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Detection of methandienone (methandrostenolone) and metabolites in horse urine by gas chromatography–mass spectrometry

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ABSTRACT

The metabolic transformation of methandienone (I) in the horse was investigated. After administration of a commercial drug preparation to a female horse (0.5 mg/kg), urine samples were collected up to 96 h and processed without enzymic hydrolysis. Extraction was performed by a series of solid–liquid and liquid–liquid extractions, thus avoiding laborious purification techniques. For analysis by gas chromatography–mass spectrometry, the extracts were trimethylsilylated. Besides the parent compound I and its C-17 epimer II, three monohydroxylated metabolites were identified: 6β-hydroxymethandienone (III), its C-17 epimer (IV) and 16β-hydroxymethandienone (V). In addition, three isomers of 6β,16-dihydroxymethandienone (VIa–c) were discovered. Apparently, reduction of the Δ^4 double bond of 16β-hyroxymethandienone (V) in the horse yields 16β,17β-dihydroxy-17α-methyl-5β-androst-1-en-3-one (VII). Reduction of the isomers VIa–c results in the corresponding 6β,16,17-trihydroxy-17-methyl-5β-androst-1-en-3-ones (VIIIa–c). The data presented here suggest that screening for the isomers of VI and VIII, applying the selected-ion monitoring technique, will be the most successful way of proving methandienone administration to a horse.

INTRODUCTION

Methandienone (I) (Fig. 1) is a commonly used anabolic agent and, thus, has been used illegally in horses (doping). Though studies have been reported on methandienone and metabolites in humans [1–6] and in rabbits [7] less is known about the fate of I in the horse. However, using double liquid chromatography with UV detection, methandienone could be quantified in urine and blood of a horse up to six days after a therapeutic dose, and confirmation was provided by liquid chromatography with single or double mass spectrometry [8]. It was further demonstrated that one of the predominant metabolites of I, 17-epimethandienone (II), originates from methandienone 17-sulphate by spontaneous hydrolysis [8]. In general, detailed information on the metabolism of I in the horse is still missing, although monohydroxy and dihydroxy compounds as well as 6-hydroxymethandienone were detected [8].

The aim of the present investigation was to develop a convenient method to investigate the metabolic fate of I in the horse. By omitting the hydrolysis of urine [3–7], extraction can be simplified because additional purification by high-performance liquid chromatography, as suggested previously [3,6,8], appears to be time-consuming and may even be unnecessary for the examination of unconjugated metabolites.

Compound I and its unconjugated metabolites have been isolated from urine on C_{18} material followed by purification of the extract employing Extrelut columns. After derivatization, analysis by gas chromatography–mass spectrometry (GC–MS) revealed abundant information about

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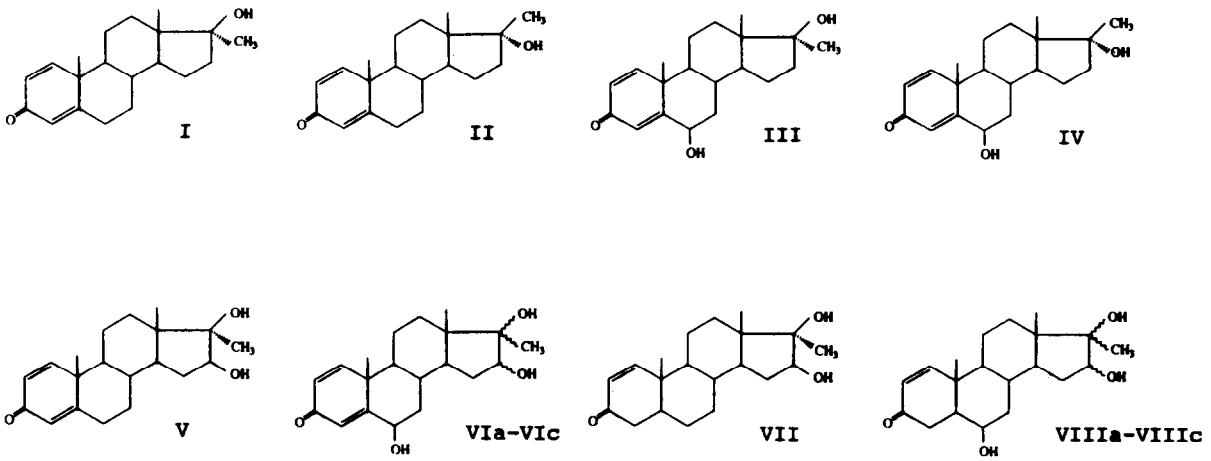

