

B (at a collision energy of 55 eV)

C (at a collision energy of 55 eV)

D (at a collision energy of 45 eV)

Fig 6 – m/z 345.25 derived product ion scan chromatograms obtained on the 5500 Q Trap (following incubation of 25 uM stanozolol with equine liver microsomes) that aid the identification of metabolite peaks (see also the spectra in Fig 5). A; total ion chromatogram, B; extracted ion chromatogram of m/z 145, which distinguishes the 4α-hydroxy-stanozolol peak at 4.01 minutes due to it's increased m/z 145 (in common with the 4β-isomer at 5.25 minutes), C; extracted ion chromatogram of m/z 97, which distinguishes the 3'-hydroxy-stanozolol peak at 5.00 minutes due to it's increased m/z 97 (in common with the postulated stanozolol N-oxide or hydroxylamine peak at 6.13 minutes) and D; extracted ion chromatogram of m/z 271, which distinguishes the 16β-hydroxy-stanozolol peak at 4.82 minutes and suggests that the peak at 4.17 minutes is 16α-hydroxy-stanozolol.
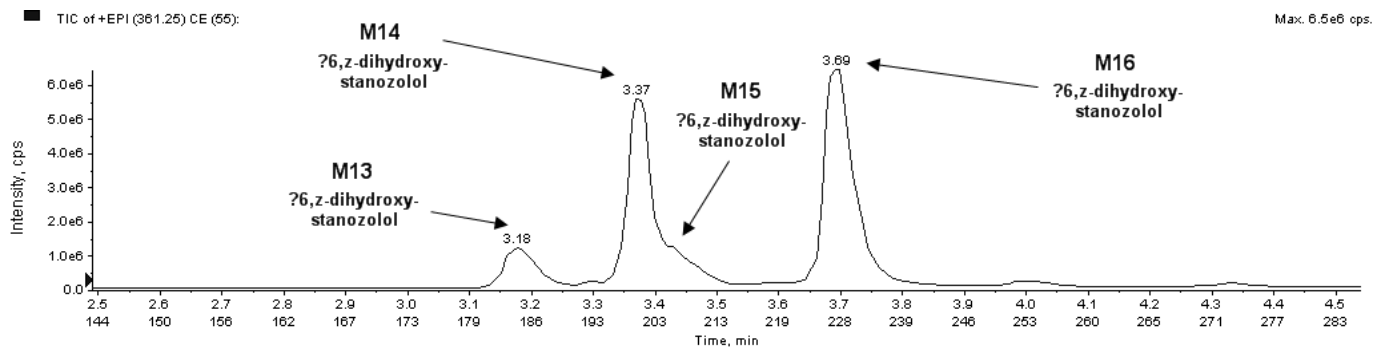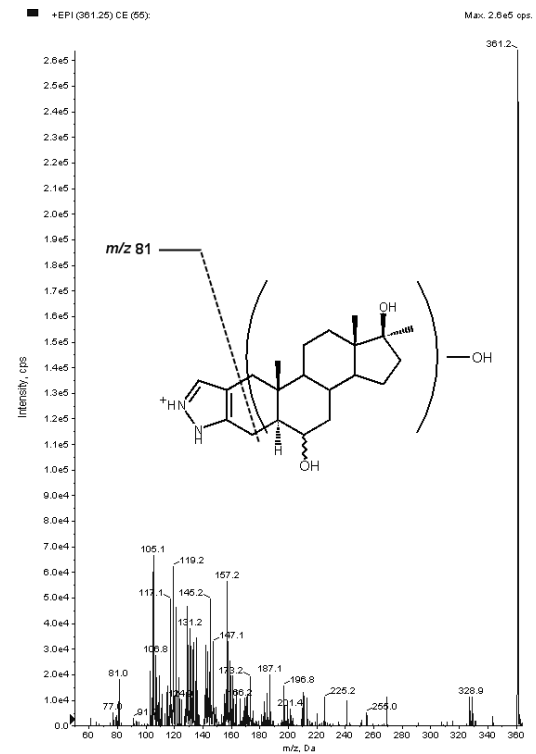
Fig 7– m/z 361.25 derived product ion scan chromatograms relating to potential di-hydroxy-stanozolol metabolites obtained on the 5500 Q Trap at a collision energy of 55 eV following incubation with 25 uM of 6α-hydroxy-stanozolol. No peaks were seen following stanozolol or 16β-hydroxy-stanozolol incubations.
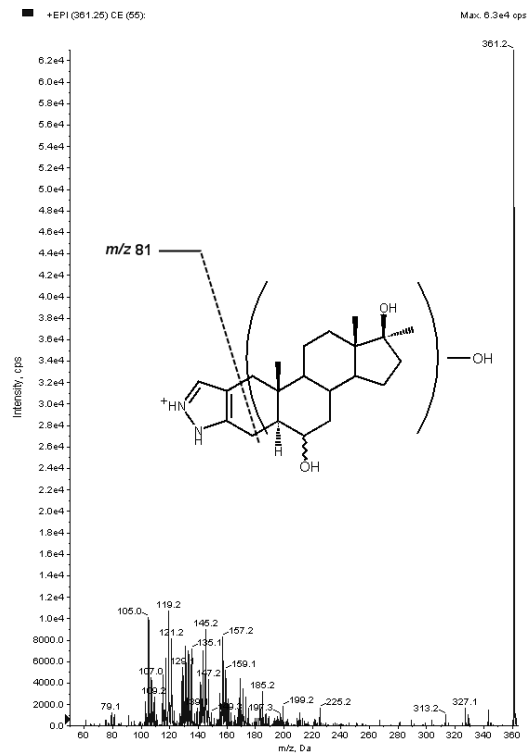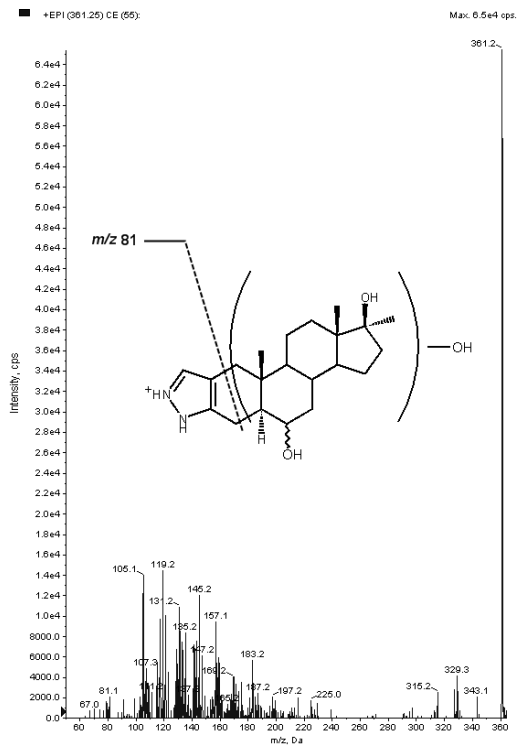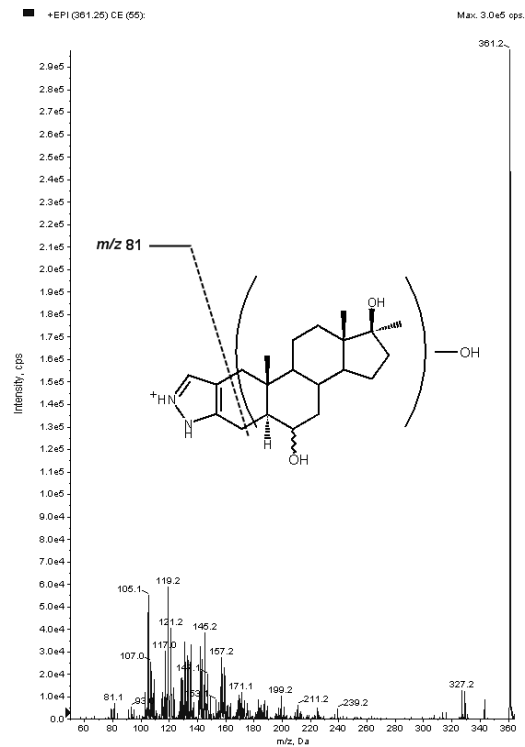
Fig 8a – m/z 361.25 derived product ion spectra relating to potential di-hydroxy-stanozolol metabolites obtained on the 5500 Q Trap at a collision energy of 55 eV following incubation with 25 uM of 6α-hydroxy-stanozolol.

C (M15)   **D** (M16)

Fig 8b – m/z 361.25 derived product ion spectra relating to potential di-hydroxy-stanozolol metabolites obtained on the 5500 Q Trap at a collision energy of 55 eV following incubation with 25 uM of 6α-hydroxy-stanozolol.

Starting by analysing fragments of parent drug stanozolol (retention time 5.89 mins), diagnostic fragments are produced at *m/z* 81, resulting from charge retention in the pyrazole portion of the molecule, at *m/z* 311, resulting from the loss of water (derived from the hydroxy group attached to C17) and at *m/z* 297 (resulting from the neutral loss of 32). The fact that D_3-stanozolol also loses 32 and forms a fragment with *m/z* 300 (and therefore retains the three deuterium atoms within the observed charged fragment) suggests that the loss of 32 relates to methanol and that the methyl group involved is derived from a site other than the deuterated C20 methyl group. The simplest explanation would be that the C18 methyl group and the hydroxy group attached to C17 are lost as methanol in a concerted mechanism leading to the introduction of a double bond between C13 and C17 in the observed charged fragment. For 3'-hydroxy-stanozolol (retention time 4.98 mins), the *m/z* of 81 that existed in the stanozolol spectra is incremented by 16 u to form *m/z* 97 due to the introduction of the 3'-hydroxy group into this pyrazole derived fragment. The product ion spectra of 6α-hydoxy-stanozolol (retention time 4.02 mins) does not produce any fragment ions that are indicative of the position of hydroxylation, but 4α- and 4β-hydroxy-stanozolol (retention times of 4.91 and 5.25 mins respectively) show a very characteristic pattern of fragmentation. The spectra of these latter two metabolites are characterised by intense fragments at *m/z* 145 and the absence of *m/z* 81. As proposed by Thevis et al. (Thevis et al. 2005), this can be explained by a loss of water at C4 leading to the introduction of a double bond between position 4 and 5, with subsequent fission between carbons 6 and 7 and also between 9 to 8 and 11 leading to a highly stable, conjugated structure. The product ion spectra of 16β-hydroxy-stanozolol (retention time 4.82 mins) is similar to 6α-hydoxy-stanozolol, except that at lower collision energies it produces a fragment at *m/z* 271 (not observed with any of the other hydroxy-stanozolol metabolite reference standards), which is proposed to result from cleavage between carbons C13/C17 and C15/C16 depicted in Fig 3.

For the equine microsomal incubations, total ion chromatograms resulting from the product ion scans of ions *m/z* 345.25 and 343.25, relating to potential hydroxy-stanozolol and keto-stanozolol metabolites respectively, are shown in Fig 4. From these chromatograms and the resulting spectra (Fig 5), it was possible to identify 11 hydroxy-stanozolol (labelled M1 to M11) and one keto-stanozolol (labelled M12) metabolite. Hydroxy metabolites M4 and M5 did not produce any fragment ions that were considered to be indicative of their position of hydroxylation, but involvement of the pyrazole ring

was considered unlikely as both metabolites retained the *m/z* 81 fragment. Metabolites M2, M6, M7, M8 and M10 however, had chromatographic retention times and mass spectra that matched reference standards of 6α-, 16β-, 4α-, 3'- and 4β-hydroxy-stanozolol respectively. Of these, metabolites M6, M7 and M8 were found to partially co-elute under the conditions used here, but could be readily distinguished from each other based on mass spectral fragmentation as shown in Fig 6.

Hydroxy metabolite M1 was the earliest eluting of the hydroxy-metabolites and was characterised by a small ion at *m/z* 287. Although this ion could not be definitively ascribed to a particular route of fragmentation, it was considered likely that if the metabolite were a 15-hydroxy-stanozolol (of unknown stereochemistry) then fission of the D-ring between carbons C13/C17 and C15/C16, analogous to that described for 16β-hydroxy-stanozolol, would be the most likely explanation for the stability of this particular fragment (see Fig 5). This explanation is consistent with results obtained by McKinney et al. (2004) where they also proposed that the earliest eluting of their observed *in vivo* hydroxy-stanozolol metabolites to be a 15-hydroxy-stanozolol. The existence of a 15-hydroxy metabolite of stanozolol in the equine was originally proposed in a previous *in vivo* study performed in our laboratories in the 1990s using electron ionisation GC-MS of trimethylsilylated stanozolol sample extracts following a stanozolol *in vivo* administration (Phil Teale – personal observation of previously unpublished data). The use of GC-MS in this previous study was highly informative as it was possible to assign the site of hydroxylation to either the 15- or 16- positions due to their different patterns of fragmentation. The purported 15-hydroxy-stanozolol produced a single intense fragment at *m/z* 231 resulting from a proposed cleavage between C14/C15 and C13/C17 and retention of the charge on the derivatised D-ring fragment of the molecule as shown in Fig 9. 16β-hydroxy-stanozolol on the other hand also contained a 218 *m/z*, resulting from a proposed cleavage between C15/C16 and C13/C17, again with retention of the charge on the derivatised D-ring fragment of the molecule. Trimethylsilylated extracts resulting from the *in vitro* incubation of stanozolol with equine liver microsomes were therefore analysed by GC-MS in the current study in order to try and detect the *m/z* 231 that had been shown by this previous study to be present following stanozolol administration. As shown in Fig 9, it was possible to identify a peak containing an intense *m/z* 231 in the post-dose *in vitro* samples. This proposed 15-hydroxy-stanozolol peak co-eluted with a peak whose retention time and spectra matched with that of a 4α-hydroxy-stanozolol reference standard, but the absence of the intense 231 *m/z* in either the reference

standard spectra or blank *in vitro* incubations provides support that this ion does relate to a potential 15-hydroxy-stanozolol-metabolite. Unfortunately, because of the inherent problems of analysing stanozolol by GC-MS (one of the reasons why the majority of the study was carried out by LC-MS), it was not possible to set the system up to reanalyse the samples using a different chromatographic method able to resolve the metabolite peaks. It was also not possible to identify any further compounds in other than the proposed 15-hydroxy- and 4α-hydroxy-stanozolol and parent stanozolol itself in the samples that were able to be run by GC-MS.
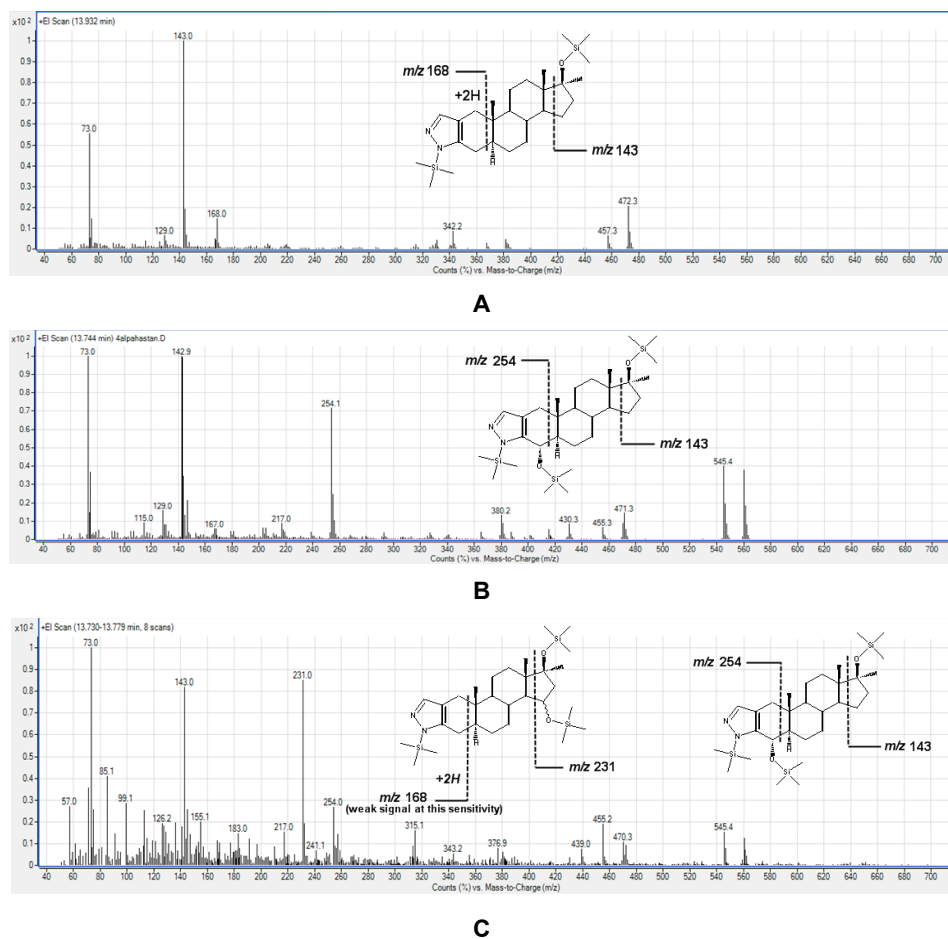
A

B

C

Fig 9 – Full scan EI spectra obtained on the Agilent 7000A for (A) stanozolol-bis-TMS reference standard (13.93 min), (B) 4α-hydroxy-stanozolol-tris-TMS reference standard (13.74 min) and (C) co-eluting 4α- and ?15-hydroxy-stanozolol-tris-TMS spectrum obtained following an incubation of 25 uM stanozolol with equine liver microsomes (13.75 min – background subtracted).

As depicted in Fig 6, hydroxy-metabolite M3 contained an *m/z* 271 fragment when ran under low energy collision conditions. *In vitro* incubations of equine liver microsomes with D$_3$-stanozolol instead of stanozolol (therefore forming deuterated version of the metabolites) support the suggestion that the *m/z* 271 fragment of 16β-hydroxy-stanozolol is produced through the route shown in Fig 5. If this is accepted, then since the spectra of M3 also contains the *m/z* 271, then the position of hydroxylation for this metabolite can only be on C16 or C20. However, if hydroxylation was in the C20 position then fragmentation would likely be very different to that observed and at least one of the deuterium atoms (known to be in the C20 position of D$_3$-stanozolol) would also expect have been replaced with hydrogen during the process of metabolism. This line of evidence, coupled with the expected absence of 17-epimerisation under the *in vitro* conditions used, therefore lead to the suggestion that M3 is 16α-hydroxy-stanozolol.

Hydroxy metabolite M9 fragments very differently to the other hydroxy-metabolites, producing an intense fragment at *m/z* 297. Incubations with D$_3$-stanozolol instead of stanozolol caused the fragment for the analogous deuterated metabolite to be shifted to *m/z* 300, indicating that the C20 methyl group is retained within the charged molecule during this fragmentation. The mechanism that was considered most likely to lead to this stable, intense fragment was via the neutral loss of the hydroxy group at C17 accompanied by a hydroxylated methyl group (as depicted in Fig 5). Three methyl groups exist within stanozolol that could be subject to hydroxylation. The C20 methyl group could not have been involved since the D$_3$-stanozolol incubation experiment showed that this methyl group was not lost during fragmentation of this metabolite. The C19 methyl group was also not considered a likely candidate since none of the lower mass fragments that are known to retain the C19 carbon were seen to be altered. This process of deduction leads to the speculation that the hydroxylation may be in the C18 position. This suggestion that is further supported by the observation that this would involve a neutral loss of the hydroxy group at C17 accompanied by a hydroxy-methyl group at C18, with the subsequent insertion of a stable double bond between carbons C13 and C17.

Hydroxy metabolite M11 produced a small fragment at *m/z* 97 fragment (Fig 5 and 6). The presence of this *m/z* 97 fragment is in common with 3'-hydroxy-stanozolol and suggests that hydroxylation of this metabolite involves one of the nitrogens on the

pyrazole ring to form either an N-oxide or a hydroxylamine, a suggestion that is backed up by the significantly later retention time of this analyte relative to the other metabolites.

Metabolite M12, a proposed keto metabolite due to its monitored M+H mass being 2 u down from a hydroxy-stanozolol, was found to produce diagnostic fragment ions at *m/z* 257 and 313, for incubations with both stanozolol (from a precursor ion of *m/z* 343.25) and D_3-stanozolol (from a precursor ion of *m/z* 346.25). This suggests that both neutral loses involved in these pathways of fragmentation included the C20 methyl group. Since the fragment at 313 involves the neutral loss of 30 u from the M+H ion of stanozolol (or 33 from the M+H ion of D_3-stanozolol), the proven inclusion of the C20 methyl group as part of this loss means that the other 15 u (or 18 in the case of D_3-stanozolol) can only be reasonably attributed to a second methyl group from elsewhere in the molecule. The most likely methyl group was considered to be that of C18, since this would then involve a neutral loss of the two methyl groups at C17 and C18, with the subsequent insertion of a stable double bond between carbons C13 and C17. The fragment at *m/z* 257 was attributed to cleavage between carbons C14/C15 and C13/C17 as shown in Fig 5 and leads to the speculation that the added ketone group exist in either the 15 or 16 positions. Support for this isomer taking 16-keto-stanozolol form was provided by incubations of equine liver microsomes with 16β-hydroxy-stanozolol instead of stanozolol. Since the hydroxy group for this alternative starting material was already known to be in the 16 position, then the observed keto-stanozolol-metabolite that was produced by the incubation was known to be in the 16-position. Since the keto-stanozolol metabolites produced by the incubations with both stanozolol and 16β-hydroxy-stanozolol had effectively identical retention time and mass spectra (data not shown), then this is very strong evidence that this metabolite is 16-keto-stanozolol.

