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The use of *in vitro* technologies and high-resolution/accurate-mass LC-MS to screen for metabolites of 'designer' steroids in the equine

Adam Clarke,[a]* James Scarth,[a] Phil Teale,[a] Clive Pearce[a] and Lynn Hillyer[b]

Detection of androgenic-anabolic steroid abuse in equine sports requires knowledge of the drug's metabolism in order to target appropriate metabolites, especially where urine is the matrix of choice. Studying 'designer' steroid metabolism is problematic since it is difficult to obtain ethical approval for *in vivo* metabolism studies due to a lack of toxicological data.

In this study, the equine *in vitro* metabolism of eight steroids available for purchase on the Internet is reported; including androsta-1,4,6-triene-3,17-dione, 4-chloro,17α-methyl-androsta-1,4-diene-3,17β-diol, estra-4,9-diene-3,17-dione, 4-hydroxyandrostenedione, 20-hydroxyecdysone, 11-keto-androstenedione, 17α-methyldrostanolone, and tetrahydrogestrinone. In order to allow for retrospective analysis of sample testing data, the use of a high-resolution (HR) accurate-mass Thermo LTQ-Orbitrap liquid chromatography-mass spectrometry (LC-MS) instrument was employed for metabolite identification of underivatized sample extracts. The full scan LC-HRMS Orbitrap data were complimented by LC-HRMS/MS and gas-chromatography-mass spectrometry (GC-MS) experiments in order to provide fragmentation information and to ascertain whether GC-MS was capable of detecting any metabolite not detected by LC-HRMS.

With the exception of 20-hydroxyecdysone, all compounds were found to be metabolized by equine liver S9 and/or microsomes. With the exception of 17α-methyldrostanolone, which produced metabolites that could only be detected by GC-MS, the metabolites of all other compounds could be identified using LC-HRMS, thus allowing retrospective analysis of previously acquired full-scan data resulting from routine equine drug testing screens. In summary, while *in vitro* techniques do not serve as a replacement for more definitive *in vivo* studies in all situations, their use does offer an alternative in situations where it would not be ethical to administer untested drugs to animals.

Keywords: *in vitro*; metabolism; equine; Orbitrap; designer steroid

Introduction

The use of androgenic-anabolic steroids (AASs) in equine sports is prohibited and effective analytical procedures are required in order to control their prohibition.[1] The detection of the majority of marketed AAS products in the equine is now possible using targeted approaches such as gas or liquid chromatography linked to tandem mass spectrometry (GC or LC-MS/MS, respectively).[2]

Recently, however, a range of steroids containing minor chemical modifications have emerged for sale.[3,4] Such 'designer' steroids are synthesized either to deliberately evade detection, or as appears more common, to enable them to be marketed freely on the Internet because their structures do not fall within the scope of legal regulations that prevent their sale. Because they often contain minor modifications compared to marketed products, some designer steroids are initially undetectable by current targeted mass spectrometric procedures. However, a number of other Internet-marketed products, including a range of aromatase inhibitors, have legitimate clinical applications, but are offered for sale claiming ergogenic effects. In this study, these compounds will also be categorized under the designer steroid 'banner' since they are deliberately targeted at performance enhancement. Because the majority of these compounds are not legitimate clinical products, their biological activity and the extent of their abuse is often difficult to determine. These steroids are prohibited by UK and international rules of horseracing as they act on the endocrine system.[5,6]

Effective control of steroid abuse usually requires knowledge of their metabolism in order to be able to target the most abundant urinary metabolites.[7] However, because designer steroids have undefined toxicological profiles, it is difficult to obtain ethical approval for *in vivo* studies. An alternative approach that has recently been used to study the metabolism of steroids is the use of *in vitro* techniques; involving incubation of the drug with *ex vivo* liver preparations.[4,8–11] Furthermore, two recent equine publications from our laboratories have shown that for both steroidal[12] and non-steroidal[13] drugs, all major *in vivo* metabolites, were also detected following *in vitro* incubation using either liver microsomes or S9. However, although the qualitative profile of metabolites compares favourably between *in vitro* and *in vivo*, the quantitative profile can be different. Other alternative techniques that have recently been developed involve the use of a rodent model transplanted with human hepatocytes[14,15] or

* *Correspondence to: Adam Clarke, HFL Sport Science Newmarket Road, Fordham, Cambridgeshire, CB7 5WW, UK. E-mail: aclarke@hfl.co.uk*

a *HFL Sport Science Newmarket Road, Fordham, Cambridgeshire, CB7 5WW, UK*

b *British Horseracing Authority, 75 High Holborn, London, WC1V 6LS, UK*

the use of invertebrate models[16,17]. However, these techniques still require the use of animal experimentation.

A further use for *in-vitro*-generated metabolites is as reference materials for confirmatory analysis in animal sports (2009 International Laboratory Accreditation Cooperation-G7 (ILAC-G7) guidelines).[18] These guidelines state that it is acceptable to use metabolite reference material generated by *in vitro* techniques where *in vivo* post-administration samples are not available (article 16.4).

Several studies on designer steroids have also been carried out in humans. For example, the *in vitro* metabolism of THG, 17α-methyldrostanolone and a range of 4,9,11-trien-3-one steroids have been studied,[19–21] while the *in* vivo human metabolism of a number of capsulated products purported to contain different steroidal products has also been reported.[22] The mass spectrometric behaviour of a range of different designer steroids has also been reported.[23]

In addition to studies on the metabolism, for example, of the designer steroid, the possibility of retrospective analysis of the data would be ideal after the discovery of the compound by doping control laboratories. The majority of existing targeted GC- or LC-MS/MS procedures do not allow for this retrospectivity.[2] However, it has been shown that recently introduced full-scan high-resolution accurate-mass LC-MS (LC-HRMS) instrumentation means that it is now possible to retrospectively analyse data from previously tested equine samples.[4,13,24,25]

In this study, the equine *in vitro* metabolism of seven Internet-marketed steroids (Figure 1) is reported. The steroids were chosen because they all featured (as of May 2010) in numerous products available for purchase on the Internet and were also available as reference grade material from various distributors. They included androsta-1,4,6-triene-3,17-dione (ATD – an aromatase inhibitor), 4-chloro,17α-methyl-androsta-1,4-diene-3,17β-diol (Halodrol – a Turinabol analogue), estra-4,9-diene-3,17-dione (Xtren – a trenbolone analogue), 4-hydroxyandrostenedione (Formestane – an aromatase inhibitor), 20-hydroxyecdysone (20-OH-E – a potentially anabolic arthropod derived ecdysteroid), 11-keto-androstenedione (adrenosterone – a potential 11β-hydroxysteroid dehydrogenase type 1 inhibitor) and 17α-methyldrostanolone (Superdrol – a drostanolone analogue). Although not freely available on the Internet, the metabolism of the designer steroid tetrahydrogestrinone (THG), previously known to have been abused in human sports,[3] was also studied because data on its metabolic disposition in the equine are lacking. The metabolism Xtren (reported previously in Scarth *et al.*[4]) was also included in the current study as a positive control.

In order to allow the retrospective analysis of sample testing data, the use of an LC-HRMS Thermo LTQ-Orbitrap instrument was employed for metabolite identification of underivatized sample extracts. The full-scan LC-HRMS Orbitrap data were complemented by HR-MS/MS and GC-MS experiments in order to provide information on the fragmentation of the metabolites and to ascertain whether GC-MS was capable of detecting additional metabolites.

