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Metabolic studies of 1-testosterone in horses

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1-Testosterone (17β-hydroxy-5α-androst-1-en-3-one), a synthetic anabolic steroid, has been described as one of the most effective muscle-building supplements currently on the market. It has an anabolic potency of 200 as compared to 26 for testosterone. Apart from its abuse in human sports, it can also be a doping agent in racehorses. Metabolic studies on 1-testosterone have only been reported for human in the early seventies, whereas little is known about its metabolic fate in horses. This paper describes the studies of *in vitro* and *in vivo* metabolism of 1-testosterone in horses, with the aim of identifying the most appropriate target metabolites to be monitored for controlling the misuse or abuse of 1-testosterone in racehorses.

Six *in vitro* metabolites, namely 5α-androst-1-ene-3α,17β-diol (T1a), 5α-androstane-3β,17β-diol (T2), epiandrosterone (T3), 16,17-dihydroxy-5α-androst-1-ene-3-one (T4 & T5), and 5α-androst-1-ene-3,17-dione (T6), were identified.

For the *in vivo* studies, two thoroughbred geldings were each administered orally with 800 mg of 1-testosterone by stomach tubing. The results revealed that the parent drug and eight metabolites were detected in urine. Besides the four *in vitro* metabolites (T1a, T2, T3, and T5), four other urinary metabolites, namely 5α-androst-1-ene-3β,17α-diol (T1b), 5α-androst-1-ene-3β,17β-diol (T1c), 5α-androstane-3α,17α-diol (T7) and 5α-androstane-3β,17α-diol (T8) were identified. This study shows that the detection of 1-testosterone administration is best achieved by monitoring the parent drug, which could be detected for up to 30 h post-administration. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: 1-testosterone; metabolism; horse; urine; doping control

Introduction

The misuse of anabolic steroids was first identified in human sports in the late 1950s. Since then, new anabolic steroids keep appearing on the black market. In most cases, these substances are marketed on the Internet, and sports regulators become aware of their misuse or abuse when they are confiscated during inspection. 1-Testosterone (17β-hydroxy-5α-androst-1-en-3-one), a synthetic anabolic steroid, has been described as one of the most effective muscle-building supplements for humans currently on the market.[1–3] It closely resembles the natural hormone testosterone, containing a C1,2-double bond instead of a C4,5-double bond. 1-Testosterone can also be viewed as a 5α-reduced version of the hormone boldenone, which cannot aromatize to estrogens. Hence the use of 1-testosterone could enhance muscle development[4] but would not result in undesirable oestrogen-related side effects such as water retention and breast development.[3] Furthermore, it is claimed that the androgenic potency of 1-testosterone is more than seven times greater than that of testosterone considering their normal hepatic metabolic processes.[3,5] 1-Testosterone has been included in the Prohibited List by the World Anti-Doping Agency (WADA) since 2004. Apart from its abuse in human sports, it can also be a doping agent in racehorses. The potential abuse of anabolic steroids in equine sports had been recognized since the late sixties, and the first prosecution for steroid misuse occurred in the UK in 1976. The metabolism of anabolic steroids in racehorses and the analytical approaches for their control in equine sports have recently been reviewed.[6–8] Contributions to the metabolism of a variety of anabolic steroids in horses have been made by the authors' laboratory, including oxymetholone and mestanolone,[9] danazol,[10] clostebol acetate,[11] mesterolone,[12] methonolone acetate,[13] 7-keto-dehydroepiandrosterone,[14] turinabol,[15] and androst-4-ene-3,6,17-trione (6-OXO).[16] Metabolic studies of 1-testosterone have been reported for human since the early seventies,[17,18] as well as an *in vitro* metabolic study in rat,[19] whereas little is known about the metabolic fate of 1-testosterone in horses. This paper describes the studies of *in vitro* and *in vivo* metabolism of 1-testosterone in racehorses, with the aim of identifying the most appropriate target to be monitored for controlling the misuse or abuse of 1-testosterone in racehorses.

Materials and methods

Materials

5α-Androstane-3β,17β-diol (T2), epiandrosterone (3β-hydroxy-5α-androstan-17-one) (T3), 5α-androst-1-ene-3,17-dione (T6), 5α-androstane-3α,17α-diol (T7) and 5α-androstane-3β,17α-diol (T8) were obtained from Steraloids (Newport, RI, USA). 1-Testosterone was purchased from Shanghai Freemen (Shanghai, China). 5α-Androst-1-ene-3α,17β-diol (T1a) and 5α-androst-1-ene-3β,17β-diol (T1c) were synthesized from 1-testosterone by

