A review of designer anabolic steroids in equine sports

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Abstract:

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

Keywords:

horse; drug testing; anti-doping; anabolic steroid; designer steroid; metabolism

History of doping in sport

Among the earliest known examples of doping in sport come from the ancient Greek and Roman cultures which are known to have held sporting competition in high regard. Competitors devoted themselves to winning at any cost and were said to have consumed numerous substances as a part of the strict diet and exercise regimes that accompanied preparation for these competitions. Alcohol, hallucinogenic mushrooms, leaves and syrups derived from opium-containing plants, bull urine, and raw animal testicles were favourites of these competitors [1,2]. Animal sports were also quite common during this time and although records relating to the doping of animal competitors during this period are scarce, it is unlikely that they would not have been subject to the same types of treatment as their human-counterparts [3,4]. Among the first known instances of doping in equine sports comes from the fifteenth century where a mixture containing anise seed, honey and red arsenic sulphide (sandarac) was reportedly given to horses as a stimulant [3], and later from an English regulation reported in 1666 banning the use of "exciting substances" in competitive races [3,5]. The first well-documented cases of doping in equine sports however came to light during the nineteenth century and in response such substances were soon outlawed [3,6]. These bans were ineffective without a means to police them however, so this lead to the rapid development of saliva-based tests to detect alkaloids such as cocaine and heroin [4–7].

Reports of anabolic steroid misuse in equine sports date back to 1941, where an eighteen-year-old standardbred US trotter named Holloway was treated with testosterone (17β-hydroxyandrost-4-en-3-one) for several months during training, and as a result reportedly regained much of his former racing ability [8]. More recently, the trainer of the US champion thoroughbred Big Brown admitted to injecting his horses with the synthetic anabolic steroid stanozolol ([1',2']-1'*H*-pyrazolo[4',5':2,3]-17α-methyl-5α-androstan-17β-ol) during the 2008 Kentucky Derby, although stanozolol was legal in US racing at the time [8]. Big Brown won the first two races of the series under the effects of stanozolol; however, the horse failed to perform as expected in the third race where he was run steroid-free. To combat the growing problem of doping in equine sports, the Horseracing Authorities of the United States of America, France, Great Britain and Ireland jointly decided in 1961 to coordinate their resources to better manage the future of the industry, as equine sports have traditionally been governed by the local authorities in each jurisdiction [9]. In 1993 the International Federation of Horseracing Authorities (IFHA) was formed and included over 60 members. They meet annually to update the International Agreement on Breeding, Racing and Wagering, first endorsed in 1974, which outlines the recommended best practice for all jurisdictions, including protocols for how to effectively address the problem of doping in equine sports [10].

Anabolic androgenic steroids

Steroids are a class of chemical compounds characterised by their tetracyclic fused ring system consisting of three cyclohexane rings (Rings A, B and C) and a cyclopentane ring (Ring D), conferring chemical stability and conformational rigidity to the molecule. Furthermore, the rings can be modified at many possible positions, and also occur as a variety of stereochemistries and degrees of unsaturation, and they occur widely in nature as lipids, hormones and other natural products [11]. The structure of anabolic steroids is typified in the example of 17α-methyltestosterone (17β-hydroxy-17α-methylandrost-4-en-3-one) as shown in Figure 1.

Figure 1

Steroid compounds can be categorised into number of subclasses, typically based on the number of carbon atoms present in their core structure. The most interesting classes in the anti-doping context include; cholestanes (C27), which are typified by cholesterol, that are the steroid precursors from which most others are ultimately derived; cholanes (C24), which primarily consist of the steroid bile acids; pregnanes (C21), which consist of the progestogens and the corticosteroids; androstanes (C19), which form the basic framework of most androgens; and estranes (C18), which form the basic framework of some androgens, and the estrogens [11,12]. Representative examples of each steroid class are shown in Figure 2.

Figure 2

Based on the World Anti-Doping Agency (WADA) detection statistics from 2014, anabolic androgenic steroids (AAS) accounted for 48% of all adverse analytical findings of banned substances in competing athletes [13]. With the wide range of performance-enhancing drugs available to athletes today, it is interesting that a significant proportion that chose to dope used steroids, which suggests that they offer benefits that other types of compounds do not. Unlike other classes of drugs, such as stimulants, which usually offer benefits only for as long as they remain active in the body, the effects of anabolic steroids can be long-lasting. Whilst the compounds themselves may no longer be present in the body, athletes can use these compounds to train; gaining muscle mass which will be retained for a period after steroid use is discontinued. Thus athletes retain the majority of the benefits without putting themselves at risk of being caught during in-competition screening. This provides the motivation for authorities to undertake out-of-competition screening, and AAS are banned at all times by WADA in competing athletes.

Anabolic steroids in equine sports

In contrast to the well-documented advantages that anabolic steroids offer to human athletes, the effects

of anabolic steroids in equine competitors are not well-established [14]. A study has shown that short-term nandrolone administration increased the glycogen content of post-exercise muscle tissue [15], however this is in contrast with two studies that found no observable changes resulting from nandrolone administration [16,17]. It has been shown that steroid administration delayed closure of epiphyseal growth plates in standardbreds, and suggested that this could lead to a potential increase to the long-term risks of injury during training [14]. Additionally, it has been suggested that testosterone may not be involved in muscle development or maintenance in horses, as horses that experienced weight loss from maladaptation to training were observed to have similar testosterone levels compared to a control group [18]. Aggressive behavioural changes resulting from anabolic steroid administrations have been noted [14,16] which could lead to more competitive horses that perform better in training and competition, however they may also lead to injury and accidents with other horses, riders or trainers. Nonetheless, even if the evidence for anabolic steroids acting as performance-enhancing substances in horses is currently unclear, there is clear evidence that anabolic steroid misuse can result in serious animal health and welfare consequences [14]. These concerns, as well as the overwhelming evidence of the effects of anabolic steroids (including designer steroids) in other species (including humans) more than warrant their banning in competitive equine sports by IFHA [10].

Designer anabolic steroids

Since the first synthesis of testosterone over 80 years ago [19], there has been substantial work aiming to produce steroid compounds with differing and useful biological properties. During the so-called "golden age" of steroid research during the 1950-60s, numerous potent analogues of testosterone were synthesised some of which were published [20–24] whilst others were patented. The majority of these compounds however were only briefly tested for their anabolic activity and were never subsequently evaluated through clinical testing or approved for sale. As a result, they were forgotten, and then subsequently rediscovered in the past decade where they have been exploited by chemists who would seek to use the knowledge in the older steroid literature and bring these compounds to market in a clandestine fashion.

Figure 3

The term "designer steroid" can be defined as any anabolic steroid which has been prepared to intentionally evade detection by anti-doping laboratories. This typically involves chemical modification to the steroid core. The term itself was first coined in 2002 where Catlin *et al.* reported the first instance of a

designer anabolic steroid compound detected in an athlete's urine [25]. This designer steroid was norbolethone (18β-homo-17β-hydroxy-19-nor-17α-pregn-4-en-3-one), a synthetic anabolic steroid compound first synthesised in 1966 and subsequently found in clinical studies to be a highly potent anabolic agent [25,26], but which had never been approved for clinical use. Two years later, the same group detected tetrahydrogestrinone (18β-homo-17β-hydroxy-19-nor-17α-pregna-4,9,11-trien-3-one; THG) another designer steroid, following analysis of a spent syringe containing an allegedly undetectable anabolic steroid which was provided anonymously to the United States Anti-Doping Agency (USADA) by a former sporting coach [27–29]. A third designer steroid, madol (17α-methyl-5α-androst-2-en-17β-ol; desoxymethyltestosterone), was also detected in a crude oily preparation received by the laboratory in 2005 [30]. A transdermal preparation called "The Cream" was also identified, which contained a mixture of testosterone and epitestosterone in a controlled ratio. At this time the testosterone-epitestosterone ratio (T/E ratio) was an important marker used within WADA laboratories to identify samples suspected of doping with testosterone, and application of "The Cream" provided an increase in testosterone levels without altering the T/E ratio, making it harder to detect [31]. These preparations were subsequently found in an investigation by the United States Federal Government to have been distributed by the Bay Area Laboratory Co-Operative (BALCO) alongside other anabolic steroids and performance enhancing substances in a secret program that supplied elite athletes with "undetectable drugs" to provide a competitive advantage [31]. Although BALCO is now defunct, its legacy has changed the way anti-doping laboratories must approach the problem of anabolic steroid abuse in the world today.