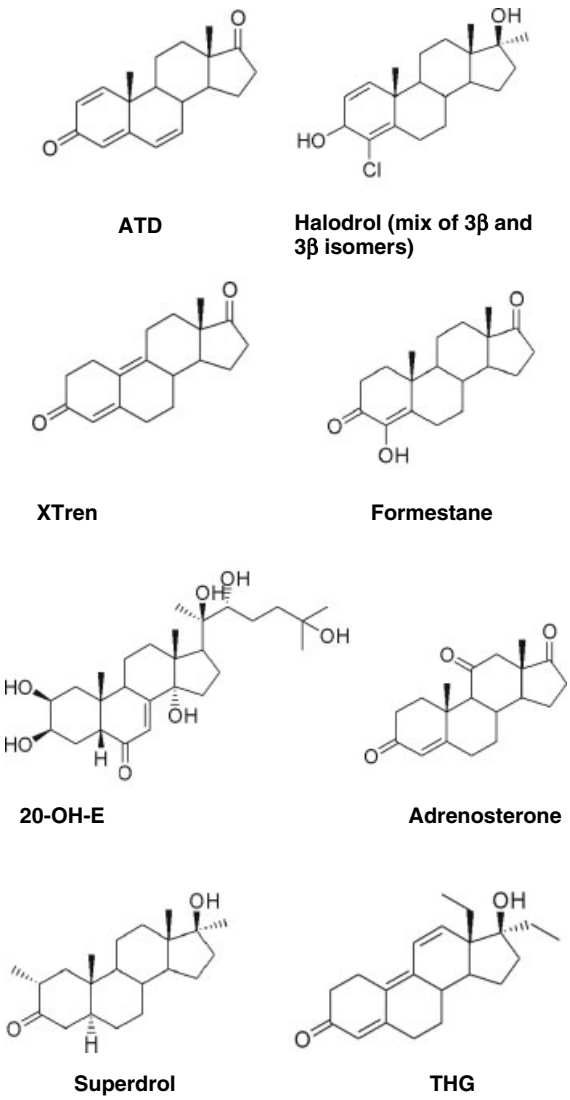

Figure 1. Structures of the eight 'designer' steroids used in this study.

Experimental

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, ethane thiol, ammonium iodide, N-methyl-N-(trimethylsilyl) -trifluoroacetamide, NADPH and NAD were obtained from Sigma-Aldrich (Dorset, UK). Estra-4,9-diene-3,17-dione and 2α,17α-Dimethyl-17β-hydroxy-5α-androstan-3-one were supplied by Toronto Research Chemicals (North York, Canada). Tetrahydrogestrinone was supplied by National Measurement Institute (Pymble, Australia). 20-hydroxyecdysone, 11-keto-adrenostenedione and 4-hydroxyandrostenedione were obtained from Sigma-Aldrich (Dorset, UK). 4-Chloro-17α-methyl-androst-1,4-diene-3,17β-diol and androsta-1,4,6-triene-3,17-dione was supplied by Great Dragon Biochemicals Co., Ltd (Hong Kong, China). 100 mm $\times$ 2.1ID 3 µm Atlantis T3 column were supplied by Waters (Wexford, Ireland). Equine liver microsomes and S9 (both at 20 mg/mL protein and from a Quarter horse) were purchased from Xenotech Llc. (Lenexa, KS, USA).

Nuclear Magnetic Resonance (NMR) analyses

As a quality control measure, each 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

($C^2H_3O^2H$) and the resulting solution (~0.5 mL) used for all NMR experiments analyzed on a Bruker Avance DRX 500. ^1H, ^{13}C and DEPT-135 spectra were acquired using standard pulse sequences. Tetrahydrogestrinone was not subjected to NMR analysis as the supplier provided a full NMR description in the certificate of analysis.

In vitro experiments

Incubation of steroids was performed separately with both S9 and microsomes of equine liver. Reaction volumes were 0.66 mL and contained drug (at 6 or 60 μM), liver S9 or microsomes (at 1 mg/mL protein), 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 3 h and 150 μL aliquots were quenched at 0 and 120 min by the addition of 300 μL of ice-cold acetonitrile. Control experiments with either no added cofactor or no enzyme were also performed.

For these experiments, phase II metabolism was not activated *in vitro* since the current methods of choice for the analysis for steroids in sports drug surveillance use deconjugation followed by analysis of the 'free' drug.[2]

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

For samples that were analyzed by LC-HRMS/MS, the quenched aliquots were then centrifuged for 5 mins t 11'000 rpm. The supernatant was transferred to a separate vial, blown down to dryness, and reconstituted in 5 μL propan-2-ol followed by 95 μL of water, before being submitted to LC-HRMS analysis on the Thermo LTQ-Orbitrap.

Once the samples had been run by LC-HRMS, the remaining aliquots were then blown down to dryness, reconstituted in 30 μL of an enol-trimethylsilyl derivatizing 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 °C for 2.5 h before being transferred to tapered vials and submitted to GC-MS analysis on a Varian 1200L.

Thermo LTQ-Orbitrap LC-HRMS and LC-HRMS/MS analyses

Initial metabolite identification was carried out using LC-HRMS and then followed up by LC-HRMS/MS in order to obtain more detailed structural information regarding the metabolites. All data were acquired and processed using Xcalibur version 2.0.7 software.

For the full-scan LC-HRMS 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 × 2.0 ID 2.5 μm HST Luna C18(2) column held at 35 °C. Mobile phase A was 0.1% acetic acid with 300 μg/litre uracil (external calibrant); mobile phase B was 0.1% acetic acid in methanol with 300 μg/litre uracil (external calibrant); and mobile phase C was 99 : 1 methanol:2 M pH 4 acetate buffer with 300 μg/litre uracil (external calibrant). The different stages of the gradient and flow rate are shown in Table 1. Ionization 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 amu was then acquired by the LTQ Orbitrap using a resolution of 30 000 at full width at half maximum (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 were first isolated in the LTQ and then fragmented at a normalized collision energy of 35, before the ions were supplied onto the Orbitrap for full-scan analysis at a resolution of 7500, 15 000 or 30 000 at FWHM. However, if the original LC-MS signal was of very low intensity, product ion scans were acquired using the LTQ without the orbitrap in order to maximize the resulting signal.

Table 1. The stages of the gradient and flow rate used on the Thermo Discovery LTQ-Orbitrap for the full scan LC-HRMS metabolite identification experiments

Time (mins)	% Mobile Phase A	% Mobile Phase B	% Mobile Phase C	Flow rate (μl/min)
0	90	10	0	400
1.00	40	60	0	400
3.49	2	98	0	400
3.50	2	23	75	400
4.00	2	23	75	400
4.20	2	23	75	500
4.50	2	23	75	500
4.51	2	98	0	500
4.99	2	98	0	500
5.00	90	5	0	400
5.50	90	5	0	400

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). Two ZB5-MSi 15m × 250 um × 0.25 um columns (Phenomenex, Macclesfield, UK, PN 7EG-G018-11) were set up in a backflushing configuration, of which deeper details have been reported previously.[26]

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 amu was acquired. Transfer line temperature was 280 °C, ion source temperature was 220 °C, electron energy was 70 eV, and the electron multiplier was used in extended dynamic range mode. Data were acquired and processed using Varian Workstation version 6.9 software.

Results and Discussion

NMR analyses

Results of the NMR analyses demonstrated consistency with the proposed structures, as summarized below for each steroid.

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

^1H spectrum (500 MHz): δ7.28 (1H, doublet; 10.1 Hz) – consistent with H-1, δ6.38 (1H, double doublet; 9.9, 2.88 Hz) – consistent with H-7, δ6.23 (2H, multiplet) – consistent with H-2 and H-6, δ6.00 (1H, broad singlet) – consistent with H-4, δ1.25 (3H, singlet) – consistent with Me-19, δ0.99 (3H, s) – consistent with Me-18, δ2.51 (2H, m), δ2.13 (2H, m), δ1.93 (1H, m), δ1.83 (1H,

m), δ1.78 (2H, m), δ1.57 (1H, m), δ1.46 (1H, m), δ1.31 (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): δ221.0 (C-17); δ188.5 (C-3); δ165.9 (C-5); δ156.1 (C-1); δ138.6, δ129.0, δ128.3, δ124.2 (C-2/C-4/C-6/C-7); δ50.1, δ49*, δ38.9 (C-8/C-9/C-14); δ49*, δ42.9 (C-10/C-13); δ49*, δ36.4, δ32.4, δ22.2 (C-11/C-12/C-15/C-16); δ21.2 (C-19); δ14.1 (C-18).

4-Chloro,17α-methyl-androsta-1,4-diene-3,17β-diol (Halodrol)

This material was supplied with the stereochemistry at C-3 undefined. The ^{1}H and ^{13}C spectra revealed the presence of both epimers, i.e. the 3α-OH and 3β-OH stereoisomers, in a ratio of approximately 1.5 : 1. For clarity, only the signals from the major isomer are reported here but in the ^{1}H spectrum, the signals from the minor isomer overlap with the former and in the ^{13}C spectrum they either overlap or are very adjacent.