Fig. 1. Structures of methandienone and its metabolites detected in horse urine. I = methandienone; II = 17-epimethandienone; III = 6β,17β-dihydroxy-17α-methyl-1,4-androstadien-3-one; IV = 6β,17α-dihydroxy-17β-methyl-1,4-androstadien-3-one; V = 16ξ,17β-dihydroxy-17α-methyl-1,4-androstadien-3-one; VIa–c = isomers of 6β,16,17-trihydroxy-17-methyl-1,4-androstadien-3-one; VII = 16ξ,17β-dihydroxy-17α-methyl-5β-androst-1-en-3-one; VIIIa–c = isomers of 6β,16,17-trihydroxy-17-methyl-5β-androst-1-en-3-one.

the metabolites of I in urine. Our newly developed extraction procedure, as well as the new knowledge obtained from metabolic patterns, facilitates the recognition of methandienone administration to horses.

EXPERIMENTAL

Reagents and materials

Analytical-grade methanol, diethyl ether, and Extrelut 3 columns were purchased from E. Merck (Darmstadt, Germany). Tri-Sil TBT [a 3:3:2 (v/v/v) mixture of N-trimethylsilylimidazole, N,O-bistrimethylsilylacetamide and trimethylchlorosilane, ready to use] was supplied by Pierce (Oud-Beijerland, Netherlands). Mega-Bond Elut columns (10 g of C_{18} material) were obtained from ICT Handelsgesellschaft (Frankfurt, Germany).

For the animal studies, the commercially available preparation Anabolikum 2.5% (Meca, Goch, Germany), containing 25 mg/ml methandienone, was used.

Apparatus

A Hewlett-Packard GC 5890 (GC capillary HP 1, 12.5 m × 0.2 mm I.D., 0.33 μm film thickness, cross-linked methyl silicone) was used. The detector and injector temperatures were kept at 290°C. Automatic injections (2 μl) were performed with a 7673 A autosampler (Hewlett-Packard) at 60°C. The temperature was increased stepwise to 200°C (40°C/min), then to 290°C at 10°C/min, then held for 5 min at 290°C. The purge-off time was 0.5 min. Helium served as the carrier gas.

The GC column was interfaced to a Hewlett-Packard MSD 5970 mass-selective detector linked to a Hewlett-Packard 59970C chem-station. The mass-selective detector was tuned every day with perfluorotributylamine (PFTBA) according to the specification of the manufacturer. The electron energy was 70 eV.

Drug screening was performed in the selected-ion monitoring (SIM) mode (see below). Full-scan electron impact (EI) mass spectra were acquired by scanning the 50–700 a.m.u. range.

Animal experiments

A female standard-bred horse (470 kg, 13 years) was treated with Anabolikum 2.5% (0.5 mg/kg, intramuscularly). Urine samples voided spontaneously or obtained by a urethral catheter were collected at 2, 4. 6, 8, 12, 24, 48, 72 and 96 h post-dose. Aliquots of 100 ml were placed in polypropylene containers and stored at −30°C until analysis.

Extraction procedure

After centrifugation (4000 *g*, 15 min) of urine, 50 ml were placed on a Mega-Bond Elut column, previously activated with methanol (50 ml) and washed with water (50 ml). To remove interfering matrix compounds, the column was washed with water (50 ml) and subsequently with 30% methanol (50 ml). Finally, the steroids were eluted with 40 ml of pure methanol. All eluents were drawn through the column under vacuum (5–7 kPa) using a Baker spe-10 apparatus (Baker, Gross-Gerau, Germany). The eluate was evaporated to dryness under vacuum (Speed-Vac concentrator, Bachofer, Reutlingen, Germany), and the residue was dissolved in 3 ml of water made alkaline (pH 9.5) by adding 150 mg of sodium hydrogencarbonate–sodium carbonate (2:1, w/w). The resulting solution was then placed on an Extrelut 3 column, which was eluted with 15 ml of diethyl ether. The eluate was evaporated to dryness under vacuum (Speed-Vac concentrator), and the residue was transferred in diethyl ether (0.5 ml) to an autosampler vial. After evaporation of the solvent the sample was derivatized.

Derivatization

The residue was added to 20 μl of Tri-Sil TBT, and the vial was tightly capped. The solution was heated at 60°C in a sand-bath for 15 min. An aliquot (2 μl) was submitted to GC–MS.

RESULTS

It is known that enzymic hydrolysis of horse urine yields numerous matrix compounds that are difficult to separate from the substances of interest [9]. On the other hand, methandienone and some of its metabolites have been reported to be excreted unconjugated in measurable amounts in the urine of different species [2,6,8]. Consequently, the method described in this paper also has the advantage of analysing the "free" fraction, which allows for the detection of unchanged methandienone as well as some of its metabolites for a long period of time in horse urine.