Despite an increase in awareness and research to combat designer steroid misuse, their usage is becoming more widespread, in part due to the ease in which these compounds can be obtained. These compounds are typically present in "dietary" or "nutritional" supplements which are widely marketed online and often contain unusual structures and substitution patterns that may render them more difficult to detect by existing analytical methods. These supplements are also often discontinued and replaced by new products when they become detectable by anti-doping laboratories or law enforcement. These structural changes to the core steroid structure also help suppliers evade legal restrictions and penalties regarding their manufacture and sale in some jurisdictions [32,33]. As a result, these designer anabolic steroids pose a number of problems. They are often prepared in a clandestine fashion and as a result there is often minimal data available detailing the purity, safety or efficacy of these products. Users are forced to rely on advice from other users reporting their outcomes in online forums, or as is common, to experiment on themselves. This environment also encourages underreporting of side-effects and health complications, often with serious consequences [34]. This is compounded by another major problem in that the labelling detailing the contents of these products is often falsified or omitted in an attempt to circumvent their control by law-enforcement [32,33]. This can hinder the work of health practitioners who are required to treat any complications that may

arise from supplement usage [34], and also prevent customs or border authorities from identifying potentially dangerous or illicit materials that may be entering their jurisdiction. With their widespread use by human athletes, it is highly likely that the problem of designer steroid misuse will find its way into the realm of equine sports as well.

Equine steroid metabolism

An understanding of the metabolism of anabolic steroid compounds is essential to develop methods for the detection of these compounds in equine sports. Anti-doping analysis typically requires the detection of known steroid markers and metabolites, as the parent compounds are often rapidly metabolised after administration. As such these metabolites form the basis of the majority of anti-doping screening protocols currently in use today. These metabolites are typically confirmed through the use of reference materials, which are pure compounds used as a positive control in analytical methods to confirm instances of anabolic steroid misuse. The identification of metabolites with reference materials involves comparison of the chromatographic retention time, mass spectrometry or other behaviour of the reference material to the identified metabolite, through a set of standardised criteria [35]. A limitation of this approach however lies in the need for knowledge of the metabolite structure in order to provide a suitable material for comparison. This is particularly important in the case of designer steroids where the structures of the metabolites may not be known, or the reference materials may not be available.

Steroid metabolites have historically been identified from urine, due to the relative ease of which samples can be obtained from competing animals, as well as the higher concentrations of drugs and drug metabolites that may be present. In recent times, blood samples have become another valuable biological matrix to detect steroid misuse, although this is less common due to the invasive sample collection required. Hair [36–38], faeces [39], and saliva [7] can also be used for the detection of drug compounds, and these matrices find use in anti-doping laboratories in some jurisdictions.

Steroid metabolism typically proceeds by two complementary pathways, named phase I and phase II metabolism. By definition these are two distinct metabolic pathways, and although they commonly occur in concert, they are known to occur independently as well. Phase I metabolic processes typically involve functional group manipulations such as hydrolysis, oxidation, reduction, or hydroxylation. Metabolism primarily occurs on the steroid A and D-rings, although if these positions are inaccessible (as is often the case in designer steroids) then the B- and C-rings can be metabolised [39,40]. Of particular interest to the equine metabolism of anabolic steroid compounds is the tendency for C3-ketone reduction, and C16-hydroxylation to occur, particularly if the C17-position is alkylated [39]. Additionally, A-ring metabolism is

commonly inhibited through extended conjugation as is seen in the case of the steroid trenbolone (17β-hydroxyestra-4,9,11-trien-3-one) which produces multiple hydroxylated metabolites [39]. The phase I metabolism of these compounds can also have an effect on their biological activity, as it has been reported in androgen bioassay studies that the metabolism of anabolic steroids can either activate or deactivate the steroid molecule [33,41].

Phase II metabolism involves the conjugation of highly polar groups to the steroid metabolites. The conjugation of small, charged or highly polar compounds to the hydrophobic steroid backbone confers an increase in the aqueous solubility of these compounds, allowing for their rapid and efficient excretion *via* the urine. The two main phase II steroid conjugation pathways are glucuronylation and sulfation; steroid glucuronide metabolites form *via* enzyme-mediated transfer of glucuronic acid from a uridine diphosphate (UDP) glucuronic acid donor, while steroid sulfate metabolites form *via* enzyme-mediated transfer of sulfate from a 3'-phosphoadenosine-5'-phosphosulfate (PAPS) donor [40]. Minor conjugates of other small molecules are also known including phosphate, sugars, and amino acids [42,43], although they are considered minor components in anti-doping analysis and are not routinely investigated. Although phase II conjugates are the primary components identified from urine, unconjugated metabolites can also be observed. Conjugation can also occur in the absence of phase I metabolism if metabolism is hindered, or if the compound already possesses suitable functionality [40].

In vitro equine steroid metabolism

Since anti-doping analysis requires knowledge of the metabolites that indicate steroid misuse, the metabolism of each steroid must be studied in order to determine which metabolites(s) may be the most suitable markers for detection. *In vivo* administration is a staple method for the study of steroid metabolism, however *in vitro* methods are gaining popularity [39]. Additionally, the Association of Official Racing Chemists (AORC) criteria currently allow for the use of *in vitro*-derived materials as standards for confirmatory analysis [35], providing additional motivation for developing *in vitro* systems to model equine steroid metabolism. The key concern regarding the use of *in vitro* methods is how accurately they reflect the metabolism observed *in vivo* and for this reason they typically accompany administration studies to allow comparison between *in vivo* and *in vitro* systems.

In order to model the liver, which is the principle organ involved in detoxification and metabolism of exogenous substances, preparations involving liver metabolic enzymes are amongst the most commonly

used *in vitro* methods [29,44–50]. Equine liver microsomes and S9 fraction are most commonly used to model equine metabolism due their ease of use and commercial availability. These enzymes are typically supplemented with a number of biological co-factors, including nicotinamide adenine dinucleotide (NADH), or nicotinamide adenine dinucleotide phosphate (NADPH) in order to promote the metabolic reactions. Most systems use an excess of these reagents [44,48], although systems have been developed which employ co-factor regeneration, in which a NADH/NADPH-generating reaction is coupled to the NADH/NADPH-dependant metabolic reactions. Typically, the reaction of glucose-6-phosphate with a glucose-6-phosphate dehydrogenase (G6PDH) enzyme is used, which regenerates NADH/NADPH as a by-product of oxidation. This allows for the use of a catalytic amount of these co-factors in the metabolism reaction [29,45,46,51]. Recent reports have also demonstrated the practicality of using homogenised whole liver tissue to perform *in vitro* studies, in an effort to closely replicate *in vivo* metabolism [51–53]. These studies have even demonstrated the ability to generate phase II metabolites without the use of expensive phase II co-factors such as UDP-glucuronic acid or PAPS [53]. The limitations of this approach are reflected in the sample preparation of the whole liver extracts, as well as potential variations in the metabolic profile due to the use of individual tissue donors. Other approaches to model equine metabolism involve microbial systems which are typically based on equine faecal bacteria, and although these are currently largely unimportant for modelling equine steroid metabolism in an anti-doping context, they may be important in other areas [54–58].

Anti-doping screening for designer anabolic steroids

Historically, steroid metabolites have been detected in analytical samples by thin layer chromatography coupled to ultra-violet detection (TLC-UV) [59]. This was largely superseded by the development of high performance liquid chromatography-ultraviolet detection (HPLC-UV) which was popular until the 1980-1990's, although it still finds use in specialised anti-doping applications [39]. With the development of gas-chromatography coupled to mass spectrometry (GC-MS), these instruments became the method of choice in anti-doping laboratories [8]. A range of other techniques have become available and occasionally find use in anti-doping laboratories alongside existing methods. Biological assays [8,60–65] can be used to detect doping directly from samples. For example, if a sample gave a positive result from an androgen receptor assay or immunoassay, it may indicate that anabolic agent(s) are present in the sample which would warrant further testing. In most cases, these bioassay techniques can be sufficiently general to allow for detection of certain steroid classes or highly specific for certain steroid compounds, although they often do not provide an opportunity to confirm the identity of any detected compounds [62]. Building on this idea, metabolomics approaches are also gaining popularity as they allow for the high-throughput detection of minor variations of a very large number of biomarkers in response to drug administration [66], making it extremely difficult to hide instances of steroid misuse. Nuclear magnetic resonance spectroscopy (NMR) occasionally finds use in anti-doping laboratories as it can be used to unequivocally determine metabolite structure, however is

severely limited in most cases as it requires extensive sample purification and large sample volumes to be effective [39].