^{1}H spectrum (500 MHz): δ5.97 (1H, d, 10.2 Hz) – consistent with H-1, δ5.68 (1H, dd, 10.2, 3.8 Hz) – consistent with H-2, δ4.40 (1H, d, 3.8) – consistent with H-3, δ0.87 (3H, s) – consistent with Me-18, δ2.99 (1H, m), δ2.09 (1H, m), δ1.83 (2H, m), δ1.60 (6H, m), δ1.29 (2H, m), δ1.20 (1H, m), δ1.14 (3H, s), δ1.12 (3H, s), δ1.05 (1H, m), and δ0.91 (1H, m) – not individually assigned but with chemical shifts consistent with all other methine, methylene and methyl protons expected.

^{13}C spectrum (125 MHz; partially assigned using DEPT-135): δ143.3, δ127.1 (C-4/C-5); δ137.7 (C-2); δ125.9 (C-1); δ82.1 (C-17); δ67.1 (C-3); δ54.5, δ51.5, δ37.7 (C-8/C-9/C-14); δ46.9, δ43.6 (C-10/C-13); δ39.2 (C-16); δ32.8, δ32.7, δ27.5, δ24.2, δ23.1 (C-6/C-7/C-11/C-12/C-15); δ26.1, δ21.1, δ14.7 (C-18/C-19/C-20).

Estra-4,9-diene-3,17-dione (XTren)

^{1}H spectrum (500 MHz): δ5.67 (1H, s) – consistent with H-4; δ1.04 (3H, s) – consistent with Me-18, δ2.95 (2H, m), δ2.48 (7H, m), δ2.10 (4H, m), δ1.85 (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); δ160.4, δ147.8 (C-5/C-10); δ127.2 (C-9); δ122.6 (C-4); δ52.6, δ40.0 (C-8/C-14); δ48.9 (C-13); δ37.9, δ36.7, δ32.7, δ31.7, δ27.7, δ26.8, δ26.1, δ22.8 (C-1/C-2/C-6/C-7/C-11/C-12/C-15/C-16); δ13.5 (C-18).

4-Hydroxyandrostenedione (Formestane)

^{1}H spectrum (500 MHz): δ3.08 (1H, m) – consistent with H-6$_{eq}$, δ1.22 (3H, s) – consistent with Me-18, δ0.92 (3H, m) – consistent with Me-19; δ2.48 (3H, m), δ2.02 (5H, m), δ1.70 (5H, m), δ1.46 (1H, double quartet, 4.2, 13.0 Hz), δ1.31 (2H, m), and δ1.02 (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): δ223.6 (C-17); δ195.3 (C-3); δ143.0, δ141.3 (C-4/C-5); δ55.9, δ52.3, δ36.1 (C-8/C-9/C-14); δ49*, δ39.1 (C-10/C-13); δ36.7, δ36.0, δ33.2, δ32.6, δ31.2, δ23.6, δ22.7, δ21.4 (C-1/C-2/C-6/C-7/C-11/C-12/C-15/C-16); δ17.4, δ14.1 (C-18/C-19).

* signals obscured by solvent signal

20-Hydroxyecdysone (20-OH-E)

^{1}H spectrum (500 MHz): δ5.79 (1H, d, 2.3 Hz) – consistent with H-7, δ3.94 (1H, d, 2.3 Hz) – consistent with H-21, δ3.82 (1H, double triplet, 11.8, 3.3 Hz) – consistent with H-3; δ3.14 (1H, triple triplet, 8.6, 2.8 Hz) – consistent with H-2, δ2.38 (2H, m), δ2.12 (1H, m), δ1.78 (13H, overlapping multiplets), δ1.42 (2H, m), δ1.28 (1H, double quartet, 4.3, 13.1 Hz), δ1.19 (3H, s), δ1.19 (3H, s), δ1.18 (3H, s), δ0.96 (3H, s), and δ0.88 (3H, s) – not individually assigned but with chemical shifts consistent with all other methine, methylene and methyl protons expected.

^{13}C spectrum (125 MHz; partially assigned using DEPT-135): δ206.4 (C-6); δ168.0 (C-8); δ122.1, (C-7); δ85.2, δ77.9, δ71.3 (C-14/C-20/C-24); δ78.4, δ68.7, δ68.5 (C-2/C-3/C-21); δ51.8, δ50.5 δ35.1 (C-5/C-9/C-17); δ49*, δ39.3 (C-10/C-13); δ49*, δ42.4, δ37.4, δ32.8, δ32.5, δ31.7, δ27.3, δ21.5 (C-1/C-4/C-11/C-12/C-15/C-16/C-22/C-23); δ29.7, δ28.9, δ24.4, δ21.0, δ18.0 (C-18/C-19/C-25/C-26/C-27).

11-Keto-androstenedione (adrenosterone)

^{1}H spectrum (500 MHz): δ5.73 (1H, s) – consistent with H-4, δ1.45 (3H, s) – consistent with Me-19, δ0.85 (3H, s) – consistent with Me-18, δ2.71 (1H, ddd, 13.7, 5.1, 3.4 Hz), δ2.52 (4H, m), δ2.36 (1H, ddd, 14.8, 4.0, 2.4 Hz), δ2.26 (3H, m), δ2.14 (4H, m), δ2.04 (1H, m), δ1.72 (2H, m), and δ1.34 (1H, 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): δ219.7, δ210.0 (C-11/C-17); δ202.3 (C-3); δ172.5 (C-5); δ124.9 (C-4); δ64.0, δ50.5, δ37.4 (C-8/C-9/C-14); δ51.6, δ39.7 (C-10/C-13); δ51.3 (C-12); δ36.9, δ35.6, δ34.4, δ33.2, δ32.1, δ22.4 (C-1/C-2/C-6/C-7/C-15/C-16); δ17.7, δ15.0 (C-18/C-19).

17α-Methyldrostanolone (Superdrol)

^{1}H spectrum (500 MHz): δ2.56 (1H, approximate septet, 6.5 Hz) – consistent with H-2, δ0.96 (3H, d, 6.5 Hz) – consistent with Me-21, δ0.86 (3H, s) – consistent with Me-18, δ2.42 (1H, dt, 0.9, 14.0 Hz), δ2.10 (1H, dd, 13.0, 6.0 Hz), δ1.98 (1H, dd, 14.1, 3.6 Hz), δ1.83 (1H, dt, 3.1, 11.9 Hz), δ1.73 (1H, dq, 6.3, 3.1 Hz), δ1.43 (12H, overlapping multiplets), δ1.19 (3H, s), δ1.12 (3H, s), δ1.05 (1H, t, 13.0 Hz), δ0.92 (1H, obscured multiplet), and δ0.72 (1H, ddd, 10.9, 7.9, 4.21) – not individually assigned but with chemical shifts consistent with all other methine, methylene and methyl protons expected.

^{13}C spectrum (125 MHz; partially assigned using DEPT-135): δ215.7 (C-3); δ82.2 (C-17); δ55.4, δ51.9, δ49.6, δ42.2, δ37.7 (C-2/C-5/C-8/C-9/C-14); δ50.0, δ45.6, δ39.2, δ32.9, δ32.7, δ29.9, δ24.3, δ22.3 (C-1/C-4/C-6/C-7/C-11/C-12/C-15/C-16); δ46.9, δ37.7 (C-10/C-13); δ26.1, δ14.9, δ14.7, δ12.7 (C-18/C-19/C-20/C-21).

