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Metabolism of methandrostenolone in the horse: a gas chromatographic–mass spectrometric investigation of phase I and phase II metabolism

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Abstract

The phase I and phase II metabolism of the anabolic steroid methandrostenolone was investigated following oral administration to a standardbred gelding. In the phase I study, metabolites were isolated from the urine by solid-phase extraction, deconjugated by acid catalysed methanolysis and converted to their *O*-methyloxime trimethylsilyl derivatives. GC–MS analysis indicated the major metabolic processes to be sequential reduction of the A-ring and hydroxylation at C6 and C16. In the phase II study, unconjugated, β-glucuronidated and sulfated metabolites were fractionated and deconjugated using a combination of liquid–liquid extraction, enzyme hydrolysis, solid-phase extraction and acid catalysed methanolysis. Derivatization followed by GC–MS analysis revealed extensive conjugation to both glucuronic and sulfuric acids, with only a small proportion of metabolites occurring in unconjugated form.

Keywords: Methandrostenolone; Steroids

1. Introduction

The anabolic steroid methandrostenolone (1) was first synthesized in 1955 by Vischer et al. [1], and in recent years has featured prominently in association with both human and equine sports. Under the current Australian rules of racing, anabolic steroids may be administered while spelling or training as long as they are clear of the horse's system by the time it is presented to race. The use of these drugs thus entails a certain element of risk, particularly in the case of the traditional injectable steroids, which tend to have long and unpredictable excretion profiles. As a result many trainers have turned to the use of shorter acting orally administered steroids, which typically have much more predictable excretion characteristics. Methandrostenolone is usually administered to horses by injection, but does possess oral activity and can also be given in the form of a paste, powder or drench. It is in this context that the development of appropriate doping control procedures for methandrostenolone is of greatest interest to Australian racing authorities.

The in vivo metabolism of methandrostenolone

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has previously been studied in humans [2–9], rats [10,11], rabbits [12] and horses [13–15]. Much of the human work has concentrated on the identification of unconjugated urinary metabolites, and the most important metabolic processes observed in this fraction are 6β-hydroxylation and 17-epimerisation. The latter has been the subject of some debate, but is now considered to arise from the spontaneous hydrolysis of 17β-sulfate conjugates in urine [13,16]. Additional processes observed for conjugated metabolites include 16-hydroxylation and sequential reduction of the dienone function on the A-ring. In the rat, a series of 17α-methylandrostanediols were reported as faecal and urinary metabolites, but without 17-epimerisation or additional hydroxylation. The rabbit study reported a similar range of metabolic processes to humans, but again with the notable exception of 17-epimerisation.

The equine metabolism of methandrostenolone has been reported to involve 6-hydroxylation, 16-hydroxylation, 17-epimerisation and reduction of the Δ^4-double bond. However these reports were based on urine samples obtained following administration by intramuscular injection, and restrict their examination to unconjugated metabolites exclusively. No work has been published concerning the equine metabolism of methandrostenolone following oral administration, and the significance of its phase II metabolism has not been examined at all. The aim of this investigation was to examine the equine urinary metabolites of methandrostenolone following oral administration with a view to identifying suitable targets for doping control analysis.

2. Experimental

2.1. Steroids

Methandrostenolone, 17α-methyl-5α-androstane-3α,17β-diol, 17α-methyl-5α-androstane-3β,17β-diol and 17β-hydroxy-17α-methyl-5β-androstan--3-one were purchased from Steraloids (Newport, RI, USA). 17α-Methyl-5β-androstane-3α,17β-diol and 17α-methyl-5β-androstane-3β,17β-diol were synthesized from 17β-hydroxy-17α-methyl-5β-androstan-3-one by the procedure of Schänzer and Donike [17].

2.2. Reagents and chemicals

Methoxylamine hydrochloride and *Escherichia coli* β-glucuronidase enzyme were purchased from Sigma (Castle Hill, NSW, Australia), *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) and *N*-trimethylsilylimidazole (TMSI) were purchased from Pierce (Rockford, IL, USA), and C_{18} Sep-Pak Vac solid-phase extraction cartridges were purchased from Waters (Rydalmere, NSW, Australia). Anhydrous methanolic hydrogen chloride solution (1 *M*) was prepared by dropwise addition of acetyl chloride (21.3 ml) to anhydrous methanol (300 ml) with stirring and cooling. All solvents were redistilled before use.