Whilst GC-MS has been the mainstay for analytical laboratories, in recent years there has been a movement towards liquid-chromatography coupled to mass spectrometry (LC-MS) which offers several advantages over GC-MS analysis [8]. GC-MS requires often laborious sample preparation including purification, hydrolysis of the phase II urinary conjugates, and chemical derivatisation to a more volatile species prior to analysis. Trimethylsilyl (TMS) ethers or other silyl derivatives are among the derivatives most widely used in laboratories due to their stability, ease of preparation, and characteristic fragmentation patterns [67]. In contrast, LC-MS analysis has the advantage of not requiring these preparatory steps allowing much higher throughput in sample analysis. Additionally, LC-MS allows the direct detection of intact phase II conjugates, which may offer advantages in the detection of some steroid compounds which are primarily excreted as these conjugates and that may otherwise only be detected indirectly by GC-MS methods after hydrolysis and derivatisation [8,46,68]. LC-MS is not a complete replacement for GC-MS however, as the analysis of some compounds is difficult with LC-MS, including the study of saturated steroids and steroid diols, which ionise poorly under the electrospray ionisation (ESI) and atmospheric-pressure chemical ionisation (APCI) conditions common to LC-MS systems [8,39]. Additionally, the characteristic fragmentation patterns observed using electron ionisation (EI) common to GC-MS systems may often provide more diagnostic information about metabolite structure compared to the softer ionisation techniques common to LC-MS [8]. However, the high energy used in EI methods which result in these characteristic fragments may also result in reduced sensitivity as the parent structure is heavily fragmented. Regardless, both analytical methods afford their own distinct advantages and anti-doping laboratories should make use of the strengths of both these techniques for the efficient screening of AAS misuse.

Recent advances in modern instrumentation which allow tandem MS experiments (MS^n) afford a greater ability to detect and identify metabolites. This is due to the ability of these systems to perform a multitude of scan types including: full scan MS, multiple reaction monitoring (MRM), product-ion or precursor-ion MS, or neutral loss experiments. This is in contrast to older technologies which often rely on full-scan and single-ion monitoring (SIM) techniques in order to achieve the required sensitivity for detection. Additionally, the recent development of affordable liquid-chromatography high resolution accurate mass spectrometry (LC-HRAM) technologies has greatly assisted anti-doping laboratories. Data obtained with these systems is of very high quality and at sufficient mass resolution that minute differences in molecular composition can be detected [69]. The increase in specificity is afforded through the use of a much narrower mass window (±10-15 ppm) for mass detection compared to standard triple quadrupole detection (±1 Da).

As a result, this can allow laboratories to undertake retrospective analysis of newly-identified compounds in historically acquired data, allowing detection of previously unidentified compounds.

The most recent advances in anti-doping research come from new developments in "untargeted" or "open" screening methods. Such methods typically attempt to screen for characteristic fragments, or fragmentation modes (such as characteristic neutral losses) of steroid compounds, rather than the steroid compound itself [70–73]. This would prevent minor structural changes to a molecule which results in a change of the molecular mass from rendering a compound undetectable, and highlight the need for follow-up testing to identify the new target compound. Another promising advancement is the Equine Biological Passport (EBP) [74], which mirrors the Athlete Biological Passport (ABP) maintained by WADA for human athletes [75]. The EBP would longitudinally monitor the concentration of certain biological compounds in the horse, and allow for the detection of changes in these levels in response to anabolic steroid misuse. This would require frequent testing to establish the steroid profile of the individual animal, and future instances of steroid misuse could be detected through changes to the established profile. Such an approach would assist greatly in establishing relevant threshold levels for endogenous compounds, as well as assisting in the detection of designer anabolic steroids. Although there are a number of technical and administrative hurdles to overcome before this can be fully realised, this approach has the potential to effectively combat the misuse of drugs in equine sports. Such methods are likely to be essential to combat the rapid increase of designer steroids, and other unknown compounds into the future.

Equine metabolism of designer steroids

The metabolism of all anabolic androgenic steroid compounds in horses is far beyond the scope of this review, however a substantial summary can be found in several reviews by Scarth *et al.* [76] (endogenous anabolic steroids in horses and other animals), Teale and Houghton [77] and Houghton *et al.* [78] (phase I and II metabolism studies of some common synthetic steroids marketed as pharmaceuticals), and Scarth *et al.* [39] (a comprehensive review of drug metabolism in horses). Instead the purpose of this review is to explore recent advances in the study of designer anabolic steroids as well as other anabolic steroids present in many of the dietary supplements available online, which have not been covered in previous reviews. A summary of the metabolism of these compounds in equine systems in presented below. The designer steroids desoxyvinyltestosterone [79], and estra-4,9-diene-3,17-dione [80] have been studied in equine systems as reviewed by Scarth *et al.* [39], and are included only briefly in this work.

Table 1: Summary of the equine metabolism of designer steroids

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

Figure 4

In humans, 11-adrenosterone is an endogenous steroid produced predominately in the adrenal cortex that exerts a mild anabolic effect [81]. It has been reported as a component of dietary supplements such as 11-OXO (ErgoPharm) [81] and is marketed as a "selective cortisol modulator" rather than as an anabolic agent. It is a known inhibitor of the enzyme 11β-hydroxysteroid dehydrogenase type 1, which is required for the formation of cortisol from cortisone [81]. Inhibition of cortisol biosynthesis may offer athletes a competitive advantage as cortisol itself is involved in a range of biological processes including fat and protein metabolism, regulation of the immune system, and responding to stress [81–83]. The endogenous concentrations of this compound in the horse is currently unknown, however based on a previously reported human *in vivo* administration [81] it has been suggested that a 11β-hydroxyandrosterone threshold of greater than 10 µg/mL, or a 11β-hydroxyandrosterone:11β-hydroxyetiocholanolone ratio of greater than 20:1, or the use of GC/IRMS may be indicative of 11-adrenosterone misuse [44].

The equine metabolism of 11-adrenosterone has only been studied by *in vitro* methods [44]. Following metabolism with equine liver microsomes and S9 fraction, one major reduced metabolite was observed by LC-MS/MS. Minor metabolites were also observed including: one minor reduced metabolite, one direduced metabolite, one trireduced metabolite, two hydroxylated metabolites, and one reduced and hydroxylated metabolite. Metabolites were tentatively assigned structures based on analysis of their LC-HRAM product-ion spectra. An additional major reduced metabolite was observed by GC-MS/MS. Although the endogenous nature of 11-adrenosterone in humans has been established, it is currently unclear if this compound is endogenous in horses. Older studies have proposed 11-adrenosterone as a metabolite of cortisol in the synovial fluid of human and equine knee joints after administration of cortisol [84], however no studies have identified it directly. Due to its potential endogenous nature, a threshold approach may be required in order to confirm misuse of this compound.

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

Figure 5

ATD is an aromatase inhibitor that has been reported as a component of dietary supplements including Attitude (SAN Nutrition) [85] and Novedex XT (Gaspari Nutrition) [2,86]. Aromatase inhibitors irreversibly and covalently bind to the active site of the P450 enzyme aromatase, which is essential in the conversion of androgens into estrogens *in vivo* [87,45]. Inhibition of this enzyme can be used to limit the endogenous

conversion of androgens such as testosterone into estrogens, in turn increasing the concentration of AAS in the body. Although aromatase inhibitors have legitimate therapeutic applications such as in the treatment of human breast and ovarian cancers [45,88], they can be exploited to gain muscle mass as they can increase the effects of endogenous or co-administered anabolic steroids. They can also potentially alleviate some of the side effects of anabolic steroid misuse [45,89].

The equine metabolism of ATD has been studied by *in vitro* methods [44]. Following metabolism with equine liver microsomes and S9 fraction, two reduced metabolites (tentatively assigned as C17-isomers), and one reduced and hydroxylated metabolite (tentatively assigned as C15, or C16-hydroxylated) were identified. Minor metabolites including three direduced metabolites, three hydroxylated metabolites, five additional reduced and hydroxylated metabolites, and three hydroxylated and direduced metabolites were also observed. The structures of the major metabolites were tentatively assigned based on their LC-HRAM product-ion spectra. In addition, one of the direduced metabolites was identified as boldenone (17β-hydroxyandrosta-1,4-dien-3-one) by comparison to a reference material. The authors recommend analysis by LC-MS over GC-MS due to the detection of several C19-nor steroid artefacts that resulted from TMS derivatisation prior to GC-MS analysis [44,90], and also due to the higher sensitivity of detection by LC-MS.

The metabolism of ATD has also been studied *in vivo* by a controlled oral administration (800 mg, 2 thoroughbred geldings) [51]. Following phase I metabolism, fourteen metabolites were identified by LC-HRAM analysis including: three reduced metabolites (two C17-reduced and C1-C2 reduced), three direduced metabolites (C1-C2 and C17-direduced, C1-C2 and C3-direduced, and boldenone), four reduced and hydroxylated metabolites, and four direduced and hydroxylated metabolites (two C5-C6 and C17 direduced with C16-hydroxylation, and two C1-C2 and C17-direduced and hydroxylated metabolites). A number of these were matched to reference materials. The identities of the phase I metabolites not matched to standards were tentatively assigned by analysis of the LC-HRAM product-ion spectra. The position of hydroxylation in these metabolites was not assigned where standards were not available, although the authors comment on the presence of MS fragments at *m/z* 149 and 167 being characteristic of D-ring hydroxylation which suggests C16-hydroxylation as a major pathway in the metabolism of these compounds [39,51]. A pair of metabolites resulting from C1-C2 and C17-direduction and hydroxylation were observed that had not been previously detected in human studies [86]. Elevated levels of testosterone were also observed which were above the threshold levels required for a positive testosterone doping result.