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reduction with sodium borohydride and cerium trichloride at The Chinese University of Hong Kong. 5α-Androst-1-ene-3β,17α-diol (T1b) was synthesized at The Chinese University of Hong Kong first with a Mitsunobu Reaction[20] of 1-testosterone to obtain 5α-androst-1-en-17α-ol-3-one, followed by reduction with sodium borohydride and cerium trichloride. [16,16,17]-d_3-5α-Androstane-3α,17β-diol (d_3-androstanediol) was synthesized in-house according to the literature.[21] d_3-Testosterone sulfate was obtained from National Measurement Institute (Sydney, Australia). β-Nicotine adenine dinucleotide (β-NAD), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, magnesium chloride ($MgCl_2$), ammonium iodide (NH_4I), dithioerythritol (DTE), ethylenediaminetetraacetic acid (EDTA), phosphate buffer, ammonium iodide (NH_4I), sodium hydroxide (pellets, analytical grade), protease (from bovine pancreases, type I), Tris (TRIZMA®) and pyridine were obtained from Sigma (St Louis, MO, USA). β-Glucuronidase (*E. Coli*) was purchased from Roche (Indianapolis, IN, USA). Anhydrous methanolic hydrogen chloride used for methanolysis was prepared according to the procedures reported previously.[22] Sodium chloride, ammonium sulfate, potassium phosphate, sulfuric acid (96%, SPUR) and potassium chloride (GR grade) were obtained from Merck (Darmstadt, Germany). Anhydrous sodium sulfate was purchased from Uni-CHEM (Guangdong, China). GR grade diisopropyl ether, acetonitrile, chloroform, n-hexane, n-heptane, methanol and ethyl acetate were provided by Merck (Darmstadt, Germany). N-Methyl-N-trimethylsilylfluoroacetamide (MSTFA) and pentafluoropropionic acid anhydride (PFPA) were obtained from Pierce (Rockford, IL, USA). Acetic anhydride was obtained from the International Laboratory Limited (San Bruno, CA, USA). C18 Sep-Pak cartridges (3 ml, 500 mg) and Supel-clean LC-Si cartridges (3 ml) were supplied by Waters (Milford, MA, USA) and Supelco (Bellefonte, PA, USA) respectively. ABS Elut Nexus cartridges (60 mg, 3 ml) were purchased from Varian (Harbor City, CA, USA).

Horse liver microsomes were isolated in-house from fresh horse liver supplied by the Equine Hospital of The Hong Kong Jockey Club. Small pieces of horse liver were homogenized in Tris/KCl buffer (0.05 M, pH 7.4). The homogeneous mixture was centrifuged at 10 000 g for 25 min. Microsomes were isolated from the supernatant by centrifugation at 105 000 g for 1 h. The pellet of microsomes obtained was then washed twice with Tris/KCl buffer (0.05 M, pH 7.4). All the preparation steps and reagents were kept at 4 °C.

Microsomal incubation

For the *in vitro* metabolism study of 1-testosterone, a mixture of freshly prepared horse liver microsomes (30 μl), β-NAD (1.5 mM), glucose-6-phosphate (7.5 mM), magnesium chloride (4.4 mM), EDTA (1 mM) and glucose-6-phosphate dehydrogenase (1 U/ml) in phosphate buffer (pH 7.4, 5 ml) was incubated with 1-testosterone (2 mg) at 37 °C for 2 h with shaking. The reaction was terminated by boiling at 100 °C for 10 min. The mixture was then centrifuged at 2100 g for 10 min. The resulting supernatant was extracted twice with ethyl acetate (5 ml), and the combined organic extract was evaporated to dryness. The residue was analyzed by gas chromatography–mass spectrometry (GC-MS) as the trimethylsilyl (TMS) or pentafluoropropionyl (PFP) derivatives. Control experiments in the absence of either (a) 1-testosterone or (b) microsomes were performed in parallel.

Derivatization for GC-MS analysis

Pentafluoropropionyl (PFP) derivatives were prepared by adding acetonitrile (100 μl) and pentafluoropropionic acid anhydride (30 μl) to the dry residue. The mixture was incubated at 60 °C for 15 min, and then evaporated to dryness at 60 °C under nitrogen. The residue was reconstituted in *n*-heptane (35 μl) for GC-MS analysis.

Trimethylsilyl (TMS) derivatives were prepared by adding MSTFA/NH_4I/DTE (30 μl, 1000:2:4, *v/w/w*) to the dry residue. The mixture was incubated at 60 °C for 15 min. The resulting solution was then injected directly into the GC-MS.

Instrumentation

An Agilent 6890N Network GC system coupled to an Agilent 5973 Network Mass Selective Detector (Agilent Technologies, Santa Clara, CA, USA) was used. For the analysis of TMS derivatives, separation was performed on an HP-1MS (~ 30 m × 0.25 mm, 0.25 μm film thickness) column. The oven temperature was set initially at 60 °C for 1 min, increased to 120 °C at 30 °C/min and held for 3 min, and increased to 320 °C at 15 °C/min, and finally held at 320 °C for 8 min. For the analysis of PFP derivatives, separation was performed on a DB-5MS (~ 30 m × 0.25 mm, 0.25 μm film thickness) column. The oven temperature was set initially at 60 °C for 1 min, increased to 320 °C at 5 °C/min, and finally held at 320 °C for 6 min. For the separation of T1a – T1c, a long GC program was employed and performed on a DB-5MS (~ 30 m × 0.25 mm, 0.25 μm film thickness) column. The oven temperature was set initially at 150 °C for 1 min, increased to 300 °C at 7.5 °C/min, and finally held at 300 °C for 5 min.

A constant helium flow of 1.2 ml/min was used for all analyses. Samples (1 μl each) were injected at 250 °C in splitless mode. All GC-MS analyses were performed in the EI mode with full-scan acquisition (40–600 amu).

