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Biotransformation of 17-alkylsteroids in the equine: gas chromatographic–mass spectral identification of ten intermediate metabolites of methyltestosterone

S.M.R. Stanley*, L. Smith, J.P. Rodgers

Jockey Club of Southern Africa, Turffontein Racecourse, Turffontein, Johannesburg, Gauteng, Republic of South Africa

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Abstract

The metabolism of the orally active anabolic steroid methyltestosterone in the equine was investigated by administration of the drug along with a tritiated radiolabel tracer. In this study some of the metabolites were identified and a radio immunoassay screen and immunoaffinity chromatography gel for methyltestosterone were also evaluated. Pathway intermediates, in particular the 17-methylandrostanediols, were studied to gain an insight into the most likely stereochemistry of the major metabolites. The predominant phase I biotransformations involve reduction of the A ring 3-oxo and 4-ene groups to yield predominantly 3β-hydroxy-5α-androstane products and hydroxylation of the steroid nucleus at several positions. Epimerisation of the 17α-methyl group also occurred. Ten steroids could be positively identified by comparison with authentic reference materials and many other triol, tetrol and pentols were also observed. Phase II metabolites and sulphate conjugates in particular, were common.

Keywords: Steroids; 17-Alkylsteroids; Methyltestosterone

1. Introduction

Rumours of the widespread abuse of the 17α-alkyl anabolic steroids in South African thoroughbred horse racing have been given substance by the seizure by the Customs Department of illegal medications containing these drugs. There has also been an increase in the number of legitimate products containing these steroids being offered for sale. They are popular because alkylation at the 17-position prevents the rapid oral inactivation that occurs with other anabolic steroids and, therefore, eliminates the need to inject the drug. Many of these steroids also have a reduced androgenic side effect [1]. The fate of these steroids is well documented in the human but very little information has been published on equine metabolism. To date the results of studies of methandrostenolone [2] and stanozolol [3] as well as a preliminary study [4] on 17α-methyltestosterone (MT) have been published. In order to screen and confirm the abuse of these substances in the horse the metabolic pathways and the stereochemistry of the major metabolites must be identified. We have investigated the biotransformation of MT (17α-methyl-17β-hydroxyandrost-4-en-3-one) in the equine with an emphasis on identifying the stereochemistry of as many metabolites as possible. To facilitate tracing the excretion of these products in

*Corresponding author.

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urine, MT as well as radiolabelled methyltestosterone (^{3}H-MT) were administered via nasogastric tube and samples collected over 96 h. Fractionation of the urine samples into non-conjugated, glucuronide and sulphate bound steroids allowed the percentage of each to be calculated from the radioactivity detected. Extracts were derivatised and analysed by GC–MS in the positive electron impact mode. Reconstructed chromatograms of the molecular ion of potential metabolites and characteristic D ring fragments (*m/z* 143, 218 and 231) were used to identify the materials of interest. Where possible those steroids detected were compared with results from authentic reference material.

A further administration of MT (without the radiolabel) provided urine samples that were used to evaluate a commercially available radio immunoassay (RIA) screen and immunoaffinity chromatography gel for confirmation of MT abuse.