2.3. Apparatus

Solid-phase extractions were performed using a Zymark (Hopkinton, MA, USA) Benchmate I laboratory robot. GC–MS data were obtained on a Shimadzu (Rydalmere, NSW, Australia) QP-5000 instrument equipped with an AS-1400 autosampler. The column was an SGE (Ermington, NSW, Australia) BPX-5 capillary column (12 m×0.22 mm I.D., 0.33 μm film thickness). The carrier gas was helium (99.999%) purchased from BOC Gases (North Ryde, NSW, Australia).

2.4. Sample preparation

2.4.1. Animal administration

An aqueous suspension of methandrostenolone (200 mg) was administered to a standardbred gelding (aged, 550 kg) by stomach tube. Urine samples were collected prior to administration, then at 2 hourly intervals for 8 h and at 24 hourly intervals for 5 days. All samples were immediately frozen and stored at −20°C. The trial was approved by the New South Wales Thoroughbred Racing Board Animal Care and Ethics Committee.

2.4.2. Extraction and hydrolysis procedure – phase I metabolic study

Aliquots of urine (5 ml) were adjusted to pH 6.8 and centrifuged to sediment particulate matter. The supernatant phase was decanted, diluted with water (5 ml) and loaded onto a C_{18} solid-phase extraction

cartridge (500 mg, 3 ml) which had previously been primed with methanol (5 ml) and water (5 ml). The cartridge was rinsed with water (5 ml), dried briefly under pressure, rinsed with hexane (5 ml), dried again and finally eluted with methanol (5 ml). The eluates were dried by evaporation at 75°C under a stream of nitrogen. Steroid conjugates were cleaved by incubating for 15 min at 60°C with anhydrous methanolic hydrogen chloride solution (1 *M*, 0.5 ml) as described by Tang and Crone [18]. The reaction was quenched by addition of sodium hydroxide solution (2 *M*, 5 ml) and the resulting mixture extracted with diisopropyl ether (5 ml). The extracts were dried by evaporation at 75°C under a stream of nitrogen.

2.4.3. Extraction and hydrolysis procedure – phase II metabolic study

Aliquots of urine (5 ml) were adjusted to pH 6.8 and unconjugated steroids were isolated by extraction with diisopropyl ether (5 ml). Residual ether was removed from the extracted urine by brief evaporation under a stream of nitrogen, after which the urine was incubated overnight at 37°C with *E. coli* β-glucuronidase enzyme (1000 U) to cleave β-glucuronide conjugates. The cleaved steroids were isolated by extraction with diisopropyl ether (5 ml), and after removal of residual ether the remaining sulfate conjugates were isolated by solid-phase extraction as described previously. The unconjugated and glucuronide fractions were further purified by washing with sodium hydroxide solution (2 *M*, 5 ml), while the sulfate fraction was treated with anhydrous methanolic hydrogen chloride solution and extracted as described previously. All three sets of extracts were dried by evaporation at 75°C under a stream of nitrogen.

2.4.4. Derivatization procedure

Dried residues were reconstituted in a solution of methoxylamine hydrochloride in pyridine (2%, w/v, 50 μl) and incubated for 30 min at 80°C. The reaction was terminated by evaporation at 75°C under a stream of nitrogen, and the dried residues were reconstituted in a solution of TMSI in MSTFA (2%, v/v, 50 μl) and incubated for a further 60 min at 80°C. Excess MSTFA was removed by evaporation at 75°C under a stream of nitrogen and the silylated residues were reconstituted in ethyl acetate (100 μl) for GC–MS analysis.