Drug administration studies

1-Testosterone (800 mg each) was administered orally to two thoroughbred geldings (489 kg and 519 kg, respectively) by stomach tubing. Pre- and post-administration urine samples were collected for analysis. Urine samples were collected at least twice daily for up to seven days post-administration.

Extraction procedures for identifying Phase 1 metabolites in post-administration urine samples

Enzyme hydrolysis

Urine (3 ml) was spiked with d_3-androstanediol (30 ng) as the internal standard and diluted with potassium phosphate buffer (pH 6.0, 0.1 M, 1 ml). A solution of protease (5 mg/ml, 60 μl) and β-glucuronidase (50 μl, *E. Coli*) was added and the urine sample was incubated at 45 °C for 1 h. The enzyme-treated urine was then diluted with potassium phosphate buffer (pH 6.0, 0.1 M, 1.6 ml) and all loaded onto a C_{18} Sep-Pak cartridge, which had been pre-washed with methanol (5 ml) and deionized water (5 ml × 2). The cartridge was rinsed with deionized water (5 ml × 2) and *n*-hexane (5 ml), then eluted with methanol (3 ml). The eluate was evaporated to dryness at 60 °C under nitrogen. Diisopropyl ether (3 ml) was added and the mixture was transferred to a 15-ml graduated centrifuge tube containing NaOH/NaCl (1 M/0.15 M, 2 ml). The organic extract was then passed through an anhydrous sodium

sulfate drying tube and evaporated to dryness under nitrogen at 60 °C. The residue was reconstituted in ethyl acetate (50 μl) and loaded onto a Supel-clean LC-Si normal-phase extraction cartridge, which had been pre-conditioned with chloroform/ethyl acetate (3 ml; 1:1, *v/v*). The cartridge was eluted with chloroform/ethyl acetate (3 ml; 1:1, *v/v*). The first 0.5 ml eluate was discarded. The next 2.5 ml of eluate was collected and evaporated to dryness under nitrogen at 60 °C. The dried residue was derivatized as TMS or PFP derivatives for GC-MS analysis.

Methanolysis

Urine (5 ml) was spiked with d_3-androstanediol (50 ng) as the internal standard and loaded onto C_{18} Sep-Pak cartridge, which had been pre-washed with methanol (5 ml) and deionized water (5 ml × 2). The cartridge was rinsed with deionized water (5 ml × 2) and *n*-hexane (5 ml), then eluted with methanol (3 ml). The eluate was evaporated to dryness at 60 °C under nitrogen. Anhydrous methanolic hydrogen chloride (1 M, 0.5 ml)[22] was added and the solution was heated at 60 °C for 10 min. Diisopropyl ether (3 ml) was added and the mixture was transferred to a 15-ml graduated centrifuge tube containing NaOH/NaCl (1 M/0.15 M, 2 ml). The mixture was then vortexed for 0.5 min and centrifuged at 1500 g for 0.5 min. The organic layer was passed through an anhydrous sodium sulfate drying tube, and then evaporated to dryness at 60 °C under nitrogen. The residue was reconstituted in ethyl acetate (50 μl) and loaded onto a Supel-clean LC-Si normal-phase extraction cartridge, which had been pre-conditioned with chloroform/ethyl acetate (3 ml; 1:1, *v/v*). The cartridge was eluted with chloroform/ethyl acetate (3 ml; 1:1, *v/v*). The first 0.5 ml eluate was discarded. The next 2.5 ml of eluate was collected and evaporated to dryness under nitrogen at 60 °C. The dried residue was derivatized as TMS or PFP derivatives for GC-MS analysis.

Extraction procedures for Phase II metabolism studies

Unconjugated steroids

Urine (5 ml) was adjusted to pH 6.8 and extracted with ethyl acetate (5 ml). The organic layer was base-washed (NaOH/ NaCl, 1 M/0.15 M, 4 ml) and centrifuged (1500 g for 0.5 min). The organic layer was passed through an anhydrous sodium sulfate drying tube, and then evaporated to dryness under nitrogen at 60 °C. The dried residue was derivatized as TMS or PFP derivatives for GC-MS analysis.

Glucuronide-conjugated steroids

The remaining aqueous portion was then adjusted to pH 6.4 and incubated for 1 h at 45 °C with β-glucuronidase from *E. Coli* (50 μl). The enzyme-hydrolysed steroids were extracted according to the procedures for the unconjugated steroids. The dried residue was derivatized as TMS or PFP derivatives for GC-MS analysis.

Sulfate and sulfate-glucuronide conjugated steroids

The remaining sulfate and sulfate-glucuronide conjugates in the aqueous layer after enzyme hydrolysis were extracted using SPE (C_{18} Sep-Pak cartridge), and eluted with a solvolysis reagent (ethyl acetate:methanol:conc. H_2SO_4, 100:20:0.2, *v/v/v*). The eluate was incubated at 55 °C for 2 h. NaOH/NaCl (1 M/0.15 M, 2 ml) was then added. The mixture was vortexed for 0.5 min. The organic layer was passed through an anhydrous sodium sulfate drying tube and evaporated to dryness under nitrogen at 60 °C. The dried residue was derivatized either as the TMS or PFP derivatives for GC-MS analysis.