Each substance shown in Fig. 1 (I–VIIIc) was readily observed in total ion chromatograms obtained from urine extracts by monitoring only a few characteristic mass fragments, which can also be used for SIM acquisitions. In particular, fragments *m/z* 143 and *m/z* 218, derived from the fission of the unmetabolized and the C-16 hydroxylated D-ring, respectively (Fig. 2), can serve as "leading" ions. Accordingly, the mass spectra of each peak detected in the ion chromatograms (Fig. 3) were analysed and, if available, compared with the corresponding mass spectra reported in the literature. Others were interpreted on theoretical grounds. Using this analytical approach, the following twelve substances were identified.

Methandienone (I) and its C-17 epimer (II). Fig. 3a displays an ion chromatogram of *m/z* 143 from the full-scan GC–MS analysis of a urine extract obtained 4 h after intramuscular administration of I. Unchanged I appears only as a minor peak (Fig. 3a, 12.68 min), which hampers the demonstration of all details of the full-scan mass spectrum of an authentic sample. Because of this, I was preferably detected by the more sensitive SIM mode (see below).

The C-17 epimer II (11.77 min) has been reported as one of the most prominent unconjugated metabolites of I in different species [2,6,8]. The mass spectrum of the trimethylsilyl ether found in this study is identical with that published previously [2,6]. The mechanism of generation of II in horse urine is independent of any enzyme action as it is produced by spontaneous hydrolysis of

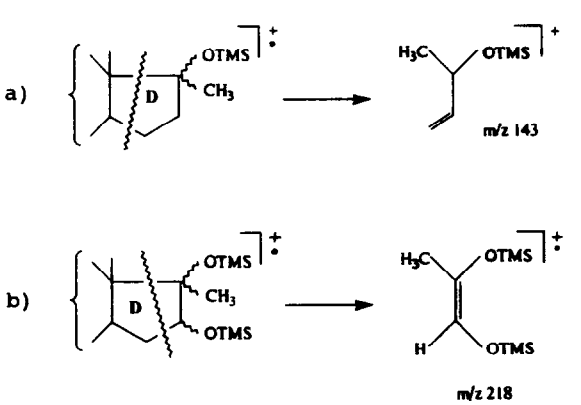

Fig. 2. Formation of the ions (a) *m/z* 143 from the unmetabolized D-ring, and (b) *m/z* 218 from 16-hydroxy metabolites.

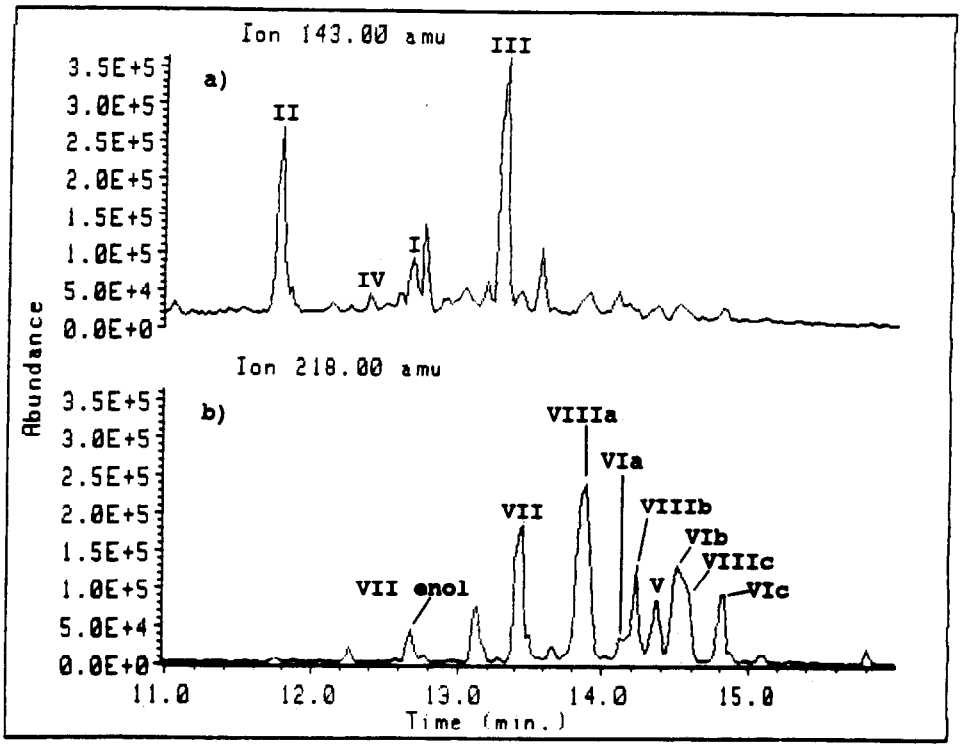

Fig. 3. Ion chromatograms of (a) *m/z* 143 and (b) *m/z* 218, from the full-scan GC–MS analysis of a urine extract 4 h after administration of methandienone to a horse. All compounds are detected as trimethylsilyl ethers.

methandienone 17-sulphate [8]. We also found that the concentrations of I and II are highly dependent on both the storage time and the temperature after collection of the urine samples. These factors may complicate quantitative analysis, as has been reported previously [8].