2. Experimental

2.1. Reagents and chemicals

Methanol and ethyl acetate B and J brand HPLC grade solvents were obtained from Baxter (Muskegon, MI, USA). Diethyl ether was purified from commercial grade (Illovo Merebank, Alrode, Johannesburg, Republic of South Africa [RSA]) by distillation after NaOH addition. Riedel-de Häen, Seelze, Germany, supplied acetic acid of >99.8% purity. Sulphuric acid (99%) was from Associated Chemical Enterprises (Johannesburg, RSA). Chlorosulphonic acid, ammonium iodide, methyl iodide, silica gel 60 [63 to 200 μm], magnesium metal ribbon and KOH were purchased from Saarchem, Krugersdorp, RSA. Hydroxymethylammonium chloride, dithioerythritol and *E. coli* β-glucuronidase enzymes were imported from Sigma (St. Louis, MO, USA). The pyridine (dried GR) used was from Merck (Darmstadt, Germany). Acros Chimica NV, Geel, Belgium supplied N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA). *Helix pomatia* β-glucuronidase/arylsulphatase enzymes were obtained from Boehringer Mannheim, Johannesburg, RSA. Undecane was imported from Tokyo Chemical Industries (Tokyo, Japan). Steraloids (Wilton, NH, USA) provided the following authenticated steroids: 17α-methyl-11α,17β-dihydroxyandrost-4-en-3-one, 17α-methyl-3α,17β-dihydroxyandrost-4-ene, 17α-methyl-3β,17β-dihydroxyandrost-4-ene, 17α-methyl-3α,17β-dihydroxy-5α-androstane, 17α-methyl-3β,17β-dihydroxy-5α-androstane, 17α-methyl-17β-hydroxy-5α-androstan-3-one, 3β-hydroxy-5α-androstan-17-one, 3α-hydroxy-5β-androstan-17-one and 3β,16α-dihyroxy-5α-androstan-17-one. Roussel Laboratories, (Johannesburg, RSA) provided authentic methyltestosterone (17α-methyl-17β-hydroxyandrost-4-en-3-one, MT). Methyltestosterone tablets of 10 mg strength were obtained from Lennons Limited (Port Elizabeth, RSA). Tritiated MT (250 μCi) was obtained from the Laboratoire D'Hormonologie (Marloie, Belgium) who also supplied the MT radio immunoassay kit and the MT immunoaffinity chromatography (IAC) gel.

2.2. Synthesis of steroids

2.2.1. 17-Methylepitestosterone (MET)

A solution of MT (0.5 g) dissolved in 10 ml of dry pyridine had 100 μl of chlorosulphonic acid added over 10 min. This was left to stir for 30 min before addition of 100 ml of water. After standing for 12 h the precipitated material was recovered by filtration. Purification of the product was achieved by liquid chromatography on a silica gel 60 column (500×10 mm I.D.) with 1:1 (v/v) ethyl acetate–chloroform mixture as eluent. The material was analysed by GC–MS of the enol ether TMS and O-methoxime TMS (*m/z* 403, 313, 298 and a base peak at 143) derivatives and by comparing the ultraviolet spectrum to MT. The retention time of this product relative to MT as the MO-TMS derivative was 0.92.

2.2.2. 17β-Methyl-3β,17α-dihydroxyandrost-4-ene

The MET was reduced with $NaBH_4$ in methanol–water (95:05, v/v) for 1 h. The 3α- and 3β-hydroxylated materials were isolated by column chromatography as described above (Section 2.2.1). These materials gave TMS spectra with *m/z* 448, 358, 301, 253, 216 and a base peak at 143.

Two steroids were produced by a Grignard synthesis to incorporate 17-methyl-17-hydroxy functionalities into authentic 17-keto steroid precursors using

a freshly prepared mixture of magnesium, methyl iodide and diethylether. These products were isolated by extraction into diethyl ether and then fractional crystallisation from methanol and water. This was followed by purification using column chromatography as described above (Section 2.2.1).

2.2.3. 17β-Methyl-3α,17α-dihydroxy-5α-androstane

This material was produced from 3α-hydroxy-5α-androstan-17-one. In this case the use of the authentic standard of the 17α-methyl steroid enabled us to distinguish between the two isomers produced.

2.2.4. 17α-Methyl-3β,16α,17β-trihydroxy-5α-androstane and 17β-methyl-3β,16α,17α-trihydroxy-5α-androstane

Produced [5,6] from 3β,16α-dihydroxy-5α-androstan-17-one by Grignard synthesis. There were two peaks in the GC–MS chromatogram with a molecular ion at *m/z* 538. They were postulated to be the 17-methyl epimers of the product, of which the faster running of the two was designated as 17β-methyl based on the behaviour of analogous steroids.