Following the incubations of equine liver microsomes with stanozolol and 16β-hydroxy-stanozolol, no di-hydroxy metabolites were detected on either the LTQ-Orbitrap or the 5500 Q trap. However, as shown in Figs 7, when the starting material for incubation was change to 6α-hydroxy-stanozolol, four peaks with M+H masses of 361.25 (corresponding to di-hydroxy-stanozolol) were detected. Since the starting material for these experiments was 6α-hydroxy-stanozolol, it is already known that one of the two hydroxy positions is at C6. However, since there is the possibility that the α-hydroxy group at position 6 could theoretically have been oxidised to a ketone and then further reduced back to a hydroxy of either α or β configuration, it is not possible to assign the

stereochemistry of position 6 to these metabolites. Also, as Fig 8 shows, the spectra of these metabolites did not produce any fragment ions that could be used to locate the position of the second hydroxylation. All that can be said is that the pyrazole ring is not likely to be involved since fragments at m/z 81 are observed and is it unlikely to involve hydroxylation at position 4 since an enhanced peak at m/z 145 is not visible. Further work would need to be performed in order to confirm the locations of the second hydroxy group, but considering the propensity of the equine to hydroxylate steroids in the 16 position, it could be speculated that one or all of these peaks could relate to 6,16-hydroxy-stanozolol metabolites.

5.3.3   In vitro enzyme inhibition studies

The most definitive way to identify the enzyme isoforms responsible for the metabolism of a drug is to incubate the drug with individually expressed recombinantly produced versions of enzymes in order to see which ones are able to produce metabolite (James Scarth, personal observation). However, since no equine CYPs are commercially available as recombinantly expressed proteins, this is not currently a viable option. A second option regularly used by the drug development industry is to incubate drug and tissue *in vitro* along with chemical inhibitors that are known to selectively inhibit particular enzymes (Bjornsson et al. 2003). A reduced production of metabolite following incubation with a particular inhibitor suggests that the inhibited isoform is responsible for metabolism. Since a lot is known about the specificity of chemical inhibitors toward a range of different human CYPs and since it is a reasonable first step to identifying similar enzymes in other species, the chemical inhibitor approach was tested for the stanozolol in the equine. Considering the possibility that CYP isoforms in the horse may have an altered specificities or rates of reaction compared to the human, a positive result does not guarantee that a homologous enzyme exists and/or is involved in stanozolol metabolism in the horse. Neither does a negative result guarantee that an analogous enzyme does not exist and/or is not involved in metabolism. However, since the chemical inhibition route is the best option until recombinantly produced versions of equine CYPs are available, this approach was considered worthwhile as the clues it provides may guide future research.

Following an initial screen of stanozolol with high concentrations of each of the chemical inhibitors (as described in the experimental section), the only chemicals that were found

to inhibit the metabolism of stanozolol were ketoconazole and quercetin (as measured by the production of 16α- and 16β-hydroxy-stanozolol since these appeared to be the major metabolites). More extensive analyses using these two inhibitors were then carried out in order to plot full IC50 profiles for the reactions. As detailed in Table 1, ketoconazole was a more potent inhibitor than quercetin. Also, the IC50 was lower for 16α-hydroxy-compared to 16β-hydroxy-stanozolol for both ketoconazole and quercetin. Quercetin was initially chosen as it is considered a relatively selective inhibitor of human CYP2C8 and ketoconazole because it is a known inhibitor of CYP3A4. However, complete selectivity toward a particular isoform is difficult to achieve and it is often necessary to analyze the overall pattern of inhibitors responses before drawing any conclusions. It is therefore pertinent to observe that in addition to being a CYP3A4 inhibitor, ketoconazole is also known to be a potent inhibitor of CYP2C8 (Bjornsson et al. 2003) The observation that both ketoconazole and quercetin were able to reduce the production of 16α-hydroxy- and 16β-hydroxy-stanozolol by almost 100% at the high concentrations is strong evidence that an enzyme related to CYP2C8 is involved in the metabolism of stanozolol in the equine. This line of evidence is also consistent with the results of a study by Komuri et al. (1993) where the authors found that a purified equine CYP with a high degree of N-terminal amino acid sequence homology to CYP2C isoforms was able to 16α-hydroxylate the androgenic/anabolic steroid testosterone. The recent cloning of the CYP2C92 gene (DiMaio Knych et al. 2009) is also relevant since this proves the existence of at least one CYP2C enzyme isoform in the horse.

Table 1 – determined IC50 values for the inhibition of 16α- and 16β-hydroxy-testosterone production from a 25 uM incubation of stanozolol with equine liver microsomes.

	Determined IC50 (µM)	
	Ketoconazole	**Quercetin**
16α-hydroxy-stanozolol	0.06 (SE +/- 0.05)	9.1 (SE +/- 5.5)
16β-hydroxy-stanozolol	0.52 (SE +/- 0.37)	19.3 (SE +/- 28.7)

5.4   Conclusion

In summary, the *in vitro* and *in vivo* phase 1 metabolism results reported herein compare well with the existing *in vivo* paradigm. Also, the flexibility offered by the in vitro techniques allowed experiments to be carried out with multiple starting materials, which significantly aided the identification of metabolite structures by GC- and LC-MS/MS and allowed a CYP isoform that may be important in equine steroid metabolism to be identified.

In general, although such *in vitro* techniques do not serve as a replacement for *in vivo* studies in all situations, they do offer the following advantages:

- The ability to reduce and refine the number of *in vivo* studies, therefore providing a significant benefit with respect to animal welfare.
- To aid metabolite identification (since they generally produce a more concentrated, cleaner extract for analysis).
- For fast reaction to potential new threats.
- For studying the mechanisms of metabolism in more detail.
- To biologically generate reference standards where no chemically synthesized metabolite standard is available.
- For studying the fate of designer drugs that have no toxicological profiles.

5.5   Acknowledgements

5.6   References

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Chapter 6: Metabolism of the 'designer' steroid estra-4,9-diene-3,17-dione in the equine and comparison to human and canine

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After:

6.1   Introduction

As discussed in chapter 4, the use of androgenic-anabolic steroids (AASs) in the majority of horseracing, greyhound racing and human sports are prohibited (Wada, 2009, Greyhound Board of Great Britain, 2009, International Federation of Horseracing Authorities, 2008). Effective doping control procedures are therefore required in order to control their prohibition. Targeted approaches such as gas and liquid chromatography linked to tandem mass spectrometry (GC and LC/MS/MS respectively) are the most commonly applied analytical methods (Biddle et al. 2009, Fragkaki et al. 2009, Pozo et al. 2009a). Using these techniques, the detection of the majority of marketed AAS products in human and animal sports is now possible (McKinney et al. 2009, Parr et al. 2010).

In 2003, however, the scandal surrounding the detection of the novel steroid tetrahydrogestrinone (THG) raised concerns about the possible use of such 'designer' steroids (Catlin et al. 2004). Designer steroids have chemical structures based on previously marketed products, but with minor modifications which make them

undetectable by the majority of current targeted mass spectrometric procedures (chapter 4). In the case of THG for example, the drug's structure is based upon gestrinone, but with the 17-alkyl side chain fully saturated such that the relative molecular mass of THG is 4 u higher than gestrinone (Catlin et al. 2004). Designer steroids are synthesized either to deliberately evade detection, or as appears more common, to enable them to be marketed freely on the Internet to customers in some countries because their structures do not fall within the scope of legal regulations that prevent the sale of defined steroidal products. An Internet search for 'designer steroids' quickly produces many hits for low-cost formulations of steroids that can be ordered and delivered directly to a consumer's home address. Since the majority of these compounds are available only on the Internet, their biological activity and the extent of their use is difficult to determine. One such steroid that currently (January 2010) features in a large number of marketed products and in Internet forums discussing its use by sportspersons (J. Scarth, personal observation) is estra-4,9-diene-3,17-dione. This compound is based on the structure of the known steroid trenbolone but differs in that the oxygen function at carbon 17 has been converted to a ketone and it is lacking the 11-12 double-bond (Fig 1). It is therefore not a pro-drug of trenbolone, but rather a structural analogue and hence its metabolism needs to be considered separately from trenbolone.
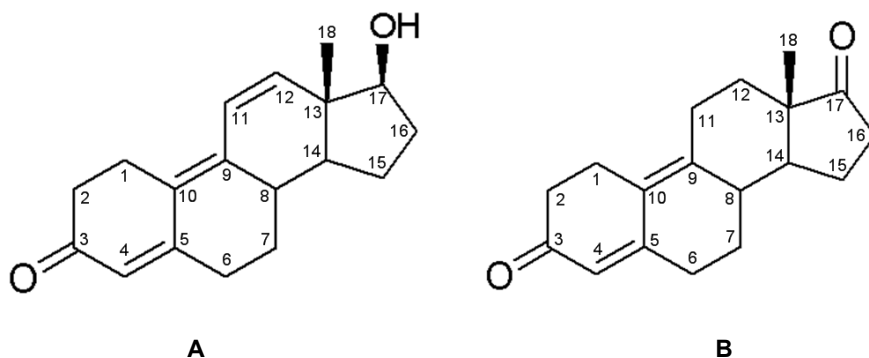

A   **B**

Fig 1 – structures of **A;** trenbolone (17β-hydroxy-estra-4,9,11-trien-3-one) and **B;** estra-4,9-diene-3,17-dione (a structural analogue, not a pro-drug, of trenbolone).

Effective control of steroids such as estra-4,9-diene-3,17-dione requires knowledge of their metabolism in order to be able to target the most abundant metabolites (Biddle et al. 2009, Houghton et al. 1979, Dumasia et al. 1983, Schänzer, 1996, Van Eenoo and

Delbeke, 2006, Poelmans et al. 2006). Because designer steroids do not have defined toxicological profiles, it is difficult to obtain ethical approval for *in vivo* administrations. *In vivo* experiments are also typically more costly than *in vitro* procedures. Therefore, attractive alternatives that have shown excellent qualitative *in vivo* correlation are the use of different *in vitro* models, involving incubation of steroids with either *ex vivo* mammalian liver preparations (Ho et al. 2005, Ho et al. 2007,Leung et al. 2004, Scarth et al. 2010a, Scarth et al. 2010b) or surrogate invertebrate models (De Wasch et al. 2002, Verheyden et al. 2007). Two recent equine publications from our laboratories for example have shown that for both steroidal (Scarth et al. 2010a) and non-steroidal (Scarth et al. 2010b) drugs, all major *in vivo* metabolites were also detected following *in vitro* incubation using either liver microsomes or S9 (as presented in chapter 5 using stanozolol as an example). It is important to point out, however, that while the qualitative profile of metabolites compares favourably between *in vitro* and *in vivo*, the quantitative profile of metabolites can vary between the two conditions. An alternative that has recently been developed is the use of rodent model transplanted with human hepatocytes (Lootens et al. 2009, Pozo et al. 2009b). Using this approach, human type metabolism can be investigated without the need for experimenting on people. In the future, it may be possible to adapt this approach to utilise equine hepatocytes. However, this technique still requires the use of animal experimentation.

To date, no studies have reported the metabolism of designer steroids in the canine and only one study has been reported in the equine. In the equine study, Kwok et al. (2006) reported the *in vivo* and *in vitro* metabolism of desoxy-vinyltestosterone and some of its analogues. In contrast to the equine and canine, relatively more studies have been carried out in the human. Levesque et al. (2005) and Gauthier et al. in (2009) used human hepatocytes to study the *in vitro* metabolism of THG and 17α-methyl-drostanolone respectively, while Rodchenkov et al. (2006) studied the *in* vivo human metabolism of a number of capsulated products purported to contain different steroidal products. In the latter study, however, reference standards for the compounds in question were not available to confirm the structures of the steroids contained in the capsules obtained on the Internet. Thevis et al. (2005a) reported the mass spectrometric behaviour of a range of different designer steroids, but this report was limited to the structures of the 'parent' steroids. Because of this limited number of studies, there still remains a large number of designer steroids are available on the Internet for which knowledge of metabolism is not available.

In addition to knowledge of the metabolism of a designer steroid, it would also be ideal to be able to retrospectively analyse sample testing data once a new steroid is discovered in order that it can be determined whether abuse of the product has occurred. Most of the existing targeted GC- or LC-MS/MS procedures do not allow for this retrospectivity (McKinney et al. 2009). Recently, however, full scan high-resolution accurate-mass LC-MS (HR-LC-MS) has been applied to the study of the metabolism and detection of drugs in doping control (Scarth et al. 2010a, Virus et al. 2008, Thevis et al. 2005b). Because full scan data are acquired, previously tested samples can be retrospectively analysed once intelligence of a new designer steroid becomes available.

In this study, the *in vitro* metabolism of estra-4,9-diene-3,17-dione is reported for the first time. This is also the first study comparing the metabolism of a designer steroid in the three major species subject to regular doping control; namely equine, canine and human. In order to allow the retrospective analysis of sample testing data, the use of a HR-LC-MS Thermo LTQ-Orbitrap instrument was employed for metabolite identification of underivatised sample extracts. The full scan HR-LC-MS Orbitrap data was then complemented by several further experiments targeted at elucidating more detailed structural information for the most abundant metabolites. These included; HR-LC-MS/MS of the underivatised metabolites, functional group selective chemical derivatisation and full scan HR-LC-MS, enzyme inhibition experiments and electron ionisation (EI) GC-MS analysis of methoxyamine-trimethylsilyl (MO-TMS) derivatives.

6.2   Experimental

6.2.1   Chemicals and reagents

Deionized water was prepared using an SG Ultrachem TWF UV system (Barsbuttel, Germany). Pyridine and all acids, bases and solvents were analytical grade and were purchased from Fisher Scientific (Loughborough, UK). Trizma base and HCl, N-Methyl-n-(trimethylsilyl)-trifluoroacetamide, sodium borohydride, methoxyamine hydrochloride, NADPH and NAD were obtained from Sigma-Aldrich (Dorset, UK). A 100 mm x 2.0 ID 2.5 μm HST Luna C18(2) column was obtained from Phenomenex (Macclesfield, UK). Equine liver S9 (at 20 mg ml^{-1} protein and from a female Quarter horse), canine liver S9 (at 20 mg ml^{-1} and from a mixed male/female Beagle pool) and human liver S9 (at 20 mg ml^{-1} and from a mixed male/female pool) were purchased from Xenotech Llc. (Lenexa,

USA). Estra-4,9-diene-3,17-dione was supplied by Toronto Research Chemicals (North York, Canada). Finasteride was obtained from Merck Share and Dohme (Granville, Australia).

6.2.2   Nuclear Magnetic Resonance (NMR) analysis of estra-4,9-diene-3,17-dione

As a quality control measure, the estra-4,9-diene-3,17-dione reference standard used for the *in* vitro incubations was examined by NMR. A sample of the standard (~3 mg) was dissolved in fully deuterated methanol (MeOH-d_4) and the resulting solution (~0.5 ml) used for all NMR experiments analysed on a Bruker Avance DRX 500. [1]H, ^{13}C and DEPT-135 spectra were acquired using standard pulse sequences and demonstrated consistency with the proposed structure, as summarised below.

[1]H spectrum (500 MHz): δ5.67 (1H, singlet) – consistent with H-4; δ1.04 (3H, s) – consistent with Me-18; δ2.95 (2H, multiplet), δ2.48 (7H, m), δ 2.10 (4H, m), δ 1.85 (1H, m), δ1.70 (1H, m), δ1.49 (1H, m) and δ1.37 (2H, m) – not individually assigned but with chemical shifts consistent with all other methine and methylene protons expected.

[13]C spectrum (125 MHz; partially assigned using DEPT-135): δ222.5 (C-17); δ202.5 (C-3); δ 160.4 and δ147.8 (C-5/C-10); δ127.2 (C-9); δ122.6 (C-4); δ52.6 and δ40.0 (C-8/C-14); δ 48.9 (C-13); δ37.9, δ36.7, δ32.7, δ31.7, δ27.7, δ26.8, δ26.1 and δ 22.8 (C-1/C-2/C-6/C-7/C-11/C-12/C-15/C-16); and δ13.5 (C-18).

Standard injections of a steroid reference solution were also performed under the LC- and GC-MS conditions that were used for the identification of metabolites (see below). The outcome of these experiments is detailed in the results section where the significance of any impurities in relation to metabolite identification is discussed.

6.2.3   In vitro incubations

Incubations of estra-4,9-diene-3,17-dione were performed with equine, canine and human liver S9 (the post-mitochondrial spin tissue fraction). The S9 fraction was preferred over microsomes since it contains a wider range of drug metabolising enzymes (Gibson and Skeet, 2001). Also, S9 and microsome fractions are currently the only tissue preparations commercially available in the equine (liver slices were not available from

the horse and would have presented practical problems regarding obtaining fresh liver from each three species at the same time). Reactions volumes were 0.66 ml and contained drug (at 15 or 150 µM), liver S9 (at 1 mg ml^{-1} protein for all species and therefore providing as level basis as possible for which to compare metabolic activity between species), NADPH (0.63 mM), NAD (0.63 mM) and pH 7.4 TRIS buffer (50 mM). Samples were incubated in a water bath at 37°C for 2 hours and 150 µl aliquots were quenched at 0 and 120 mins by the addition of 300 µl of ice-cold acetonitrile. Control experiments with either no added cofactor or no S9 were also performed in order to check that metabolism was responsible for producing any metabolites observed.

If required, it is possible to activate phase 2 metabolic pathways in liver S9 tissue. However, phase 2 metabolism was not activated *in vitro* since the current methods of choice for the analysis for steroids in sports drug surveillance generally use deconjugation followed by analysis of the 'free' drug using either GC- or LC-MS/MS (Biddle et al. 2009, Fragkaki et al. 2009, Pozo et al. 2009, McKinney et al. 2009). While the choice of whether to activate phase 2 metabolism or not may also have a small influence on the quantitative profile of phase 1 metabolites (due to a sequestering effect of the conjugating moiety), this effect is generally only small (J. Scarth, personal observation) and does not affect the qualitative profile of metabolites.

6.2.4   Enzyme inhibition experiments

Estra-4,9-diene-3,17-dione was also incubated under the above conditions, but in the presence of finasteride (at 50 µM). The rationale for adding finasteride is because it is a known inhibitor of the 5α-reductase type 2 (Vis and Schröder, 2009) and 5β-reductase (Drury et al. 2009) enzymes. Therefore, if incubation with this inhibitor causes a fall in the production of any mono-reduced metabolites, then this would be supporting evidence that reduction occurs at a site distinct from the 5-position.

6.2.5   Preparation of samples for LC- or GC-MS analysis

Sample were analysed by LC/MS either underivatised or following chemical modification to form methoxyamine derivatives of the ketone groups (as an aid to structural elucidation). In both cases, the quenched aliquots from the *in vitro* incubations were centrifuged for 5 mins at 11,000 rpm, the supernatant transferred to a separate vial and

blown down to dryness at 60°C. For the underivatised samples, these were simply reconstituted in 5 µl propan-2-ol followed by 95 µl of water and then submitted to LC-MS analysis on the Thermo LTQ-Orbitrap. For the derivatised samples, 50 µl of methoxyamine in pyridine (1 M) was added and the samples heated at 60°C for 30-mins. Samples were then blown down to dryness at 60 $^{\circ}$C, reconstituted in 5 µl propan-2-ol followed by 95 µl of water and then submitted to LC-MS analysis on the Thermo LTQ-Orbitrap.

For samples that were analysed by GC-MS as their methoxyamine-trimethylsilyl (MO-TMS) derivatives, the quenched aliquots from the *in vitro* incubations were centrifuged for 5 mins at 11,000 rpm, the supernatant transferred to a separate vial and blown down to dryness at 60°C. 50 µl of methoxyamine in pyridine (1 M) was added and the samples heated at 60°C for 30-mins. Samples were then blown down to dryness at 60 $^{\circ}$C, reconstituted in 50 µl of N-Methyl-n-(trimethylsilyl)-trifluoroacetamide (MSTFA) and heated at 60^{0}C for 2-hours before being submitted to GC-MS analysis on the Varian 1200L.

6.2.6   Chemical reduction of estra-4,9-diene-3,17-dione

Estra-4,9-diene-3,17-dione was reduced by mixing 0.5 mg of the steroid with 500 µl methanol, 100 µl water and 1 mg of sodium borohydride in a 3 ml glass vial suspended in a beaker containing ice. The mixture was then left to react for 30-minutes. 50 µl aliquots of the reaction mixture were removed to separate glass vials at 0, 10, 20 and 30 minutes. To each aliquot was then immediately added 2 ml of 0.02 M HCl and 4 ml diethyl ether before samples were capped and rotary mixed for 10-minutes. The organic layer was then removed to a clean glass vial, blown down to dryness at 60 $^{\circ}$C, reconstituted in 10 µl propan-2-ol followed by 190 µl of water and submitted for HR-LC-MS analyses under the same conditions that were used for the analyses of the *in vitro* incubates.

6.2.7   Thermo LTQ-Orbitrap HR-LC-MS and HR-LC-MS/MS analyses

Initial metabolite identification was carried out using HR-LC-MS and then followed-up by HR-LC-MS/MS in order to obtain more detailed structural information regarding the

metabolites. All data was acquired and processed using the Xcalibur version 2.0.7 software.