6β,17β-Dihydroxy-17α-methyl-1,4-androstadien-3-one (III) and its C-17 epimer (IV). In humans the metabolic hydroxylation of I at C-6 was reported to yield 6β-hydroxymethandienone (III) as well as its C-17 epimer IV [4,6]. Both compounds were detected in the ion chromatogram of *m/z* 143 (Fig. 3a) as their bis-trimethylsilylated derivatives, at 13.33 and 12.39 min, respectively. Their mass spectra are very similar, and coincide with previously published data (mass peaks and relative intensities) [6]. In some experiments, the urinary concentration of epimer IV was too low to obtain a full-scan mass spectrum.

Contrary to the fragmentation pattern suggested by Dürbeck and Büker [2], the ion *m/z* 281 is likely to be formed by cleavage of the C-9–C-10 and the C-6–C-7 bonds, with concomitant migration of the 17-TMS group to the 3-oxo function [5].

16ξ,17β-Dihydroxy-17α-methyl-1,4-androstadien-3-one (V). Besides hydroxylation at C-6, hydroxylation at C-16 was also observed. The mass spectrum of the bis-trimethylsilyl ether V (Fig. 4) found at 14.35 min (Fig. 3b) exhibits a molecular ion at *m/z* 460 and other diagnostic ions at *m/z* 445 ($M^+ - 15$), 370 ($M^+ - 90$), 355 (445 − 90), 315 (445 − $CH_3(CH)_2OTMS$), 231 and 218. The last two fragments mark the hydroxylation at C-16. Formation of the ion *m/z* 315 was interpreted according to previously published data [2]. Fragment *m/z* 147 ($((CH_3)_3SiO^+Si(CH_3)_2$ points to the presence of two vicinal OTMS groups. The mechanism of this fragmentation, involving the formation of a cyclic oxonium ion intermediate, was reported previously [10]. Fragment *m/z* 117 may be regarded as a

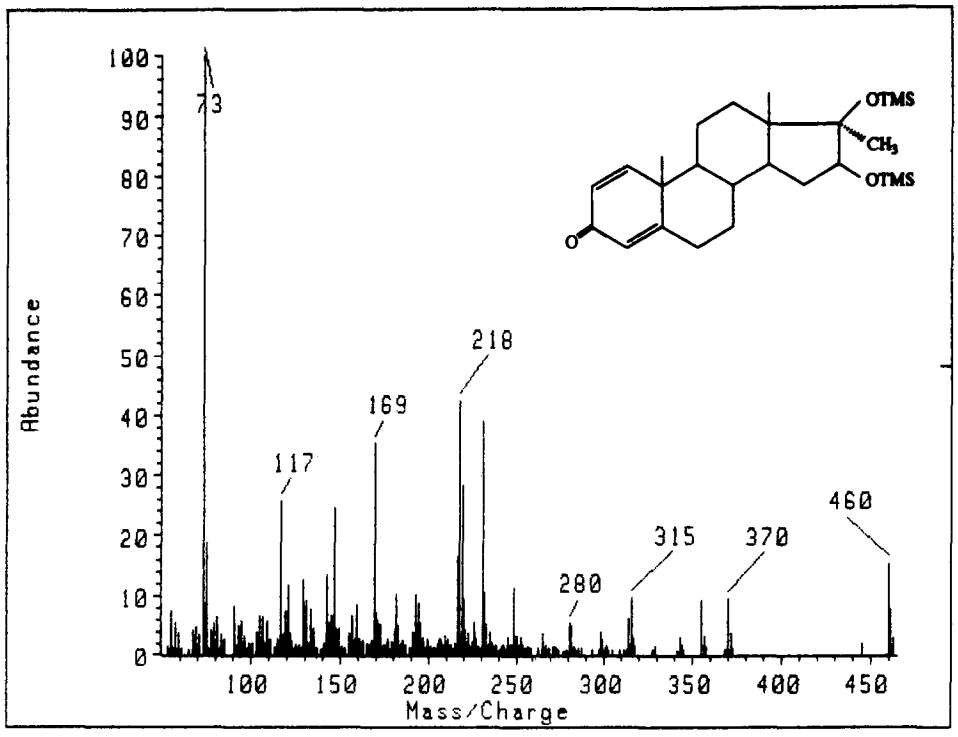

Fig. 4. EI mass spectrum of bis-trimethylsilylated 16ξ,17β-dihydroxy-17α-methyl-1,4-androstadien-3-one (V).

product of the cleavage of the C-16–C-17 bond with migration of the C-14 proton to C-17, yielding $CH_3CH=^+OTMS$. As a consequence, the ions *m/z* 143 and *m/z* 130, which are characteristic for ring D of trimethylsilylated methandienone, were not formed. No isomers were detected.