Phase II metabolites were observed directly as a mixture of sulfate and glucuronide conjugates, and a minority were identified by comparison to the products generated from *in vitro* metabolism with homogenised horse liver [35]. Phase II metabolites were also identified indirectly by hydrolysis of the fractionated glucuronide and sulfate metabolites. Parent ATD was excreted primarily unconjugated, whilst one C17-reduced metabolite was identified as the sulfate conjugate, and the remaining C17-reduced metabolite as the glucuronide conjugate. The authors comment that the C17-glucuronide is likely to be the C17α-stereochemistry, which agrees with observations reported by many as summarised by Scarth *et al.* [39]. Sulfate conjugates were also identified for boldenone, and for the C1-C2 and C17-direduced metabolite which were tentatively assigned C17β-stereochemistry. Glucuronide conjugates were observed for the C1-C2 and C3-direduced, and both two C5-C6 and C17 direduced and hydroxylated metabolites. The remaining metabolites were observed as mixtures of both sulfate and glucuronide conjugates. Some metabolites were identified up to 77 hr post-administration, and the authors recommend 17β-hydroxyandrosta-1,4,6-trien-3-one, and 17β-hydroxyandrosta-4,6-dien-3-one as potential target analytes for screening due to their long detection windows, and commercial availability.

As a part of this study, a comparative phase I *in vitro* metabolism was performed using homogenised equine liver. Twelve of the metabolites observed *in vivo* were identified *in vitro* in addition to both boldione (androsta-1,4-diene-3,17-dione) and epiboldenone (17α-hydroxyandrosta-1,4-dien-3-one) which were identified by comparison to reference materials. Interestingly, elevated levels of testosterone were not observed *in vitro* suggesting that testosterone is not a direct metabolite of ATD but rather a result of aromatase inhibition. These results, along with the *in vivo* study agree well with the previously discussed *in vitro* study reported by Clarke *et al.* [44].

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

Figure 6

3α-Chloro-17α-methyl-5α-androstan-17β-ol is an anabolic steroid containing C3-chlorination, which was identified alongside the 3β-chloro isomer (5:2 mixture) in red-and-black capsules containing white powder seized in 2012 by law-enforcement in Queensland, Australia [29]. This compound had not been previously reported in the literature, and it appears to be the first instance of a C3-halogenated anabolic steroid intended for doping purposes.

Owing to concerns about its chemical reactivity and potential toxicity, this compound was only studied using *in vitro* systems [29]. Yeast, HEK293 and HuH7 androgen receptor bioassays have found the potency of the major 3α-chloro isomer to be similar to testosterone (87-147% potency), whilst the 3β-isomer gave much lower potency (2-9%). Acute cellular toxicity was not observed in yeast and HEK293 cell lines. *In vitro* metabolism studies on the 3α-isomer using human and equine liver S9 fraction identified differences in metabolism which may be useful for doping control. Equine *in vitro* metabolism afforded 3α-chloro-17α-methyl-5α-androstane-16α,17β-diol as the sole observed metabolite, the structure of which was matched against synthetic reference material. The relative abundance isotope pattern of ^{35}Cl and ^{37}Cl (3:1) confirmed retention of C3-chlorination in the equine metabolite. The stereochemistry of the C16-hydroxylation was supported by ^{1}H NMR, and the failure to form a C16-C17 cis-acetonide derivative, with the C16α-C17α-isomer used as a control to demonstrate the efficiency of the transformation. Human *in vitro* metabolism afforded 17α-methyl-5α-androstane-3α,17β-diol, lacking C3-chlorination, which was not observed in the equine system. This metabolite was confirmed by comparison to both the 3α-hydroxy and 3β-hydroxy reference materials, and is a known metabolite of a number of other methylated anabolic steroid compounds such as methyltestosterone, and mestanolone (17β-hydroxy-17α-methylandrostan-3-one) [39]. As such this metabolite is likely detectable by existing methods. The authors recommend laboratories monitor for the 3α-chloro-17α-methyl-5α-androstane-16α,17β-diol, and 17α-methyl-5α-androstane-3α,17β-diol metabolites in routine screening to detect the misuse of this compound.

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

Figure 7

Halodrol is a 4-chlorinated steroid structurally similar to clostebol (4-chloro-17β-hydroxyandrost-4-ene-3-one), and turinabol (4-chloro-17β-hydroxy-17α-methylandrosta-1,4-diene-3-one) [39,44] which has been found in a variety of dietary supplements such as Halodrol (Gaspari Nutrition), Zeus (BioArmor) and Iron Dragon (BioArmor). These supplements typically list only the 3β-hydroxy isomer on their labelling but usually contain a mixture of both stereoisomers.

The equine metabolism of this compound has only been studied using *in* vitro systems [44]. A mixture of halodrol isomers (3:2, α:β) was subjected to phase I metabolism using both equine liver microsomes and S9 fraction. Metabolism identified the C3-oxidised metabolite (turinabol) and three oxidised and hydroxylated metabolites as major metabolites. Minor metabolites were observed including, two A-ring reduced and C3-oxidised metabolites, one hydroxylated metabolite, one A-ring reduced and hydroxylated metabolite, two

additional oxidised and hydroxylated metabolites, one dihydroxylated metabolite, one reduced and dihydroxylated metabolite, one direduced and dihydroxylated metabolite, and two oxidised and dihydroxylated metabolites. Metabolites were tentatively assigned based on their LC-HRAM, or GC-MS/MS product-ion spectra. The parent compounds (3α/β,17-diols), and the major oxidised and hydroxylated metabolites were reported to ionise poorly under APCI conditions which are commonly used for LC-MS analysis, however ionised well under GC-MS (+EI) conditions after TMS derivatisation. There were minor differences reported between the two metabolism systems used, with equine liver S9 appearing to give more of the major metabolites, which the author suggested may highlight the importance of cytosolic enzymes in the metabolism of these compounds. The predominate product was the C3-oxidised metabolite, which corresponds to the synthetic anabolic steroid turinabol, which has been previously studied *in vivo* in the horse [39,91]. A number of the key metabolites of halodrol reported in this study match those reported for the *in vivo* metabolism of turinabol. As such, the authors recommend that monitoring for turinabol misuse would likely be suitable for the detection halodrol misuse.

20-Hydroxyecdysone (2β,3β,14α,20β,22α,25-hexahydroxy-5β-cholest-7-en-6-one)

Figure 8

20-Hydroxyecdysone is an ecdysteroid hormone which is present naturally in numerous invertebrate and plant species. It is essential for moulting and reproduction in many arthropod species, and is also present as an insecticide in some plant species where it disrupts the development of insect pests that would feed upon them [92]. Reports in the older steroid literature have suggested that ecdysteroids may exert a small anabolic effect in several mammal species [92], although more recent studies offer conflicting reports of their anabolic effects [93,94]. Nonetheless, 20-hydroxyecdysone has been found in dietary supplements such as Oxybolin 250 (High-Tech Pharmaceuticals) and Ecdy-Bolin (Truly Huge Supplements). These supplements are often marketed as “natural”, “plant-based”, or “low-testosterone” alternatives to other anabolic steroid-containing body-building supplements. Whilst it is unclear whether supplements containing 20-hydroxyecdysone would offer a competitive advantage, they are currently banned in competition IFHA [10].

The equine metabolism of 20-hydroxyecdysone has only been studied by *in vitro* methods [44]. Only the parent compound was observed after incubation with equine liver microsomes and S9 fraction. GC-MS/MS analysis afforded a complex mixture of partial derivatisation products and no characteristic fragmentation information, likely due to the multiple potential sites for silylation. It is known that complete silylation of related ecdysteroid compounds is slow, due to the hindered tertiary hydroxyl groups C14 and C20, with

optimal silylation occurring only after extended reaction times [95]. Related ecdysteroid compounds are known to be rapidly excreted with only minor metabolic changes in humans [92,95] which could rationalise the lack of phase I metabolism observed in this study. The authors also suggest that phase I metabolism was expected to be a minor pathway compared to the phase II metabolism that would predominate *in vivo* and was not investigated as a part of this study. They also raise concerns over potential accidental dietary consumption through animal feed, as ecdysteroid compounds are known to be present in many plant species [92] , potentially requiring a threshold approach for detection. The authors also observed minor levels of desoxy-, dehydro-, and hydroxy-metabolite impurities in their control *in vitro* incubations, suggesting the presence of these minor components in the commercial 20-hydroxyecdysone preparation. This could call into question other studies which identify these as metabolites [95,96]. The authors recommend monitoring for the unconjugated parent compound, or its likely phase II conjugates for the detection of 20-hydroxyecdysone misuse.