2.3. Animal administrations

One mare of 482 kg was given 250 μCi with 1 g of MT and another of 490 kg given 0.5 g MT by nasogastric tube. Samples were obtained by means of 20 G polypropylene catheter and were frozen within 30 min of being collected. Storage in polypropylene bottles was at −20°C before being thawed shortly prior to extraction.

2.4. Urine extraction

Samples (20 ml) of the acquired urine samples were extracted with 2×20 ml of diethyl ether to isolate the free steroids. The remaining aqueous phase had nitrogen bubbled through it for 30 min to remove the residual ether before 200 μl of a suspension containing 4000 units of β-glucuronidase (*E. coli*) were added to each sample after they had been adjusted to pH 6.8. This was left overnight at 37°C. The steroids were collected on two C_{18} cartridges (Sep-Pak, Waters, Milford, MA, USA) as follows. The Sep-Pak was conditioned with 5 ml of methanol and 3 ml of water before 10 ml of sample were applied. After washing with 5 ml of water the compounds of interest were eluted with 5 ml of 1% acetic acid in methanol. Fractionation into previously glucuronide and the sulphate bound steroids was performed [7] on a Bond Elut Certify II cartridge (Varian, Harbor City, CA, USA). The C_{18} extracted material was reconstituted in 100 μl of methanol followed by 5 ml of 0.2 *M* pH 3 acetate buffer (100 ml water, 1140 μl acetic acid with pH adjusted to 3.0 with KOH), after being pooled and evaporated to dryness. Cartridge conditioning was with 5 ml of methanol and pH 3 buffer. The sample was applied at <2 ml/min and the solid-phase washed with 5 ml of pH 3 buffer and 10 ml of 5% methanol in water. The glucuronide fraction was eluted with 10 ml of ethyl acetate. The solvolysis mix (ethyl acetate–methanol 9:1 with 100 μl of H_2SO_4 per 100 ml) was used to elute sulphate bound steroids which were then solvolysed at 50°C for 2 h. One per cent aliquots were removed for beta radiation counting before the three fractions were dried under oxygen-free-nitrogen (OFN).

2.5. Derivatisation

2.5.1. O-Methoxime trimethylsilyl (MO-TMS)

After reconstituting the samples in 50 μl of an 8% solution of hydroxymethylammonium chloride in pyridine they were heated at 80°C for 30 min. Pyridine was removed with oxygen-free-nitrogen (OFN) at temperatures <40°C and 50 μl of MSTFA were added. The sample was again heated at 80°C for 1 h. The MSTFA was removed under OFN and the residue reconstituted in 50 μl of undecane for injection.

2.5.2. Enol trimethylsilyl (E-TMS)

MSTFA, dithioerythritol and ammonium iodide (1000 μl, 2 mg, 4 mg) were mixed together and heated at 80°C until they dissolved. Fifty microlitres of this mixture were added to each sample prior to heating at 60°C for 15 min. The samples were ready for injection.

2.6. Gas chromatography–mass spectrometry

A Finnigan MAT (San Jose, CA, USA) Magnum ion trap mass spectrometer with a AS200S autosampler and Varian (Sunnyvale, CA, USA) 3200 GC was used at a source temperature of 220°C and a scan range of 132 to 650 amu at 1 s per scan (3 μScans). The background mass setting was 70 amu and the mass defect was 50 mmu/100 amu. The GC oven fitted with a 30 m SE-30 (0.25 mm I.D., 0.25 μm) Alltech (Deerfield, IL, USA) Econocap column which was programmed from 105°C to 205°C at 40°C/min, then to 240°C at 2.5°C/min. The final ramp segment was 8°C/min to 300°C with a hold of 4 min. The injection and transfer line were set to 260°C. The carrier gas was helium of 99.999% purity from Fedgas (Alrode, Johannesburg, Republic of South Africa).