6β,16,17-Trihydroxy-17-methyl-1,4-androstadien-3-ones (VIa–c). Three isomers of dihydroxylated methandienone, Vla–c, were detected as their tris-trimethylsilyl ethers at 14.16, 14.52, and 14.82 min (Fig. 3b). Their mass spectra [6] exhibit the molecular ion *m/z* 548, and ions at *m/z* 117, 147, 218, and 231, indicating hydroxylation of I at C-16. Fragments at *m/z* 209 and 281 confirm hydroxylation at C-6 [2], whereas the configuration at C-6, C-16 and C-17 could not be determined by MS only.

16ξ,17β-Dihydroxy-17α-methyl-5β-androst-1-en-3-one (VII). Reduction of the Δ^4 double bond and hydroxylation at C-16 yields metabolite VII, detected at 13.45 min (Fig. 3b) as its bis-trimethylsilyl ether. Only one isomer was detected. Characteristic ions in the mass spectrum of VII (Fig. 5) are *m/z* 462 (M^+), 447 ($M^+ - 15$), 372 ($M^+ - 90$), 357 (447 − 90), 316 ($M^+ - CH_3C(OH)CHOTMS$) and 267 (357 − 90). Again, ions *m/z* 147, 218 and 231 indicate the C-16 hydroxylated D-ring.

In addition, the tris-trimethylsilylated enol of VII was also found (Fig. 3b, 12.27 min). Its mass spectrum (Fig. 6) exhibits the molecular ion *m/z* 534 and other characteristic fragments at *m/z* 444 ($M^+ - 90$), 429 (444 − 15), 231, 218, 147 and 117. Ion *m/z* 194 is generated by fission of the C-9–C-10 and C-6–C-7 bonds [5]. This fragment was also detected in the spectrum of bis-trimethylsilylated VII. Assuming migration of the 17-TMS group to the 3-oxo function [5], formation of ion *m/z* 316 may occur by fission of the C-15–C-16 and C-13–C-17 bonds and loss of $CH_3C(OH)CHOTMS$.

6β,16,17-Trihydroxy-17-methyl-5β-androst-1-en-3-ones (VIIIa–c). Hydroxylation of I at C-6