2.4.5. GC–MS analysis

Sample injections were made in split/splitless mode with an injector temperature of 250°C. The column was initially held at 150°C for 1 min, then ramped at 15°C/min to 300°C and held for a further 4 min. Head pressure was programmed to maintain a constant flow-rate of 0.5 ml/min and the purge valve remained off for 1 min before opening to a split of 50:1. The MS transfer line was set at 275°C and ionization energy at 70 eV. Full scan electron ionization mass spectra were obtained over the range 100–700 u with a scan rate of 1000 u/s.

3. Results and discussion

The electron ionization mass spectra of the trimethylsilylated derivatives of a number of human methandrostenolone metabolites have been examined in detail by Dürbeck and Büker [6]. As is characteristic of 17-methyl-17-trimethylsiloxysteroids, many of these spectra were found to be dominated by a D-ring fragment at *m/z* 143. The mechanism of formation of this fragment was first investigated by Middleditch et al. [19], and was shown to involve a breaking of the C13–C17 and C14–C15 bonds with concomitant transfer of a hydrogen atom from C16 to C13. A less intense *m/z* 130 fragment can be explained by a similar mechanism where the C13–C17 and C15–C16 bonds are broken and the hydrogen is transferred from C14 to C13 (Fig. 1).

Under the same conditions 17-methyl-16,17-bis-(trimethylsiloxy)steroids show a similar pair of intense ions at *m/z* 218 and 231, although their relative intensities are reversed. Massé et al. [20] demonstrated that each of these fragments contains both of the D-ring trimethylsiloxy groups, and thus is explicable in terms of the aforementioned mechanisms. Additional ions characteristic of silylated 17-methyl-16,17-dihydroxysteroids include *m/z* 117 and 147. The former derives from C17 together with its substituents and a hydrogen transferred from C14 [15], while the latter is formed via a cyclic oxonium ion intermediate from the vicinal trimethylsiloxy groups [21].

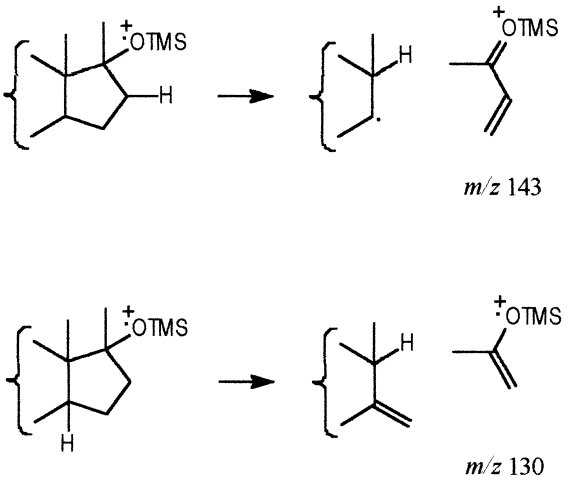


Fig. 1. D-Ring fragmentations of 17-methyl-17-trimethylsiloxy-steroids.

Given that the most important metabolic processes known to affect the D-ring of methandrostenolone are 16-hydroxylation and 17-epimerisation, a search for peaks featuring a strong *m/z* 143 or 218 fragment should provide a reasonably comprehensive survey of its major metabolites. A number of possible metabolites were identified in this way, and the most abundant were characterized by their mass spectra. A typical GC trace is shown (Fig. 2), and a full list of proposed metabolites appears in Table 1. The highest concentrations of most metabolites were observed from 2 to 24 h post-administration, with no significant concentrations being detected beyond 72 h.

3.1. Methandrostenolone (1) and 17-epimethandrostenolone (2)

A very weak peak was identified as methandrostenolone by comparison with an authentic standard. It appeared from 2 to 8 h post-administration and was present in the urine as a mixture of sulfate and glucuronide conjugates. An earlier eluting peak with an identical mass spectrum was assumed to be the 17-epimer. It too appeared from 2 to 8 h, but was unconjugated, which is consistent with its proposed origin by hydrolysis of methandrostenolone sulfate. Both peaks were extremely weak in comparison to the other identified metabolites.