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

Figure 9

In human medical practice, formestane is a pharmaceutical aromatase inhibitor which irreversibly and covalently binds to the active site of the P450 enzyme aromatase [45]. After an adverse analytical finding in 2011, the endogenous nature of formestane in horses was investigated after concerns were raised that it could potentially be a metabolite of androst-4-ene-3,17-dione, an intermediate in testosterone biosynthesis [45,76]. Additionally, it is a potential metabolite of 4,17β-dihydroxyandrost-4-en-3-one. Analysis of the data obtained during routine screening for 269 equine urine samples showed that formestane was not present endogenously. Following this, an *in vivo* controlled oral administration study of formestane was undertaken (800 mg, 2 thoroughbred geldings) [45].

After phase I metabolism, the parent compound as well as seven metabolites were identified as follows: one reduced metabolite (4,17β-dihydroxyandrost-4-en-3-one) which was matched to a reference material, two direduced metabolites (proposed as androst-4-ene-3α,4,17β-triol, and androst-4-ene-3β,4,17β-triol) which were matched to the products derived from partial reduction of formestane with sodium borohydride, and an additional four direduced metabolites (four of the possible 3,4-dihydroxy-5-androstan-17-one metabolites) which were tentatively identified by comparison to literature data [89,97]. Elevated levels of testosterone or other androgens not considered to be metabolites of formestane were not identified in this study. The structures of the phase II metabolites were determined through hydrolysis of the fractionated glucuronide and sulfate metabolites to their corresponding phase I metabolites. Formestane,

4,17β-dihydroxyandrost-4-en-3-one, and three of the 3,4-dihydroxy-5-androstan-17-one metabolites were excreted primarily as glucuronide conjugates, whilst androst-4-ene-3α,4,17β-triol, androst-4-ene-3β,4,17β-triol, and the remaining 3,4-dihydroxy-5-androstan-17-one metabolite were excreted as a mixture of glucuronide and sulfate metabolites. In addition to identifying the key metabolites, the excretion profiles of formestane and 4,17β-dihydroxyandrost-4-en-3-one were studied. Peak excretions of 40-44 µg/mL and 7-11 µg/mL respectively were observed at 5.6-6.3 hr post-administration, falling to below the limits of detection at 29 hr and 34 hr post-administration respectively. In addition, formestane was observed in plasma peaking at 6-10 hr post-administration, and falling below the limits of detection 34 hr post-administration. No other formestane metabolites were observed in equine plasma. As a part of this study, a comparative *in vitro* study was also undertaken. Phase I metabolism using equine liver microsomes prepared from whole liver tissue afforded the parent compound and seven metabolites, identified as follows: 4,17β-dihydroxyandrost-4-en-3-one, five direduced metabolites (androst-4-ene-3α,4,17β-triol, androst-4-ene-3β,4,17β-triol, and three of the possible 3,4-dihydroxy-5-androstan-17-one metabolites), and one trireduced metabolite. The majority of the metabolites observed *in vitro* matched those observed *in vivo* after hydrolysis of the phase II conjugates.

A separate *in vitro* study has also been performed by Clarke *et al.* [44], who identified a similar metabolite profile. LC-HRAM and GC-MS/MS analysis observed several hydroxylated metabolites, in addition to metabolites similar to those reported above. Metabolites were not identified by comparison to reference materials in this study, but instead were tentatively assigned by analysis of their MS data. Based on observations from both studies, the authors recommend monitoring for the parent compound and 4,17β-dihydroxyandrost-4-en-3-one to detect formestane misuse.

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

Figure 10

Furazadrol is a derivative of dihydrotestosterone containing an isoxazole ring fused to the steroid A-ring. It has been reported as a component of dietary supplements such as Orastan-A (Gaspari Nutrition) [32] and Furazadrol (Axis Labs) [33] predominately as the tetrahydropyranyl ether. In both cases, these supplements had incorrect labelling of the content information [32,33]. Furazadrol has been reported to exert anabolic activity in the older literature [21,98], and also in more recent yeast and human HuH7 androgen bioassays [33].

Following an equine *in vivo* controlled oral administration (200 mg, 1 thoroughbred gelding) [46], furazadrol was excreted primarily as the sulfate and glucuronide conjugates without phase I metabolism, which were detectable up to 24 hr post-administration by LC-HRAM analysis. Additional minor metabolites were also observed including a hydroxylated sulfate metabolite, two oxidised and hydroxylated sulfate metabolites, epifurazadrol glucuronide, and an oxidised and hydroxylated glucuronide metabolite. No unconjugated furazadrol metabolites were observed. The identity of furazadrol 17-sulfate, furazadrol 17-glucuronide, and epifurazadrol 17-glucuronide was confirmed by comparison to synthetic reference materials [99,100], and the identity of the other metabolites was tentatively assigned through analysis of the LC-HRAM product-ion spectra. The sites of hydroxylation for minor metabolites were not identified in this study. Further structural confirmation was performed through enzymatic hydrolysis of the fractionated sulfate and glucuronide metabolites, with analysis of the corresponding phase I metabolites as above. Hydrolysis of the sulfate fraction was achieved through use of *Pseudomonas aeruginosa* arylsulfatase, which is a purified enzyme with sulfatase activity and no alternative activities as are commonly found in commercial sulfatase preparations [101]. This enzyme was observed to completely hydrolyse all the *in vivo* steroid sulfate metabolites identified in this study. The major urinary metabolites (furazadrol 17-sulfate and furazadrol 17-glucuronide) were quantified in equine urine, to determine the detection window and limits of detection for these analytes. The authors recommend monitoring for these analytes or their hydrolysed phase I counterpart furazadrol for the detection of furazadrol misuse.

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

Methasterone (17β-hydroxy-2α,17α-dimethyl-5α-androstan-3-one)

Figure 11

Methasterone is a dimethylated analogue of dihydrotestosterone, and the C17-methylated analogue of drostanolone. It has been reported in the older steroid literature to exert a strong anabolic effect in rats [102,103], and predicted to be a potent anabolic agent in a more recent computational study [104]. It has been reported as a component of the dietary supplements Superdrol (Anabolic Xtreme) [34], S-drol (Black China Labs) [105], and Methasterone (Legal Gear) [2]. Methylated steroids such as these typically have the advantage of being orally bioavailable at the cost of higher toxicity to the liver and kidneys [39]. As a dimethylated steroid, methasterone has additional risks and has been reported to be involved in a number of serious health complications in humans including cases of severe jaundice, and immunoglobulin A (IgA) nephropathy [34,106,107].

The equine metabolism of methasterone has only been studied using *in vitro* methods [44]. Following metabolism with equine liver microsomes and S9 fraction, two C3-reduced metabolites were the predominate metabolites identified. Additionally, two minor hydroxylated metabolites and six minor reduced and hydroxylated metabolites were also observed. Metabolites were tentatively assigned based on their GC-MS/MS product-ion spectra as LC-MS/MS analysis afforded poor ionisation efficiency for the target analytes. The authors consider the use of GC-MS/MS essential for the detection of the two reduced metabolites (2α,17α-dimethyl-5α-androstane-3α/β,17β-diol) which may indicate methasterone misuse.

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

Figure 12

Oxyguno is an analogue of clostebol and 11-adrenosterone, and contains C17-methylation typical of orally bioavailable anabolic steroids [39]. It has been reported as a constituent of the dietary supplement Oxyguno (Spectra Force Research) [33,47,69] and has been reported in the older steroid literature to exert significant anabolic activity [108], as well as more recently in yeast, human HEK293, and human HuH7 androgen bioassays [33].