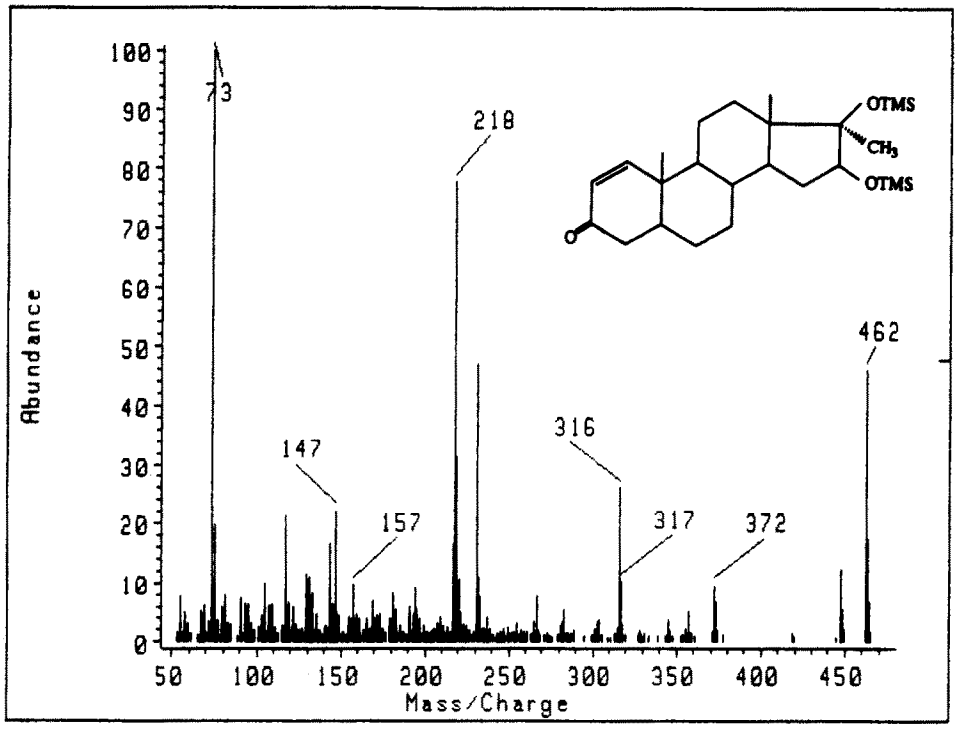

Fig. 5. EI mass spectrum of bis-trimethylsilylated 16ξ,17β-dihydroxy-17α-methyl-5β-androst-1-en-3-one (VII).

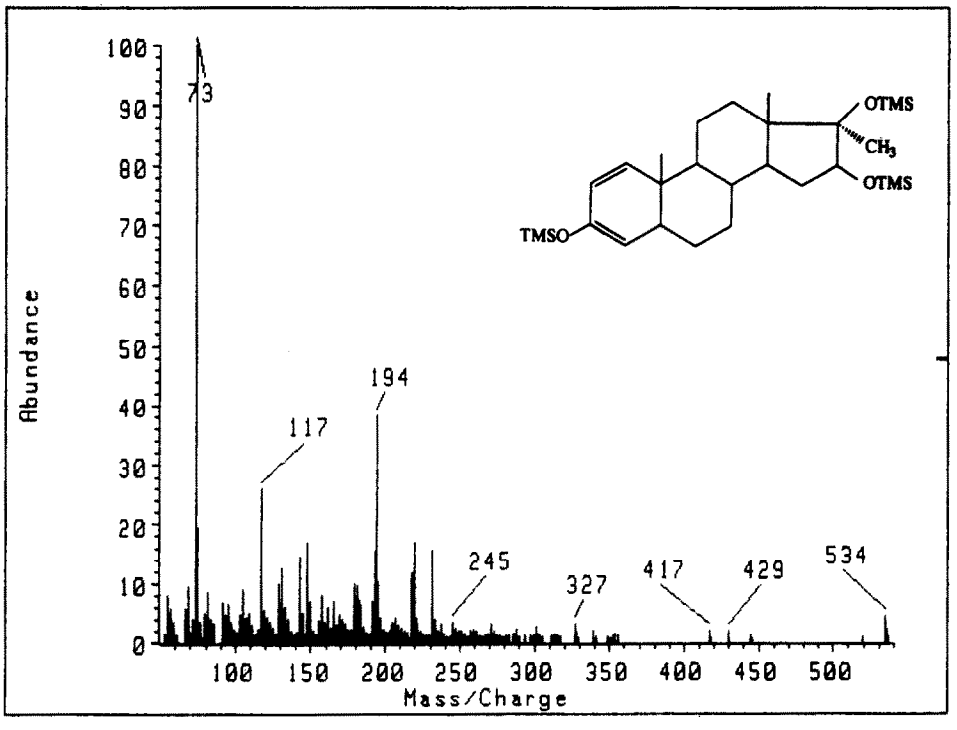

Fig. 6. EI mass spectrum of the tris-trimethylsilylated enol of 16ξ,17β-dihydroxy-17α-methyl-5β-androst-1-en-3-one (VII).