Quantification of 1-testosterone in post-administration urine

The concentrations of total 1-testosterone (free and conjugated) in post-administration urine samples were quantified as follows. A calibrator (60 ng/ml 1-testosterone), a quality control urine sample (15 ng/ml 1-testosterone), and a blank urine sample were prepared and analyzed with selected post-administration urine samples. Urine (6 ml) was spiked with d_3-testosterone sulfate (585 ng, equivalent to 360 ng free d_3-testosterone) as the internal standard and transferred to a 15-ml graduated centrifuge tube containing about 1.5 g ammonium sulfate. The mixture was vortexed and centrifuged at around 1500 g for 10 min; 5 ml of this mixture was then loaded onto an ABS Elut Nexus cartridge. The cartridge was washed with deionized water (3 ml) and *n*-hexane (3 ml), and then eluted with chloroform (2 ml) and methanol/ethyl acetate (5:95, *v/v*, 3 ml). The combined eluate was evaporated to dryness at 60 °C under nitrogen. Anhydrous methanolic hydrogen chloride (1 M, 0.5 ml)[22] was added and the solution was heated at 60 °C for 10 min. Diisopropyl ether (3 ml) was added and the mixture was transferred to a 15-ml graduated centrifuge tube containing NaOH/NaCl (1 M/0.15 M, 2 ml). The mixture was then vortexed for 0.5 min and centrifuged at around 1500 g for 0.5 min. The organic layer was passed through an anhydrous sodium sulfate drying tube, and then evaporated to dryness at 60 °C under nitrogen. The dried residue was derivatized as PFP derivatives for GC-MS analysis.

A 1-level internal standard calibration curve was set up using the Agilent ChemStation software based on the peak area ratios of 1-testosterone to d_3-testosterone (internal standard). The concentrations of 1-testosterone in post-administration urine samples were quantified using this calibration curve. The measured concentrations of the quality control samples must give a deviation of less than ±20 % from its theoretical value. The limit of detection was estimated to be 5 ng/ml (based on signal-to-noise ratio greater than 3).

Results and discussion

In vitro biotransformation studies

Six metabolites, namely 5α-androst-1-ene-3α,17β-diol (T1a), 5α-androstane-3β,17β-diol (T2), epiandrosterone (T3), 16, 17-dihydroxy-5α-androst-1-en-3-one (T4 and T5), and 5α-androst-1-ene-3,17-dione (T6), were detected in the *in vitro* biotransformation experiment with 1-testosterone. None of these metabolites were detected in the control experiments in the absence of either 1-testosterone or microsomes. Their structures are shown in Figure 1. Of these metabolites, T1a, T2, T3, and T6 were confirmed with authentic reference standards. The TMS derivative of T1a (Figure 2a) has a molecular ion at *m/z* 434, representing the bis-TMS derivative of reduced 1-testosterone. The mass spectrum of T6, a characteristic metabolite of 1-testosterone in human,[18] is shown in Figure 3.

T4 and T5 were tentatively identified by mass spectral interpretation as a pair of 16,17-dihydroxy-5α-androst-1-en-3-one epimers, which have not been reported previously as metabolites of 1-testosterone in any species. Both TMS derivatives of T4 and T5 showed a molecular ion at *m/z* 448, indicating that they could be mono-hydroxylated 1-testosterone. An interpretation of the

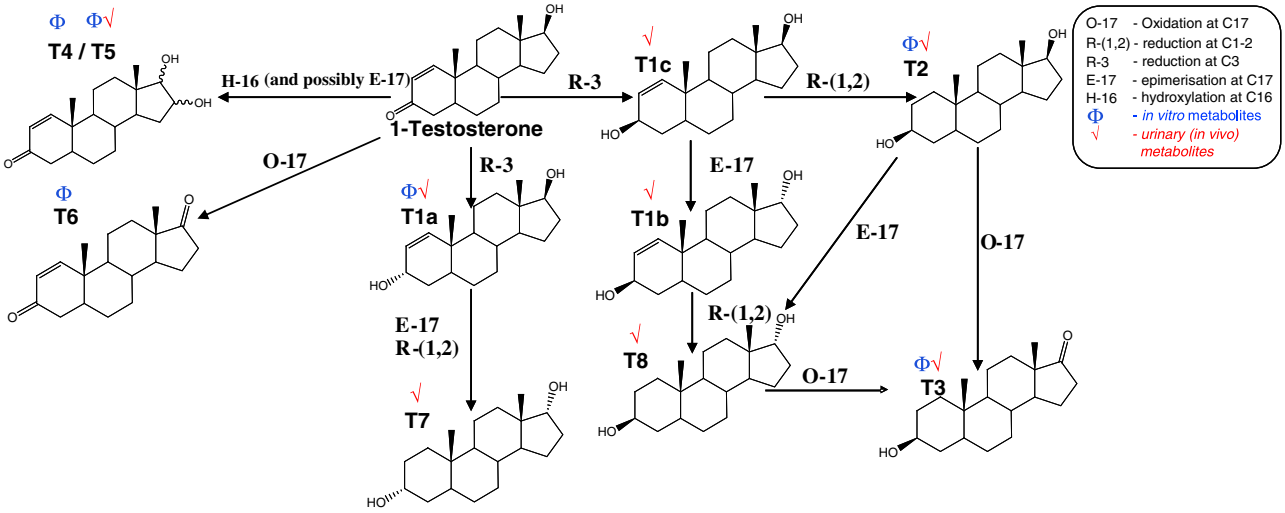

Figure 1. Proposed metabolic pathway of 1-testosterone in the horse.