Following an equine *in vivo* controlled oral administration (52.5 mg twice daily for 2 days, 2 thoroughbred geldings) [47], oxyguno has been reported to be excreted as a range of unconjugated, sulfate-conjugated and glucuronide-conjugated metabolites which were detectable up to 12 hr post-administration. After phase I metabolism, the parent compound as well five novel metabolites were identified as follows: two C11-reduced metabolites, a C3 and C4-direduced metabolite, a C20-hydroxylated metabolite with C3, C4 or C11-

direduction, and C17-epi-oxyguno. The identities of these metabolites were tentatively assigned by analysis of their GC-MS/MS product ion spectra. The two C11-reduced metabolites and the hydroxylated and direduced metabolite were observed as glucuronide conjugates, whilst the C3 and C4-direduced metabolite was observed as both glucuronide and sulfate conjugates. The C17-epi-oxyguno metabolite was observed to arise from the sulfate conjugate. Epimerisation at the tertiary centre is a known metabolic pathway in C17-methylated steroids, and occurs *via* hydrolysis of a tertiary sulfate metabolite [109–111]. The tentative identities of these metabolites were established by LC-HRAM analysis of the intact conjugates, with further structural confirmation afforded by GC-MS/MS analysis of the enol-TMS ether derivatives obtained from the hydrolysis of the fractionated glucuronide and sulfate metabolites. The excretion of free oxyguno was also quantified to establish an elimination profile and determine a suitable detection window. Excretion peaked at 1-3 h and 2 h in blood and urine respectively, and fell below the limit of detection at 7 h and 12 h respectively. The authors recommend monitoring for the parent compound, or the direduced metabolite 4-chloro-3,17β-dihydroxy-17α-methyl-5α-androstan-11-one for the detection of oxyguno misuse.

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

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

Figure 13

Δ1-Testosterone is a synthetic anabolic steroid which closely resembles the structure of testosterone, substituting the C4-C5 double bond for a C1-C2 double bond. It can also be viewed as a 5α-reduced form of boldenone, another anabolic steroid which is well-known for its abuse in sports [39]. It has been reported to be a component of dietary supplements such as 1-androsterone (Advanced Muscle Science) and 1-AD (ErgoPharm) which typically contain Δ1-testosterone in addition to one or more of the following

compounds: 5α-androst-1-ene-3,17-dione, 5α-androst-1-ene-3,17-diol, or 3β-hydroxy-5α-androst-1-en-17-one, all of which are metabolised to Δ1-testosterone *in vivo* [112,113].

Following an equine *in vivo* controlled oral administration (800 mg, 2 thoroughbred geldings) [114], Δ1-testosterone has been reported to be excreted as a range of unconjugated, sulfate-conjugated and glucuronide-conjugated metabolites which were detectable up to 72 hr post-administration. After phase I metabolism, the parent compound as well as eight metabolites were identified as follows: four reduced metabolites (5α-androst-1-ene-3α,17β-diol, 5α-androst-1-ene-3β,17α-diol, 5α-androst-1-ene-3β,17β-diol, and epiandrosterone (3β-hydroxy-5α-androstan-17-one)), three doubly reduced metabolites (5α-androstane-3β,17β-diol, 5α-androstane-3α,17α-diol, and 5α-androstane-3β,17α-diol), and one hydroxylated metabolite. The parent compound, 5α-androst-1-ene-3α,17β-diol, and the hydroxylated metabolite were found to be excreted primarily as sulfate conjugates, whilst 5α-androst-1-ene-3β,17α-diol and 5α-androst-1-ene-3β,17β-diol were excreted primarily as glucuronide conjugates. The remaining metabolites were observed as a mixture of both sulfate and glucuronide conjugates. The identities of the phase I metabolites were confirmed by comparison to synthetic reference materials where available, and the NIST spectral database, or tentatively assigned through a combination of their MS behaviour and relative elution order. Hydroxylation at C16 is a common pathway for steroid metabolism in the horse [39], and the authors rationalise the observed hydroxylated metabolite on this basis. The metabolites of Δ1-androgens are also known to have similar mass spectra, which may complicate analysis [115]. The identity of the phase II metabolites was further confirmed through the hydrolysis of the fractionated glucuronide and sulfate metabolites to their corresponding phase I metabolites and their identities assigned as stated above. A number of the observed metabolites were identified up to 72 hr post-administration, however the authors comment that these are also potential *in vivo* metabolites for endogenous steroid compounds such as testosterone [76]. The metabolites containing the C1-C2 double bond are characteristic of administration of Δ1-testosterone, but were observed only 2-6 hr post-administration. The authors recommend monitoring for the parent compound, which can be detected at low levels (5-9 ng/mL) up to 30 hr post-administration. Additionally, longer term detection may be possible by adopting thresholds for the endogenous metabolites, or using longitudinal monitoring of the steroid profile.

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

vitro metabolites appear to correlate with the phase I metabolites observed after hydrolysis of the phase II metabolites observed *in vivo*.

Tetrahydrogestrinone (18β-homo-17β-hydroxy-19-nor-17α-pregna-4,9,11-trien-3-one) and structural analogues

Figure 14

Tetrahydrogestrinone (THG) was the second "designer" steroid ever reported and was identified during the analysis of a spent syringe containing an allegedly undetectable anabolic steroid which was provided anonymously to USADA in 2004 [27–29]. It can be produced chemically *via* a one-step reduction of gestrinone, which is a legally available progestin, and was originally produced with the express intention of bypassing current screening protocols [27]. Since its initial discovery, it has been reported in several studies to exert strong activity in yeast [28,63,116,117] and mammalian AR CALUX [61] androgen bioassays. After its discovery, it was specifically banned in competition by WADA rather than relying on the phrasing *"…and other substances with a similar chemical structure or biological effect(s)."* [118] as it represented a whole new class of threat to anti-doping analysis.

Following an equine *in vivo* controlled oral administration (25 µg/kg, 10 geldings) [119], THG has been reported to be excreted unmetabolised. An excretion profile was established for both plasma and urinary excretion. In plasma, concentrations peaked between 1-2 hr post-administration and were below the limit of detection at 24 hr post-administration. In urine, concentrations peaked at 3-6 hr post-administration and were below the limit of detection 48 hr post-administration.

In a separate *in vitro* study using equine liver microsomes and S9 fraction [44], it has been reported that metabolism of THG favours formation of hydroxylated metabolites. In this study, two major hydroxylated metabolites were observed, alongside one oxidised and hydroxylated metabolite. Additional minor metabolites were observed including a reduced metabolite, a reduced and hydroxylated metabolite, a dihydroxylated and direduced metabolite, a dihydroxylated and oxidised metabolite, two dihydroxylated and reduced metabolites, and two dihydroxylated and direduced metabolites. The sites of hydroxylation, oxidation, and reduction were not identified in this study. There appears to be little difference in the metabolites observed from incubation with equine liver microsomes or S9 fraction. The minor metabolites have relative ion abundances up to three orders of magnitude less than the primary metabolites. GC-MS analysis was complicated by the presence of numerous artefactual products resulting from enol-TMS ether derivatisation. Alternate derivatisation conditions for the formation of the enol-TMS derivatives, or the use

of alternate derivatives such as TMS ether or methyloxime-TMS ether (MOX-TMS) may alleviate some of the problems associated with derivatising conjugated enone systems [67]. Alternatively, the authors recommend LC-MS as being most suitable for the detection of THG and many of its metabolites.

In another *in vitro* metabolism study using equine liver microsomes and S9 fraction [48], the phase I metabolism and phase II glucuronylation of THG was reported. Following phase II metabolism, two hydroxylated metabolites were observed, alongside THG glucuronide, and two hydroxylated glucuronide metabolites. Metabolites were identified by analysis of the LC-MS/MS spectra. Glucuronylation of the tertiary alcohol was observed only at low levels, presumably reflecting the sterically hindered nature of this position. The positions of hydroxylation or glucuronylation were not identified in this study. Additionally, the *in vitro* metabolism of several related steroid 4,9,11-trienes were reported in this study. The metabolism of gestrinone (18β-homo-17β-hydroxy-19-nor-17α-pregna-4,9,11-trien-20-yn-3-one), trenbolone (17β-hydroxyestra-4,9,11-trien-3-one), and altrenogest (17β-hydroxy-17α-(prop-2-enyl)estra-4,9,11-trien-3-one, allyltrenbolone) was reported but have been covered in previous reviews and are subject to routine screening in equine anti-doping laboratories [39,50,120,121]. The equine metabolism of dihydrogestrinone (18β-homo-17β-hydroxy-19-nor-17α-pregna-4,9,11,20-tetraen-3-one) has not been previously reviewed and following *in vitro* metabolism, a reduced and hydroxylated metabolite was observed alongside dihydrogestrinone glucuronide. Although reduction could occur at a number of positions, it presumably occurs at the terminal alkene to afford hydroxylated THG, as the extended conjugation in the A-C rings typically resists metabolism [39]. This compound is likely to be encountered as an impurity in preparations containing THG resulting from the incomplete the hydrogenation of gestrinone [27]. The equine metabolism of propyltrenbolone (17β-hydroxy-17α-propylestra-4,9,11-trien-3-one) has also not been previously reviewed and following *in vitro* metabolism, three hydroxylated metabolites were observed alongside propyltrenbolone glucuronide, and three hydroxylated glucuronide metabolites.