For the full scan HR-LC-MS metabolite identification experiments, 10 µl of sample was introduced onto a Thermo Accela autosampler/HPLC linked to a Thermo Discovery LTQ-Orbitrap. Chromatography was carried out using a Phenomenex 100 mm x 2.0 ID 2.5 µm HST Luna C18(2) column held at 35°C. Flow rate was 400 µl min^{-1} until 4.0 mins, at which time it was increased linearly to 500 µl min^{-1} at 4.20, before being held at this flow rate until 4.99 mins, reduced back to 400 µl min^{-1} by 5.00 mins and then held at this flow rate until the end of the run. Mobile phase A was 0.1% acetic acid with 300 µg l^{-1} uracil (external calibrant), mobile phase B was 0.1% acetic acid in methanol with 300 µg l^{-1} uracil (external calibrant) and mobile phase C was 99:1 methanol:2 M pH 4 acetate buffer with 300 µg l^{-1} uracil (external calibrant). Mobile phase A was 90% at 0 mins, dropping to 40% at 1.0 mins and then 2% at 3.49 mins, before being held at 2% until 4.99 mins when it was increased to 90% over 0.01 mins and held at 90% until 5.5 mins. Mobile phase B was 10% at 0 mins, rising to 60% at 1.0 mins, 98% at 3.49 mins, before dropping to 23% at 3.50 mins, held at 23% until 4.50 mins when it was increased to 98% over 0.01 mins, held at 98% until 4.99 mins, dropped to 10% over 0.01 mins and then held at 10% until 5.5 mins. Mobile phase C was 0% from 0 to 3.49 mins, before being increased to 75% over 0.01 mins at 3.49 mins, held at 75% until 4.50 mins, dropped back to 0% over 0.01 mins and then held at 0% until 5.50 mins. Sample ionisation was carried out in the positive mode using the electrospray source at a capillary temperature of 200°C, a sheath gas flow of 40 units, an auxiliary gas flow of 5 units and an electrospray voltage of 4.5 kV. Full scan centroid data over a range from 100 to 550 u was then acquired by the LTQ Orbitrap using a resolution of 30,000 at full width at half maximum height (FWHM).

For the follow-up HR-MS/MS experiments, instrumental conditions were the same as the above, except that the chosen MS/MS precursor ions (see results section for details) were first isolated in the LTQ section of the instrument and then fragmented at a normalised collision energy of 35, before the resulting ions were supplied (via a C-trap) onto the Orbitrap itself for full scan centroid analysis at a resolution of 30,000 at FWHM.

6.2.8   Varian 1200L GC-MS analyses

1 µl of sample was introduced into a Varian 1177 split-splitless injector held at 260°C. The injector split was initially closed, before being opened after 1.00 mins at a split ratio of to 50:1 for the remainder of the run-time. Chromatography was carried out using a Varian CP-3800 gas chromatograph with helium as the carrier gas (1.3 ml min^{-1}). A Varian VF-5MS 30m x 250 um x 0.25 um column was initially held at 180°C for 1.00 minutes and then ramped by 20°C min^{-1} to 250°C at 4.50 minutes, ramped by a further 5.0^{o}C min^{-1} to 310°C at 16.50 minutes and then held at 310°C until 30.00 minutes.

Mass spectrometry was carried out using a Varian 1200L triple quadrupole mass spectrometer operated in the positive electron ionization (EI) mode. Full scan centroid data between 50 and 600 u was acquired. Transfer line temperature was 280°C, ion source temperature was 230°C, electron energy was 70 eV and the electron multiplier was used in extended dynamic range mode. Data were acquired and processed using the Varian Workstation version 6.9 software.

6.3   Results and Discussion

6.3.1   Comparison of the full scan high-resolution accurate-mass LC-MS data between equine, canine and human

Underivatised compounds detected by HR-LC-MS were assigned as metabolites when a peak was observed at a mass tolerance of 5 ppm in an extracted ion chromatogram from the post-dose *in vitro* incubations that was not present in the pre-dose or control incubations. Investigated metabolic transformations were pre-defined and included single or multiple events (including combinations) of reduction, oxidation, hydroxylation, carboxylation, demethylation and dehydration. The total ion chromatograms were also inspected for the presence of any addition peaks that appeared in the post-dose samples. Using this strategy, 11 putative metabolites of estra-4,9-diene-3,17-dione were identified in the 150 µM estra-4,9-diene-3,17-dione incubations across the different species. The results from the 15 and 150 µM incubations produced comparable metabolite profiles in all species, but some of the more minor metabolites were only visible in the 150 µM incubations due to their low concentrations. Therefore, only the results from the 150 µM incubations will be discussed herein.

All eleven of the putative metabolites were detected in the equine incubations, with instrumental responses generally much higher than in the other species (Fig 2, 3 and 4 and Table 1). Although the estra-4,9-diene-3,17-dione reference standard was shown by NMR to be relatively pure, the high sensitivity of the LTQ-Orbitrap allowed the detection of two small impurities corresponding to hydroxylated versions of the 'parent' steroid at *m/z* 287.1642 (peaks at 2.80 and 2.98 mins in Fig 2D). Peak intensities of these two impurities compared to that of the parent steroid in a drug standard solution (with relative abundances therefore not affected by enzymatic metabolism) were 0.5 and 0.1 % respectively. No further impurities were identified by either LC- or GC-MS. Because these impurity peaks did not increase in size between the pre- and post-dose incubations, they were not assigned as metabolites. However, when considering any potential reduced-hydroxy metabolites appearing in the post-dose samples *in vitro*, the possibility arises that one or more of these may have derived from the hydroxylated impurities rather than directly from the parent steroid. However, any such reduced-hydroxy compounds detected may still have relevance for detection of estra-4,9-diene-3,17-dione abuse since analysis by our laboratories of marketed capsules containing estra-4,9-diene-3,17-dione have demonstrated that these impurities are also present in these products (data not shown) and hence would also be expected to contribute to the metabolic profile *in vivo*.

The major metabolite (M1) detected in all species, and therefore the most suitable candidate for screening of estra-4,9-diene-3,17-dione abuse, was a mono-reduced metabolite with *m/z* 273.1849. The remainder of the metabolites detected were much less abundant, with none reaching more than 20% of the instrumental response of M1 (Fig 3 and 4). A second, later eluting, mono-reduced metabolite, M2, was also present in each species. M3 was unique to the equine and corresponded to a di-reduced metabolite with *m/z* 275.2006. M4 and M5 were assigned as hydroxy-metabolites at *m/z* 287.1642. M4 was common to all species, but M5 was unique to the equine. It is important to note that M5 was observed to elute between two large impurities with the same elemental composition (as discussed earlier). Therefore, the failure to observe a discernable peak for M5 in the canine and human experiments does not necessarily mean that no metabolite was present, only that it was not large enough to be distinguished (unlike the equine). M6 to M11 were assigned as mono-reduced-hydroxy-metabolites at *m/z* 289.1798. M6, M8 and M10 were common to all species, but M7 and M11 were only detected in the equine. M9 was only detected in the equine and human. Since the inter-

species profile of metabolites M6 and M10 compared well with the inter-species profile of the major reduced metabolite M1, it is possible that these two metabolites are the reduced versions of the two hydroxylated reference standard impurities discussed earlier. As will be discussed in the following section, M5 and M8 produced unique fragment ions on MS/MS, distinct from those of the hydroxylated impurity peaks, providing strong evidence that M8 is a true metabolite of estra-4,9-diene-3,17-dione rather than a metabolite of a hydroxylated impurity.

Table 1 – summary of the HR-LC-MS results obtained on the LTQ-Orbitrap for the different metabolites identified from the equine, canine and human S9 incubations (following incubation at 150 uM steroid and 1 mg ml^{-1} protein), their proposed structures, theoretical mono-isotopic masses and retention times (see text for more details and results of experiments using auxiliary techniques that help confirm the structures).

* M5 was observed to elute between two large impurities with the same elemental composition. Therefore, the failure to observe a discernable peak for M5 in the canine and human experiments does not necessarily mean that no metabolite was present, only that it was not large enough to be distinguished (unlike the equine).

** The positions of hydroxylation in M6, M7, M9, M10 and M11 could not be established. The site of reduction in these compounds could not be confirmed, but is proposed to be in either position 3 or 17 for each due to their detection as mono-MO derivatives (Figure 5).

| Metabolite peak observed in an extracted ion chromatogram when using a mass tolerance of 5 ppm (proposed structures appear in brackets)	Theoretical mass (M+H$^+$)	Retention time (mins)	Peak height in equine S9	Peak height in canine S9	Peak height in human S9
Estra-4,9-diene-3,17-dione (parent drug)	271.1693	3.37	N/A	N/A	N/A
M1 (17-hydroxy-estra-4,9-dien-3-one)	273.1849	3.57	4.5×10^7	6.8×10^6	1.6×10^7
M2 (17-hydroxy-estra-4,9-dien-3-one or 3-hydroxy-estra-4,9-dien-17-one)	273.1849	3.79	1.2×10^6	2.0×10^5	1.0×10^5
M3 (estra-4,9-diene-3,17-diol)	275.2006	3.65	3.1×10^4	No peak	No peak
M4 (A-, B- or C-ring hydroxy-estra-4,9-diene-3,17-dione)	287.1642	2.69	1.2×10^6	1.3×10^6	3.3×10^6
M5 (15 or 16-hydroxy-estra-4,9-diene-3,17-dione)	287.1642	2.85	8.0×10^5	No peak*	No peak*
M6 (reduced and hydroxylated estra-4,9-diene-3,17-dione)**	289.1798	2.65	9.1×10^5	3.1×10^4	6.0×10^5
M7 (reduced and hydroxylated estra-4,9-diene-3,17-dione)**	289.1798	2.76	2.8×10^5	No peak	No peak
M8 (15 or 16, 17-dihydroxy-estra-4,9-dien-3-one)	289.1798	2.94	3.4×10^6	2.6×10^4	1.2×10^5
M9 (reduced and hydroxylated estra-4,9-diene-3,17-dione)**	289.1798	3.05	5.0×10^5	No peak	3.0×10^4
M10 (reduced and hydroxylated estra-4,9-diene-3,17-dione)**	289.1798	3.15	7.9×10^4	2.2×10^4	2.5×10^4
M11 (reduced and hydroxylated estra-4,9-diene-3,17-dione)**	289.1798	3.22	8.0×10^4	No peak	No peak
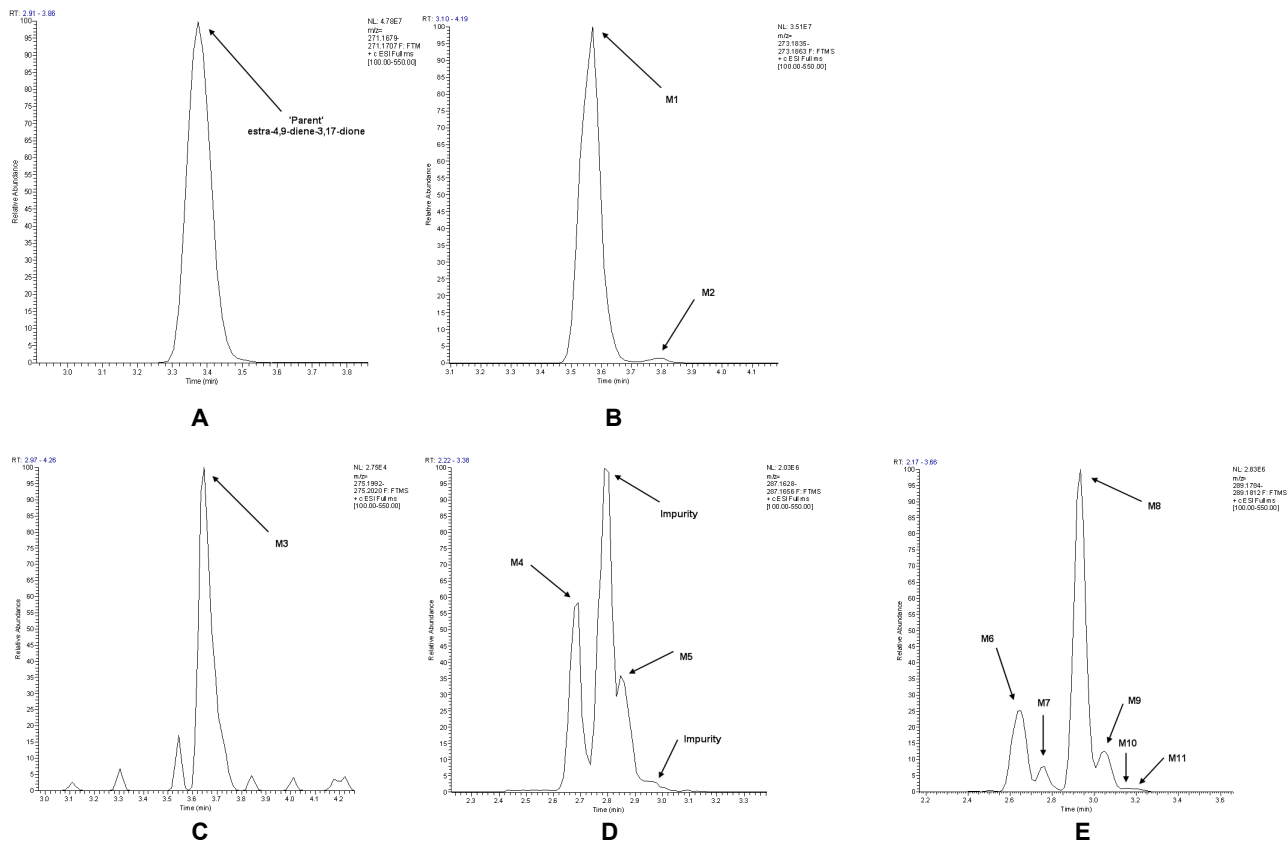
*Fig 2– extracted HR-LC-MS ion chromatograms at a mass tolerance of 5 ppm following incubation of equine liver S9 with estra-4,9-diene-3,17-dione (150 µM) for **A;** 'parent' estra-4,9-diene-3,17-dione, **B;** M1 and M2 (mono-reduced at m/z 273.1849), **C;** M3 (di-reduced at m/z 275.2006), **D;** M4 and M5 (hydroxylated at m/z 287.1642), **E;** M6 to M11 (mono-reduced and hydroxylated at m/z 289.1798).*
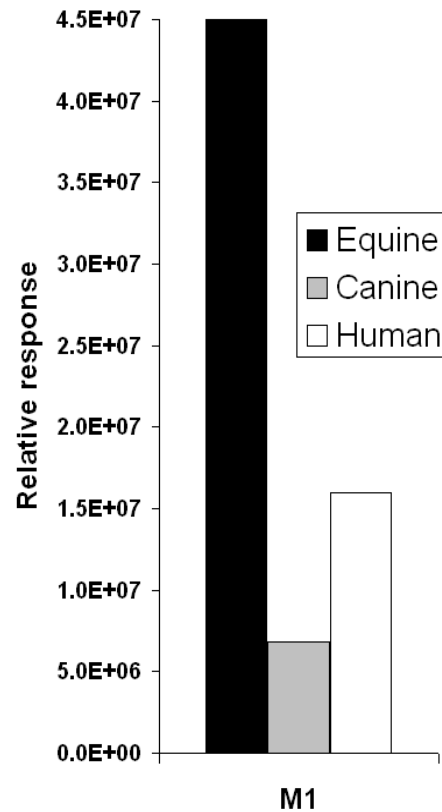
Fig 3 – relative HR-LC-MS responses of the major mono-reduced metabolite (M1 – m/z 273.1849) following the equine, canine and human liver S9 incubations (1 mg ml^{-1} protein for all species) with estra-4,9-diene-3,17-dione (150 µM).
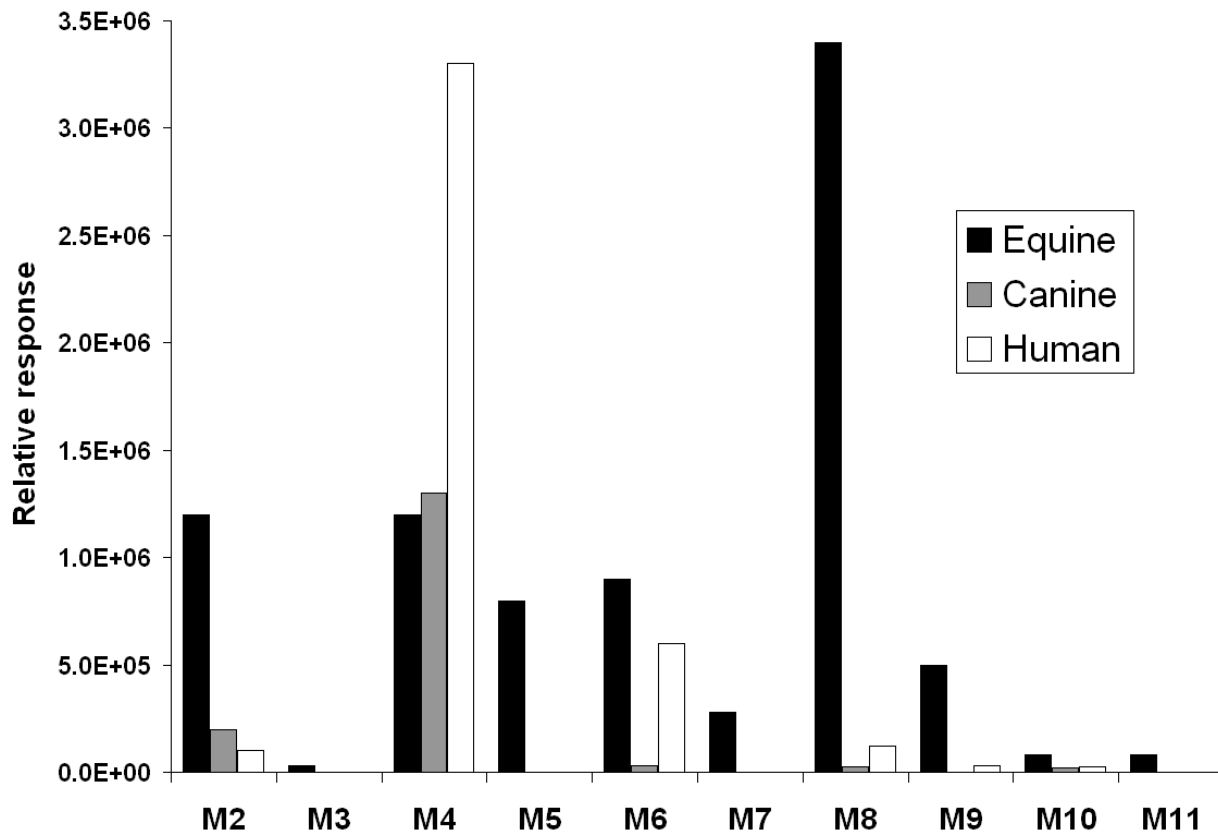
Fig 4 - relative HR-LC-MS responses of the minor metabolites following the equine, canine and human liver S9 incubations (1 mg ml^{-1} protein for all species) with estra-4,9-diene-3,17-dione (150 µM). M2 - mono-reduced (m/z 273.1849), M3 - di-reduced (m/z 275.2006), M4 and M5 - hydroxylated (m/z 287.1642), M6 to M11 – mono-reduced and hydroxylated (m/z 289.1798). See also a note in legend to Table 1 regarding M5.

Further structural elucidation of each of the metabolites, including positional information on the sites of reduction and hydroxylation, could not be gained from underivatised HR-LC-MS data alone. A more detailed consideration of the metabolite structures will be presented in the following section, where alternative analytical approaches were employed.

In terms of overall trends between the species, reductive metabolism in the canine appeared to be less significant that the other two species, while reductive metabolism in the equine was especially active. The equine was also unique in producing the di-reduced metabolite M3 and also relatively large quantities of hydroxy-metabolite M5 and hydroxy-reduced metabolite M8.

6.3.2   *Structural characterisation of the metabolites produced in vitro*

A deuterated estra-4,9-diene-3,17-dione reference standard or other analogous steroids with a 4,9-diene structure were not commercially available at the time that this study was carried out. The class of steroids with structures closest to estra-4,9-diene-3,17-dione that might therefore be useful in guiding possible metabolic pathways were the 4,9,11-triene steroids trenbolone, altrenogest, gestrinone and THG. For each of these steroids, the presence of the extended conjugated double-bond system inhibits reductive metabolism of the A-ring in all species studied to date. Trenbolone, for example, is metabolised principally to its 17α-isomer in both the horse (Houghton, 1992) and in man (De Boer et al. 1991). The horse also produces several 16-hydroxy-metabolites (Houghton, 1992). Altrenogest is similar to trenbolone, except that it contains a 17-α-alkyl subsistent and has been shown to directly conjugate to glucuronide or sulfate in the horse, with no phase 1 metabolises having been observed (Lampinen-Salomonsson et al. 2006). The metabolism of THG and gestrinone have been studied in the human and both proceed through hydroxylation at position 18, with hydroxylation at the position 16 also having been proposed as a minor pathway for THG (Lévesque et al. 2005) Data regarding the metabolism of steroids with 4,9,11-triene structures in the canine are currently lacking. Considering the results of the aforementioned studies, it might therefore be expected that the A-ring reductive metabolism of estra-4,9-diene-3,17-dione might also be inhibited and that reduction at position 17 might instead be more significant.