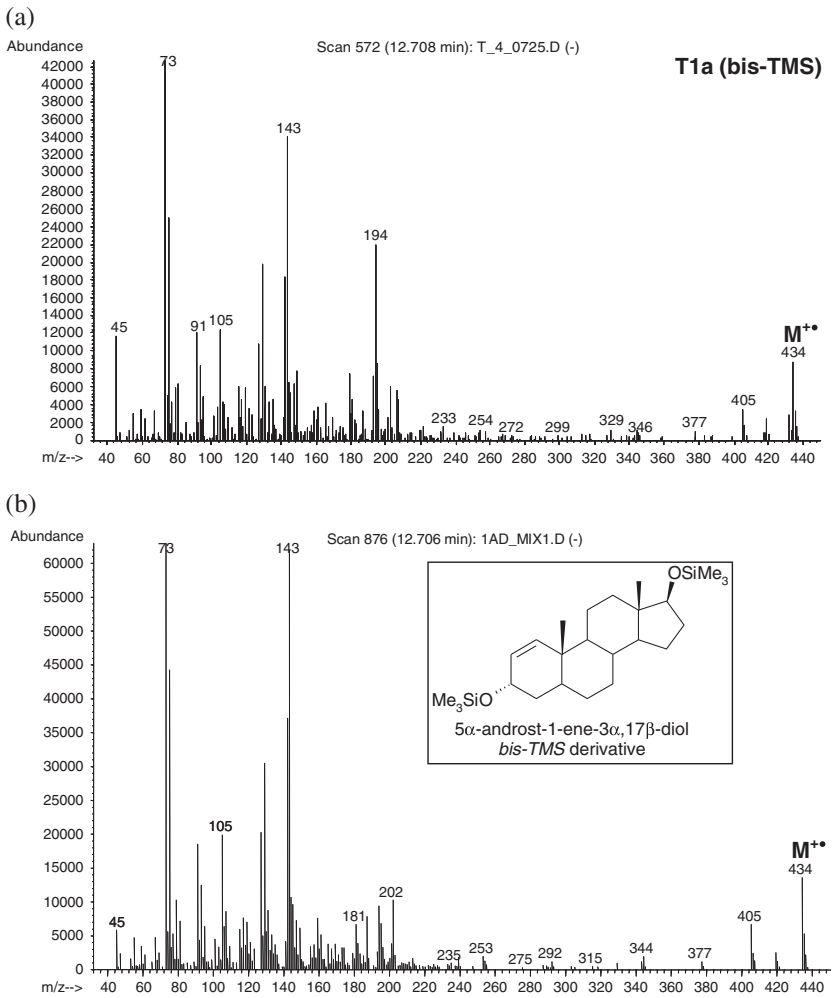

Figure 2. EI mass spectra of the bis-TMS derivative of 5α-androst-1-ene-3α,17β-diol (T1a) from (a) the incubation mixture of 1-testosterone with horse liver microsomes; (b) reference standard of 5α-androst-1-ene-3α,17β-diol.

mass spectrum of the bis-TMS derivative of 16,17-dihydroxy-5α-androst-1-en-3-one is shown in Figure 4. The presence of a significant *m/z* 147 ion, $(CH_3)_3Si{-}O^+{=}Si(CH_3)_2$, presumably formed from two trimethylsilyloxy moieties in close proximity through a cyclic oxonium ion intermediate,[23,24] suggested that the hydroxylation site was close to C17. Possible hydroxylation sites include

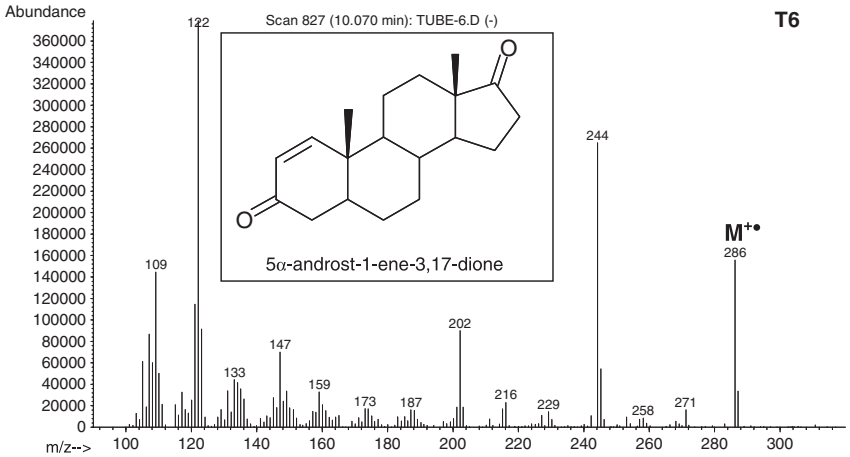

Figure 3. EI mass spectrum of 5α-androst-1-ene-3,17-dione (T6) from the incubation mixture of 1-testosterone with horse liver microsomes.