In vitro analyses

Underivatized compounds detected by LC-HRMS were assigned as metabolites when a peak was observed at a mass tolerance of 5 ppm in an extracted ion chromatogram from the samples incubated with substrate that was not present in the control incubations. TMS-derivatized compounds detected by GC-MS were assigned as metabolites when a peak was observed in

* signal obscured by solvent signal

Table 2. Summary of the metabolites detected by LC-HRMS and GC-MS following incubation of ATD (60 μM) with equine liver S9 or microsomes

LC-HRMS results

Reaction observed (mass tolerance of 5 ppm)	MS^2 product ions (% of base peak)	RT (mins)	Mol ion	Mol ion theoretical *m/z*	Relative abundance (%) S9	Relative abundance (%) Mics
Parent compound	* 265.1588(100), 187.1116(82), 173.0959(34), 171.0804(24), 147.0804(24), 161.0959(21), 223.1118 (17), 209.0961(13), 159.0803(10)	3.14	[M+H]+	283.1693	N/A	N/A
Reduction	* 171.0804(100), 267.1744(74), 147.0804(23), 173.0959(17), 197.0961(15), 211.1118(13), 133.1012(10)	3.35	[M+H]+	285.1849	100.00	100.00
Reduction	* 267.1745(100), 171.0804(51), 147.0804(26), 173.0959(22), 133.1011(18), 197.0961(18), 159.1168(13), 211.1118(13), 151.1116(11), 185.0961(11)	3.55	[M+H]+	285.1849	51.85	80.65
Di- reduction	Insufficient sensitivity	3.38	[M+H]+	287.2006	1.19	1.74
Di- reduction	Insufficient sensitivity	3.58	[M+H]+	287.2006	1.19	1.68
Di- reduction	Insufficient sensitivity	3.68	[M+H]+	287.2006	0.11	0.16
Hydroxylation	Insufficient sensitivity	2.68	[M+H]+	299.1642	0.22	0.06
Hydroxylation	Insufficient sensitivity	2.75	[M+H]+	299.1642	1.07	0.39
Hydroxylation	Insufficient sensitivity	2.93	[M+H]+	299.1642	2.11	0.77
Hydroxylation and reduction	Insufficient sensitivity	2.57	[M+H]+	301.1798	1.85	0.32
Hydroxylation and reduction	Insufficient sensitivity	2.60	[M+H]+	301.1798	0.30	0.35
Hydroxylation and reduction	* 265.1588(100), 171.0803(53), 283.1693(33), 147.0803(27), 239.1431(18), 173.0959(17), 209.0961(24), 225.1274(23), 223.1118(22), 159.0803(18)	2.73	[M+H]+	301.1798	44.44	13.87
Hydroxylation and reduction	Insufficient sensitivity	2.89	[M+H]+	301.1798	1.89	0.48
Hydroxylation and reduction	Insufficient sensitivity	3.01	[M+H]+	301.1798	9.26	4.84
Hydroxylation and reduction	Insufficient sensitivity	3.27	[M+H]+	301.1798	1.89	0.65
Hydroxylation and di-reduction	Insufficient sensitivity	2.46	[M+H]+	303.1955	0.19	0.06
Hydroxylation and di-reduction	Insufficient sensitivity	2.77	[M+H]+	303.1955	0.70	0.27
Hydroxylation and di-reduction	Insufficient sensitivity	3.15	[M+H]+	303.1955	0.33	0.14

GC-MS results

Reaction observed	EI spectra (% of base peak)	RT (mins)	Relative abundance (%) (exc. *m/z* 73) S9	Relative abundance (%) (exc. *m/z* 73) Microsomes
Parent compound (C19-nor artefact)	73(100), 229(21), 412(20), 168/169(20), 182(18), 196(17), 216(13)	10.72	N/A	N/A
Reduction (C19-nor artefact)	73(100), 309(27), 324(19), 229(18), 147(16), 414(12), 129(12)	10.60	62.73	66.67
Reduction (C19-nor artefact)	73(100), 414(28), 229(26), 309(20), 283(20), 324(12), 129(10)	10.98	100.00	100.00
Reduction and hydroxylation (C19-nor artefact)	73(100), 229(31), 147(20), 309(20), 283(20), 322(12), 209(11), 502(10)	13.36	27.27	8.33

* MS^2 acquired using LTQ-Orbitrap at 15 000 FWHM.

an unit resolution extracted ion chromatogram from the samples incubated with substrate that was not present in the control incubations. Investigated metabolic transformations were predefined 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 additional peaks. The results from the 6 and 60 μM incubations produced comparable metabolite profiles in all species, but some of the more minor metabolites were only visible in the 60 μM incubations due to their lower concentrations in the 6 μM samples. Therefore, only the results from the 60 μM incubations will be discussed herein. The results are shown in Tables 2–9, with responses given for each compound relative to the most abundant metabolite peak observed. Since

Table 3. Summary of the metabolites detected by LC-HRMS and GC-MS following incubation of Halodrol (60 µM) with equine liver S9 or microsomes

LC-HRMS results

Reaction observed (mass tolerance of 5 ppm)	MS^2 product ions (% of base peak)	RT (mins)	Mol ion	Mol ion theoretical *m/z*	Relative abundance (%) S9	Relative abundance (%) Mics
Parent compound	Insufficient sensitivity	3.90	[M+Na]+	359.1748	N/A	N/A
Parent compound	Insufficient sensitivity	4.05	[M+Na]+	359.1748	N/A	N/A
3-oxidation	* 317.1669(100) 149.1323(40) 207.0727 (10)	3.83	[M+H]+	335.1772	100.00	100.00
A-Ring reduction, 3-oxidation	Insufficient sensitivity	3.85	[M+H]+	337.1929	0.15	–
A-Ring reduction, 3-oxidation	Insufficient sensitivity	4.03	[M+H]+	337.1929	0.19	0.20
Hydroxylation	Insufficient sensitivity	3.52	[M+NH4]+	370.2149	0.85	0.08
A-Ring reduction and hydroxylation	Insufficient sensitivity	3.83	[M+H]+	355.2035	0.25	0.18
Oxidation and hydroxylation	Insufficient sensitivity	2.96	[M+H]+	351.1722	0.37	0.36
Oxidation and hydroxylation	Insufficient sensitivity	3.06	[M+H]+	351.1722	0.74	0.69
Oxidation and hydroxylation	* 333.1618(100), 315.1511(82),	3.16	[M+H]+	351.1722	20.37	14.48
Oxidation and hydroxylation	* 333.1616(100), 315.1511(65), 279.1147(25), 261.1042(20)	3.33	[M+H]+	351.1722	9.26	5.52
Oxidation and hydroxylation	* 315.1512(100), 333.1618 (90), 303.1510(20), 147.1167(15)	3.38	[M+H]+	351.1722	34.81	22.07
Di-hydroxylation	Insufficient sensitivity	2.79	[M+NH4]+	386.2098	0.10	0.17
Reduction and di-hydroxylation	Insufficient sensitivity	3.14	[M+H]+	371.1984	0.12	0.06
Di-Reduction and di-hydroxylation	Insufficient sensitivity	3.52	[M+H]+	373.2140	0.07	0.04
Oxidation and di-hydroxylation	Insufficient sensitivity	2.48	[M+H]+	367.1671	0.44	0.13
Oxidation and di-hydroxylation	Insufficient sensitivity	2.81	[M+H]+	367.1671	0.37	0.13

GC-MS results

Reaction observed	EI spectra (% of base peak)	RT (mins)	Relative abundance (%) (exc. *m/z* 73) S9	Relative abundance (%) (exc. *m/z* 73) Microsomes
Parent compound	143(100), 73(90),193(22), 130(12), 355(12), 265(10)	12.09	N/A	N/A
Parent compound	143(100), 73(90),193(22), 355(12), 265(10)	13.09	N/A	N/A
3-oxidation	73(100), 143(75), 240(50), 373(10)	14.12	100.00	100.00
Oxidation and hydroxylation	73(100), 240(12), 566(10), 463(10)	15.56	4.00	3.08
Oxidation and hydroxylation	143(100), 73(90), 315(25), 243(15)	15.20	42.00	31.54

* MS^2 acquired using LTQ-Orbitrap at 15 000 FWHM.