2.7. RIA, immunoaffinity chromatography of MT and measurement of radiolabel tracer

The RIA kit and the immunoaffinity gel were used according to the manufacturers instructions. Urine samples acquired after MT administration were extracted with diethyl ether prior to RIA.

A mixture of 40 ng of MT plus ^{3}H-MT was extracted using the IAC gel in order to determine percentage recovery by comparison to a ^{3}H-MT calibration curve.

2.8. β-Radiation measurement

All eluents from SPE of the ^{3}H-MT post-administration urine samples, IAC extraction and liquid–liquid partition phases were measured for radioactivity. One per cent aliquots of each were mixed with 900 μl of water and 7 ml of Ultima Gold XR (BV Chemical Operations, Groningen, Netherlands) scintillation cocktail and were measured in a Packard Minaxi Tricarb series 4000 liquid scintillation spectrometer for β-radiation. A single-label efficiency correlation curve was generated for disintegrations per minute (DPM) conversion. The spectrometer was normalised and time termination for sample measurement was set at 20 min, 2% terminator unchanged, with radionuclide region 1 selected.

3. Results and discussion

Samples collected from a mare who had been administered 250 μCi tritiated methyltestosterone (^{3}H-MT) plus 1 g of MT via a nasogastric tube gave the radiolabel excretion profile shown in Fig. 1a. Radioactivity was detectable above background in the urine up to and including the 36-h sample. A second mare was administered 0.5 g MT by nasogastric tube and the collected urine analysed by radio immunoassay for MT. The profiles (Fig. 1a,b) were similar and suggest that RIA would be a suitable screening method for detecting the metabolites. These graphs show that the majority of the material of interest is excreted in the first 12 h. Urine specimens containing the radiolabel were then extracted for the unbound (free) steroids by a diethyl ether liquid–liquid partition. The remaining aqueous phase was hydrolysed using β-glucuronidase enzymes from *Escherichia coli* followed by isolation of the steroids using a C_{18} solid-phase extraction (SPE) cartridge. The steroids were separated into sulphate (S) and previously glucuronide (G) bound metabolites using a mixed mode SPE technique. Measuring the radioactivity of these fractions shows (Fig. 1c) that the major metabolic components of these urines are sulphate bound conjugates. Free steroids represent a relatively minor percentage.

A commercially available MT immunoaffinity chromatography (IAC) gel was also used to extract the samples. However, this gave very low recoveries (<15%) for the radiolabel from *Helix pomatia* β-glucuronidase/aryl sulphatase hydrolysed post-administration urine. In comparison, the IAC recovery of a 40 ng standard (MT/^{3}H-MT) mixture spiked into equine urine was greater than 85%. The low yield from MT administration samples may be a result of exceeding the limited loading capacity of the gel combined with metabolic changes to the MT decreasing the affinity of the antibody for these compounds. Therefore this gel was found not to be suitable for our purposes.

The fractions were derivatised to form O-methoxime trimethylsilyl (MO-TMS) products and analysed by GC–MS in the positive electron impact (EI) mode. Chemical ionisation was used to confirm the identity of the molecular ion and, in some cases, enol trimethylsilyl (E-TMS) derivatives of ketosteroids