C12, C15, C16, and C18. However, as the mass spectra of the bis-TMS derivatives of these two isomers closely resembles that of 16α,17β-dihydroxyandrost-4-en-3-one from the NIST mass spectral library (Figure 5),[25] their hydroxylation position was tentatively assigned at C16. The absolute configurations of the two hydroxyl groups at C16 and C17 have not been determined.

Oral administration studies: (i) Phase I metabolism

Urine samples from the oral administration of 1-testosterone were collected for up to seven days post-administration and analysed for 1-testosterone and its metabolites. The parent drug and eight metabolites (T1a–T1c, T2, T3, T5, T7, and T8) were detected (Figure 1).

The *in vivo* metabolites T1a, T2, T3 and T5 were also found in the *in vitro* studies. 5α-Androst-1-ene-3β,17α-diol (T1b), 5α-androst-1-ene-3β,17β-diol (T1c), 5α-androstane-3α,17α-diol (T7) and 5α-androstane-3β,17α-diol (T8) were only detected in post-administration urine samples. T1b and T1c, together with their stereoisomer T1a, have not been reported previously as urinary metabolites of 1-testosterone in any species. These stereoisomers have similar bis-TMS mass spectra, and their identities were confirmed with reference standards synthesized (Figures 6 and 7). T1a–T1c could be separated by GC-MS using a long GC program (Figure 8). 5α-Androst-1-ene-3α,17α-diol, another stereoisomer of T1a–T1c, was not observed in both *in vitro* and *in vivo* studies. The absolute configurations of T1a–T1c could be further substantiated from the elution order of their bis-TMS derivatives on methylsilicone phase by comparing the elution order of bis-TMS derivatives of reference standards of the four 5α-androstane-3,17-diols with similar structures. The elution order (with increasing retention time) for the four reference standards is 3α,17α-diol 3α,17β-diol < 3β,17α-diol < 3β,17β-diol. Similar observations of

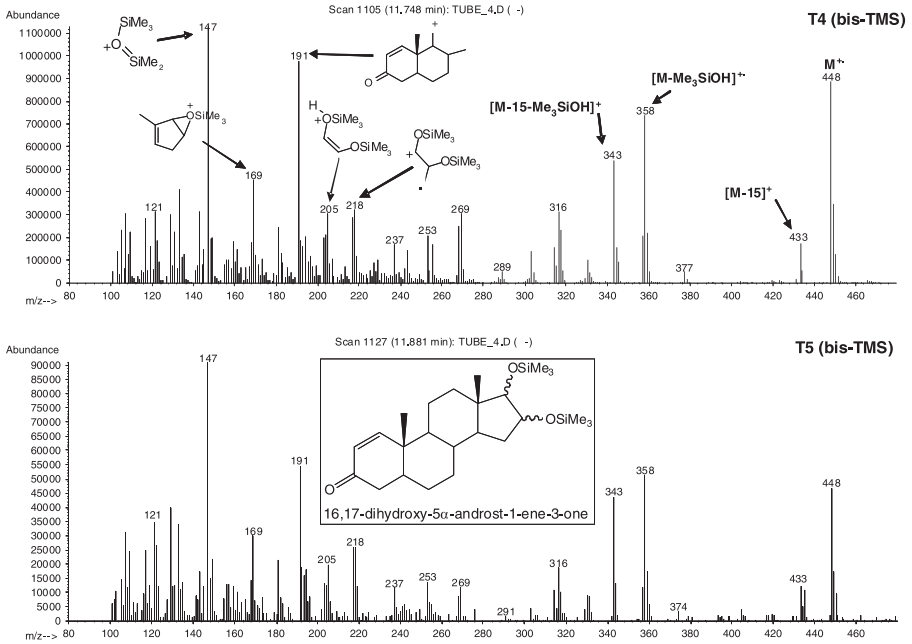

Figure 4. EI mass spectra of the bis-TMS derivatives of two 16,17-dihydroxy-5α-androst-1-en-3-one (T4 and T5) from the incubation mixture of 1-testosterone with horse liver microsomes.

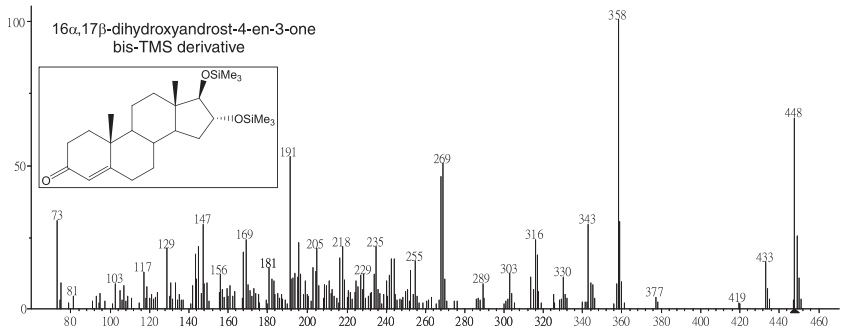

Figure 5. Abbreviated EI mass spectrum of the bis-TMS derivative of 16α,17β-dihydroxyandrost-4-en-3-one from the NIST Mass Spectral Library.