Since the equine produced all eleven of the major metabolites observed by HR-LC-MS and at generally higher concentrations than the other species, the follow-up analytical approaches targeted at elucidating the structures of the different metabolites were conducted using equine incubations.
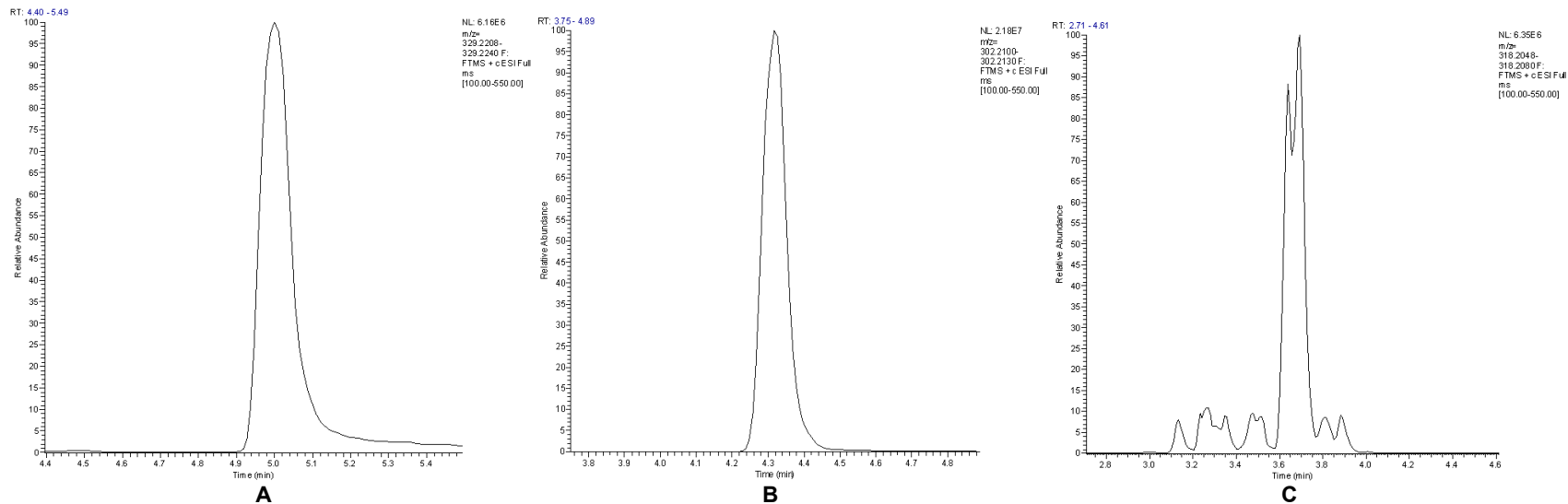
RT: 4.40 - 5.49

NL: 6.16E6
m/z=
329.2208-
329.2240 F:
FTMS + c ESI Full
ms
[100.00-550.00]

A

RT: 3.75 - 4.89

NL: 2.18E7
m/z=
302.2100-
302.2130 F:
FTMS + c ESI Full
ms
[100.00-550.00]

B

RT: 2.71 - 4.61

NL: 6.35E6
m/z=
318.2048-
318.2080 F:
FTMS + c ESI Full
ms
[100.00-550.00]

C

*Fig 5 – extracted HR-LC-MS ion chromatograms at a mass tolerance of 5 ppm following incubation of equine liver S9 with estra-4,9-diene-3,17-dione (150 μM) followed by ketone group derivatisation with methoxyamine (MO). **A;** 'parent' estra-4,9-diene-3,17-dione (bis-MO derivative at m//z 329.2224), **B;** mono-reduced metabolite M1 (mono-MO derivative at m//z 302.2115) and **C;** mono-reduced-hydroxy metabolites (mono-MO derivative at m//z 318.2064). Note that the resolution of the syn and anti MO isomers is apparent in **C;** something that was not observed in either **A** or **B).***
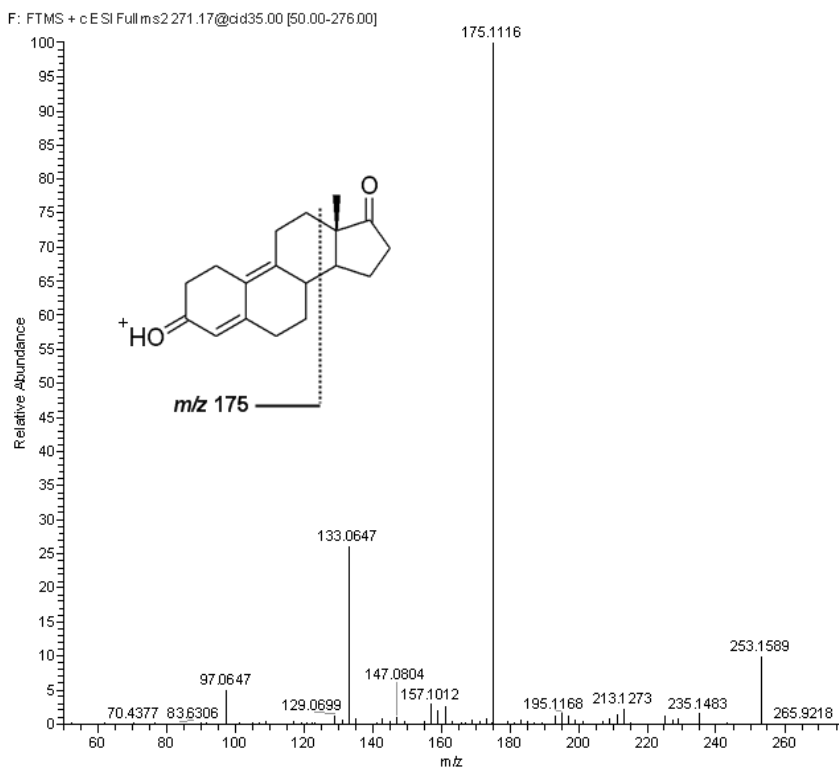
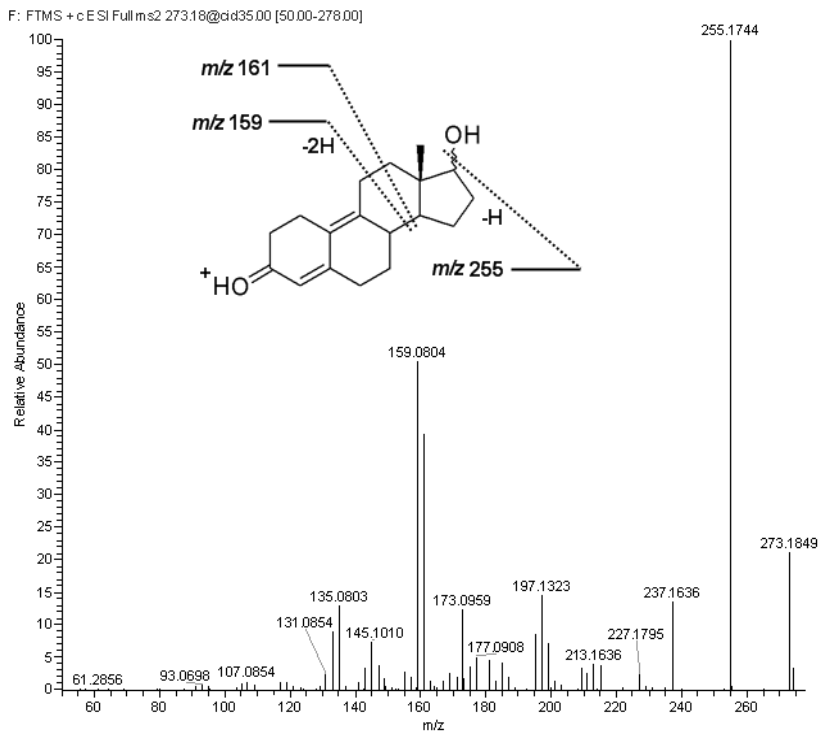Fig 6a – HR-LC-MS/MS spectrum and proposed diagnostic ion fragmentation pathways for estra-4,9-diene-3,17-dione.

Fig 6b – HR-LC-MS/MS spectrum and proposed diagnostic ion fragmentation pathways for 17z-hydroxy-estra-4,9-dien-3-one (M1) following incubation of estra-4,9-diene-3,17-dione with equine liver S9 (150 µM). 'z' indicates unknown stereochemistry.
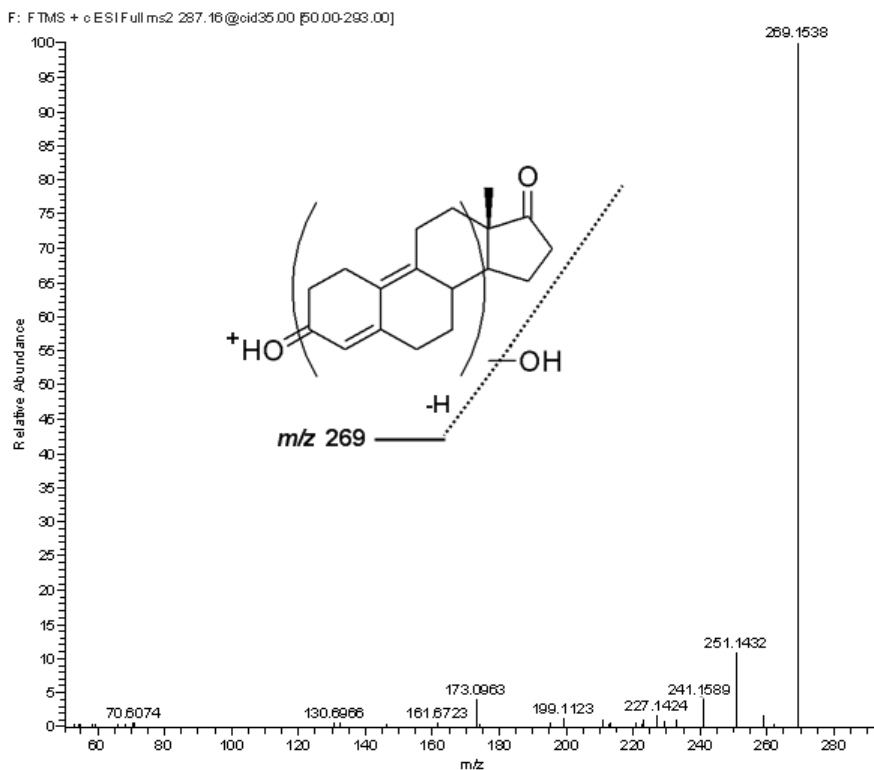
Fig 6c – HR-LC-MS/MS spectrum and proposed diagnostic ion fragmentation pathways for z-hydroxy-estra-4,9-diene-3,17-dione (M4), following incubation of estra-4,9-diene-3,17-dione with equine liver S9 (150 μM). 'z' indicates unknown stereochemistry.
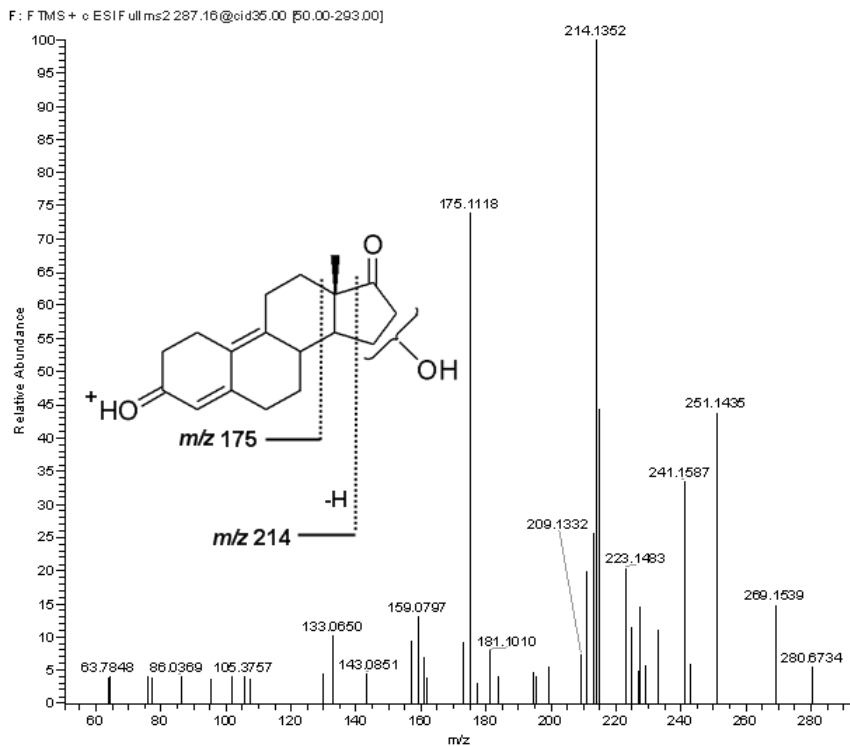
Fig 6d – HR-LC-MS/MS spectrum and proposed diagnostic ion fragmentation pathways for 15z/16z-hydroxy-estra-4,9-diene-3,17-dione (M5) following incubation of estra-4,9-diene-3,17-dione with equine liver S9 (150 µM). 'z' indicates unknown stereochemistry.
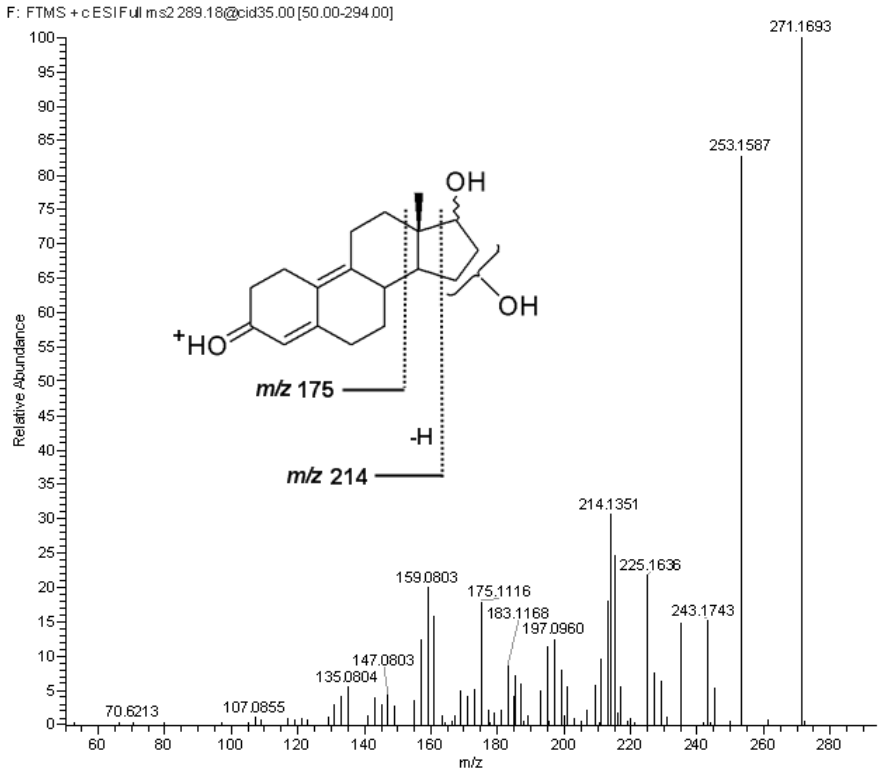
Fig 6e – HR-LC-MS/MS spectrum and proposed diagnostic ion fragmentation pathways for 15z/16z,17z-dihydroxy-estra-4,9-dien-3-one (M8) following incubation of estra-4,9-diene-3,17-dione with equine liver S9 (150 µM). 'z' indicates unknown stereochemistry.
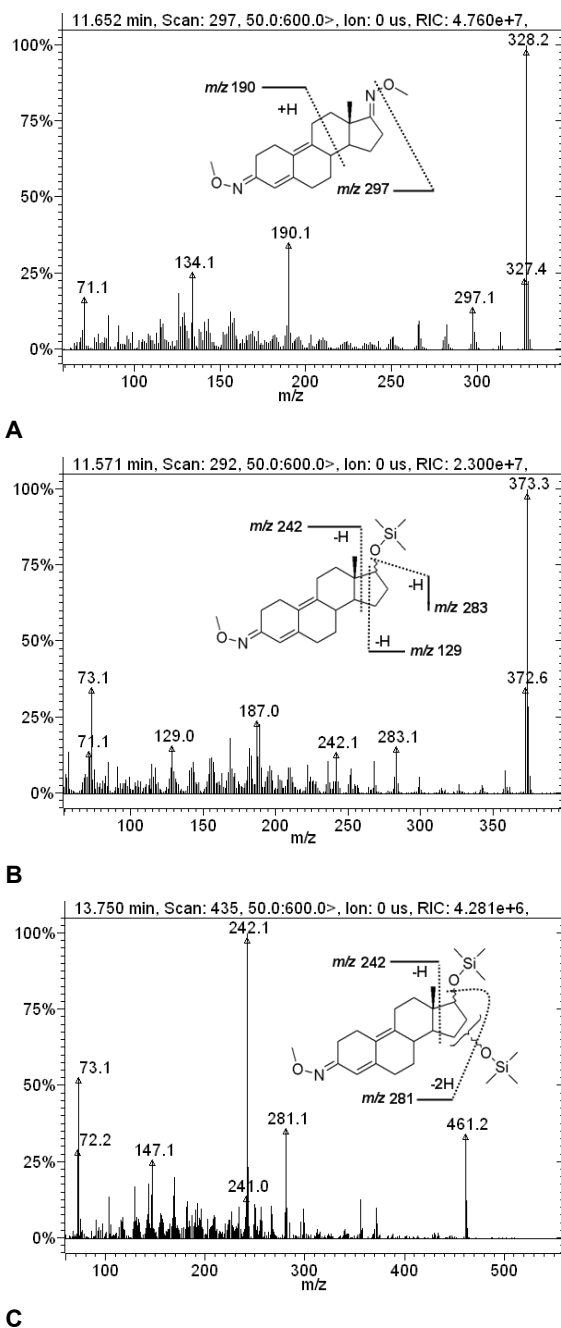
A

B

C

*Fig 7 – EI-GC-MS spectra and proposed diagnostic ion fragmentation pathways for **A**; estra-4,9-diene-3,17-dione bis-MO, **B**; 17z-hydroxy-4,9-dien-3-one MO-TMS (M1) and **C**; 15z/16z,17z-dihydroxy-4,9-dien-3-one MO-bis-TMS (M8) following incubation of estra-4,9-diene-3,17-dione with equine liver S9 (150 µM). 'z' indicates unknown stereochemistry.*

Steroids with an intact 3-keto,4-ene double-bond system ionise well in positive ion electrospray LC-MS due to resonance stabilisation, whereas reduction at the 3 or 5 positions significantly reduces the ionisation efficiency (Higashi et al. 2005). The first clue as to the structure of the major mono-reduced metabolite M1 therefore came from the strength of the HR-LC-MS signal that was observed for this analyte. The strong signal M1 (Fig 2B) suggests that reduction of metabolite M1 occurs at a site distinct from positions 3 or 5, namely at either the 9 or 17 positions. Further support for the lack of reduction at position 5 came from the enzyme inhibition experiments with the 5α-reductase type 2/5β-reductase inhibitor finasteride, which failed to inhibit the formation of metabolite M1 (data not shown).

Evidence for the reduction of metabolite M1 being at one of positions 3 or 17, rather than position 9, came from the chemical derivatisation experiments with methoxyamine (MO). MO derivatives will only form with ketone groups, not hydroxyl functions, and can therefore be used to ascertain whether reduction occurs at a carbon-carbon double bond or on a ketone. As shown in Fig 5, metabolite M1 formed only a mono-MO derivative, suggesting that one of the ketone groups had been reduced.

Another clue as to the structure of metabolite M1 was provided by the chemical reduction experiment of estra-4,9-diene-3,17-dione with sodium borohydride. Under methanolic-aqueous conditions, sodium borohydride is known to lead to preferential reduction of the 17-keto group in 3,17-diketo-4-ene steroids because the 3-keto function is protected to some extent through it's chemical conjugation (Goncharova and Grinenko, 1976, Kohler et al. 2007). Hence, the observation that metabolite peak M1 had the same chromatographic retention time on HR-LC-MS as the major mono-reduced product (>95%) from the chemical reduction of estra-4,9-diene-3,17-dione with sodium borohydride is strong supporting evidence for the site of reduction of M1 being at position 17 (data not shown).

Final support for the proposition of reduction at position 17 in metabolite M1 came from the full-scan mass spectra obtained by HR-LC-MS/MS and EI-GC-MS. Any proposed HR-LC-MS/MS fragments discussed in the following section were found to be within 1 ppm of their theoretical mono-isotopic masses, therefore giving increased confidence in the assignment of product ion structures. As shown in Fig 6A, 'parent' estra-4,9-diene-3,17-dione (precursor ion *m/z* 271) was proposed to fragment under HR-LC-MS/MS

through cleavage at carbons 8-14 and 12-13, forming a highly stable conjugated product ion at *m/z* 175. Metabolite M1 (precursor ion 273) on the other hand (Fig 6B) was proposed to fragment through cleavage at carbons 8-14 and 11-12 to form a product at *m/z* 161 and through the further loss of two hydrogens to form an observed product ion at *m/z* 159. Under low resolution EI-GC-MS conditions, the bis-MO derivative of estra-4,9-diene-3,17-dione (molecular ion *m/z* 328) produced a diagnostic fragment ion at *m/z* 190 (Fig 7A), proposed to have formed through cleavage between carbons 8-14 and 11-12. For metabolite M1, a molecular ion at *m/z* 373 was observed, consistent with an MO-TMS derivative of a metabolite reduced at one of the keto-functions (Fig 7B). Support for the reduction occurring at position 17 came from the diagnostic fragment ions at *m/z* 129 and 242, each proposed to be generated through cleavage between carbons 14-15 and 13-17, but with the charge residing on the A/B/C-ring portion of the molecule for *m/z* 242 and on the D-ring moiety in the case of *m/z* 129. Further support for the proposed fragment at *m/z* 242 taking this structure is provided by the published spectrum of trenbolone-MO-TMS, which produces an analogous fragment at *m/z* 240, which is two u down on that of M1 because of the introduction of trenbolone's double bond at position 11 (Marques et al. 2007).