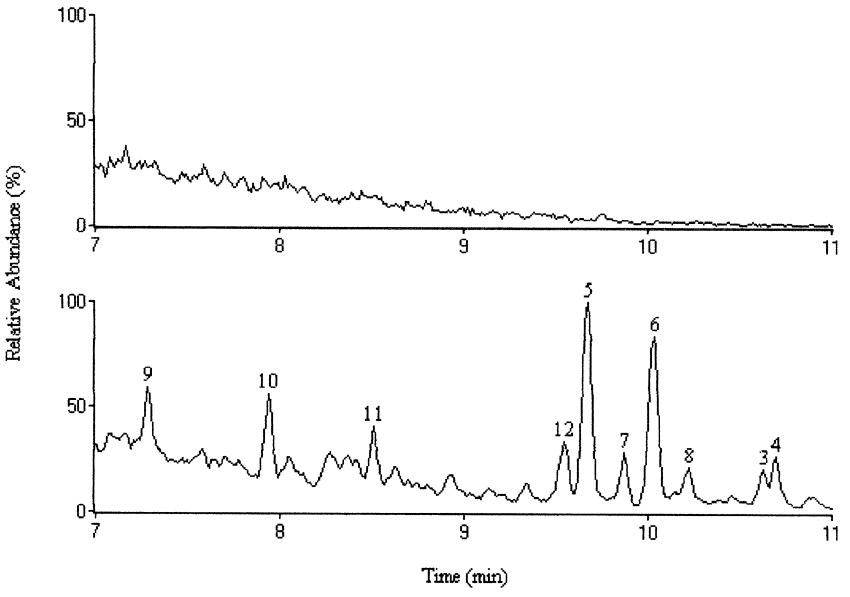
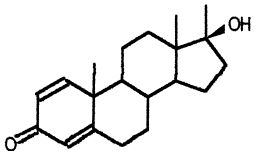
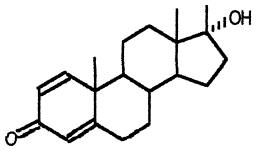
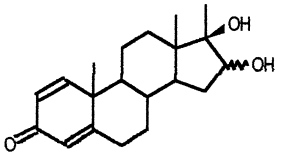
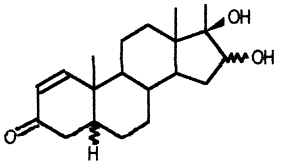
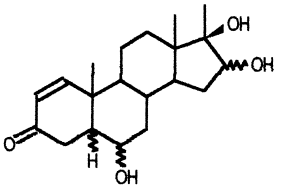
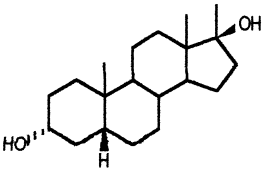
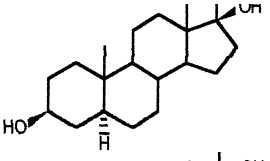
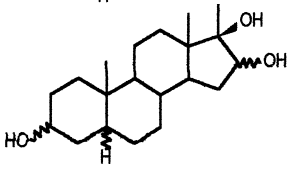


Fig. 2. Summed multi-ion chromatograms for *m/z* 143 and 218 from control (top) and 4 h post-administration (bottom) urine. Metabolite peaks are numbered as mentioned in the text. Compounds 1 and 2 are too weak to be seen in this trace.

Table 1
Methandrostenolone and its equine urinary metabolites

Compound	Structure	Relative retention time[a]	Base peak[b]	Conjugation[c]
1		1.000	143	G,S
2		0.970	143	F
3		1.433	218	S
4		1.440	218	S
5		1.307	218	G,S
6		1.353	218	G,S
7		1.332	218	G,S
8		1.376	218	G,S
9		0.992	143	G,S
10		1.078	143	G,S
11		1.152	218	G,S
12		1.290	218	G,S

[a] Retention times are for the derivatized compounds and are relative to derivatized methandrostenolone (1) (7.4 min).
[b] Base peaks are for the derivatized compounds.
[c] F, G and S indicate free steroids, β-glucuronide conjugates and sulfate conjugates, respectively.