Table 4. Summary of the metabolites detected by LC-HRMS following incubation of XTren (60 µM) with equine liver S9 or microsomes. GC-MS results are not reported since the TMS derivatization led to a number of artefactual products that meant sensitivity was very poor and that interpretation of the data was difficult. Data regarding the LC-MS/MS and MO-TMS spectra of XTren and its metabolites have been reported previously[4]

LC-HRMS results

Reaction observed (mass tolerance of 5 ppm)	RT (mins)	Mol ion	Mol ion theoretical *m/z*	Relative abundance (%) S9	Relative abundance (%) Mics
Parent compound	3.19	[M+H]+	271.1693	N/A	N/A
Reduction	3.40	[M+H]+	273.1849	100.00	100.00
Reduction	3.60	[M+H]+	273.1849	2.00	2.00
Di-reduction	3.46	[M+H]+	275.2006	0.07	0.09
Hydroxylation	2.53	[M+H]+	287.1642	0.34	0.34
Hydroxylation	2.71	[M+H]+	287.1642	0.29	0.34
Reduction and hydroxylation	2.52	[M+H]+	289.1798	1.20	0.72
Reduction and hydroxylation	2.63	[M+H]+	289.1798	0.80	0.41
Reduction and hydroxylation	2.78	[M+H]+	289.1798	4.00	3.28
Reduction and hydroxylation	2.89	[M+H]+	289.1798	0.40	0.33
Reduction and hydroxylation	3.00	[M+H]+	289.1798	0.11	0.11
Reduction and hydroxylation	3.05	[M+H]+	289.1798	0.05	0.04

Table 5. Summary of the metabolites detected by LC-HRMS and GC-MS following incubation of formestane (60 μM) with equine liver S9 or microsomes

LC-HRMS results

Reaction observed (mass tolerance of 5 ppm)	MS^2 product ions (% of base peak)	RT (mins)	Mol ion	Mol ion theoretical *m/z*	Relative abundance (%) S9	Relative abundance (%) Mics
Parent Compound	* 285.1(100), 267.1(40), 173.1(30), 239.1(20), 249.1(15), 113.2(15)	3.55	[M+H]+	303.1955	N/A	N/A
Reduction	#* 287.1(100), 269.1(80), 213.2(45), 223.1(15), 259.1(15)	3.22	[M+Na]+	327.1930	40.43	68.94
Reduction	Insufficient sensitivity	3.47	[M+Na]+	327.1930	8.52	17.12
Reduction	Insufficient sensitivity	3.64	[M+Na]+	327.1930	6.67	20.61
Reduction	Insufficient sensitivity	3.93	[M+Na]+	327.1930	2.43	6.80
Di-reduction	#* 271.2(100), 289.2(85), 253.2(55), 247.1(50), 249.1(40), 199.0(25), 275.1(15), 225.1(12)	3.20	[M+Na]+	329.2087	100.00	100.00
Di-reduction	Insufficient sensitivity	3.39	[M+Na]+	329.2087	7.84	10.32
Di-reduction	Insufficient sensitivity	3.50	[M+Na]+	329.2087	9.94	10.52
Tri-reduction	Insufficient sensitivity	3.05	[M+Na]+	331.2243	0.81	–
Tri-reduction	Insufficient sensitivity	3.26	[M+Na]+	331.2243	1.35	–
Hydroxylation	Insufficient sensitivity	3.98	[M+Na]+	341.1723	2.40	10.23
Reduction and hydroxylation	Insufficient sensitivity	2.68	[M+Na]+	343.1880	3.64	4.23
Reduction and hydroxylation	Insufficient sensitivity	3.05	[M+Na]+	343.1880	1.88	6.77
Reduction and hydroxylation	Insufficient sensitivity	3.49	[M+Na]+	343.1880	0.78	–
Di-reduction and hydroxylation	Insufficient sensitivity	2.90	[M+Na]+	345.2036	2.93	2.79

GC-MS results

Reaction observed	EI spectra (% of base peak)	RT (mins)	Relative abundance (%) (exc. *m/z* 73) S9	Relative abundance (%) (exc. *m/z* 73) Microsomes
Parent compound	73(100), 518(91), 147(22)	12.82	N/A	N/A
Reduction	73(100), 520(52)	9.85	17.04	13.72
Reduction	73(100), 520(39)	12.28	7.59	2.56
Reduction	73(100), 520(72)	12.62	50.00	25.58
Reduction	73(100), 520(89), 147(25)	13.03	100.00	100.00
Di-reduction	73(100), 522(75), 147(25)	12.88	33.33	11.16

* MS^2 acquired using LTQ only (therefore only unit resolution). # The MS^2 data was collected for the protonated forms of the compounds as the sodium adduct MS^2 data produced insufficient data due to the charge on the molecule being lost with the dissociation of the sodium ion on fragmentation.

Table 6. Summary of the LC-HRMS data for 20-OH-E. No metabolites could be identified using this technique. Furthermore, GC-MS results are not shown because interpretation of the data was complicated by the multiple derivatization products and the non-diagnostic fragmentation of the resulting molecules

LC-HRMS results

Reaction observed (mass tolerance of 5 ppm)	MS^2 product ions (% of base peak)	RT (mins)	Mol ion	Mol ion theoretical *m/z*	Relative abundance (%) S9	Relative abundance (%) Mics
Parent compound	* 445.2946(100), 371.2216(72), 463.3052(36), 427.2841(19), 303.1953(17)	2.42	[M+H]+	481.3160	N/A	N/A

* MS^2 acquired using LTQ-Orbitrap at 30 000 FWHM.

authentic reference standards were not available for comparison, the responses of the different metabolites cannot be considered as fully quantitative and can only be described in 'relative response' terms (being affected by parameters such as ionization efficiency and extent of fragmentation).

Because the primary aim of this study was to identify suitable target metabolites for inclusion in equine doping control drug screens, the metabolite structures were not fully rationalized beyond the assignment of their molecular mass and elemental composition. However, in order to demonstrate that *in-vitro*-

Table 7. Summary of the metabolites detected by LC-HRMS and GC-MS following incubation of adrenosterone (60 μM) with equine liver S9 or microsomes

LC-HRMS results

Reaction observed (mass tolerance of 5 ppm)	MS^2 product ions (% of base peak)	RT (mins)	Mol ion	Mol ion theoretical *m/z*	Relative abundance (%) S9	Relative abundance (%) Mics
Parent compound	* 265.1587(100), 283.1693(70), 255.1744(15) 175.1273(15)	2.97	[M+H]+	301.1798	N/A	N/A
Reduction	** 267.17(100), 285.1758(85), 257.1812(20), 227.1342(12)	3.08	[M+H]+	303.1955	100.00	100.00
Reduction	Insufficient sensitivity	3.28	[M+H]+	303.1955	3.14	–
Di-reduction	Insufficient sensitivity	3.13	[M+H]+	305.2111	0.11	0.05
Tri-reduction	** 271.2057(100), 289.2162(55), 253.1650(50)	3.18	[M+H]+	307.2268	1.93	1.54
Hydroxylation	Insufficient sensitivity	2.64	[M+H]+	317.1747	0.62	0.30
Hydroxylation	Insufficient sensitivity	2.55	[M+H]+	317.1747	0.07	–
Reduction and hydroxylation	* 265.1589(100), 283.1696(15) 275.1643(25), 301.1800(25), 225.1275(20), 121.0647(20)	2.62	[M+H]+	319.1904	3.66	1.60

GC-MS results

Reaction observed	EI spectra (% of base peak)	RT (mins)	Relative abundance (%) (exc. *m/z* 73) S9	Relative abundance (%) (exc. *m/z* 73) Microsomes
Parent compound	73(100), 501(51), 169(18), 516(10)	11.37	N/A	N/A
Reduction	73(100), 387(95), 193(31), 208(25), 518(20), 428(11)	11.65	100.00	100.00
Reduction	73(100), 503(76), 169(18), 518(15), 323(10)	11.80	64.52	78.85

* MS^2 acquired using LTQ-Orbitrap at 7500 FWHM. ** MS^2 acquired using LTQ-Orbitrap at 30 000 FWHM.

generated data can be used beyond an initial screen if necessary, the results acquired for ATD were subjected to a more complete analysis. In the event that a urine or plasma sample tests positive for one of the steroid metabolites in the future, *in vitro* samples could be used to allow a more stringent analysis in order to more accurately determine the likely structure/s. However, it is important to note that ILAC guidelines for confirming the presence of prohibited compounds do not require the precise structure of a metabolite, only that '. . .the analytical data from it are sufficient to fully justify its identity as a metabolite of the substance administered or incurred'.[18]

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

The LC-HRMS and GC-MS results for ATD and its equine metabolites detected in the current study are summarized in Table 2.