Although the aforementioned results provide very strong evidence for the reduction of metabolite M1 occurring at position 17, the stereochemistry of the resulting hydroxy function remains unconfirmed at the current time. However, since sodium borohydride is known to lead to the selective reduction of the 17 ketone group to the corresponding 17β-hydroxy isomer (Goncharova and Grinenko, 1976, Kohler et al. 2007), the correlation of the chromatographic retention time of metabolite M1 with that of the major mono-reduced product following sodium borohydride reduction of estra-4,9-diene-3,17-dione is highly suggestive of M1 taking the 17β-hydroxy form. While it is unknown whether 17α and 17β isomers of metabolite M1 would resolve under the chromatographic conditions reported herein, extensions of the LC gradient and/or the use of an alternative HSS-T3 polar embedded column failed to resolve metabolite M1 peak into two peaks, therefore suggesting a single stereochemistry for this metabolite.

It was not possible to postulate exact positional information on the site of reduction of the minor metabolites M2 and M3 because the analytical sensitivity did not allow for the acquisition of quality HR-LC-MS/MS or EI-GC-MS/MS spectra. However, the absence of an analytical signal for a bis-MO derivative of M2 under HR-LC-MS conditions suggests

that reduction occurred at one of either the 3 or 17 positions. Also, the absence of an analytical signal for a mono- or bis-MO derivative of di-reduced metabolite M3 under HR-LC-MS conditions suggests that reduction occurred at both the 3 and 17 positions.

Hydroxy-metabolites metabolites M4 and M5 were not visible under EI-GC-MS conditions; possibly because of limiting sensitivity. However, good quality HR-LC-MS/MS spectra were able to be obtained (Fig 6C and 6D respectively). It might be speculated that a likely site of hydroxylation for metabolite M4, which was common to all three species, might be at position 6, since metabolism in this position is prevalent for androgenic-anabolic steroids in all three species (Houghton, 1992, Williams et al. 2000). However, the exact position of hydroxylation of metabolite M4 could not be located because of the lack of any diagnostic fragments. All that can be said with any certainty is that the absence of any characteristic D-ring fragments for metabolite M4 (in contrast to that observed for metabolite M5) suggests that hydroxylation occurred somewhere on the A/B/C-rings. For metabolite M5 on the other hand, hydroxylation was proposed at either position 15 or 16 on the D-ring. Evidence for this proposal was gained through the observation that *m/z* 175 present in the 'parent' drug spectrum was also present in the spectrum of M5, therefore ruling out hydroxylation on the A/B/C-rings. Another diagnostic fragment that was observed for metabolite M5 and lead to the speculation of a 15/16 hydroxy-metabolite was at *m/z* 214. This fragment is proposed to occur through cleavage between carbons 14-15 and 13-17 as shown in Fig 6D. The suggestion of a 15/16 hydroxy-metabolite is consistent with previous published data in the equine, where D-ring hydroxylations are particularly prevalent compared to other species (Houghton, 1992), occurring at both carbons 15 and 16 (McKinney, 2009, Houghton and Dumasia, 1979, Scarth et al. 2010a, Houghton, 1992).

Mono-reduced-hydroxy-metabolites metabolites M6, M7, M9, M10 and M11 were not visible under EI-GC-MS or HR-LC-MS/MS conditions; again, possibly because of limiting sensitivity. However, good quality EI-GC-MS and HR-LC-MS/MS spectra were able to be obtained for metabolite M8 (Figs 6E and 7C respectively). Under HR-LC-MS/MS conditions, the same *m/z* 175 and 214 fragments that were observed in the spectrum of hydroxy-metabolite M5 were also present in the spectrum of M8, suggesting that metabolite M8 was the analogous 17 reduced version of this compound. Under EI-GC-MS conditions, the same *m/z* 242 fragment that was observed in the spectrum of reduced metabolite M1 was also present in the spectrum of M8, suggesting that

metabolite M8 was the analogous 15/16-hydroxylated version of this compound (and therefore consistent with the HR-LC-MS/MS data). Lastly, the observation that all of the reduced-hydroxy-metabolites produced only a mono-MO derivative when derivatised and analysed by HR-LC-MS/MS is consistent with each of them bearing either a 3 or a 17-reduced oxygen function (Fig 5).

6.4   Conclusion

In summary, the *in vitro* metabolism of estra-4,9-diene-3,17-dione has been reported for the first time. This is also the first study comparing the metabolism of a designer steroid in the three major species subject to sport's doping control; namely equine, canine and human. The major metabolite detected in all species was proposed to be an isomer of 17-hydroxy-estra-4,9-dien-3-one. Less significant metabolic pathways in all species included hydroxylation and reduction followed by hydroxylation. Reductive metabolism in the canine was less significant than in the other two species, while the equine was unique in producing a doubly-reduced metabolite proposed to be an isomer of estra-4,9-diene-3,17-diol and also relatively large quantities of D-ring hydroxy and hydroxy-reduced metabolites. These proposed metabolic pathways are summarised in Fig 8.

In the future, chemical synthesis of the proposed metabolites, or alternatively the scaling up of the *in vitro* incubations in order to allow the acquisition of quality MS/MS and/or NMR spectra for the minor metabolites, may allow for more precise stereochemistry to be assigned to the metabolites. For the time being however, the currently reported data are sufficient to allow for the screening and confirmation of the observed metabolites in laboratories with the relevant analytical equipment (ILAC-G7, 2009).

Estra-4,9-diene-3,17-dione is one of many designer steroids that are currently (2010) marketed for sale on the Internet. A next step would therefore be to apply the analytical approaches developed herein to a wider range of these steroids.
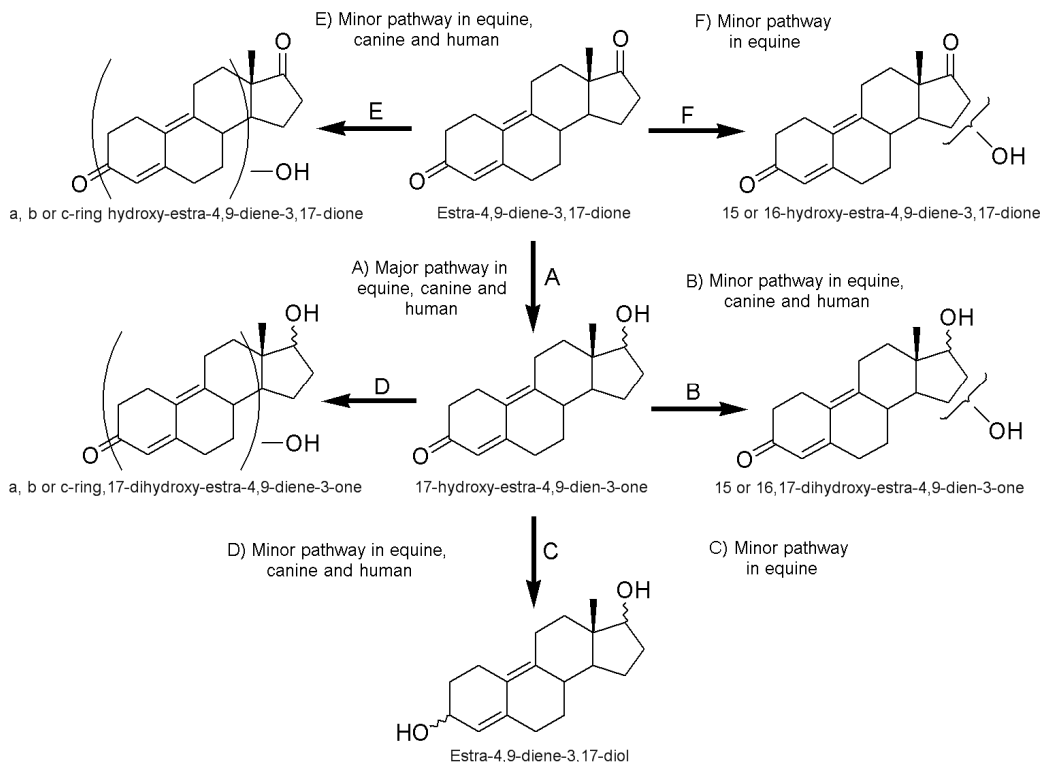
Fig 8 – *proposed pathways of estra-4,9-diene-3,17-dione metabolism in equine, canine and human.*

6.5 Acknowledgements

6.6 References

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CHAPTER 7: GENERAL DISCUSSION

7.1   Main accomplishments

7.1.1   Positioning of the research

7.1.1.1   Steroid abuse in food production and animal sports

As discussed in the introduction, steroids have been used to boost the mass and quality of animal carcasses in food production for economic reasons (Heitzman, 1975, Lone, 1997, Kay, 2010). The majority of these steroids are anabolic-androgenic steroids (AASs) such as nandrolone, boldenone, stanozolol and testosterone. However, in some species oestrogens and progestagens may also produce anabolic effects. Although there a number of steroid preparations authorised for use in countries such as the USA, the use of growth promoters is banned within the EU (EU Council Directive 96/22/EC).

In addition to their use in food production, steroids may also be used in competitive human and animal sports in order to improve performance. The range of steroids used for this purpose is generally limited to the AASs. Because of their potential to affect performance, the use of AASs in the majority of horseracing, greyhound racing and human sports is prohibited (IFHA, 2008, GBGB, 2009, FEI, 2010, WADA, 2009a). Protection of the welfare of individual competitors is another reason for prohibiting these substances; an aspect that takes increased importance in animal sports where trainers decide on the animal's behalf what substances are administered.

In order to enforce the ban on hormone use in food production, EU Council Directive 96/23/EC (and EU Commission Decision 2002/657/EC) lays down the requirements for residue testing. Enforcement of the ban on steroid use in competitive sports is not regulated in law in the same way as in food production, but guidelines regarding the analytical methods that must be followed by individual laboratories when confirming cases of steroid abuse have been produced by both the animal (AORC, 2003, ILAC-G7, 2009) and human authorities (WADA, 2009b).

As discussed in the introduction, the types of steroid used in food production and competitive sports fall into three broad classes:

'Exogenous' steroids are known marketed 'classical' steroids, such as stanozolol. These contain synthetic structures that are thought not to occur naturally. Detection of this class of steroids is relatively straightforward since a purely qualitative demonstration of the presence of these synthetic steroids is all that is required in order to determine abuse.

'Endogenous' steroids are also known marketed steroids, such as testosterone, but contain structures that are known to exist naturally. Detection of the abuse of 'endogenous' steroids is more complicated because they are, by definition, 'natural' to some extent and so a simple qualitative demonstration of their presence is insufficient to indicate abuse (chapter 1). However, the classification of a steroid as 'endogenous' is a grey area and there are some steroids that may be considered 'semi'-endogenous.

'Designer' steroids are previously unmarketed steroids that contain synthetic structures that are thought not to occur naturally. Designer steroids have chemical structures based on previously marketed products, but with minor modifications which make them undetectable by the majority of targeted mass spectrometric procedures (chapter 4).

Detection of the abuse of 'classical' synthetic steroids such as stanozolol in veterinary species is nowadays relatively straightforward. However, detection of the abuse of 'endogenous' (naturally occurring) and 'designer' (containing novel structures) steroids is more complicated. Within the horseracing industry, analytical methods are already available to detect the abuse of the majority of endogenous AASs. However, the same is not true for the majority of endogenous steroids in other food producing animals. 'Designer' steroids could in theory be abused in both horseracing and food production, but at present the majority of work on designer steroids has been commissioned by sports regulatory authorities.

The range of analytical methods used for the detection of steroid abuse in food production and animal sports was discussed in detail in the introduction and in chapters 1 and 4. The following section summarises the application of such methods to the detection of endogenous and designer steroids. It also explains the context and reasons for using the approaches adopted in the experimental chapters of this thesis.

7.1.1.2   The detection of endogenous steroid abuse in food production

The majority of approaches attempting to detect the abuse of endogenous steroids in food production are based on the direct measurement of steroids and/or their metabolites. The methods used can broadly be separated into qualitative 'marker metabolite' and quantitative 'threshold concentration' approaches. The idea of the marker metabolite method is to identify a compound that is uniquely detected following the administration of a steroid, but which is not found in untreated animals. For example, Le Bizec et al 2006 and Destrez et al. 2009 have proposed the use of boldenone sulphate as a qualitative marker metabolite to demonstrate the abuse of boldenone in cattle. Alternatively, the idea of using a threshold comprises the determination of a concentration above which it is considered statistically unlikely that a result could be produced 'naturally.' Of course, it is important that the concentrations derived from population studies are relevant to the concentrations observed following administration. If they are too high then they may be above those produced following steroid administration and will be useless. An example of the threshold approach derives from the existing EU guidelines for the detection of testosterone in bovine plasma (as proposed by Heitzman 1994). Both the marker metabolite and concentration threshold approaches have their limitations. In each case, analyses of large populations of animals are required in order to validate the approach. The use of marker metabolites and concentration thresholds for the detection of endogenous steroids was discussed in more detail in chapter 1.

As an alternative to the direct measurement of endogenous steroids in biological matrices, a range of assays based on detecting the biological *effects* of steroids have been developed. These assays can be broadly split into the areas of 'biosensors' and 'biomarkers'. Biosensors utilise biological techniques to detect the presence of steroidal activity in a sample *ex vivo* (outside of the body), whereas biomarker techniques aim to monitor activity through perturbation of 'normal' *in vivo* physiological parameters.

Biosensors show a great deal of promise for detecting steroid abuse; especially where designer steroids are concerned (chapter 4). However, their use is likely to be restricted to screening rather than confirmation since the structure of the steroid is typically not identified unequivocally. Also, they are less useful for discerning the abuse of

endogenous steroids since, by definition, these compounds are natural and some biological activity is therefore always present.

In certain respects, biosensors have been applied to residue surveillance for years in the form of immunoassays (Mooney et al. 2009a). Beyond the established field of immunoassay, several biologically based measurement technologies have recently been developed and are commonly classified as 'biosensor' technologies. Two such techniques are surface plasmon resonance (SPR) and potentiometric detection, where antibody-antigen binding can be measured quickly and assay times therefore reduced. For example, an SPR based immunobiosensor has recently been developed to detect the abuse of steroids through a reduced sex hormone-binding globulin binding capacity in bovine plasma (Mooney et al. 2009b). A further application in the biosensor field utilises knowledge of the endogenous biological receptor responsible for the pharmacological effect of the drug (i.e. Peters et al. 2010). The principle of this approach is that all drugs sharing a common method of action should be detectable through measuring the response of a recombinantly produced version of the relevant receptor. Some examples of the different receptor assays were given in chapter 1.

The rapidly advancing applications of 'omics' related technologies, which allow the simultaneous analysis of a large number of components within a biological system, have huge potential to transform the way drug residue surveillance detection is performed. The idea behind applying the 'omics' approaches is not to detect the presence of a drug directly, but instead to be able to detect it's cumulative biological effect (biomarker) within the animal through either targeted (pre-defined profiling) or untargeted (global profiling) approaches. One of the key advantages of the biomarker approach is that no matter what method of doping is used, then changes in the mRNA (transcriptomics), protein (proteomics) or metabolite (metabolomics) profiles should be detectable for drugs with common pharmacology. The definition of a 'normal' versus 'suspect' biomarker profile depends on whether 'latitudinal' or longitudinal' comparisons are applied – both of which are currently being investigated (Scarth et al. 2006). Latitudinal comparisons rely on analysing large populations of individual subjects and defining a 'normal' range, a value outside of which is considered suspect. Longitudinal measurements on the other hand, could use a change in an animal's own unique biomarker profile over time to indicate whether external perturbation had occurred. Whether biomarker approaches are suitable as confirmatory techniques or just as screening tools remains to be determined.

However, further studies are clearly warranted in order to investigate their great potential. Some examples of the different types of biomarker approaches were given in chapter 1.

A further alterative technique for detecting the use of endogenous steroids is based on gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS). GC-C-IRMS relies on detecting differences in the relative ^{12}C and ^{13}C composition of steroids between the natural and synthetic states. Synthetic steroids are typically synthesized from a single C3 plant (often soy), while the natural diet of the bovine is usually a mixture of both C3 and C4 plants (Balizs et al. 2005). The terms C3 and C4 refer to the type of metabolic pathway used by the plant in synthesising organic compounds during photosynthesis, utilising either 3 or 4 carbon-chain metabolites respectively. The significance of this lies in the fact that the two types of pathway display differing degrees of discrimination against ^{13}C and thus result in different ^{13}C to ^{12}C ratios. C4 plants have lower discrimination against ^{13}C than C3 plants, resulting in higher ^{13}C to ^{12}C ratios in C4 plant material (Balizs et al. 2005). Since steroids produced within the body will derive carbon from both C3 and C4 plant material of dietary sources, the resulting ^{13}C to ^{12}C ratio will be lower after exogenous steroid administration (mainly C3 plant material derived) relative to the endogenous state.

Geographical location has been cited as an important factor determining the degree of discrimination provided by GC-C-IRMS; mainly because of differences in feeding practices. In the UK for example, animals are typically fed a much higher base of C3 plants than in Europe, leading to a lower difference in the δ^{13}C values between administered steroids and the ERC (Mason et al. 1998). However, as studies by Buisson et al. 2005 and Hebestreit et al. 2006 have shown (chapter 1), δ^{13}C values in animals fed a C3 diet may still be sufficiently different following administration of testosterone to allow detection of the abuse of this steroid.

The GC-C-IRMS method requires substantial sample preparation prior to analysis including hydrolysis, solid phase extraction, liquid-liquid extraction and HPLC fractionation steps, as the influence of matrix interferences need to be minimised. Suitable derivatisation of the extracts followed by separation using gas chromatography further purifies the extract before introduction into a furnace. The furnace then combusts the introduced sample, which is analysed alongside a reference gas by mass

spectrometry in order to determine the relative proportions of ^{13}C and ^{12}C (Prevost et al. 2004). The laborious nature of the sample preparation technique currently makes the technique unsuitable for use as a screening tool (Piper et al. 2010), but it has already found use as a confirmatory technique for testosterone abuse in human sports (Saudan et al. 2006). The executive summary of the ISOSTER project GRD1-2001-40085 in 2006 (ISOSTER, 2006) showed that the GC-C-IRMS method for detecting testosterone abuse in bovine urine was successfully validated in several European laboratories. The use of GC-C-IRMS for the detection of endogenous steroids was discussed in more detail in chapter 1.

The majority of injectable steroid preparations contains steroids in esterified forms. The direct detection of steroid esters in matrices from an animal may, therefore, be indicative of steroid abuse. Depending on the matrix in question, contamination issues also need to be considered carefully in order to eliminate environmental contact as a possible cause of false non-compliant results. The detection of intact steroid esters in hair has attracted the most attention in literature for this purpose and has already found use in some European laboratories as a confirmatory technique for detecting natural steroid abuse (see for example Marcos et al. 2004, Nielen et al. 2006, Boyer et al. 2007, Anielski, 2008, Stolker et al. 2009 and Duffy et al. 2010). Until recently, the detection of intact steroid esters in plasma was hampered by their typically low concentrations in this matrix relative to analytical limits of detection (LOD) (Kim et al. 2000, Hooijerink et al. 1994). However, effective analytical methods based on the detection of intact testosterone, nandrolone and boldenone esters below 10 pg ml^{-1} in equine plasma have recently been developed (Gray et al. 2010). The use of steroid esters for the detection of endogenous steroids was discussed in more detail in chapter 1.

The above discussions suggested that some techniques may be suitable for both screening and confirmation whereas others may only be suitable for one of these uses. The choice of which technique to use in each situation is dictated by a number of practical, political and economic factors, which are likely to vary in different parts of the world. This subject is taken up further in the future perspectives section of this discussion, but there is currently no consensus on what is the most appropriate approach. The literature review reported in chapter 1 suggested that the use of threshold concentrations may be suitable for detection in the UK. The experiments reported in chapters 2 and 3 were therefore designed to validate thresholds for detecting some

important endogenous steroids in the porcine and bovine since these are two particularly important food producing species in the UK.

7.1.1.3   *The detection of designer steroid abuse in animal sports*

As with any AAS, if testing is based on blood or hair then detection of the 'parent' steroid may be appropriate. However, if detection is to be based on excreted matrices such as urine and faeces, knowledge of the metabolism is typically required in order to direct detection protocols toward the appropriate target analyte. With this in mind, several strategies have been proposed to detect the abuse of known or unknown designer steroids in biological samples taken from humans and animals.

One approach that has been proposed by several authors is based on predicting the pattern of fragmentation under EI-GC-MS or LC-MS/MS conditions (Thevis et al. 2005a, Fragkaki et al. 2009). Using this approach, a variety of scan functions can be used to screen for the presence of common fragments or neutral losses from a range of possible steroid structures in biological samples. If these initial screening analyses produce a suspicious result, then this may trigger a more in depth analysis of the sample in order to determine the possible structure of any unknown component.