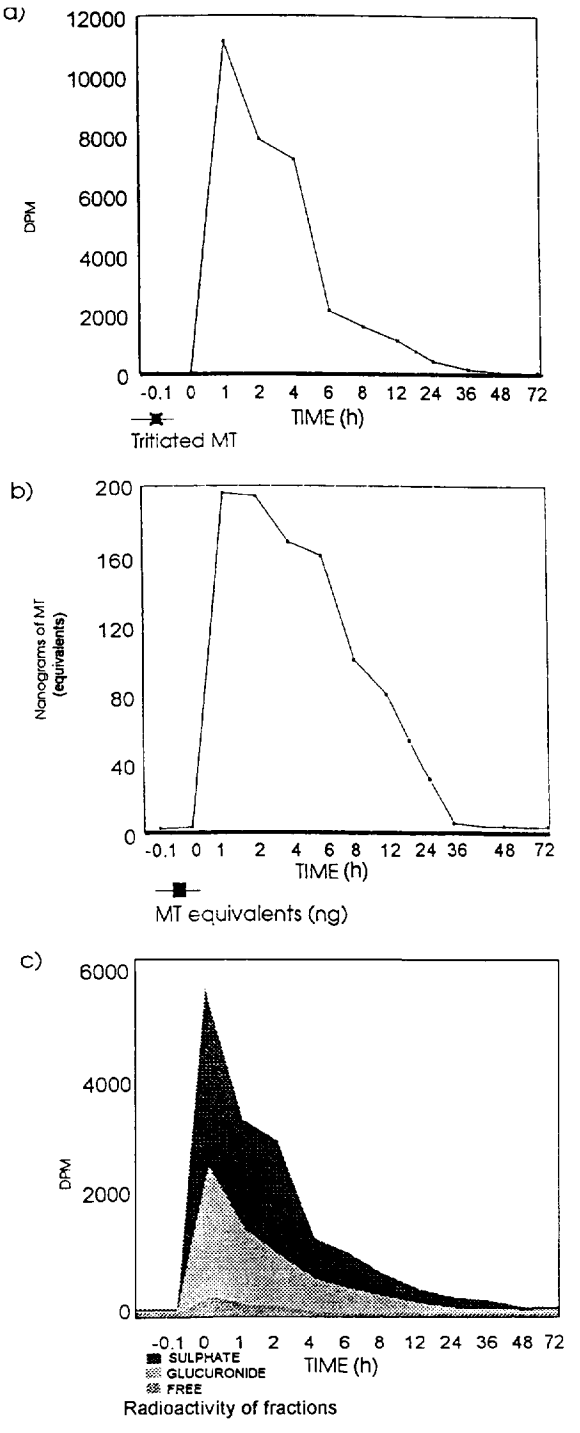

Fig. 1. Time elution profiles for MT and ^{3}H-MT. (a) Radiolabel (DPM) found in acquired urines samples after ^{3}H-MT administration, (b) RIA results for MT post-administration urine, (c) radioactivity (DPM) of the separated fractions.

were also formed to provide collateral evidence. The type of metabolite detected was dependant upon the particular isolate (Table 1). The data also shows the presence of many tri-, tetra- and possibly penta-hydroxy steroids in the post administration urine samples and these represent the major metabolites.

3.1. 17α-Methyltestosterone (I)

A GC–MS peak (Fig. 2) corresponding in retention time and spectral characteristics to the MO-TMS derivative of MT was detected in the free and glucuronide fractions. The spectrum of the MO-TMS derivative of this compound has a $M^{+\cdot}$ ion at 403 as well as characteristic $[M-CH_3]^{+\cdot}$ and $[M-Si(CH_3)_3OH]^{+\cdot}$ fragments (M−15 and M−90, respectively) common to all TMS derivatised hydroxylated steroids. The base peak is *m/z* 143 which is a charged 3 carbon enone substituted structure produced from MT and is indicative of an unaltered D ring.

3.2. 17β-Methylepitestosterone (II)

A peak with the same spectrum as MT but with a shorter retention time was observed in the free fraction from samples collected up to 4 h. This was postulated to be 17β-methyl epitestosterone (MET) because 17-epimers have previously been encountered in the study of methandrostenolone metabolism in the horse [2]. This was later confirmed by synthesis [5,8] of a 17β-methyl-17α-hydroxyandrost-4-en-3-one standard which matched the GC–MS characteristics of this compound.

3.3. Reduction of the 4-ene group of MT

3.3.1. 17α-Methyl-17β-hydroxy-5α-androstan-3-one (III)

A small amount of this material (MO-TMS derivatised) was present in the diethyl ether extract of the samples up to and including the 4-h collection.