3.2. 16,17β-Dihydroxy-17α-methylandrosta-1,4-dien-3-ones (3, 4)

16-Hydroxylation is an important equine metabolic pathway for 17α-alkyl anabolic steroids [15,22–26], and a pair of 16-hydroxymethandrostenolone metabolites was detected in this study. The mass spectra showed *m/z* 489 (M^+), 474 ($M-CH_3$), 458 ($M-OCH_3$), 399 (M−TMSOH), 384 (M−TMSOH−CH_3), 368 (M−TMSOH−OCH_3), 309 (M−2xTMSOH), 294 (M−2xTMSOH−CH_3), 278 (M−2xTMSOH−OCH_3), 231 (D-ring), 218 (D-ring), 147 (D-ring) and 117 (D-ring) (Fig. 3a). Both were sulfated in the urine and were detectable from 2 to 8 h post-administration. Edlund et al. [13] also observed two isomers of this structure in the horse and suggested that one was the 17-epimer of the other. However given the requirement that 17-epimers are desulfated at C17, the extensive conjugation observed in this study makes this seem improbable. A more likely hypothesis is epimeric hydroxylation at C16, with the two isomers being 16α,17β-dihydroxy-17α-methylandrosta-1,4-dien-3-one and 16β,17β-dihydroxy-17α-methylandrosta-1,4-dien-3-one. The latter has been confirmed as a methandrostenolone metabolite in the rabbit [12].

3.3. 16,17β-Dihydroxy-17α-methylandrost-1-en-3-ones (5, 6)

The in vivo reduction of the A-ring of 4-en-3-one anabolic steroids is well established, with formation of the saturated alcohol being the most common consequence. However the presence of a Δ^1-double bond is known to inhibit the mechanism, and leads to the formation of a variety of partially reduced metabolites [7]. In the horse, Hagedorn et al. [15] reported reduction of the Δ^4-double bond only, and Δ^4-reduced compounds constituted an abundant class of metabolites in this study as well. In conjunction

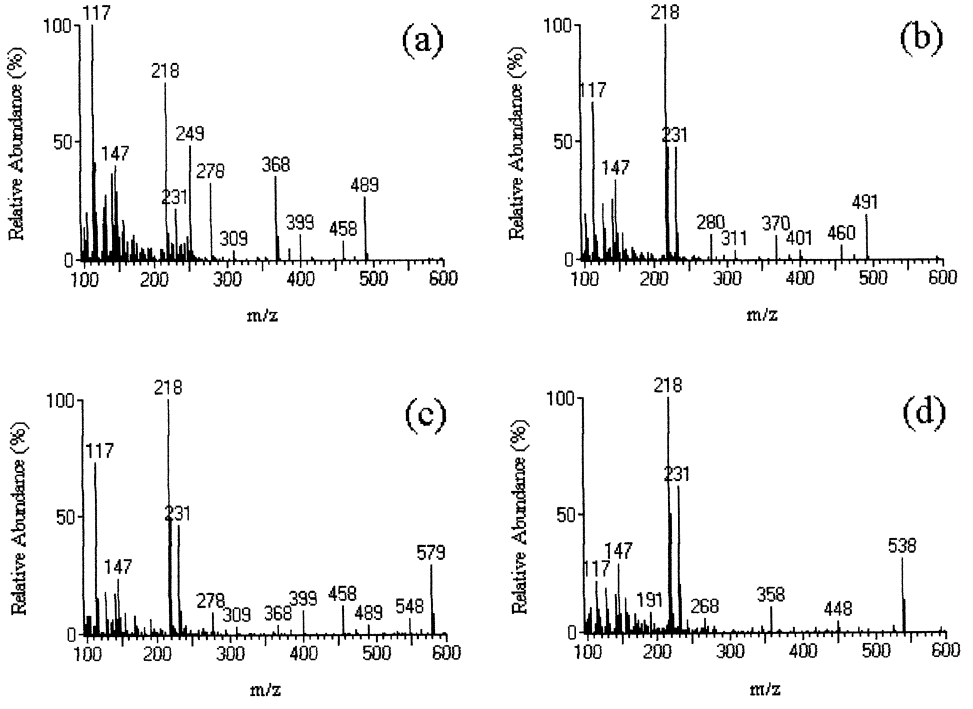


Fig. 3. EI mass spectra of derivatized methandrostenolone metabolites: (a)=16,17β-dihydroxy-17α-methylandrosta-1,4-dien-3-one (4); (b)=16,17β-dihydroxy-17α-methylandrost-1-en-3-one (5); (c)=6,16,17β-trihydroxy-17α-methylandrost-1-en-3-one (7); (d)=17α-methylandrostane-3,16,17β-triol (12).