Another approach is to use bioassay guided fractionation of samples, followed up by in depth analysis of any fractions that produce positive bioassay results. One such approach has been published by Peters et al. (2010) and uses a yeast expressed androgen receptor assay to highlight sample fractions that produce androgenic pharmacological effects. If the bioassay produces a suspicious result, then this may trigger a more in depth analysis. An alternative bioassay based on ELISA has been reported by Hungerford et al. (2005). This approach utilises an antibody that recognises steroids that contain a 17α-methyl,16β,17β-dihydroxy epitope, therefore theoretically producing a suspicious result for any 17α-methyl steroid that undergoes 16β-hydroxylation as part of it's metabolism. Since 16β-hydroxylation is a major pathway of phase 1 metabolism of such steroids, especially in equine animals (Houghton, 1992), this again highlights a suspicious sample for further work to identify any unknown molecules.

While the above approaches are based on the detection of designer steroids through screening for the presence of the steroid or metabolite itself, an alternative method that is currently being investigated is the use of endogenous 'biomarkers' (Scarth et al. 2006, Mooney et al. 2009a). The theory of the biomarker approach states that regardless of structure, the majority of AASs are likely to produce their pharmacological actions through similar means such as through binding to the androgen receptor or antagonising the cortisol receptor. Therefore, as long as they produce similar pharmacological effects, designer steroids may just as likely as classical steroids induce a change in endogenous biomarker profile.

Each of the above methods (mass spectrometric fragmentation prediction, bioassays or biomarkers) are considered most suitable for identifying new designer steroids that regulatory authorities are not aware previously existed, such as tetrahydrogestrinone (THG). However, the extent of abuse of this type of designer steroid is hard to predict and it is anticipated that it would be limited to a small group of elite individuals who are able to obtain access to a small number of rogue chemists. The designer steroids that appear to be the most widely available are those marketed on the Internet. Although these steroids contain novel structures in order to enable them to be marketed freely on the Internet to customers in some countries (because their structures do not fall within the scope of legal regulations that prevent the sale of defined steroidal products), the regulatory authorities are able to maintain an awareness of their emergence through Internet searches. For these steroids, it is therefore possible to purchase materials that can then be used for analytical method development purposes.

While it is theoretically possible to conduct *in vivo* metabolism studies using Internet available designer steroids, the fact that these compounds typically lack toxicological characterisation makes this approach difficult to justify on ethical grounds. Several other approaches have therefore been reported for producing metabolite information where reference standards of designer steroids are available. Peters et al. (2009) proposed an *in silico* (computer modelling) approach to identify the presence of designer steroids and/or their metabolites in samples using a range of possible transformations of existing steroid structures. Lootens et al. (2009) and Pozo et al. (2009) have recently developed a rodent model transplanted with human hepatocytes. Using this approach, human type metabolism can be investigated without the need for experimenting on people. In the future, it may be possible to adapt this approach to utilise equine hepatocytes. However,

this technique still requires the use of animal experimentation. Attractive alternatives that have shown excellent qualitative *in vivo* correlation are the use of different *in vitro* models, involving incubation of steroids with either *ex vivo* mammalian liver preparations (Ho et al. 2005, Ho et al. 2007a and 2007b, Leung et al. 2004) or surrogate invertebrate models (De Wasch et al. 2002, Verheyden et al. 2007), which negate the requirement for mammalian *in vivo* experimentation. To date, several designer steroid metabolism studies have been carried out in the human. Levesque et al. (2005) and Gauthier et al. in (2009) used human hepatocytes to study the *in vitro* metabolism of THG and 17α-methyl-drostanolone respectively, while Rodchenkov et al. in (2006) studied the *in vivo* human metabolism of a number of capsulated products purported to contain different steroidal products. To date, however, no studies have reported the metabolism of designer steroids in the canine and only one study has been reported in the equine (Kwok et al. 2006).

In this context, the objective of part 2 of this thesis was to review the equine steroid metabolism literature (chapter 4), to assess the suitability of *in vitro* techniques for conducting equine steroid metabolism studies (chapter 5) and to use the developed *in vitro* methods to study the equine metabolism of a novel 'designer' steroid (chapter 6).

7.1.2   Main research findings

Because endogenous and designer steroids cannot be easily detected using existing protocols, the work described in this thesis focussed on the development of analytical methodologies for the detection of their abuse in food production and animal sports respectively. However, rather than focussing purely on the specific areas of expertise developed in the author's own laboratory, a major facet of these works was consultation and collaboration with experts in other organisations around the world in order to develop the most 'rounded' approaches possible (summarised in the literature reviews in chapters 1 and 4). The following sections (7.1.2.1 and 7.1.2.2) summarise the main findings of the research presented in this thesis.

7.1.2.1   The detection of endogenous steroid abuse in food production using threshold concentrations

The following points summarise the main findings in part 1 of this thesis:

- In chapter 1, a review on the presence, metabolism and detection of endogenous anabolic-androgenic steroids in mammalian food producing animals was presented. This was the first comprehensive review of the topic to be published and was invaluable in guiding the research in the remainder of part 1 of this thesis. From this review, the concentration threshold approach was considered suitable for the detection of endogenous steroid abuse in the UK. However, it is not necessarily advocated as a unilateral international approach because of differences in farming practices, animal populations, laboratory facilities and possible patterns of drug abuse, etc.

- In chapter 2, an analytical biomarker approach for the detection of nandrolone abuse in the porcine was developed and validated. The method was based on the quantification of the nandrolone metabolite 19-noretiocholanolone in the unconjugated (free) fraction of urine using GC-MS/MS. The method was then applied to large populations of untreated UK animals in order to establish threshold concentrations for regulating nandrolone abuse. At a false non-compliance rate of 1 in 10,000 of the normal population, the suggested confirmatory thresholds (7,502 pg ml^{-1} for boars and 19,200 pg ml^{-1} in gilts) are able to detect the abuse of nandrolone for several weeks following administration of this steroid. Alternative concentration thresholds were also presented at lower non-compliance rates to serve for use in screening assays prior to confirmation by other techniques should this be required.

- In chapter 3, an analytical biomarker approach for the detection of nandrolone, boldenone, testosterone, progesterone and oestradiol was developed and validated. Using a single aliquot of urine, metabolite markers of the AASs and progestagens were extracted for GC-MS/MS analysis, while oestrogens were removed into a separate fraction using an extractive derivatisation with dansyl chloride and analysed using LC-MS/MS. The methods have since been applied to urine samples from a large population of untreated male and female animals

in order to produce data for establishing screening and confirmatory concentration thresholds, the results of which will be published separately.

7.1.2.2   The detection of designer steroid abuse in animal sports using in vitro models

The following points summarise the main findings in part 2 of this thesis:

- In chapter 4, a review on the metabolism and detection of endogenous anabolic-androgenic steroids in the equine was presented. This review highlighted that detection of the abuse of designer steroids is challenging and at present few data are available in the equine. From this review, the development of an *in vitro* model was chosen for assessing the metabolism of Internet available designer steroids so that information regarding the metabolites produced can be inserted into routine screening methods. However, this is not necessarily advocated as a unilateral international approach since it is only suitable for steroids in which a source of reference material can be found.

- In chapter 5, the suitability of using *in vitro* techniques in place of animal administrations for conducting qualitative metabolism experiments was assessed using stanozolol in the equine as an example. Using high resolution/accurate mass full scan analysis on an Orbitrap LC-MS system, equine liver microsome and S9 *in vitro* fractions were found to generate all the major phase-1 metabolites observed following *in vivo* administrations. Along with other studies conducted by the author correlating the *in vitro* and *in vivo* metabolites from a wide range of non-steroidal drugs in the equine (Scarth et al. 2010), this chapter suggested that *in vitro* techniques could serve as a viable alternative to identify suitable target metabolites when *in vivo* administrations are not possible.

- In chapter 6, the *in vitro* methods developed in chapter 5 were used to study the metabolism of a novel designer steroid, namely estra-4,9-diene-3,17-dione, in the equine. The study was also conducted with canine and human tissue, which was the first time that the metabolism of a designer steroid in the three major species subject to sport's doping control was compared. The information

regarding the equine target metabolites have since been included in the routine screening methods used at the author's laboratory in order to detect any potential abuse of this steroid.

7.2   Contribution to scientific knowledge

The following sections (7.2.1 and 7.2.2) summarise in more detail the range of different contributions that the research in this thesis makes to scientific knowledge.

7.2.1   Endogenous steroids in food producing animals

In part 1, chapter 1, the extensive literature regarding the presence and metabolism of endogenous AASs in food producing animals and the methods used to detect their abuse was reviewed. The wide variation of the natural and post-steroid administration metabolite profiles for the different compounds was highlighted in this chapter. It also reiterated the challenging nature of 'proving' the abuse of endogenous steroids, which revolves around the fact that a simple qualititative demonstration of the presence of these steroids is typically insufficient. Importantly, it was also evident that that the more sensitive analytical methods become, the more steroids are discovered to be endogenous at low concentrations (Table 1). Nandrolone, for example, was once thought to be solely exogenous, but is now known to occur naturally in a number of animal species. A wide range of different approaches for their detection have, therefore, been proposed by authors, including threshold, marker metabolite and biomarker approaches, the detection of exogenous steroid esters in plasma/hair and the use of GC-C-IRMS. During the preparation of this chapter and prior to the start of the analytical phase of this thesis, many international colleagues inside and outside of Europe were consulted for their opinions so that the author could make an informed decision on how to best tackle the challenges. It was obvious from these experiences that there is no single correct answer on how best to tackle the potential abuse of endogenous steroids. Much of the decision making process involves consideration of political, economic and other practical factors rather than just scientific ideals. In this respect, the use of the concentration threshold approach was considered most applicable at the current time to detect steroid abuse within the UK. The remainder of part 1 of this thesis, therefore, set out to develop analytical methods prior to applying them to large animal populations in order to produce data from which to derive possible thresholds.

Table 1 – summary of the endogenous occurrence of androgenic-anabolic steroids in mammalian meat-producing animal species. NMP = National Monitoring Programme, therefore not controlled with respect to steroid abuse.

Steroid	Details of endogenous presence in different species					
	Bovine	**Ovine**	**Porcine**	**Equine**	**Cervine**	**Caprine**
Testosterone	Ubiquitous in males and females at varying concentrations	Ubiquitous in males and females at varying concentrations	Ubiquitous in males and females at varying concentrations	Ubiquitous in males and females at varying concentrations	Ubiquitous in males and females at varying concentrations	Ubiquitous in males and females at varying concentrations
Nandrolone and related 19-nor androgens	Epinandrolone detectable during pregnancy. Most other studies find no nandrolone or related metabolites in males, but some find trace amounts in male and female urine i.e. following casualty (Kennedy et al. 2009)	Epinandrolone detectable during pregnancy. Two reports published on the endogenous presence of urinary nandrolone, epinandrolone and 19-norandrostenedione in male + female UK and Austrian populations	Nandrolone and 19-norandrostenedione detected in urine and some other matrices of animals of all sexes (including intersex animals) at different concentrations	Detected in urine of pregnant mares and at high concentrations in stallions (probably as a byproduct of the high concentration of aromatisation in the testes) but not in geldings or fillies	Urinary epinandrolone detected in a pregnant red deer, but no other animals studied. One of 35 urines from an Australian NMP contained epinandrolone but not nandrolone	Epinandrolone, but not nandrolone, detected in urine during pregnancy, while neither analyte detected in non-pregnant females. Studies on endogenous concentrations in males is lacking
Boldenone and related 1-dehydro androgens	Boldenone and related compounds have been detected in urine and faeces, possibly secondary to their formation by gut bacteria. Some phase 1 + 2 metabolites only detected after boldenone admin. Boldenone precursors also detected in wood crates from calves housing	Insufficient data to draw any conclusions, although 2 of 961 urines from an Australian NMP contained low concentrations of epiboldenone	Boldenone detected in urine and some other matrices of boars, cryptorchids, gilts and barrows at different concentrations, but not above the LOD in sows or an intersex animal	Boldenone detected at low concentrations in the urine of stallions, but not geldings or fillies	Insufficient data to draw any conclusions, although zero of 35 urines from an Australian NMP contained boldenone or epiboldenone	Insufficient data to draw any conclusions

In chapter 2, an analytical biomarker approach for the detection of nandrolone abuse in the porcine was developed and validated. The method was based on the quantification of the nandrolone metabolite 19-noretiocholanolone in the unconjugated (free) fraction of urine using analysis by GC-MS/MS. This analyte was selected because it has been shown to be a major metabolite following nandrolone administration, but not in untreated animals (Roig et al. 2007, Ventura et al. 2008) (see proposed pathways in Figure 1).
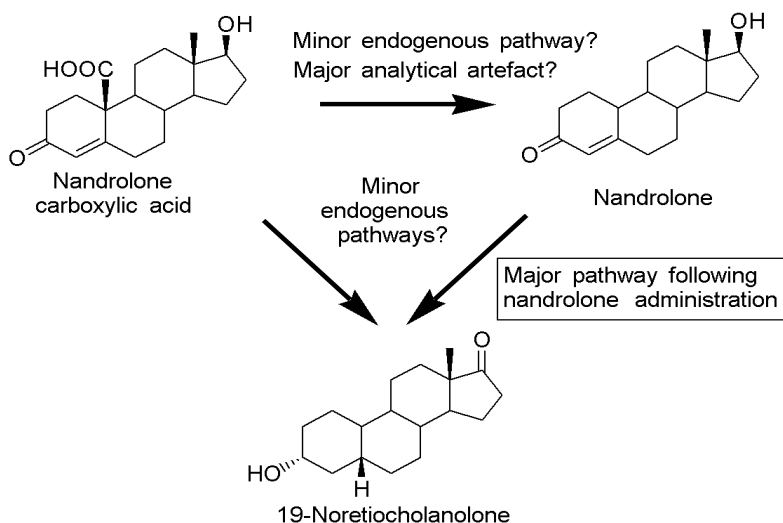
Figure 1 – diagram depicting some of the possible metabolic pathways leading to the presence of 19-noretiocholanolone in porcine urine.

The LOD (13.2 pg ml^{-1}) was significantly lower than previously reported porcine methods, the most sensitive of which was 100 pg ml^{-1} (Roig et al. 2007). This increase in sensitivity was achieved through the use of an extensive sample clean-up (providing a reduced matrix background), the application of a PTV injector (allowing a large proportion of the sample to be injected) and analysis by GC-MS/MS (providing enhanced selectivity). When applied to a population of untreated animals, 19-noretiocholanolone distributions in boars and gilts were bimodal, with a small number of concentrations in each sex around the 1,000 pg ml^{-1} region and the majority of concentrations closer to the lower end of the calibration range. This was the first study to demonstrate that 19-noretiocholanolone is endogenous in the porcine and hence would require a concentration threshold rather than a qualitative marker metabolite approach as proposed previously (Roig et al. 2007, Ventura et al. 2008). The detection of low concentrations of 19-noretiocholanolone in porcine urine is, perhaps, not surprising since

it might be expected that a small amount of the proposed nandrolone carboxylic acid precursor might be converted into nandrolone metabolites *in vivo.* Indeed, the analogous situation is observed in the equine, where very low concentrations of the major equine nandrolone metabolite 5α-estrane-3β,17α-diol can be detected in the urine of stallions (Teale and Houghton, 2010).

Statistical analysis of the population data was carried out in order to suggest screening and confirmatory thresholds for this steroid in the urine of boars and gilts. Because of the non-normal distribution of the data, it was necessary to use a non-parametric method of statistical analysis and the Chebyshev inequality was considered the most suitable as it makes minimal assumptions about the population distribution and produces conservative thresholds relative to methods based on normally distributed data (Roy Macarthur, personal communication). At a false non-compliance rate of 1 in 10,000 of the normal population, the suggested confirmatory thresholds are 7,502 pg ml^{-1} for boars and 19,200 pg ml^{-1} in gilts. To put these thresholds into context, in a recent study administering 2 mg/kg nandrolone laurate via intra-muscular injection to six boars aged 8-10 weeks, the mean free fraction 19-noretiocholanolone concentration at the last time-point of the study (15 days following administration) was 28,400 pg/mL, with a range of 9,600 to 53,600 pg/mL (Ventura et al. 2008). The suggested thresholds should therefore be able to detect the abuse of nandrolone for a significant time period in most treated animals and lead to rates of both low false compliance and non-compliance. The validation of these thresholds is significant as there are currently no other published methods available for the detection of nandrolone abuse in the porcine. Thresholds for screening may be set at a lower probability, but there then needs to be a secondary mechanism for confirmation if the confirmatory threshold is not also breeched. Typically, this may include follow-up analyses using gas chromatography carbon isotope mass spectrometry (GC-C-IRMS) (Prévost et al. 2004), detection of an intact steroid ester (Boyer et al. 2007) or an on-farm inspection (Jack Kay, personal communication). In an ideal world, a confirmatory threshold would also be suitable as a screening threshold, but this requires that the threshold is able to produce both low rates of false compliance and non-compliance; an ideal that is seldom achieved.

In chapter 3, an analytical biomarker approach for the detection of nandrolone, boldenone, testosterone, progesterone and oestradiol in the bovine was developed and validated. Using a single aliquot of urine, metabolite markers of the AASs and

progestagens were extracted for GC-MS/MS analysis, while epioestradiol was removed into a separate fraction using an extractive derivatisation with dansyl chloride and analysed using LC-MS/MS. The high sensitivity of this method was achieved through the use of an extensive sample clean-up (providing a reduced matrix background), the application of a PTV injector for GC-MS/MS (allowing a large proportion of the sample to be injected) and the separation of epioestradiol into a separate derivatised fraction for LC-MS/MS (enhancing sensitivity in the positive electrospray mode through the addition of the high proton affinity dansyl moiety). Using a standard addition calibration line approach in pooled bovine urine, the method was linear between the endogenous concentrations and those augmented with 3,000 pg ml^{-1}. The determined LODs were in several instances lower than those published previously using mass spectrometric based methods (with the exception of 5α-pregnane-3β,20α-diol, for which no data was available for comparison) (Table 2).

Table 2 – determined LODs using the current method in comparison to those reported previously (to the best of the author's knowledge).

Steroid	Current LOD (pg ml^{-1})	Lowest previously reported mass spectrometric based LOD/CCα (pg ml^{-1})
5β-androstane-3α,17β-diol	94.1	LOD = 500 (Biddle et al. 2003)
5α-estrane-3β,17α-diol	10.9	LOD = 500 (Biddle et al. 2003). Pinel et al. 2010 reported 5α-estrane-3β,17α-diol excretion profiles, but no LOD/CCα.
Epinandrolone	19.8	CCα = 30 (Pinel et al. 2008)
19-Noretiocholanolone	32.4	CCα = 20 (Pinel et al. 2008)
17β-Hydroxy-5β-androst-1-ene-3-one	160.7	LOD = 50 (Le Bizec et al. 2006)
Epiboldenone	10.5	LOD = 10 (Arts et al. 1996 using high resolution MS)
5α-pregnane-3β,20α-diol	12.2	N/A – no published LOD/CCα
Epioestradiol	14.8	CCα = 40 (Pinel et al. 2008)

With the exception of the boldenone metabolite 17β-hydroxy-5β-androst-1-en-3-one, all metabolites were shown to be endogenous at some concentration in pooled urine from untreated steers. The observation that testosterone, progesterone and oestradiol metabolites were detected is not surprising since the testosterone, progesterone and oestradiol are known to be endogenous in this species (Scarth et al. 2009). In contrast, the fact that epiboldenone and the nandrolone metabolites 5α-estrane-3β,17α-diol,

epinandrolone and 19-noretiocholanolone were detected in the pooled UK steer urine is perhaps more significant. Epiboldenone has previously been shown to be endogenous in animal populations in other countries (De Brabander et al. 2004) and epinandrolone been detected in the urine of injured male cattle and pregnant females (Kennedy et al. 2009). However, the results from this thesis suggest that certain nandrolone metabolites are also endogenous at low concentrations in uninjured steers. Possible traces of 17β-hydroxy-5β-androst-1-en-3-one have been reported previously in the urine of untreated bovine animals (Le Bizec et al. 2006), but it's identity as an endogenous compound has yet to be confirmed unambiguously. The analytical methods presented herein have since been applied to urine samples from a large population of untreated male and female animals in order to produce data for establishing screening and confirmatory concentration thresholds, the results of which will be published separately in due course.

The adoption of the threshold concentration approaches for detecting steroid abuse in veterinary species as proposed in part 1 of this thesis is currently being considered by the relevant UK regulatory authorities. While it is anticipated that these thresholds may find use as either screening and/or confirmatory techniques, they may only form part of a wider portfolio of techniques that are applied in different situations. These may also include marker metabolites, 'omics' biomarker approaches, GC-C-IRMS, detection of intact steroid esters in plasma/hair and on farm visits. This subject is taken up further in the future perspectives section of this discussion.

7.2.2 Designer steroids in animal sports

In part 2, chapter 4, the metabolism of AASs in the equine and the methods used to detect their abuse was reviewed. The trends within the equine metabolism data were also compared to those of the human, porcine and bovine, highlighting some interesting differences between these different species. This chapter exemplifies the need for performing metabolism studies in order that urinary based detection methods are targeted toward the most appropriate marker metabolites. Figure 2 summarises the major pathways of AAS metabolism in the horse.