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

Figure 15

Trestolone is a C7-methylated analogue of nandrolone, which itself is a C19-norsteroid analogue of testosterone. Owing to the lack of the C19-methyl substituent, this compound is more resistant to metabolism by aromatase enzymes [122], increasing its potential half-life in the body. Dietary supplements labelled to contain trestolone such as TR3ST (Olympus Labs) and 7-MENT Alpha (Wyked Labs) have become available in recent years. It has been shown in bioassays with HeLa cells transfected with the human androgen receptor [123], human AR CALUX bioassays [124,125] and *in vivo* rat models [125–127] to be a potent

androgen, as well as exhibit strong binding to the human progesterone receptor [123]. Trestolone has also been recently explored for use as a human male contraceptive as it has been shown to inhibit spermatogenesis without inducing androgen deficiency [126,128]. It has also been shown to inhibit equine and human steroid aromatase enzymes [49].

As a part of a study of human and equine steroid aromatase enzymes, the equine metabolism of trestolone has been studied by *in vitro* methods [49]. Although an appropriate choice for the study of the steroid aromatase pathway, the placental microsomes employed in this study are unlikely to reflect the metabolism afforded by liver enzymes. Following incubation of tritium labelled trestolone with equine placental microsomes, four metabolites were detected. Two of the detected metabolites were determined to be estrogenic as they were extracted in a phenolic extraction assay. Additionally, they matched TLC retention factors and GC-MS fragmentation with reference standards for 7α-methylestradiol, and 7α-methylestrone. The identities of the two remaining metabolites were not determined, although they were hypothesised to be intermediate compounds in androgen aromatisation, such as 1-hydroxytrestolone. These metabolites were not identified in control experiments utilising 4-hydroxyandrostenedione, a known aromatase enzyme inhibitor [49]. Additional kinetic experiments also showed that trestolone competitively inhibited the aromatisation of androstenedione and testosterone, suggesting that it may also function as an aromatase inhibitor. As a part of this study, incubations were also performed using a purified equine testicular P450 aromatase enzyme. After metabolism, these experiments also showed the presence of 7α-methylestradiol, as well as the two intermediates identified above, which were matched to the previous experiment. The authors recommend monitoring for the parent compound, alongside 7α-methylestradiol, and 7α-methylestrone for the detection of trestolone misuse. Additionally, alteration of the endogenous steroid profile due to aromatase inhibition could likely be detected through a threshold approach, or longitudinal monitoring of the equine steroid profile.

Future Directions

As laboratories respond to new threats, the directions of equine anti-doping research will shift, and will vary into the future. The importance of alternate testing matrices is gaining attention, with many jurisdictions incorporating these samples into routine testing. Hair has been reported to be a potentially long-term marker of steroid misuse [37,39] and these samples are easily acquired and processed in the laboratory. However, the use of hair as a matrix for drug detection could be problematic in jurisdictions that do not enforce out-of-competition drug use as the long excretion profiles of steroid compounds in hair could cast uncertainty as to whether a steroid was administered in- or out-of-competition. Blood is also

becoming a more common sample matrix, and it has been suggested that the levels of drug compounds in the bloodstream may indicate the potential for pharmacological effects *in vivo* [39]. Blood excretion profiles have been shown to be short, especially when compared to urinary excretion [47,119], which may limit its suitability to anti-doping laboratories. However, since the administered drugs can often be directly detected unmetabolised in blood [129] or hair [37], the ability to detect drug precursors of endogenous steroids arises and the need to conduct equine metabolism studies is reduced. Urine is still likely to remain a valuable analytical matrix as it is routinely used in the majority of currently available methods, and many of the important analytical thresholds for endogenous substances have only been determined in urine. The use of both GC-MS and LC-MS technologies is likely to be vital for adequate coverage of AAS metabolites in anti-doping screening as these techniques each provide advantages for certain target analytes.

The rapid increase in the prevalence of designer anabolic steroids present in "dietary" and "nutritional" supplements available online containing untested and unapproved anabolic agents, or the more recent emergence of selective androgen receptor modulators (SARMs) [58,130,131], is likely to pose a significant threat to the integrity of the industry if left unchecked. Both drug classes present a number of problems for anti-doping laboratories resulting from their widely variable structures that will need to be addressed in the future. Due to ethical concerns over the potential detrimental effects to the health and welfare of animal subjects, *in vitro* techniques are rapidly gaining attention as tools to study the metabolism of these compounds. These techniques, as highlighted by multiple examples throughout this review, are rapidly improving, and can more faithfully generate a metabolic profile representative of *in vivo* systems which will greatly assist anti-doping analysis [45–47,51,114]. Currently, AORC criteria allow for the use of *in vitro*-derived materials as standards for confirmatory analysis [35], and improvements to *in vitro* techniques may allow for the direct detection and confirmation of *in vivo* phase II metabolites by LC-MS/MS methods [39].

Recent improvements in untargeted and open screening methods show that these methods are also gaining popularity. These methods screen for characteristic fragments, or fragmentation modes of metabolite families rather than targeting individual drug metabolites, and they have been demonstrated to be suitable for the detection of unknown compounds in analytical samples [70–73]. The recent advancements made in affordable LC-HRAM and MS^n technologies also have greatly assisted the translation of these methods into routine screening as they allow for the acquisition of high-quality data which may be retrospectively analysed once new compounds are identified or new methods are developed. The development of more powerful computational packages provided by manufacturers also has the potential to make metabolomics approaches practical enough to be undertaken routinely in laboratories.

The final promising advancement is the development of the Equine Biological Passport [74], which mirrors the Athlete Biological Passport maintained by WADA for human athletes [75]. This approach would build a baseline profile from the routine sample analysis of all samples from an individual competing horse. Any future instances of doping would then be detected by abnormal changes between the baseline and a subsequent sample. Although there are a number of technical and administrative hurdles to overcome before this can be realised, this approach has the potential to effectively combat the misuse of anabolic steroids, and other doping agents in equine sports now and into the future.

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Figures and Tables

Table 1: Summary of the equine metabolism of designer steroids

Steroid	Phase I and Phase II metabolites	Comments
11-Adrenosterone (Ref [44])	Predominately C3/C11/C17 mono/di/trireduced. Minor hydroxylated metabolites. Phase II metabolism not studied.	Recommended analyte(s): Reduced 11-adrenosterone, 11β-hydroxyandrosterone threshold of greater than 10 µg/mL, or a 11β-hydroxyandrosterone:11β-hydroxyetiocholanolone ratio of greater than 20:1 have been suggested based on human studies. *In vitro* metabolism only. 11-Adrenosterone and its metabolites are potentially endogenous, may require threshold for detection.
ATD (Ref [44,51])	Predominately C17-reduced. Minor C16-hydroxylated and mono/direduced metabolites. C17β-sulfate and C17α-glucuronide metabolites. Mixture of both sulfate and glucuronide minor metabolites.	Recommended analyte(s): 17β-hydroxyandrosta-1,4,6-trien-3-one, and 17β-hydroxyandrosta-4,6-dien-3-one. Elevated testosterone observed *in vivo*, suggesting aromatase inhibition. Boldenone observed as an *in vivo* metabolite.
3α-Chloro-17α-methyl-5α-androstan-17β-ol (Ref [29])	3α-Chloro-17α-methyl-5α-androstan-16α,17β-diol identified as the sole equine metabolite. Phase II metabolism not studied.	Recommended analyte(s): 3α-Chloro-17α-methyl-5α-androstane-16α,17β-diol, and 17α-methyl-5α-androstane-3α,17β-diol. *In vitro* metabolism only. Potential elimination of C3-

		chloride to form madol.
Desoxyvinyltestosterone (Ref [39,79])	A number of A-ring enones were identified alongside minor A-ring hydroxylated, and A-ring reduced and dihydroxylated metabolites. Also identified metabolites resulting from reduction and hydroxylation of the vinyl group. Predominately glucuronide conjugated	<u>Recommended analyte(s):</u> 5α,17α-pregn-20-ene-2α/β,3α/β,17β-triol, and 5α,17α-pregn-1-ene-3α/β,17β,20α/β,21-tetraol. A number of analogues were also studied *in vitro.*
estra-4,9-diene-3,17-dione (Ref [39,80])	Major C17-reduced. Phase II metabolism not studied.	<u>Recommended analyte(s):</u> 17α/β-hydroxyestra-4,9-dien-3-one *In vitro* metabolism only.
Halodrol (Ref [44])	Turinabol observed as the major metabolite. Minor C3-oxidised and hydroxylated metabolites. Phase II metabolism not studied.	<u>Recommended analyte(s):</u> Turinabol (4-chloro-17β-hydroxy-17α-methylandrosta-1,4-diene-3-one). *In vitro* metabolism only.
20-Hydroxyecdysone (Ref [44])	No phase I metabolism observed. Phase II metabolism not studied.	<u>Recommended analyte(s):</u> 20-Hydroxyecdysone. *In vitro* metabolism only. Phase II metabolism likely to predominate *in vivo.*
Formestane (Ref [44,45])	Predominately C17-reduced, with minor C3/C17 direduction. Predominately glucuronide conjugated.	<u>Recommended analyte(s):</u> Formestane, and 4,17β-dihydroxyandrost-4-en-3-one. No aromatase inhibition observed *in vivo.*
Furazadrol (Ref [46])	Major *in vivo* metabolites: furazadrol sulfate and furazadrol glucuronide. Minor hydroxylated and, oxidised and hydroxylated metabolites also observed. Predominately sulfate conjugated.	<u>Recommended analyte(s):</u> Furazadrol, furazadrol 17-sulfate, and furazadrol 17-glucuronide.
Methasterone (Ref [44])	Predominately C3-reduced. Minor hydroxylated metabolites. Phase II metabolism not studied.	<u>Recommended analyte(s):</u> 2α,17α-dimethyl-5α-androstane-3α/β,17β-diol.