3.4. Reduction of the 3-keto group of MT

Three peaks with the a prominent *m/z* 448 molecular ion were evident in the reconstructed ion

Table 1
Identified methyl testosterone metabolites

Fraction	Metabolite	Name	Molecular ion[a]	Fragment ion[b] *m/z*
Free	I	17α-Methyl testosterone	403	143
	II	17β-Methyl epitestosterone	403	143
	III	17α-Methyl-17β-hydroxy-5α-androstan-3-one	405	143
	IV	17α-Methyl-3α,17β-dihydroxyandrost-4-ene	448	143
	V	17α-Methyl-3β,17β-dihydroxyandrost-4-ene	448	143
	VI	17β-Methyl-3β,17α-dihydroxyandrost-4-ene	448	143
	VIII	17β-Methyl-3α,17α-dihydroxy-5α-androstane	450	143
	IX	17α-Methyl-3α,17α-dihydroxy-5α-androstane	450	143
Glucuronide	VII	17α-Methyl-3β,17β-dihydroxy-5α-androstane	450	143
	VIII	17β-Methyl-3α,17α-dihydroxy-5α-androstane	450	143
	IX	17β-Methyl-3α,17α-dihydroxy-5α-androstane	450	143
Sulphate	VII	17α-Methyl-3β,17β-dihydroxy-5α-androstane	450	143
	VIII	17β-Methyl-3α,17α-dihydroxy-5α-androstane	450	143
	IX	17β-Methyl-3α,17α-dihydroxy-5α-androstane	450	143
	X	17α-Methyl-3α,16α,17β-trihydroxy-5α-androstane	538	218+231

[a] MO-TMS derivative.
[b] Major D ring ion.

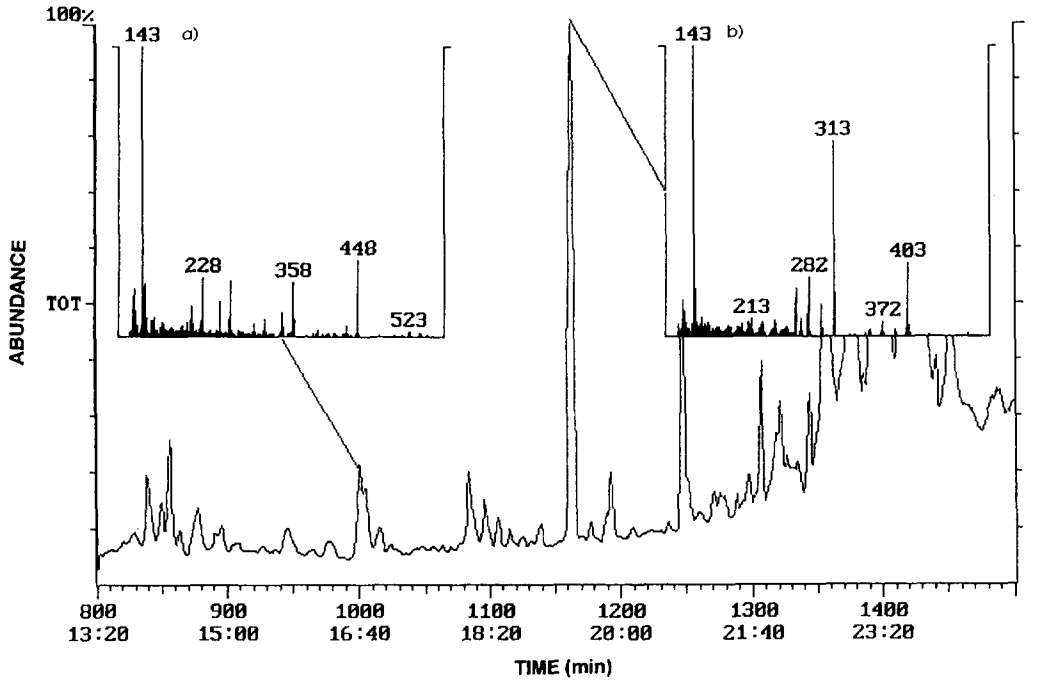

Fig. 2. Total ion chromatogram with spectra of the MO-TMS derivatised free fraction of the 4-h post-administration sample. (a) Metabolite V; (b) MT.