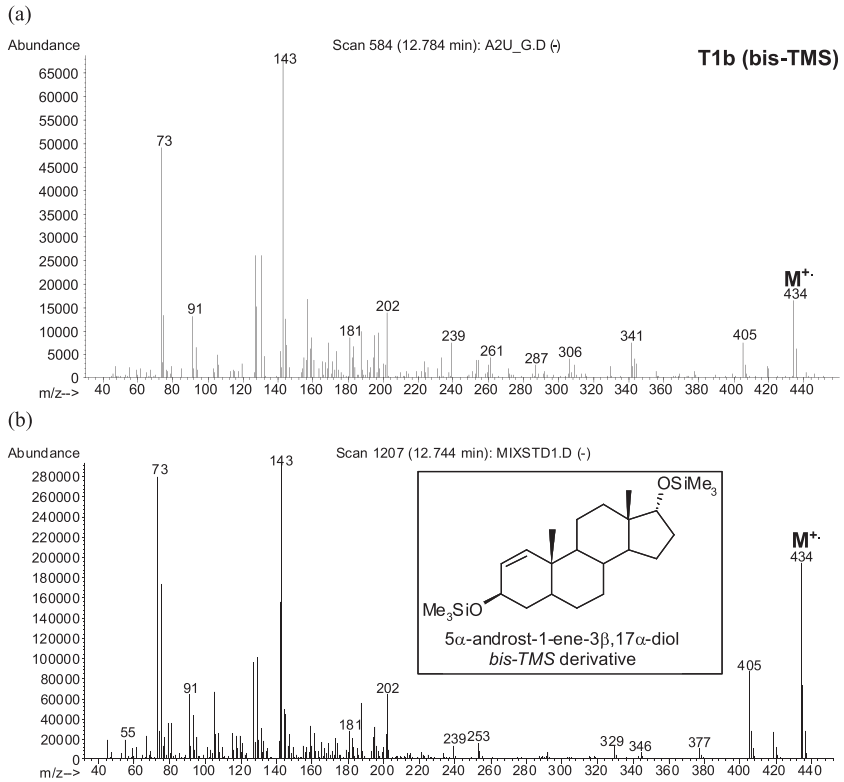

Figure 6. EI mass spectra of the bis-TMS derivative of 5α-androst-1-ene-3β,17α-diol (T1b) from (a) a post-administration urine sample; (b) reference standard of 5α-androst-1-ene-3β,17α-diol.

the GC elution order of steroid stereoisomers with hydroxyl groups at both C3 and C17 have also been reported by other research groups.[26–29] The identities of T7 and T8 were confirmed with commercially available reference standards.

A proposed metabolic pathway for 1-testosterone in the horses is shown in Figure 1. The major phase I metabolic processes of 1-testosterone were reduction of the 3-keto group to yield T1a and T1c and hydroxylation at C16 to give T5. T1b was formed possibly from epimerization of the 17-hydroxy group of T1c. The formation of T8 and T2 involved reduction of T1b and T1c respectively at the C1,2-double bond. Oxidation at C17 of T8 or T2 could lead to T3. The formation of T7 involved epimerization of the 17-hydroxyl group of T1a and reduction of its C1,2-double bond.

Oral administration studies: (ii) Phase II metabolism

To study the Phase II metabolism of 1-testosterone, the unconjugated, glucuronide-conjugated, sulfate and sulfate-glucuronide conjugated metabolites were isolated and deconjugated by sequential treatment with glucuronidase and solvolysis. The results are summarized in Table 1. The parent drug, T1a and T5 were found to be excreted as sulfates (or sulfate-glucuronides), while T1b and T1c were conjugated as glucuronides. The other four *in vivo* metabolites (T2, T3, T7, and T8) were found in both the glucuronide and sulfate fractions (see Table 1 for the major form of conjugation). T1a–T1c were found to be unstable upon methanolysis.

Oral administration studies: (iii) Analytes of choice for screening

One of the objectives of this study was to identify screening targets for controlling the misuse of 1-testosterone in racehorses. The longest detection periods of 1-testosterone and its metabolites in post-administration urine by GC-MS after enzyme hydrolysis, methanolysis, or solvolysis are thus summarized in