with 16-hydroxylation, two compounds were observed with mass spectra showing m/z 491 (M^+), 476 ($M-CH_3$), 460 ($M-OCH_3$), 401 (M−TMSOH), 386 ($M-TMSOH-CH_3$), 370 ($M-TMSOH-OCH_3$), 311 (M−2xTMSOH), 296 ($M-2xTMSOH-CH_3$), 280 ($M-2xTMSOH-OCH_3$), 231 (D-ring), 218 (D-ring), 147 (D-ring) and 117 (D-ring) (Fig. 3b). Both isomers appeared as a mixture of sulfate and glucuronide conjugates from 2 to 24 h post-administration, and achieved the highest concentrations of any metabolites from 2 to 8 h. The appearance of two isomers may be due to variable configurations at either C5 or C16. The 5β-isomers have been confirmed as methandrostenolone metabolites in the rabbit [12].

3.4. 6,16,17β-Trihydroxy-17α-methylandrost-1-en-3-ones (7, 8)

Another pair of 16-hydroxylated Δ^4-reduced metabolites were detected, but with an additional hydroxylation site other than C16. Hagedorn et al. [15] observed a number of metabolites of this type, and were able to positively identify some of them as being hydroxylated at C6 by comparison of their mass spectra with literature. C6 is the primary hydroxylation site for methandrostenolone in humans and rabbits, and it can be assumed that it is also the most likely site in the horse. The mass spectra had m/z 579 (M^+), 564 ($M-CH_3$), 548 ($M-OCH_3$), 489 (M−TMSOH), 474 ($M-TMSOH-CH_3$), 458 ($M-TMSOH-OCH_3$), 399 (M−2xTMSOH), 384 ($M-2xTMSOH-CH_3$), 368 ($M-2xTMSOH-OCH_3$), 309 (M−3xTMSOH), 294 ($M-3xTMSOH-CH_3$), 278 ($M-3xTMSOH-OCH_3$), 231 (D-ring), 218 (D-ring), 147 (D-ring) and 117 (D-ring) (Fig. 3c). Both isomers were mostly conjugated as a mixture of sulfates and glucuronides, although a significant quantity of each was unconjugated. They could be detected from 2 to 8 h post-administration and were the most abundant metabolites observed in the free fraction. It was not possible to determine the stereochemistry of the 6-hydroxy function by mass spectrometry, but the 6β-orientation seems more likely by analogy with other species. Thus the appearance of two isomers again can be attributed to variation at either C5 or C16.

3.5. 17α-Methylandrostane-3,17β-diols (9, 10)

Fully reduced metabolites have not previously been reported for methandrostenolone in the horse, although they are known in humans, rats and rabbits. Two metabolites were identified in the present study as 17α-methyl-5β-androstane-3α,17β-diol (9) and 17α-methyl-5α-androstane-3β,17β-diol (10) by comparison with authentic reference standards encompassing all four possible A-ring stereochemistries. Both sulfate and glucuronide conjugation were apparent, but the two isomers showed different excretion dynamics. The 5α-metabolite appeared from 4 to 24 h post-administration with the highest concentration at 8 h. The 5β-metabolite however was detectable from 4 to 72 h with a peak at 24 h. It was the longest lasting metabolite, and achieved the highest concentration of any metabolite from 24 to 72 h.