The S9 and microsomal incubations produced broadly the same results, with by far the most intense signals in both the LC-HRMS and GC-MS analyses corresponding to two reduced-ATD isomers and one reduced-hydroxy-ATD metabolite. The GC-MS results were characterized by the artefactual loss of the 19-methyl group from each compound; an observation also reported in a previous human study.[27] As rationalized in Figures 2 and 3, it is proposed that both of the reduced-ATD isomers bear a 17-hydroxy function, therefore corresponding to the 17β and 17α isomers. This is consistent with results obtained in a recent human study, which also found reduction at position 17 to be a major pathway of metabolism.[27] The GC-MS results were particularly useful in rationalizing the structure of the major reduced-hydroxy-ATD metabolite, which produced a pseudo-molecular ion at *m/z* 502 (Figure 3). The same A, B, and C ring fragments that were detected in the GC-MS spectrum of ATD and its 17-reduced metabolites were also present in the spectrum of the reduced-hydroxy-ATD metabolite, suggesting that hydroxylation occurred on either the D-ring or the 18-methyl group. The 18-methyl group was not considered a likely candidate since the characteristic *m/z* 103 loss from a TMS derivatized hydroxy-methyl groups was not observed.[28] By process of deduction, this therefore leads to the speculation that the site of hydroxylation takes either the 15 or 16 positions, both of which are common pathways of steroid metabolism in the horse.[29] It was also noted that the mass and retention time of one of the di-reduced-ATD metabolites analyzed by LC-HRMS matched those of a boldenone reference standard. Again, this is comparable with the results of the previous human study, which also detected boldenone as a metabolite of ATD.[27]

Since LC-MS analysis was found to offer superior sensitivity compared to GC-MS, the use of LC-MS would appear to be the most effective way of screening for the abuse of ATD in the equine. As ATD and its metabolites are not known to be endogenous in the equine, a qualitative approach is considered sufficient for detection of the misuse of this steroid.

4-Chloro,17α-methyl-androsta-1,4-diene-3,17β-diol (Halodrol)

The LC-HRMS and GC-MS results for Halodrol and its equine metabolites detected in the current study are summarized in Table 3.

Table 8. Summary of the metabolites detected by LC-HRMS and GC-MS following incubation of Superdrol (60 µM) with equine liver S9 or microsomes

LC-HRMS results

Reaction observed (mass tolerance of 5 ppm)	MS^2 product ions (% of base peak)	RT (mins)	Mol ion	Mol ion theoretical *m/z*	Relative abundance (%) S9	Relative abundance (%) Mics
Parent compound	Insufficient sensitivity	4.42	[M+H]+	319.2632 (H^+)	N/A	N/A
Hydroxylation	Insufficient sensitivity	3.94	[M+Na]+	357.2400 (Na^+)	100.00	100.00
Reduction and hydroxylation	Insufficient sensitivity	3.89	[M+Na]+	359.2556 (Na^+)	31.35	12.75
Reduction and hydroxylation	Insufficient sensitivity	4.05	[M+Na]+	359.2556 (Na^+)	12.40	4.61
Reduction and hydroxylation	Insufficient sensitivity	4.19	[M+Na]+	359.2556 (Na^+)	6.19	1.97

GC-MS results

Reaction observed	EI spectra (% of base peak)	RT (mins)	Relative abundance (%) (exc. *m/z* 73) S9	Relative abundance (%) (exc. *m/z* 73) Microsomes
Parent compound	143(100), 73(50), 432(61), 141(42), 157(20), 156(18), 216(15), 332(14), 419(12)	12.12	N/A	N/A
Position 3 reduction	143(100), 73(42), 130(26), 449(17), 269(14)	10.65	92.00	96.43
Position 3 reduction	143(100), 73(62), 449(24), 130(14), 269(10)	11.72	100.00	100.00
Hydroxylation	143(100), 73(68), 460(27), 535(12)	13.72	4.40	2.46
Hydroxylation	73(100), 218(27), 231(27), 550(10)	14.65	0.56	0.39
Position 3 reduction and hydroxylationHydroxylation	143(100), 73(64), 157(25), 332(25), 130(14), 372(13)	12.46	11.60	2.21
Position 3 reduction and hydroxylation	218(100), 73(82), 372(24), 462(18), 552(10)	12.72	1.20	0.43
Position 3 reduction and hydroxylation	218(100), 231(71), 73(64), 462(18)	13.20	0.96	0.54
Position 3 reduction and hydroxylation	73(100), 218(68), 231(45), 462(18), 372(15), 552(11)	14.07	1.32	0.61
Position 3 reduction and hydroxylation	73(100), 143(94), 372(22), 130(16)	14.17	6.00	0.71
Position 3 reduction and hydroxylation	218(100), 231(80), 73(75), 372(26), 552(12)	14.36	1.04	0.39

The parent compound 3α and 3β isomers ionized poorly under API conditions and produced more intense signals using GC-MS. However, its metabolites typically ionized well under API conditions and therefore a larger number of metabolites were identified using LC-HRMS. For a small number of metabolites, the responses obtained from the S9 were much larger than those of the microsomes, suggesting that cytosolic enzymes might be important in the metabolism of Halodrol. By far the most abundant metabolite observed by both LC-HRMS and GC-MS was an oxidized metabolite (−2H) proposed to be 3-keto-Halodrol (otherwise known as Turinabol). This is significant since it is therefore likely that Halodrol is a pro-drug for Turinabol (a 3-keto analogue of Halodrol) and that its metabolism could be very similar to this steroid.

In light of the above results, the most suitable method for screening for the Halodrol would likely be through detection of its metabolites by LC-MS. However, as the parent compound produced a more intense signal using GC-MS, this method of detection could also be used to support the LC-MS data. Since Halodrol and its metabolites are not known to be endogenous in the equine, a qualitative approach is considered sufficient for detection of the misuse of this steroid.

While the metabolism of Halodrol has not been reported previously in the equine, the *in vivo* and *in vitro* metabolism of the structural analogue (and one of Halodrol's proposed metabolites) Turinabol has been studied.[10] It is pertinent to note that of the large number of additional metabolites that were detected by LC-HRMS in the current study; many had elemental compositions matching those discovered in the previous metabolism study of Turinabol. Also, a number of A-ring reduced metabolites were detected in the current study; all of which were only observed *in vivo* and not *in vitro* in the previous report.[10]

Estra-4,9-diene-3,17-dione (XTren)

The LC-HRMS for XTren and its equine metabolites detected in the current study are summarized in Table 4.

The results shown in Table 4 are in a broad agreement with those reported previously.[4] These control results therefore demonstrate that the experimental system was working adequately. GC-MS results are not reported since the TMS derivatization led to a number of artefactual products, decreasing the sensitivity and making the data interpretation difficult. Data regarding the GC-MS analysis of MO-TMS derivatized XTren metabolites and the LC-HRMS/MS analysis of underivatized XTren metabolites have been reported previously.[4]

In light of the results from both the current and previous studies, the most effective way of screening for the abuse of XTren

Table 9. Summary of the metabolites detected by LC-HRMS following incubation of THG (60 µM) with equine liver S9 or microsomes. GC-MS results are not reported since the TMS derivatization led to a number of artefactual products that meant sensitivity was very poor and that interpretation of the data was difficult

LC-HRMS results

Reaction observed (mass tolerance of 5 ppm)	MS^2 product ions (% of base peak)	RT (mins)	Mol ion	Mol ion theoretical *m/z*	Relative abundance (%)	
					S9	Mics
Parent compound	* 295.2008(100), 225.1267(28),241.1574(20), 239.1443(18)	4.08	[M+H]+	313.2162	N/A	N/A
Reduction	Insufficient sensitivity	4.23	[M+H]+	315.2319	0.37	1.00
Hydroxylation and oxidation	* 309.1849(100), 291.1743(18), 280.1459(12), 125.0595(11), 237.1281(10), 239.1420(10), 241.1319(10)	3.60	[M+H]+	327.1955	8.26	7.00
Hydroxylation	* 311.2000(100), 293.1899(89), 285.1851(71), 267.1743(28), 237.1325(27), 241.1583(25), 239.1457(16)	3.43	[M+H]+	329.2111	100.00	100.00
Hydroxylation	* 311.2004(100), 293.1902(72), 227.1425(58), 241.1575(32), 237.1311(19), 239.1434(12)	3.67	[M+H]+	329.2111	22.09	20.77
Reduction and hydroxylation	Insufficient sensitivity	2.88	[M+H]+	331.2268	5.35	4.38
Di-hydroxylation and di-oxidation	Insufficient sensitivity	3.28	[M+H]+	341.1747	0.41	–
Di-hydroxylation and oxidation or di-hydroxylation, reduction and Di-Oxidation or carboxylation	Insufficient sensitivity	3.58	[M+H]+	343.1904	2.44	2.77
Reduction and di-hydroxylation	Insufficient sensitivity	2.61	[M+H]+	347.2217	0.58	0.77
Reduction and di-hydroxylation	Insufficient sensitivity	3.05	[M+H]+	347.2217	0.59	0.85
Di-reduction and di-hydroxylation	Insufficient sensitivity	3.06	[M+H]+	349.2373	0.22	0.08
Di-reduction and di-hydroxylation	Insufficient sensitivity	3.22	[M+H]+	349.2373	0.47	0.35

* MS^2 acquired using LTQ-Orbitrap at 7500 FWHM.

would appear to be through the use of LC-MS. As XTren and its metabolites are not known to be endogenous in the equine, a qualitative approach is considered sufficient for detection of the misuse of this steroid.