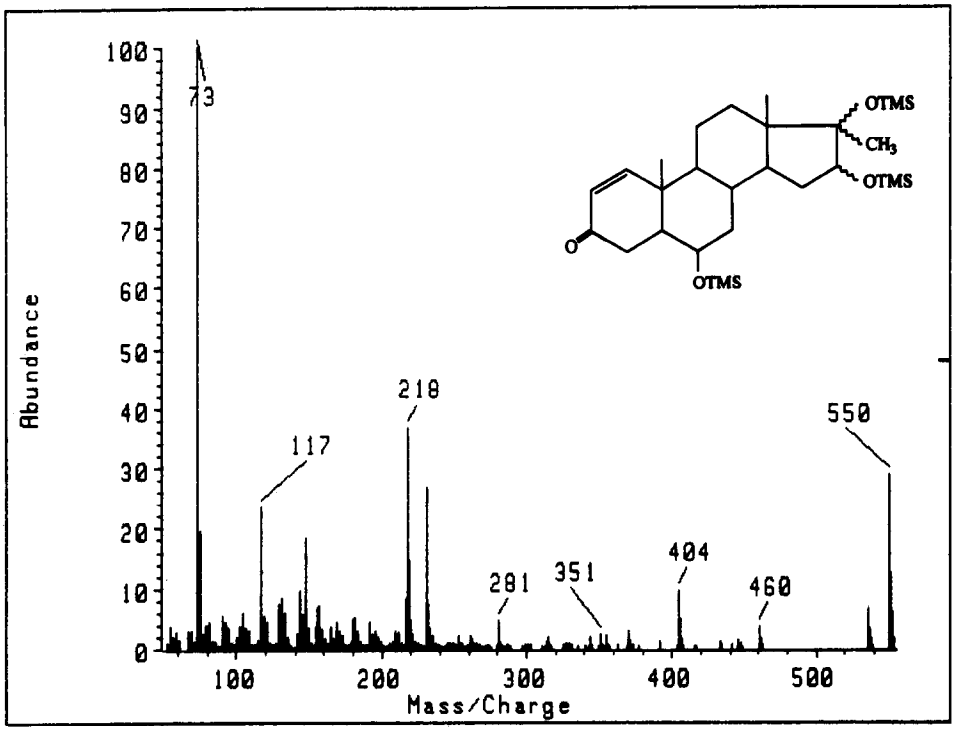

Fig. 7. EI mass spectrum of tris-trimethylsilylated 6β,16,17-trihydroxy-17-methyl-5β-androst-1-en-3-one (isomer VIIIa).

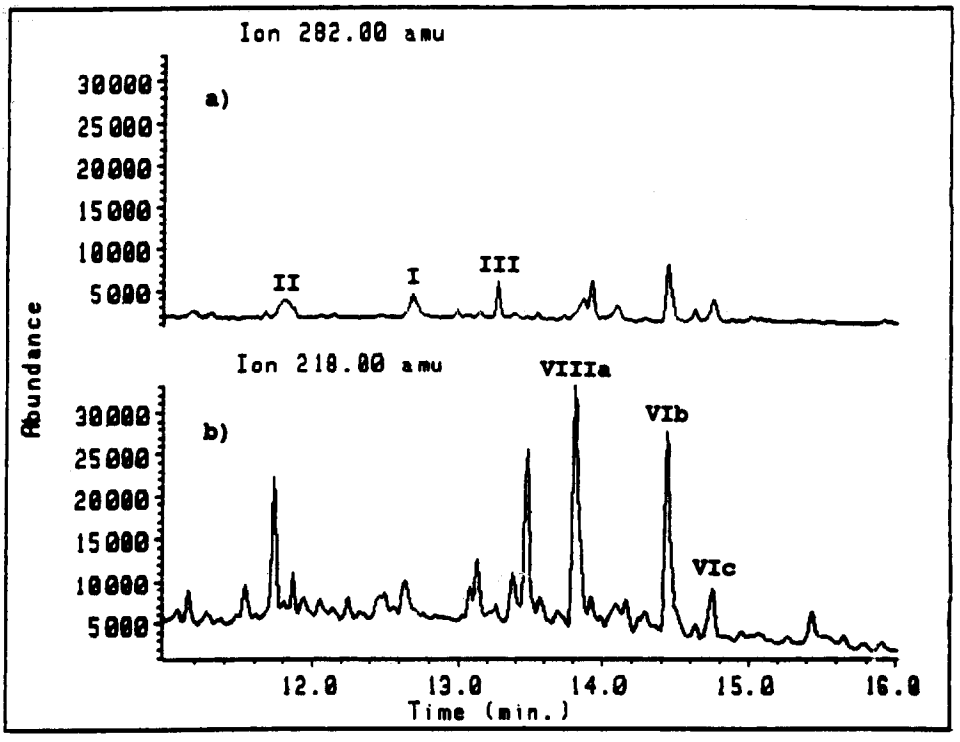

Fig. 8. Ion chromatograms of (a) *m/z* 282 and (b) *m/z* 218, from the SIM analysis of a urine extract 96 h after methandienone administration to a horse; all compounds detected as trimethylsilyl ethers.

and C-16 and reduction of the Δ^4 double bond yields the three isomers VIIIa–c, which are detected as their tris-trimethylsilyl derivatives at 13.90, 14.23 and 14.59 min in the ion chromatogram (Fig. 3b). Although chromatographic resolution of VIIIc was partially achieved, all structural details provided by the mass spectrum of VIIIa (Fig. 7) are confirmed. Typical mass fragments found in the spectra of VIIIa–c are *m/z* 550 (M^+), 535 ($M^+ - 15$), 460 ($M^+ - 90$), 404 ($M^+ - CH_3C(OH)CHOTMS$), 281, 231, 218, 147 and 117, which are generated according to the above-mentioned mechanisms. However, the configuration at C-6, C-16 and C-17 could not be assigned by MS.

Detection of methandienone and metabolites by SIM

Depending on the storage conditions of the samples, I, II, VI and VIII were recovered up to 96 h after dosing, using the more sensitive SIM technique with the ions *m/z* 282 and 218 (Fig. 8).