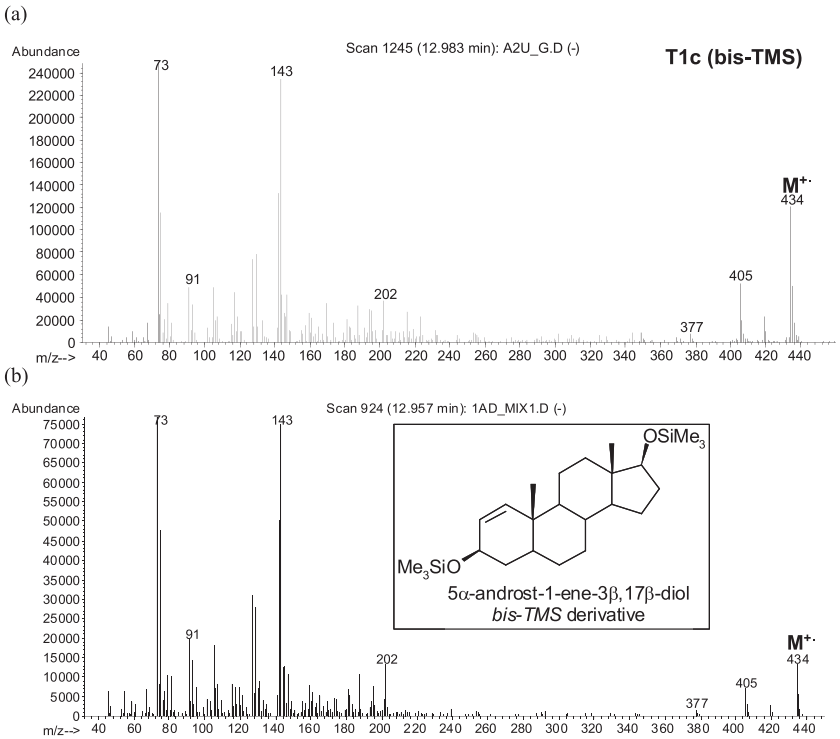

Figure 7. EI mass spectra of the bis-TMS derivative of 5α-androst-1-ene-3β,17β-diol (T1c) from (a) a post-administration urine sample; (b) reference standard of 5α-androst-1-ene-3β,17β-diol.

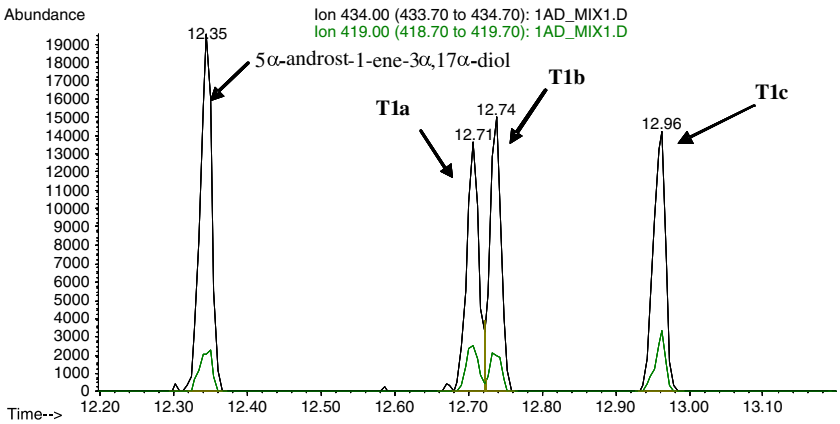

Figure 8. Extracted-ion chromatograms of bis-TMS derivatives of four reference standards of 5α-androst-1-ene-3,17-diols (a longer GC programme on a DB-5MS column was used for resolving these stereosiomers).

Table 1. Detection periods by GC-MS and the major form of conjugation for 1-testosterone and its urinary metabolites

Metabolite	Horse A	Horse B	Major Form of Conjugation
1-Testosterone	30 h	30 h	Sulfate
5α-Androst-1-ene-3α,17β-diol (T1a)	2 h	3 h	Sulfate
5α-Androst-1-ene-3β,17α-diol (T1b)	2 h	3 h	Glucuronide
5α-Androst-1-ene-3β,17β-diol (T1c)	2 h	3 h	Glucuronide
5α-Androstane-3β,17β-diol (T2)	51 h	72 h	Sulfate
Epiandrosterone (T3)	30 h	30 h	Sulfate
16,17-Dihydroxy-5α-androst-1-en-3-one (T5)	6 h	6 h	Sulfate
5α-Androstane-3α,17α-diol (T7)	30 h	Not detected	Glucuronide
5α-Androstane-3β,17α-diol (T8)	47 h	30 h	Glucuronide

Table 1. T1a–T1c and T5 all contained a double bond at the C1,2 position, and were therefore urinary metabolites characteristic of 1-testosterone administration. However, their detectable times after oral administration of 800 mg of 1-testosterone were relatively short (2–6 h post-administration). While 5α-androstane-3β,17β-diol (T2) could be detected for up to 72 h in one horse, it is not a diagnostic target for 1-testosterone administration as it is also a metabolite of other anabolic steroids such as testosterone. The detection of 1-testosterone administration is best achieved by monitoring the parent drug. The peak concentrations (5.7–6.4 μg/ml) of 1-testosterone were observed at 1.7–2.8 h post-administration, and it could be detected at around 5–9 ng/ml for up to 30 h post-administration.

Conclusion

We have studied the *in vitro* biotransformation of 1-testosterone using horse liver microsomes, and the *in vivo* metabolism of 1-testosterone in horses. For the *in vitro* study, six metabolites, namely 5α-androst-1-ene-3α,17β-diol (T1a), 5α-androstane-3β, 17β-diol (T2), epiandrosterone (T3), two 16,17-dihydroxy-5α-androst-1-en-3-one (T4 & T5), and 5α-androst-1-ene-3,17-dione (T6), were detected. For the drug administration study, the parent drug and eight urinary metabolites were observed. These include T1a, T2, T3, and T5 (also identified *in vitro*) as well as 5α-androst-1-ene-3β,17α-diol (T1b), 5α-androst-1-ene-3β,17β-diol (T1c), 5α-androstane-3α,17α-diol (T7) and 5α-androstane-3β,17α-diol (T8). A metabolic pathway for 1-testosterone in horses has been postulated. The major biotransformation pathways observed for 1-testosterone in horses were: hydroxylation at the C16, reduction of A-ring enone group, and oxidation and epimerization at C17. The parent drug could be detected for up to 30 h post-administration and is the preferred target for the identification of 1-testosterone administration in horses.

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