		In vitro metabolism only.
Oxyguno (Ref [47])	Predominately C3/C11 mono/direduced. Minor C20-hydroxylation and C17-epimerisation. C3 sulfate and glucuronide. C11 glucuronide only. Proposed C17-epi-oxyguno sulfate.	<u>Recommended analyte(s):</u> Oxyguno, and 4-chloro-3,17β-dihydroxy-17α-methyl-5α-androstan-11-one. *In vitro* metabolism appears limited in its ability to predict *in vivo* metabolism.
Δ1-Testosterone (Ref [114])	Range of mono/direduced metabolites. Minor hydroxylated and reduced metabolites. Mixture of sulfate and glucuronide conjugates.	<u>Recommended analyte(s):</u> Δ1-Testosterone. Some metabolites are potentially endogenous.
Tetrahydrogestrinone (Ref [44,48,119])	Excreted unmetabolised *in vivo.* Primarily hydroxylated *in vitro.* Hydroxylated glucuronide metabolites observed *in vitro.*	<u>Recommended analyte(s):</u> Tetrahydrogestrinone. Related steroid 4,9,11-trienes also studied *in vitro* [48].
Trestolone (Ref [49])	7α-methylestradiol, 7α-methylestrone, and two unidentified metabolites. Phase II metabolism not studied.	<u>Recommended analyte(s):</u> Trestolone, 7α-methylestradiol, and 7α-methylestrone. *In vitro* metabolism only. Aromatase inhibitor.

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

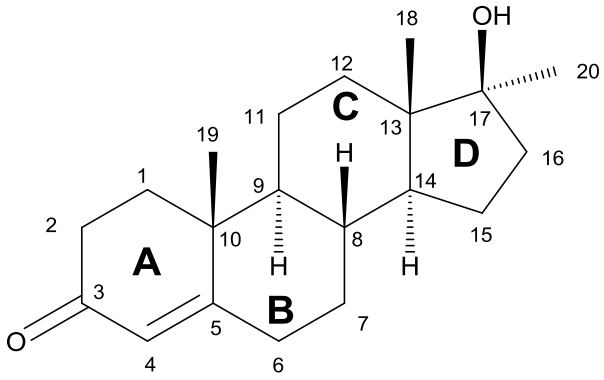
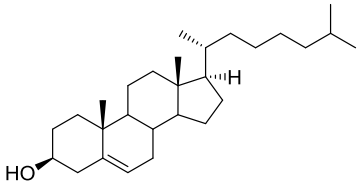
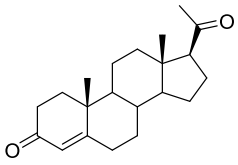


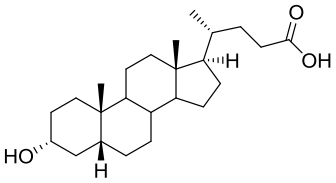
Figure 2: Representative examples of the major steroid classes



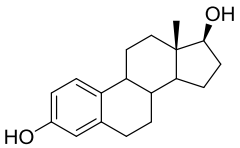
cholesterol (C27)



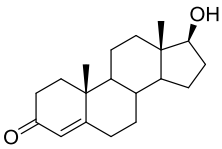
progesterone (C21)



lithocholic acid (C24)

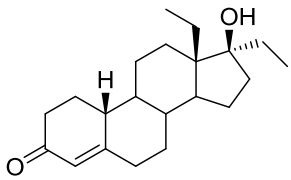


estradiol (C18)

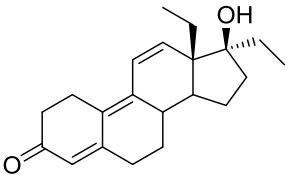


testosterone (C19)

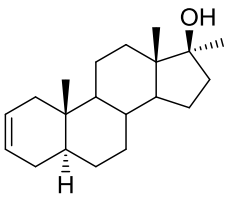
Figure 3: Structures of the first "designer" steroids: norbolethone, tetrahydrogestrinone, and madol



norbolethone



tetrahydrogestrinone



madol

Figure 4

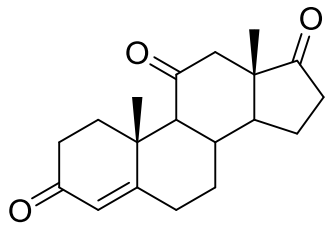


Figure 5

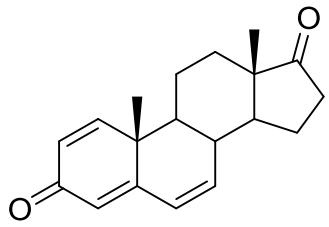


Figure 6

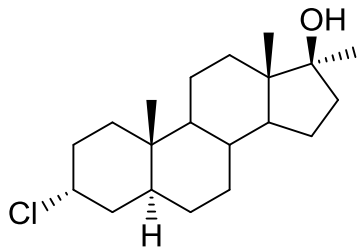


Figure 7

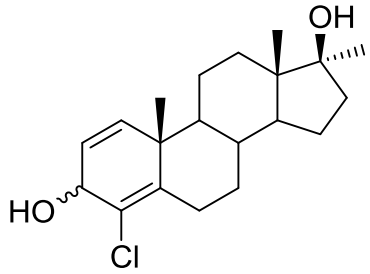


Figure 8

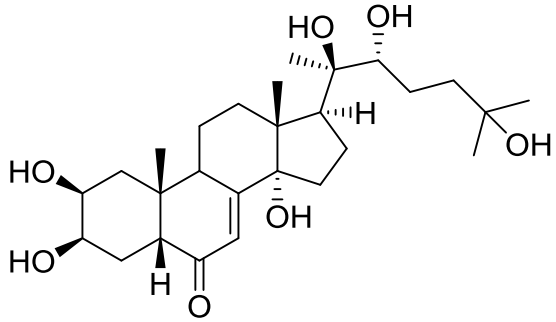


Figure 9

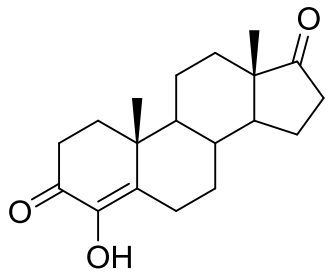


Figure 10

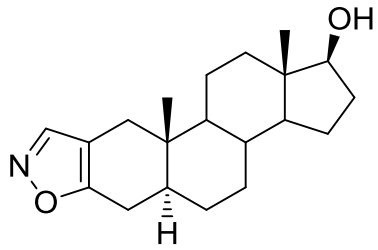


Figure 11

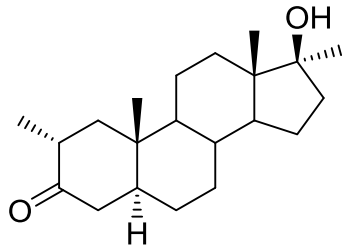


Figure 12

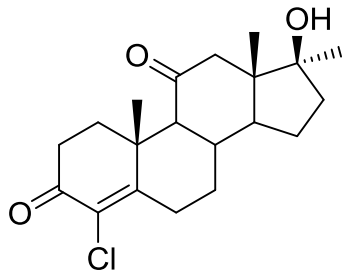


Figure 13

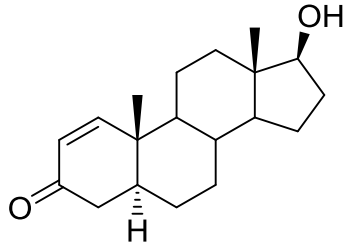


Figure 14

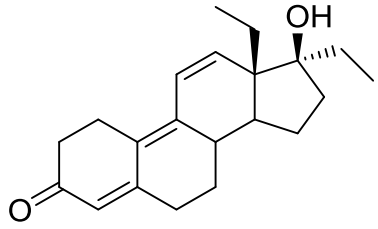


Figure 15