One of the biggest perceived current threats in terms of AAS use in animal sports is the possible abuse of designer steroids, particularly those readily available for purchase from Internet sites. The remainder of part 2 therefore set out to develop *in vitro* methods for

studying the metabolism of Internet available designer steroids in order to identify marker metabolites for inclusion in drug screening procedures. An *in vitro* rather than *in vivo* approach was necessary because of the ethical constraints of administering previously untested substances to healthy animals.
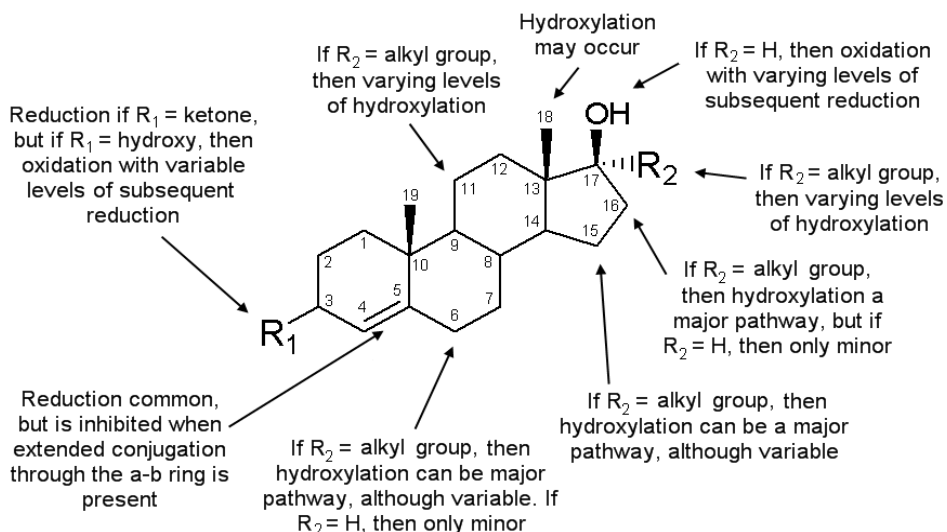

Figure 2 – summary of some of the common pathways of AAS metabolism in the horse.

In chapter 5, equine liver/lung microsomes and S9 tissue fractions were used to study the *in vitro* metabolism of the AAS stanozolol. The results were also correlated with those obtained following an *in vivo* administration of the steroid. The major aim of this study was to assess the feasibility of using *in vitro* techniques in place of animal administrations for conducting qualitative metabolism experiments. Using high resolution/accurate mass full scan analysis on an Orbitrap LC-MS system, equine liver microsome and S9 *in vitro* fractions were found to generate all the major phase 1 metabolites observed following *in vivo* administrations. The results also correlated well with previously published data regarding the metabolism of stanozolol, which proposed that 16α-, 16β- and 15-hydroxystanozolol were the major equine phase 1 metabolites (Muck and Henion 1990, McKinney et al. 2004). Additionally, analysis of the liver microsomal incubates using a shallower LC gradient combined with various MS/MS functions on a Sciex 5500 Q trap instrument allowed the identification of a number of

phase 1 metabolites previously unreported in the equine or any other species. Figure 3 summarises the range of different hydroxystanozolol isomers that were identified. Comparison between liver and lung S9 metabolism showed that the liver was the major site of metabolic activity in the equine. Furthermore, using chemical enzyme inhibitors that are known to be selective for particular isoforms in other species suggested that an enzyme related to CYP2C8 may be responsible the production of 16-hydroxy-stanozolol metabolites in the equine. This suggestion is also consistent with the results of a study by Komuri et al. (Komuri et al. 1993) where the authors found that a purified equine CYP with a high degree of N-terminal amino acid sequence homology to CYP2C isoforms was able to 16α-hydroxylate the androgenic/anabolic steroid testosterone. Along with other studies conducted by the author correlating the *in vitro*/*in vivo* metabolism of a wider range of non-steroidal drugs in the equine (Scarth et al. 2010), this chapter suggested that *in vitro* techniques could serve as a viable alternative to identifying suitable target metabolites when *in vivo* administrations are not possible.
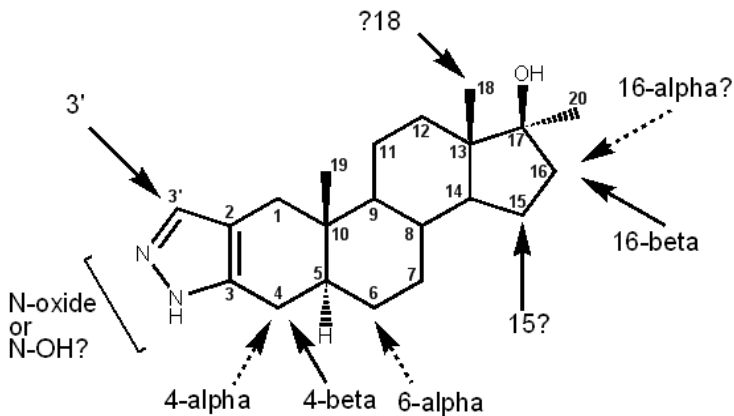

Figure 3 – structure of stanozolol with carbon numbering indicated. Arrows point to the sites of mono-hydroxylation produced in the equine liver microsomal fractions that could either be confirmed by reference standards or suggested (shown by a ?) based on pattern of fragmentation.

In chapter 6, the *in vitro* methods developed in chapter 5 were used to study the metabolism of a novel designer steroid, namely estra-4,9-diene-3,17-dione, in the equine. The study was also conducted with canine and human tissue, which was the first time that the metabolism of a designer steroid in the canine was studied and was also

the first time that the metabolism of a designer steroid in all three major species subject to sport's doping control was compared. The *in vitro* metabolism of several designer steroids has been reported previously using human tissues (Levesque et al. 2005, Kuuranne et al. 2008, Gauthier et al. 2009), but only one previous study in the equine has been published (Kwok et al. 2006). The methods presented in chapter 6 extended those used in the previous equine study by employing the use of the liver S9 fraction in order to increase the range of enzymes involved. Also, in order to permit the retrospective analysis of sample testing data, the use of high resolution/accurate mass full scan analysis on an Orbitrap LC-MS system was employed for metabolite identification of underivatised sample extracts. The full scan Orbitrap data were complemented by several further experiments targeted at elucidating more detailed structural information for the most abundant metabolites. These included; MS/MS of the underivatised metabolites, functional group selective chemical derivatisation followed by full scan LC-MS, enzyme inhibition experiments and full scan electron ionisation GC-MS analysis of methoxyamine-trimethylsilyl derivatives.

The major metabolite detected in all species, and therefore the most suitable candidate for screening of estra-4,9-diene-3,17-dione abuse, was proposed to be an isomer of 17-hydroxy-estra-4,9-diene-3-one. Less significant metabolic pathways in all species included hydroxylation and reduction followed by hydroxylation. Reductive metabolism in the canine was less significant than in the other two species, while the equine was unique in producing a di-reduced metabolite (proposed to be an isomer of estra-4,9-diene-3,17-diol) and also relatively large quantities of D-ring hydroxy and hydroxy-reduced metabolites.
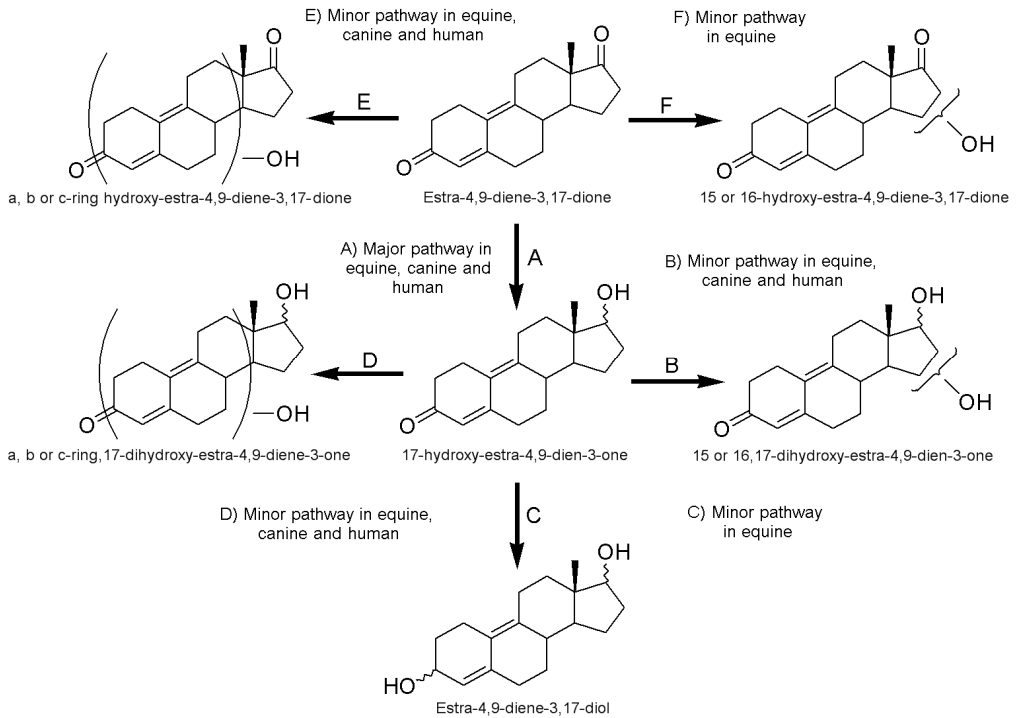
Figure 4 – proposed pathways of estra-4,9-diene-3,17-dione metabolism in equine, canine and human.

The proposed metabolism of estra-4,9-diene-3,17-dione is summarised in Figure 4. These pathways are consistent with those of the 4,9,11-triene analogues (as opposed to 4,9-diene) trenbolone, altrenogest and THG. For each of these steroids, the presence of the extended conjugated double-bond system inhibits reductive metabolism. Trenbolone, for example, is metabolised principally to its 17α-isomer in both the horse (Houghton, 1992) and in man (De Boer et al. 1991). The horse also produces several 16-hydroxy-metabolites (Houghton, 1992). Altrenogest is similar to trenbolone, except that it contains a 17-α-alkyl subsistent and has been shown to directly conjugate to glucuronide or sulfate in the horse, with no phase 1 metabolises having been observed (Lampinen-Salomonsson et al. 2006). The metabolism of THG and gestrinone have been studied in the human and both proceed through hydroxylation at position 18, with hydroxylation at the position 16 also having been proposed as a minor pathway for THG (Levesque et al.

2005). Data regarding the metabolism of steroids with 4,9,11-triene structures in the canine are currently lacking.

The information regarding the equine target metabolites of the designer steroid estra-4,9-diene-3,17-dione that was reported in part 2 of this thesis has since been included in the routine drug screening methods used at the author's laboratory in order to detect any potential abuse of this steroid.

7.3 Future perspectives

While the author has attempted to tackle a number of analytical challenges in the current thesis, the work has its limitations and a number of unanswered questions and areas for future research inevitably remain. The following section serves to highlight some areas of research that may follow on from these studies.

7.3.1 The detection of endogenous steroid abuse in food production

With regard to the porcine results reported in chapter 2, the observation of only very low concentrations of 19-nortiocholanolnoe in the urine of untreated animals is difficult to reconcile if nandrolone itself is truly endogenous in this species. As proposed in chapters 1 and 2, this situation could be explained if the routinely detected nandrolone is in fact predominantly an analytical artefact from the degradation of a testosterone-19-carboxylic acid (as demonstrated in the equine by Houghton et al. 2007). If this is the case, then the presence of a small amount of endogenous 19-noretiocholanolone in urine (as observed herein) may be expected if a small proportion of the testosterone-19-carboxylic acid is degraded to nandrolone in the body and subject to metabolism in the liver. Further experiments may therefore be justified in order to confirm this hypothesis. Also, the reasons for the existence of a bimodal distribution of 19-noretiocholanolone concentrations in the urine of boars and gilts are currently unknown. Further experiments to probe the cause of the varying concentration of this analyte may therefore be warranted in order to establish the underlying physiology and determine the function, if any, of the secreted 19-norandrogens.

The bovine methods developed in chapter 3 have since been applied to urine samples from a large population of untreated male and female animals. The data produced will be

used in a similar way to that from the porcine studies in proposing screening and confirmatory concentration thresholds. The results of these studies will be published separately in due course, but are also likely to pose a number of questions and therefore areas for future research. As described earlier, epiboldenone and the nandrolone metabolites 5α-estrane-3β,17α-diol, epinandrolone and 19-noretiocholanolone were found to be endogenous in the pooled steer urine used for the current study. Although a number of theories have been put forward regarding the origin of 1-dehydrosteroids in the bovine (chapter 1), further work would be required in order to clarify the origin of epiboldenone in the urine from UK bovine animals. Further work would also be required in order to determine the origin of the nandrolone metabolites in these populations, since nandrolone metabolites have only been detected endogenously in injured males or pregnant females to date (Kennedy et al. 2009).

For both the porcine and bovine results reported in this thesis, there were limitations with regard to the demographic of the animal populations used. For example, they were limited to certain age ranges, geographical location (UK), sexes (no castrated porcine animals or uncastrated bovine animals were available), disease and pregnancy states as well as concomitant medications such as those relating to immunocastration or oestrous synchronisation. Furthermore, there are also several other animal species in the UK that are considered meat producing species. Cervine, equine and caprine species were not considered here for economic reasons (only a finite resource was available). However, ovine populations were studied and the results from these studies in relation to nandrolone and boldenone detection will be reported separately in due course. In addition to the aforementioned limitations, the range of steroids considered in each species was not exhaustive. For example, while the bovine studies focussed on androgens, oestrogens and progestagens, they did not consider corticosteroids. Also, the porcine studies focussed solely on detecting nandrolone abuse. Further studies concerning a wider range of steroid/species combinations may therefore be warranted. Also, the current studies were limited to urine as the target matrix. While this was considered suitable for testing on-farm and at slaughter within the UK, it may not be applicable elsewhere in the world and would not be suitable for testing imported meat products. Since the beginning of the current study, increases in analytical sensitivity in various laboratories have meant that further compounds have also been identified as endogenous. For example, it is now suspected that prednisolone (Arioli et al. 2010, Bredehöft et al. 2010) and thiouracil (Vanden Bussche et al. 2010) may occur 'naturally'

in some species; possibly through biosynthesis within bodily tissues or through bacterial conversion in the gut/excreta. However, further work is required in order to clarify the precise origin of these compounds.

As mentioned earlier, the adoption of threshold concentration approaches for detecting steroid abuse in veterinary species is currently being considered by the relevant UK regulatory authorities. This process will involve lengthy discussion with parties possessing the relevant practical, political, economic and legal knowledge. A major consideration is whether the concentration thresholds carry enough legal weight for use as formal confirmatory methods or if they will instead be restricted as screening tests. If thresholds are used for either purpose then the degree of statistical confidence required at each stage will need to be agreed. A false non-compliance rate of 1 in 10,000 was proposed in the current thesis for use in confirmation since this is already deemed to give acceptable confidence in the analogous area of horseracing doping control (chapter 4 and Houghton and Crone, 2000). If used for screening, then a lower false non-compliance rate of 1 in 1,000 was proposed in order to reduce the number of false compliance results, but still produce only a relatively low number of false non-compliant results requiring follow-up analyses. However, the relevant regulatory authorities would need to agree the statistical confidence required at each stage in order to strike the appropriate balance between false compliant and false non-compliant results. If threshold concentrations are not used for formal confirmation (or at least not on their own), viable alternatives may include marker metabolite, 'omics' biomarker approaches, GC-C-IRMS, detection of intact steroid esters in plasma/hair and on-farm visits. With the exception of on-farm visits, however, these alternative confirmatory approaches may also require more research and discussion before they can be used in the UK.

7.3.2   The detection of designer steroid abuse in animal sports

The structures of the *in vitro* synthesised metabolites of estra-4,9-diene-3,17-dione proposed in chapter 5 are, at present, only putative. In the future, chemical synthesis of the proposed metabolites, or alternatively the scaling up of the *in vitro* incubations in order to allow the acquisition of quality MS/MS or NMR spectra for the minor metabolites, may allow for more precise stereochemistry to be assigned to the metabolites. Also, estra-4,9-diene-3,17-dione is one of many designer steroids that are currently (2010) marketed for sale on the Internet. Further efforts are therefore underway in our

laboratory (and other laboratories around the world) to use these *in vitro* techniques to study the metabolism of a wider range of designer steroids in human, equine and canine species. As discussed earlier in chapter 5, the degree of *in vivo / in* vitro qualitative correlation with regard to drug metabolism has in general been found to be good. However, if any tested samples from humans or animals are found in the future to contain evidence for the presence of designer steroid metabolites, these results may further assist in determining the true extent of correlation and help further refine the techniques.

One limitation of the *in vitro* approach used in this thesis is that it can only be applied to Internet available designer steroids since these are the only types of designer steroid for which materials can be purchased for use in experimentation. For compounds which may initially be unknown to regulatory authorities, such as THG, the problem of not knowing their existence is significant as it implies that the *in vitro* approach is not viable. For these steroids, further evaluation of the alternative research methods described in section 7.1.1.3 may, therefore, be warranted in order to ensure that these compounds do not go 'undetected.' One significant recent development with respect to the detection of all designer steroids is the increasing adoption of full scan HR-LC-MS (chapters 4, 5 and 6). Using this technique, data can theoretically be retrospectively analysed once information about the existence of a new steroid is found. If aliquots of the original sample matrix are stored for long periods of time, it may even be feasible to retest the sample in order to confirm the presence of any suspicious retrospective findings.

The *in vitro* method used for the equine designer steroid metabolism studies in this thesis utilised liver S9 fractions from Quarter horse. However, the majority of horses racing in the UK are of the Thoroughbred breed. In the future, it may therefore be useful to produce liver material from this breed to see if this improves the *in vivo / in vitro* correlation. In this respect, it would also be interesting to extend the range of tissues used for this purpose to include those such as kidney, intestine and faeces. Recent research has also focused on extending the *in vitro* methods to allow the production of phase 2 steroid metabolites in order that they can be used for method development and for use as reference standards (Taylor et al. 2010). While the current thesis focussed on applying *in vitro* methods to study the metabolism of designer steroids, there are also other classes of designer drugs for which these approaches may be applicable. For

example, Hudson et al. (2010) have recently reported the finding of a number of designer cannabinoid compounds in 'herbal high' products sold in the UK.

In addition to abuse in sports, it is also possible that designer steroids may be used as growth promoting agents in food production. *In vitro* techniques could also be useful for studying metabolism in these animals if they are deemed to be a threat to the consumer.

7.3.3   Beyond endogenous and designer steroids

Whilst endogenous and designer steroids are two of the most challenging classes of growth promoters for detection purposes in food production and horseracing, new chemical entities are constantly being developed by pharmaceutical companies. In this respect, it is pertinent to note that a range of other anabolic agents are currently being tested for their clinical efficacy, including non-steroidal selective androgen receptor modulators (SARMs), myostatin inhibitors, growth hormone secretagogues (in addition to the existing problem of recombinant growth hormone itself) and a range of possible macromolecule based therapies including 'gene' therapy. While the approaches described in this thesis may be applicable to some of these new challenges, they will no doubt be ineffective against others. Appropriate solutions to these forthcoming challenges may therefore require a whole new range of approaches from a fresh crop of enthusiastic young scientists!

Of course, the above considerations are all dependent on the different hormone bans remaining in place. It is possible, in theory at least, that one or more of the bans could be overturned on political or scientific grounds. This would no doubt mean that the requirements for residue testing would change significantly. However, the bans remain in place for the foreseeable future (and so therefore do the efforts of the doping chemists).

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SUMMARY

Despite many years spent trying to develop approaches for the control of steroid abuse in food production and competitive sports, numerous obstacles still remain and research efforts therefore continue. When the appropriate sample collection and analytical procedures are employed, detection of the abuse of 'classical' synthetic steroids such as stanozolol in veterinary species is nowadays relatively straightforward. However, detection of the abuse of 'endogenous' (naturally occurring) and 'designer' (containing novel structures) steroids is not straightforward and many analytical challenges remain to be tackled.

Within the horseracing industry, analytical methods are already available to detect the abuse of the majority of endogenous anabolic-androgenic steroids (AAS). However, the same is not true for the majority of endogenous steroids in other food producing animals. 'Designer' steroids could in theory be abused in both horseracing and food production, but at present the majority of work on designer steroids has been commissioned by sports regulatory authorities. One reason that this class of compounds has received more attention in sports doping control is because of the proven use of designer steroids such as THG by a number of athletes. Another factor may relate to the fact that human and animal sports typically involve single individuals looking to gain marginal advantages for significant financial and/or sociological gain, whereas food production involves large herds of animals with smaller financial return relative to the risk. These differences in return relative to risk could, therefore, be considered to make the abuse of 'exotic' treatments such as designer steroids more likely in competitive sports compared to food production. In light of the aforementioned discussion, the aim of this thesis was to develop novel analytical approaches for the detection of 'endogenous' steroid abuse in food-production (part 1) and of 'designer' steroid abuse in animal sports (part 2). The primary focus of this work related to AASs such as nandrolone, boldenone, testosterone and their synthetic 'designer' analogues. However, the important natural steroids oestradiol and progesterone were also considered in relation to the detection of their abuse in the bovine (chapter 3) because it is not just AASs that may be abused in food production (unlike in competitive sports).