The reduction of the methandrostenolone A-ring in humans has been investigated in detail by Massé et al. [7]. Based on the presence of partially reduced 1-en-3-one and 1-en-3-ol intermediates in the urine, these workers concluded that the reduction occurs discretely in the order Δ^4-double bond, 3-ketone and Δ^1-double bond. Furthermore, the relative intensities of the various metabolites suggested the first two steps to be significantly faster than the third. In the horse, the Δ^4 reduction remains rapid as evidenced by the abundance of 1-en-3-one intermediates. However reduction of the 3-ketone is obviously much slower, presumably due to inhibition by the Δ^1-double bond. In fact the absence of any other partially reduced intermediates suggests that the final two reductions may even occur in the opposite order to humans, with reduction of the 3-ketone being inhibited completely by the Δ^1-double bond. Slow reduction of the latter would then be followed by rapid reduction of the former, resulting in very low levels of saturated 3-one intermediates. Hence the only significant reduction products are the 1-en-3-ones and saturated 3-ols (Fig. 4).

3.6. 17α-Methylandrostane-3,16,17β-triols (11, 12)

A pair of fully reduced 16-hydroxylated metabolites was also detected. Metabolites of this type have not previously been reported for methandrostenolone in any species, although they are known in the horse

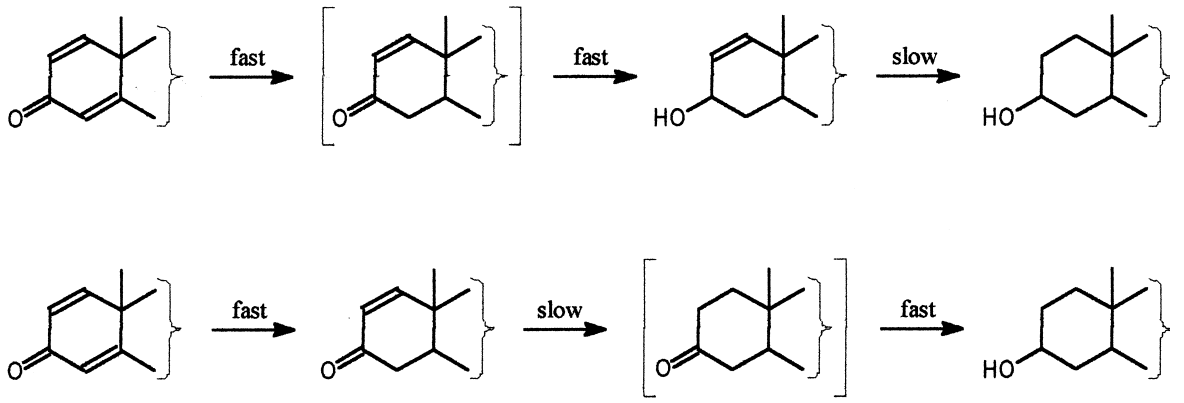


Fig. 4. Proposed order of reduction of the methandrostenolone A-ring in humans (top) and horses (bottom). Compounds in square brackets are observed at low levels or not at all.

as metabolites of 17α-methyltestosterone [22]. The mass spectra had m/z 538 (M^+), 448 (M−TMSOH), 358 (M−2xTMSOH), 268 (M−3xTMSOH), 231 (D-ring), 218 (D-ring), 147 (D-ring) and 117 (D-ring) (Fig. 3d). Both isomers were conjugated as a mixture of sulfates and glucuronides, and were detectable from 4 to 48 h post-administration with the highest concentrations at 8 h.

4. Conclusion

In the horse, methandrostenolone undergoes extensive phase I and phase II metabolism following oral administration. The major phase I processes are hydroxylation at C6 and C16 together with sequential reduction of the dienone function on the A-ring. The most rapid of these are the hydroxylations and the reduction of the Δ^4-double bond, which were observable immediately after administration. The reduction of the 3-ketone and Δ^1-double bond are slower and do not appear until about 4 h post-administration. However these metabolites are excreted for a significantly longer period and constitute the bulk of metabolites observed from 24 to 72 h. Phase II metabolism took the form of both sulfation and β-glucuronidation and heavily affected all of the major metabolites. The conjugated metabolites appeared relatively stable in the urine, and the formation of 17-epimerised artifacts by the spontaneous hydrolysis of 17-sulfate conjugates did not appear to be a significant process. Synthetic work aimed at confirming the stereochemistry of several of the major metabolites is currently being undertaken.

Acknowledgements

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