4-Hydroxyandrostenedione (Formestane)

The LC-HRMS and GC-MS results for the marketed aromatase inhibitor, Formestane, and its equine metabolites detected in the current study are summarized in Table 5.

A wider range of metabolites were detected using LC-HRMS, but the GC-MS peaks corresponding to the four major reduced and the most abundant di-reduced-Formestane metabolites produced relatively larger peaks. An isomer of di-reduced formestane produced the most abundant signal by both LC-HRMS and GC-MS; followed by the four isomers of reduced-Formestane. For a small number of metabolites, the responses obtained from the S9 were much higher than those of the microsomes, suggesting that cytosolic enzymes might be important in the metabolism of Formestane.

In light of the results described above, the most effective approach for detecting Formestane abuse would appear to be a combination of GC-MS and LC-MS methods.

Although the equine metabolism of Formestane has not previously been reported, a recent study on its *in vivo* metabolism in the human has been published.[30] Formestane was found to be extensively metabolized, forming a wide range of products resulting from reduction and isomerization at positions 3, 4 and 17. Formestane is known to be a minor metabolite of the endogenous steroid androstenedione in other species,[31] but the range of endogenous concentrations of Formestane and its metabolites in the equine are currently unknown. Further studies may therefore be required in order to determine whether Formestane is endogenously produced or if it may be derived from dietary intake.

20-Hydroxyecdysone (20-OH-E)

20-OH-E was readily detected by LC-HRMS and its chromatographic and mass spectrometric properties are summarized in Table 6. No GC-MS results are shown because interpretation of the data was complicated by the multiple derivatization products and the non-diagnostic fragmentation of the resulting molecules. As shown in Table 6, no metabolites of 20-OH-E could be identified from the enzymatic incubations using either LC- or GC-MS.

In light of the observation that the GC-MS analysis of 20-OH-E was complicated by multiple derivatization products and, therefore, poor sensitivity, the use of LC-MS-based methods is considered most suitable for use in screening for this compound. Because of the hydrophilic nature of 20-OH-E, it seems reasonable to assume that a significant amount of this drug would be excreted unchanged or conjugated in urine. Therefore, detection based on parent compound may be sufficient; a theory which is consistent with *in vivo* studies in the bovine.[32]

Although the equine metabolism 20-OH-E has not been reported previously, its metabolism has been studied *in vivo* in several other species. For example, human metabolism is reported to proceed mainly through deoxygenation at positions 2, 14 and 20, although it has been noted that parent compound can be detected for a longer duration than any of the metabolites.[33] In the bovine however, metabolism is reported to proceed via a combination of deoxygenation at position 14 and hydroxylation at position 26.[32] It has also been reported that ecdysteroids are natural steroidal products produced by invertebrates and plants.[34] It is currently unknown what the ranges of 'natural' concentrations of ecdysteroids resulting from dietary intake in mammals (including the equine) are, but they are likely to vary depending on diet.

In the current study it was noted that a number of low level (<1% relative to the parent compound) desoxy, dehydro and hydroxy impurities were detected in the control incubations (data not shown). It would therefore be interesting to determine what proportion of the compounds identified in previously reported human and bovine studies were 'true' metabolites as compared to excreted drug impurities.

11-Keto-androstenedione (adrenosterone)

The LC-HRMS and GC-MS results for adrenosterone and its equine metabolites detected in the current study are summarized in Table 7.

While a wider range of metabolites were detected using LC-HRMS, the GC-MS peak corresponding to one of the two major reduced-adrenosterone metabolites produced a relatively high response. For a small number of metabolites, the responses obtained from the S9 were much larger than those of the microsomes, suggesting that cytosolic enzymes might be important in the metabolism of adrenosterone.

In light of the results described above, the most effective approach for detecting adrenosterone abuse would appear to be a combination of GC-MS and LC-MS methods.

While the equine metabolism adrenosterone has not been reported previously, a recent study on its metabolism *in vivo* in the human has been published.[35] In this study, increased concentrations of 11β-hydroxy-androsterone, 11β-hydroxy-etiocholanolone, 11-oxoandrosterone and 11-oxoetiocholanolone were observed following adrenosterone administration. Furthermore, 3α,17β-dihydroxy-5β-androstan-11-one, 3α-hydroxyandrost-4-ene-11,17-dione and 3α,11β-dihydroxyandrost-4-en-17-one were identified as minor metabolites. Due to the endogenous nature of this analyte, the authors recommended screening criteria for the detection of adrenosterone abuse of 11β-hydroxy-androsterone greater than 10 µg/mL in urine (specific gravity adjusted to 1.02) or an 11β-hydroxy-androsterone:11β-hydroxy-etiocholanolone ratio of greater than 20 : 1. The authors also suggested that gas chromatography-combustion-isotope ratio mass spectrometry be used for confirmatory analysis. The range of endogenous concentrations of adrenosterone and its metabolites in the equine are currently unknown.

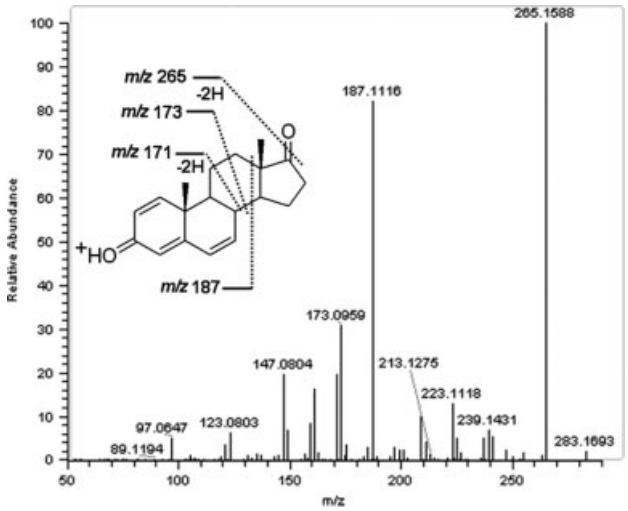

Figure 2a. LC-HRMS/MS spectrum (3.14 min) of ATD.

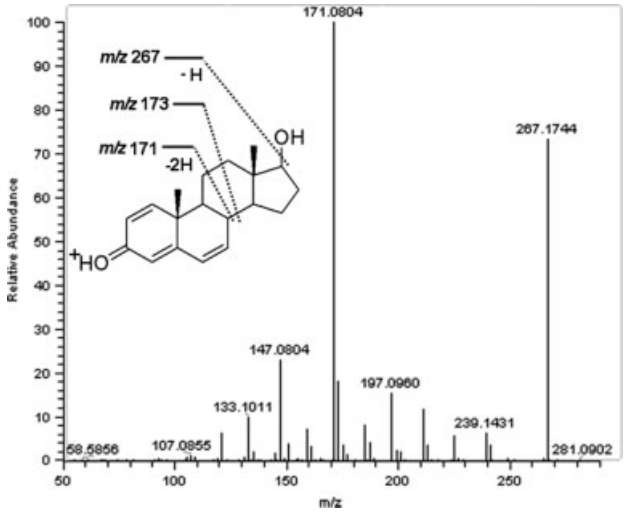

Figure 2b. LC-HRMS/MS spectrum (3.35 min) of reduced-ATD following incubation liver equine liver S9 (60 µM). A second peak at 3.55 min with a qualitatively similar spectrum was also present (see text for details).

17α-Methyldrostanolone (Superdrol)

The LC-HRMS and GC-MS results for Superdrol and its equine metabolites detected in the current study are summarized in Table 8.

Two reduced metabolites of Superdrol detected by GC-MS were by far the most significant reaction products detected. For a small number of the remaining metabolites, the responses obtained from the S9 were much larger than those of the microsomes, suggesting that cytosolic enzymes might be important in the metabolism of Superdrol.