In part 1, chapter 1, the extensive literature regarding the presence and metabolism of endogenous AASs in food producing animals and the methods used to detect their

abuse was reviewed. The wide variation of the natural and post steroid administration metabolite profiles for the different compounds was highlighted in this chapter. It also reiterated the challenging nature of 'proving' the abuse of endogenous steroids, which revolves around the fact that a simple qualititative demonstration of the presence of these steroids is typically insufficient. Importantly, it was also evident that the more sensitive analytical methods become, the more steroids are discovered to be endogenous at low concentrations. Nandrolone, for example, was once thought to be solely exogenous, but is now know to occur naturally in a number of animal species. A wide range of different approaches to detection have therefore been proposed by authors, including threshold and biomarker approaches, the detection of exogenous steroid esters in plasma/hair and the use of GC-C-IRMS. During the preparation of this chapter and prior to the start of the analytical phase of this thesis, many international colleagues inside and outside of Europe were consulted for their opinions so that the author could make an informed decision on how to best tackle the challenges. It was obvious from these experiences that there is no single correct answer on how best to tackle the potential abuse of endogenous steroids. Much of the decision making process involves consideration of political, economic and other practical factors rather than just scientific ideals. In this respect, the use of the concentration threshold approach was considered most applicable at the current time to detection of abuse within the UK. The remainder of part 1 of this thesis therefore set out to develop analytical methods prior to applying them to large animal populations in order to produce data from which to derive possible thresholds.

In chapter 2, an analytical biomarker approach for the detection of nandrolone abuse in the porcine was developed and validated. The method was based on the quantification of the nandrolone metabolite 19-noretiocholanolone in the unconjugated (free) fraction of urine using analysis by GC-MS/MS. The lower and upper limits of quantification of the assay were 25 and 3,000 pg ml^{-1} respectively. The limit of detection was calculated as 13.2 pg ml^{-1}, which is significantly lower than previously reported methods. When applied to a population of untreated animals, 19-noretiocholanolone distributions in boars and gilt were bimodal, with a small number of concentrations in each sex at around the 1,000 pg ml^{-1} region and the majority of concentrations closer to the lower end of the calibration range. This was the first study to demonstrate that 19-noretiocholanolone is endogenous in the porcine and hence would benefit from a threshold approach. Statistical analysis of the data using a statistical method based on the Chebyshev inequality was carried out in

order to suggest screening and confirmatory thresholds for this steroid in the urine of boars and gilts. The adoption of particular thresholds, however, will be at the discretion of the individual regulating authorities. At a false non-compliance rate of 1 in 10,000 of the normal population (a standard number adopted within the horseracing industry and therefore extended to the food production area), the suggested confirmatory thresholds (7,502 pg ml^{-1} for boars and 19,200 pg ml^{-1} in gilts) are able to detect the abuse of nandrolone for several weeks following administration of this steroid.

In chapter 3, an analytical biomarker approach for the detection of nandrolone, boldenone, testosterone, progesterone and oestradiol was developed and validated. Using a single aliquot of urine, metabolite markers of the AASs and progestagens were extracted for analysis by GC-MS/MS analysis, while oestrogens were removed into a separate fraction using an extractive derivatisation with dansyl chloride and analysed using LC-MS/MS. Using a standard addition calibration line approach in pooled bovine urine, the method was found to be more sensitive for some steroids compared to previously published methods. Limits of detection for the different analytes ranged from 10.5 to 160.7 pg ml^{-1}, and the method was linear between the endogenous concentrations and those augmented with 3,000 pg ml^{-1}. With the possible exception of the boldenone metabolite 17β-hydroxy-5β-androst-1-en-3-one, all metabolites were shown to be endogenous at some concentration in both male and female bovine urine samples. The methods have since been applied to urine samples from a large population of untreated male and female animals in order to produce data for establishing screening and confirmatory concentration thresholds, the results of which will be published separately in due course.

In part 2, chapter 4, the metabolism of AASs in the equine and the methods used to detect their abuse was reviewed. The trends within the equine metabolism data were also compared to those of the human, porcine and bovine, highlighting some interesting differences between the species. This chapter exemplifies the need for performing metabolism studies in order that urinary based detection methods are targeted toward the most appropriate marker metabolites. One of the biggest perceived current threats in terms of AAS use in animal sports is the possible abuse of designer steroids, particularly those readily available for purchase from Internet sites. The remainder of part 2 therefore set out to develop *in vitro* methods for studying the metabolism of Internet available designer steroids in order to identify marker metabolites for inclusion in drug screening

procedures. An *in vitro* rather than *in vivo* approach was necessary because of the ethical constraints of administering previously untested substances to healthy animals.

In chapter 5, equine liver/lung microsomes and S9 tissue fractions were used to study the metabolism of the AAS stanozolol and the results correlated with those following *in vivo* administration of the steroid. The major aim of this study was to assess the feasibility of using *in vitro* techniques in place of animal administrations for conducting qualitative metabolism experiments. Using high resolution/accurate mass full scan analysis on an Orbitrap LC-MS system, equine liver microsome and S9 *in vitro* fractions were found to generate all the major phase-1 metabolites observed following *in vivo* administrations. Additionally, analysis of the liver microsomal incubates using a shallower LC gradient combined with various MS/MS functions on a Sciex 5500 Q trap instrument allowed the identification of a number of phase 1 metabolites previously unreported in the equine or any other species. Comparison between liver and lung S9 metabolism showed that the liver was the major site of metabolic activity in the equine. Furthermore, using chemical enzyme inhibitors that are known to be selective for particular isoforms in other species suggested that an enzyme related to CYP2C8 may be responsible the production of 16-hydroxy-stanozolol metabolites in the equine. Along with other studies conducted by the author correlating the *in vitro* and *in vivo* metabolites from a wide range of drugs in the equine, this chapter suggested that *in vitro* techniques could serve as a viable alternative to identifying suitable target metabolites when *in vivo* administrations are not possible.

In chapter 6, the *in vitro* methods developed in chapter 5 were used to study the metabolism of a novel designer steroid, namely estra-4,9-diene-3,17-dione, in the equine. The study was also conducted with canine and human tissue, which was the first time that the metabolism of a designer steroid in the three major species subject to sport's doping control was compared. In order to permit the retrospective analysis of sample testing data, the use of high resolution/accurate mass full scan analysis on an Orbitrap LC-MS system was employed for metabolite identification of underivatised sample extracts. The full scan Orbitrap data was complemented by several further experiments targeted at elucidating more detailed structural information for the most abundant metabolites. These included; MS/MS of the underivatised metabolites, functional group selective chemical derivatisation followed by full scan LC-MS, enzyme inhibition experiments and full scan electron ionisation GC-MS analysis of

methoxyamine-trimethylsilyl derivatives. The major metabolite detected in all species and, therefore, the most suitable candidate for screening of estra-4,9-diene-3,17-dione abuse was proposed to be an isomer of 17-hydroxy-estra-4,9-diene-3-one. Less significant metabolic pathways in all species included hydroxylation and reduction followed by hydroxylation. Reductive metabolism in the canine was less significant than in the other two species, while the equine was unique in producing a di-reduced metabolite (proposed to be an isomer of estra-4,9-diene-3,17-diol) and also relatively large quantities of D-ring hydroxy and hydroxy-reduced metabolites. The information regarding the equine target metabolites of estra-4,9-diene-3,17-dione has since been included in the routine drug screening methods used at the author's laboratory in order to detect any potential abuse of this steroid.

In conclusion, the work conducted in this thesis provides food residue and animal sports drug surveillance laboratories with some new analytical approaches to use in the fight against endogenous and designer steroid abuse in veterinary species. Work is ongoing in the author's laboratory (and in collaboration with other researchers around the world) to try and build on these approaches and to further enhance their applicability.

SAMENVATTING

Ondanks de jarenlange ontwikkelingen in de controle op steroïd misbruik in de voedselproductie en de competitie sport, blijven er nog vele obstakels over en dienen de onderzoeksinspanningen verder gezet. Wanneer de geschikte monstername en juiste analytische procedures worden gebruikt, is de detectie van "klassieke" synthetische steroïden zoals stanozolol in (nuts)dieren vandaag de dag vrij rechtlijnig. De detectie van het misbruik van "endogene" (natuurlijk voorkomende) en "designer" steroïden (steroïden met nieuwe structuren) is echter nog niet zo eenduidig en er blijven op dit punt nog verschillende analytische uitdagingen.

Binnen de wereld van het paardenrennen zijn reeds analytische methodes beschikbaar om het misbruik van de meeste endogene anabole-androgene steroïden (AAS) te detecteren. Dit is echter niet het geval voor de meeste endogene steroïden bij nutsdieren. "Designer" steroïden kunnen in theorie zowel worden misbruikt in paardenrennen als voedselproductie. Het meeste onderzoek op het gebied van "designer" steroïden gebeurt op dit ogenblik eerder in opdracht van de regelgevende instanties op sportgebied. In het licht van deze vaststellingen bestond het doel van deze thesis erin om nieuwe analytische benaderingen te ontwikkelen voor de detectie van het misbruik van "endogene" steroïden in de voedselproductie en van "designer" steroïd misbruik in de sport. Dit werk concentreert zich in eerste instantie op AAS's zoals nandrolone, boldenone, testosteron, stanozolol, trenbolone en hun analogen. Omdat niet alleen AASs kunnen worden misbruikt in voedselproductie (in tegenstelling met competitie sport) werden de belangrijke natuurlijke steroïden oestradiol en progesteron echter ook beschouwd in relatie tot het opsporen van hun misbruik bij het rund.

In hoofdstuk 1, wordt een overzicht gegeven van de uitvoerige literatuur die voorhanden is met betrekking tot de aanwezigheid en het metabolisme van endogene AAS's bij nutsdieren en van de methodes voor het opsporen van hun misbruik. De nadruk werd hierbij gelegd op het brede bereik aan metabole profielen van verschillende natuurlijke en exogeen toegediende steroïden . Een belangrijk leiddraad daarbij is het feit dat het kwalitatieve aantonen van de aanwezigheid van deze steroïden -op zich- onvoldoende is om het misbruik van endogene steroïden te bewijzen. Naarmate immers de analytische methodes gevoeliger werden, werd de endogene oorsprong van verschillende steroïden

bij lagere concentraties aangetoond. Nandrolone, bijvoorbeeld, werd vroeger als uitsluitend exogene component bestempeld. Nu is echter geweten dat dit steroïd natuurlijk voorkomt in een aantal species. Daarom wordt in de literatuur een brede waaier van verschillende benaderingen voor de detectie van hun misbruik voorgesteld: "drempelwaarde" en "biomerker" benaderingen, de detectie van exogene steroïd esters in plasma/haar en het gebruik van GC-C-IRMS. Voor de start van de analytische fase van deze thesis werden verschillende internationale collega's, binnen en buiten Europa geconsulteerd ter voorbereiding van dit onderzoek. De auteur probeerde, aan de hand van hun opinies, een gefundeerde beslissing te nemen hoe deze uitdaging best kon worden aangepakt. Vanuit deze ervaring werd besloten dat het probleem van het potentieel misbruik van endogene steroïden niet op één enkele maar op verschillende manieren diende aangepakt te worden. Bij een groot deel van het beslissingsproces waren immers naast wetenschappelijke, ook politieke, economische en andere praktische overwegingen betrokken. Het gebruik van een concentratiedrempel benadering wordt, vandaag de dag beschouwd als meest toepasbaar om het misbruik binnen het Verenigd Koninkrijk te bestrijden. In het vervolg van deze thesis werden daarom eerst analytische methodes ontwikkeld, alvorens deze toe te passen op grote dierpopulaties. Uit deze gegevens kunnen mogelijke drempelwaarden worden afgeleid.

In hoofdstuk 2 werd een analytische biomerker benadering voor de detectie van nandrolone misbruik bij het varken ontwikkeld en gevalideerd. De methode baseerde zich op de kwantificatie van het nandrolone metaboliet 19-noretiocholanolone in de ongeconjugeerde (vrije) fractie van urine door analyse met GC-MS/MS. De laagste en hoogste limieten van kwantificatie van deze bepaling waren respectievelijk 25 and 3,000 pg ml^{-1}. De detectielimiet werd berekend als 13.2 pg ml^{-1}, en is gevoelig lager dan bij vroeger gepubliceerde methodes. Wanneer deze methode werd toegepast op een populatie onbehandelde dieren, bleken de 19-noretiocholanolone distributies in beren en jonge zeugen (gelten) bimodiaal. Een klein aantal concentraties bij elke sexe bevinden zich rond de 1,000 pg ml^{-1}, terwijl de meerderheid van de concentraties dicht bij het laagste deel van het calibratie bereik lag. Dit was de eerste studie die aantoonde dat 19-noretiocholanolone endogeen is bij het varken en dat een "drempelwaarde" aanpak dus noodzakelijk is. Statistische analyse van de gegevens met een statistische methode gebaseerd op de Chebyshev ongelijkheid werd uitgevoerd om screenings- en confirmatie drempelwaarden voor te stellen voor dit steroïd in de urine van beren en jonge zeugen. Het gebruiken van deze bijzondere screening drempelwaarden wordt

overgelaten aan de individuele regelgevende autoriteiten. Met de voorgestelde drempelwaarden voor confirmatie (7,502 pg ml^{-1} voor beren en 19,200 pg ml^{-1} voor jonge zeugen) kan het misbruik van nandrolone worden gedetecteerd gedurende verschillende weken na de toediening van dit steroïd met een vals niet-conforme verhouding van 1 op 10,000 van de normale populatie (een standaardwaarde aangenomen binnen de paardenrennen en daarom uitgebreid naar de voedselproducerende sector).

In hoofdstuk 3, werd een analytische biomerker benadering voor de detectie van nandrolone, boldenone, testosteron, progesteron en oestradiol ontwikkeld en gevalideerd. Gebruik makend van een enkel monster urine werden de metaboliet merkers van de AAS's en de progestagenen geëxtraheerd voor analyse met GC-MS/MS. De oestrogenen werden uit dezelfde portie geëxtraheerd en na derivatisatie met dansylchloride tot een afzonderlijke fractie en geanalyseerd met LC-MS/MS. Door gebruik te maken van een standaardadditie calibratiecurve in een mengsel van runderurines, werden detectielimieten bereikt die varieerden tussen 10.5 tot 160.7 pg ml^{-1}. De methode was lineair tussen de endogene concentraties en deze waaraan 3,000 pg ml^{-1} werd toegevoegd. Met mogelijke uitzondering van het boldenone metaboliet 17β-hydroxy-5β-androst-1-en-3-one, werd aangetoond dat alle metabolieten endogeneen waren bij bepaalde concentraties en dit in urinestalen van zowel mannelijke als vrouwelijke dieren. De methodes werden nadien toegepast op urine monsters van een grote populatie onbehandelde mannelijke en vrouwelijke dieren. Dit liet toe concentratie drempelwaarden voor screening en confirmatie vast te leggen. Deze resultaten zullen ten gepaste tijde worden gepubliceerd.

In hoofdstuk 4, wordt een overzicht gegeven van het metabolisme van de AAS's bij het paard en van de methodes die worden gebruikt om hun misbruik op te sporen. De gegevens over het metabolisme bij het paard werden ook vergeleken met deze bij de mens, het varken en het rund. Daarbij vielen enkele belangrijke verschillen op tussen de verschillende species. Dit hoofdstuk benadrukt de noodzaak aan metabolisatie studies zodat de detectiemethodes zich kunnen toespitsen zijn op de meest geschikte merkermetabolieten (bij analyse van urine). Een van de belangrijkste recente gevaren bij gebruik van AAS in de sportwereld is het mogelijk misbruik van "designer" steroïden (in het bijzonder deze vlot verkrijgbaar via het Internet). In het laatste deel van dit hoofdstuk worden daarom *in vitro* testen opgezet om het metabolisme van, via het Internet beschikbare, "designer" steroïden te bestuderen. Op die manier kunnen merker

metabolieten worden geïdentificeerd die dan in de screening procedures kunnen worden inge-bouwd. Een *in vitro* in plaats van een *in vivo* benadering was noodzakelijk omwille van de ethische beperkingen voor het toedienen van deze "ongeteste" producten aan gezonde dieren.

In hoofdstuk 5, werden lever/long microsomen van het paard en S9 weefsel fracties gebruikt om het metabolisme van het AAS stanozolol te bestuderen. De resultaten werden gecorreleerd met deze uit volgend uit de *in vivo* toediening van het steroïd om de haalbaarheid van *in vitro* technieken in plaats van dierproeven te beoordelen. Door gebruik te maken van hoge resolutie/accurate massa "full scan" analyse op een Orbitrap LC-MS systeem, werd gevonden dat paarde-lever microsomen en S9 *in vitro* fracties dezelfde belangrijke fase I metabolieten genereren als bij *in vivo* toediening. Aanvullend werden lever microsomale incubaten geanalyseerd met een langere LC gradient, gecombineerd met verschillende MS/MS functies op een Sciex 5500 Q trap instrument. Dit liet de identificatie van een aantal nieuwe fase I metabolieten, nog niet beschreven bij het paard en andere species, toe. Vergelijking tussen lever en long S9 metabolisme toonde aan dat de lever de belangrijkste plaats was van metabole activiteit bij het paard. Er werd verder gebruik te maken van chemische enzyme inhibitoren, bekend voor hun selectiviteit voor particuliere isovormen in andere species. Daaruit volgde dat een enzyme, gerelateerd aan CYP2C8, verantwoordelijk kan zijn voor de vorming van 16-hydroxy-stanozolol metabolieten bij het paard. Ook in andere experimenten uitgevoerd door de auteur, werden *in vitro* en *in vivo* metabolieten van een brede waaier geneesmiddelen voor het paard vergeleken. Daaruit kon worden besloten dat *in vitro* technieken kunnen dienen als een geschikt alternatief om geschikte target metabolieten te identificeren wanneer *in vivo* toedieningen onmogelijk zijn.

In hoofdstuk 6, werden de *in vitro* methodes, ontwikkeld in hoofdstuk 5 gebruikt om het metabolisme van een nieuw "designer" steroïd, estra-4,9-diene-3,17-dione, bij het paard te bestuderen. De studie werd ook uitgevoerd met weefsel afkomstig van honden en mensen. Het was de eerste maal dat het metabolisme van een "designer steroïd" in de drie belangrijkste species voor de controle op sportdoping werd vergeleken. Om de retrospectieve analyse van de data van monsters toe te laten en zo de metabolieten in niet gederivatiseerde monsters te identificeren, werd gebruik gemaakt van hoge resolutie/accurate massa full scan analysis op een Orbitrap LC-MS system. De full scan Orbitrap data werden aangevuld met verschillende verdere experimenten om meer

gedetailleerde structurele informatie te bekomen van de meest voorkomende metabolieten. De gebruikte technieken waren: MS/MS van de ongederivatiseerd metabolieten, selectieve chemische derivatisatie van functionele groepen gevolgd door 'full scan' LC-MS, enzyme inhibitie experimenten en 'full scan' electronimpact ionisatie GC-MS analyse van methoxyamine-trimethylsilyl derivaten. Het belangrijkste gedetecteerde metaboliet in alle species is een isomeer van 17-hydroxy-estra-4,9-diene-3-one. Deze molecule is dan ook de meest geschikte kandidaat voor screening op het misbruik van estra-4,9-diene-3,17-dione. Minder significante metabo-lische wegen in alle species zijn hydroxylatie en reductie gevolgd door hydroxylatie. Het reductive metabolisme bij de hond was minder uitgesproken dan bij de twee andere species, terwijl het paard uniek was in het produceren van een di-gereduceerd metaboliet (een isomeer van estra-4,9-diene-3,17-diol) en ook relatief grote hoeveelheden van D-ring hydroxy- en hydroxy-gereduceerde metabolieten. De informatie betreffende "target" metabolieten van estra-4,9-diene-3,17-dione bij het paard is sinds dit onderzoek gebruikt in de routine drug screening van het laboratorium van de auteur om potentieel misbruik van dit steroïd te detecteren.

Als conclusie kan worden gesteld dat het onderzoek vervat in deze thesis de controle laboratoria op het gebied van residuen in voeding en sportdoping (bij dieren) voorziet van enkele nieuwe analytische benaderingen die kunnen worden gebruikt in de strijd tegen endogeen en "designer" steroïd misbruik bij nutsdieren. In het laboratorium van de auteur wordt -in samenwerking met andere wetenschappers van alle nationaliteiten- gewerkt om deze nieuwe benaderingen uit te testen en uit te bouwen om zo hun toepasbaarheid verder te verhogen.

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Nationality: British

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2009 - present - Principal Scientist - responsible for scientific developments within HFL's animal sports drug screening procedures. Also responsible for researching and developing new methods for detecting the abuse of protein therapeutics in horseracing.

2006 - 2009 - Senior Scientist – performed research on endogenous steroids for the control of their abuse in food production. Also acted as the company's scientific training coordinator.

2003 - 2006 - Senior Scientist - established a range of in vitro drug discovery ADME assays to offer as a service to pharmaceutical companies performing drug development.

2001 - 2003 - Senior Scientist - acted as a Study Director for pre-clinical and clinical bioanalytical and biomarker studies.

2000 - 2001 - Scientist - performed drug metabolism research to support the control of drug abuse in greyhound racing.

1999 - 2000 - Assistant Scientist – carried out research on natural steroids to support the control of steroid abuse in horseracing.

1997 - 1999 - Assistant Scientist - performed follow-up work arising from suspicious results from drug screening procedures in horse and greyhound racing.

National Institute of Agricultural Botany, Cambridge - 1996-1997

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Peer reviewed publications:

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