In this regard, screening for the isomers of VI and VIII will be most useful for detecting administration of methandienone to a horse.

DISCUSSION

Since only a few details of the biotransformation of I in the horse are known, a method was developed to provide more information on the metabolism of this anabolic steroid.

Solid-phase extraction of urine, which was not hydrolysed by enzymes, allowed the processing of as much as 50 ml per column, yielding residues that were subsequently purified by liquid–liquid extraction on Extrelut columns under weakly basic conditions. This convenient procedure avoids laborious and time-consuming steps for further purification, and direct submission of the derivatized material to GC-MS proved feasible. By searching for characteristic mass fragments, *i.e.* *m/z* 143 (unmetabolized D-ring) and *m/z* 218 (metabolites bearing C-16 hydroxy group), compounds I-VIIIc were reliably detected.

Employing the guide ion *m/z* 143, 17-epimethandienone (II) was more readily detectable than I itself, because of the higher amounts present and a better chromatographic resolution. However, the most important metabolite characterized by *m/z* 143 is the 6β-hydroxy compound III. Only traces of the C-17 epimer IV were recovered under the conditions used.

The ion profile of *m/z* 218 confirmed the presence of some further metabolites. In addition, their mass spectra exhibited the ions *m/z* 231, 147 and 117, confirming the substitution pattern of the ring D.

On theoretical grounds, four isomers of 16-hydroxymethandienone, including the C-17 epimers, may be expected, but only one was detected. Analogously to what was found in the rabbit, this metabolite was correlated to 16β,17β-dihydroxy-17α-methyl-1,4-androstadien-3-one (V) [7]. Interestingly, this metabolite, found here in horse urine, is missing in humans [6].

Hydroxylation of I at C-6 and C-16 yields three isomers, VIa–c, in equine urine. The configuration of the asymmetric carbon atoms cannot be determined by their mass spectra alone. However, after methandienone administration to humans, Schänzer *et al.* [6] detected in the unconjugated fraction of a urine extract 6β,16β-dihydroxy-17-epimethandienone, 6β,16α-dihydroxymethandienone and 6β,16β-dihydroxymethandienone, which were eluted in that order on an SE-54 capillary column (5% phenylmethylsilicone, cross-linked). The same structure may be attributed to compounds VIa–c generated by the horse. Moreover, 6α-hydroxy metabolites have not been observed in any species after administration of I [2,4,6–8]. Therefore, the 6β configuration of VIa–c can be assumed.

Another important metabolic pathway of I in the horse appears to be the reduction of the Δ^4 double bond and simultaneous hydroxylation at C-6 and C-16. We thus propose that reduction of 16β-hydroxymethandienone (V) leads to 16β,17β-dihydroxy-17α-methyl-5β-androst-1-en-3-one (VII).

Moreover, reduction of the 6,16-dihydroxylated isomers VIa–c yields the corresponding compounds VIIIa–c which, to our knowledge, have not been reported before as metabolites of I.

CONCLUSION

A method for the isolation and detection of methandienone in non-hydrolyzed horse urine is presented. All compounds discussed were identified by their EI mass spectra after trimethylsilylation and GC separation. Thus, the data reported here for the metabolic fate of I may also simplify the screening for methandienone administered to horses.

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REFERENCES

1 H. W. Dürbeck, I. Büker, B. Scheulen and B. Telin, *J. Chromatogr.*, 167 (1978) 117.
2 H. W. Dürbeck and I. Büker, *Biomed. Mass Spectrom.*, 7 (1980) 437.
3 I. Björkhem, O. Lantto and A. Löf, *J. Steroid Biochem.*, 13 (1980) 169.
4 R. Masse, C. A. Ayotte and R. Dugal, *J. Chromatogr.*, 489 (1989) 23.
5 R. Masse, H. Bi, C. Ayotte, P. Du, H. Gelinas and R. Dugal, *J. Chromatogr.*, 562 (1991) 323.
6 W. Schänzer, H. Geyer and M. Donike, *J. Steroid Biochem. Mol. Biol.*, 38 (1991) 441.
7 J. F. Templeton, C.-J. C. Jackson and J. W. Steele, *Eur. J. Drug Metab. Pharmacokin.*, 9 (1984) 229.
8 P. O. Edlund, L. Bowers and J. Henion, *J. Chromatogr.*, 487 (1989) 341.
9 E. Houghton, M. C. Dumasia and P. Teale, *Analyst*, 113 (1988) 1179.
10 S. Sloan, D. J. Harvey and P. Vouros, *Org. Mass Spectrom.*, 5 (1971) 789.