Because of the low responses obtained when using LC-HRMS, it is considered especially important to use GC-MS to detect the abuse of Superdrol in the equine. As Superdrol and its metabolites are not known to be endogenous in the equine, a qualitative

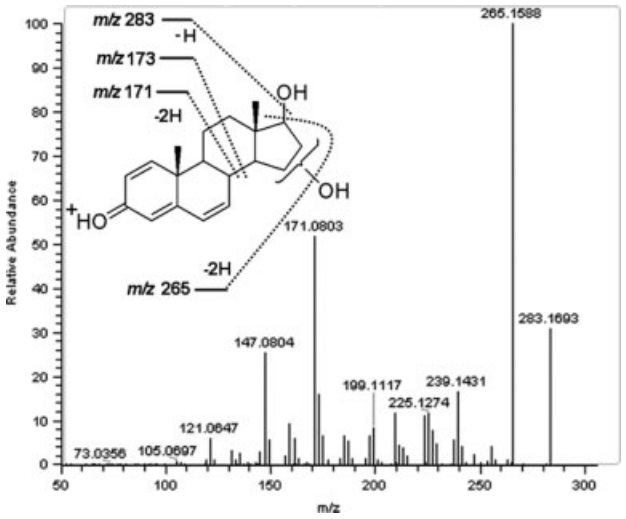

Figure 2c. LC-HRMS/MS spectrum (2.73 min) of reduced-hydroxy-ATD following incubation liver equine liver S9 (60 µM).

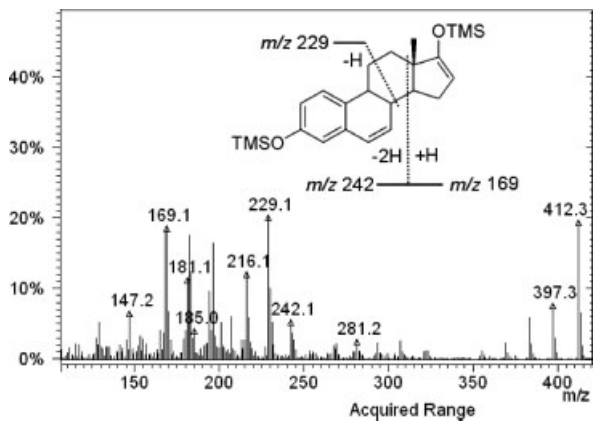

Figure 3a. GC-MS spectrum (10.72 min) of ATD-di-TMS artefact.

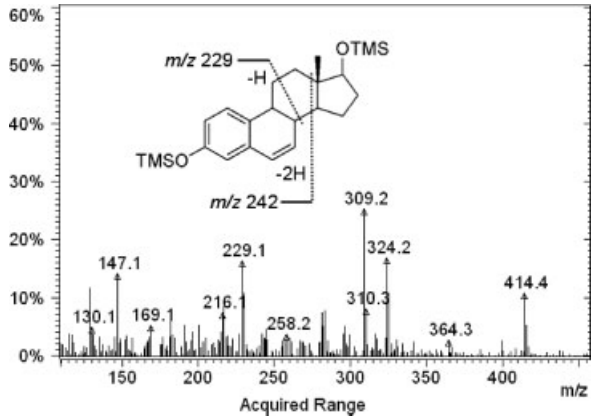

Figure 3b. GC-MS spectrum (10.60 min) of reduced-ATD-di-TMS artefact following incubation liver equine liver S9 (60 µM). A second peak at 10.98 min with a qualitatively similar spectrum was also present (see text for details).

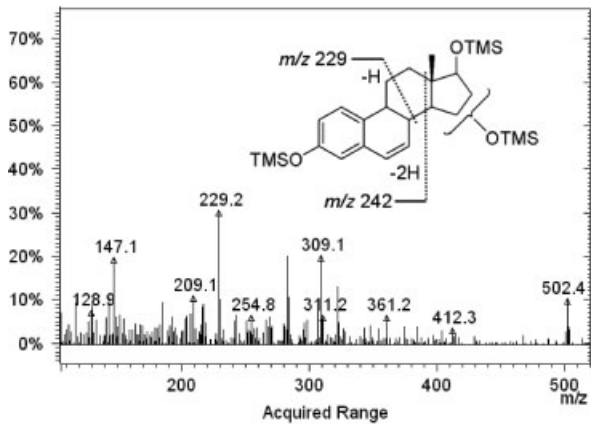

Figure 3c. GC-MS spectrum (13.36 min) of reduced-hydroxy-ATD-tri-TMS artefact following incubation liver equine liver S9 (60 µM).

approach is considered sufficient for detection of the misuse of this steroid.

While the equine metabolism of Superdrol has not previously been reported, two studies in the human have recently been published.[20,22] In one study, Superdrol was found to be extensively metabolized *in vitro*, forming a wide range of products resulting from reduction at position 3 and hydroxylation in positions 2, 12, and 16.[20] In a separate *in vivo* study, however, Superdrol was reported to be excreted as a mixture of 3-reduced and intact parent compound; with the relative proportions of each varying between different subjects.[22] The results of this *in* vivo human are therefore consistent with those obtained *in vitro* in the current study.

Tetrahydrogestrinone (THG)

The LC-HRMS results for THG and its equine metabolites detected in the current study are summarized in Table 9.

GC-MS results are not reported since the TMS derivatization led to a number of artefactual products that meant sensitivity was very poor and interpretation of the data was difficult. The detection of several reduced metabolites is interesting given the general inhibition of reductive metabolism in steroids with extended conjugated systems such as in THG. In general, the reductive metabolism of the A-ring is inhibited in these compounds.[4,19,29,36] However, the responses for the reduced metabolites were only very small in comparison with the major hydroxy-THG metabolites.

In light of the observation that the GC-MS analysis of THG was complicated by poor sensitivity and the production of artefacts, the use of LC-MS-based methods is considered most suitable for use in screening for THG and its metabolites. Since THG and its metabolites are not known to be endogenous in the equine, a qualitative approach is considered sufficient for detection of the misuse of this steroid.

The pharmacokinetics of THG in the equine has been reported previously,[37] but details of its metabolic fate are lacking. The metabolism of THG has, however, been studied in human hepatocytes, where metabolism occurred predominantly through hydroxylation at position 18, but with hydroxylation at position 16 also as a minor pathway.[19]

Conclusion

Because of their ease of availability for purchase on the Internet and because of their often novel structures, designer steroids pose a potential threat to equine sports. The approach presented

herein utilizes a combination of *in vitro* screening and LC-HRMS detection in order to produce metabolic information. LC-HRMS is considered the most valuable overall analytical approach because of its ability to screen for a wide number of analytes and to allow the retrospective analysis of full scan data. However, GC-MS approaches are still required for some compounds with low ESI response.

While many of the metabolites detected during the current study were produced in similar quantities in both S9 and microsomal liver fractions, there were a small number of compounds which could be detected in only the S9 incubations. It is therefore recommended that both microsomal and S9 incubations be carried for such drugs in the future in order to guarantee the widest possible coverage of metabolites.

For seven of the eight steroids studied in the current work, metabolites were produced following *in vitro* incubation. Although the detection of parent compound is often more suitable for analysis of drugs in plasma, oral doses of anabolic-androgenic steroids can often lead to significant concentrations of metabolites in this matrix too, because of first pass metabolism in the liver. Therefore, in the absence of *in vivo* metabolism data, it would seem sensible to utilize the full capabilities of full scan LC-HRMS analysis and screen for parent compound plus its metabolites in both urine and plasma. The only steroid that was not found to be subject to metabolism was 20-OH-E.

A number of the steroids studied have structures that are not known to be endogenous, namely ATD, Halodrol, Xtren, Superdrol, and THG. For these steroids, a purely qualitative demonstration of the presence of these compounds or their metabolites would therefore be considered sufficient to demonstrate their abuse. For adrenosterone, Formestane, and 20-OH-E, however, further studies would be required to determine any 'natural' ranges of these steroids resulting from either endogenous production or dietary intake.

The analytical data produced during this study has subsequently been inserted into routine LC-HRMS and GC-MS screens in our laboratories in order to monitor the possible abuse of these designer steroids. Also, a retrospective analysis of previously acquired LC-HRMS data is currently underway in order to determine whether any abuse has occurred. Since new designer steroid products are entering the Internet market all the time, it is recommended that the current approach be extended to cover other compounds as they become available.

Acknowledgement

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