chromatogram from the free fraction. These steroids were identified as TMS ethers:

3.4.1. 17α-Methyl-3α,17β-dihydroxyandrost-4-ene (IV)

This is a minor product and was only detected up to 4 h post administration.

3.4.2. 17α-Methyl-3β,17β-dihydroxyandrost-4-ene (V)

This is the most significant product from the reduction of the 3 keto group of MT. The majority of this was found in the free fraction (Fig. 2) with a small amount of this steroid also encountered in the S fraction.

3.4.3. 17β-Methyl-3β,17α-dihydroxyandrost-4-ene (VI)

A steroid corresponding to the 17β-methyl epimer of V was observed in the free fraction of urine collected during the first 4 h.

3.5. Reduction of both the 3 keto and the 4 ene groups of MT

The 17-methyl-androstanediols were evident to a small extent in the free extract with a preponderance in the S and G fractions.

3.5.1. 17α-Methyl-3β,17β-dihydroxy-5α-androstane (VII)

This is the primary methyl-androstanediol metabolite and was found in the glucuronide and sulphate isolates. It had a retention time corresponding to that of the standard. The spectrum of the TMS derivative has a major fragment ion at m/z 435 $[M^+ - CH_3]$. This steroid is probably produced by a pathway (Fig. 3) analogous to the catabolism of testosterone [9] where 3β,17α-dihydroxy-5α-androstane and 3β,17β-dihydroxy-5α-androstane as either glucuronide or sulphate conjugates predominate as metabolites.

3.5.2. 17β-Methyl-3α,17α-dihydroxy-5α-androstane (VIII)

This 17-methyl epimer of V is found in small quantities in all three fractions.

3.5.3. 17α-Methyl-3α,17β-didihydroxy-5α-androstane (IX)

The almost identical RT values of 17α-methyl-3α,17β-dihydroxy-5α-androstane and 17α-methyl-3α,17β-dihydroxy-5β-androstane under most GC–MS conditions have been described by Massé et al. [5] who overcame the problem of identifying these compounds by using the relative intensities of their common fragment ions. On the same basis a minor metabolite which is found in both the free, S and G fractions was assigned the above configuration by comparison to the standard.

3.6. Hydroxylation of MT and its metabolites

The major biotransformation products appear to result from the addition of hydroxyl groups to MT and intermediate metabolites. Studies published to date indicate that the most common points of attachment to the steroid nucleus are the 6, 15 and 16 positions [2,3,10,11]. The presence of poly-hydroxylated metabolites in the urine was observed from reconstructed ion chromatograms (RIC) at the relevant selected masses. The following compounds were found in the urine extracts and were postulated to be products of these pathways.

3.7. Monohydroxylation of I, II [MO-TMS $M^{+\cdot}$, m/z 491] and III [MO-TMS $M^{+\cdot}$, m/z 493]

The free fraction contains seven steroidal compounds comprising the addition of a single hydroxyl group to either I, II or III. Two of these compounds have spectra with a m/z 231 ion produced from a hydroxylated D ring [12]. Furthermore, because an α-β hydroxy ketone fragment of m/z 218 was also produced, the hydroxyl can be identified as occurring at the 16 rather than the 15 position [4]. The remainder have a prominent m/z 143 base ion indicating an unchanged D ring and, therefore, that the point of metabolism is either the A, B or C ring. The absence of the 11α-hydroxy MT metabolite was established by comparing the data with that of an authentic standard and suggests that alpha hydroxylation at the 11 position is unlikely during the metabolism of MT.

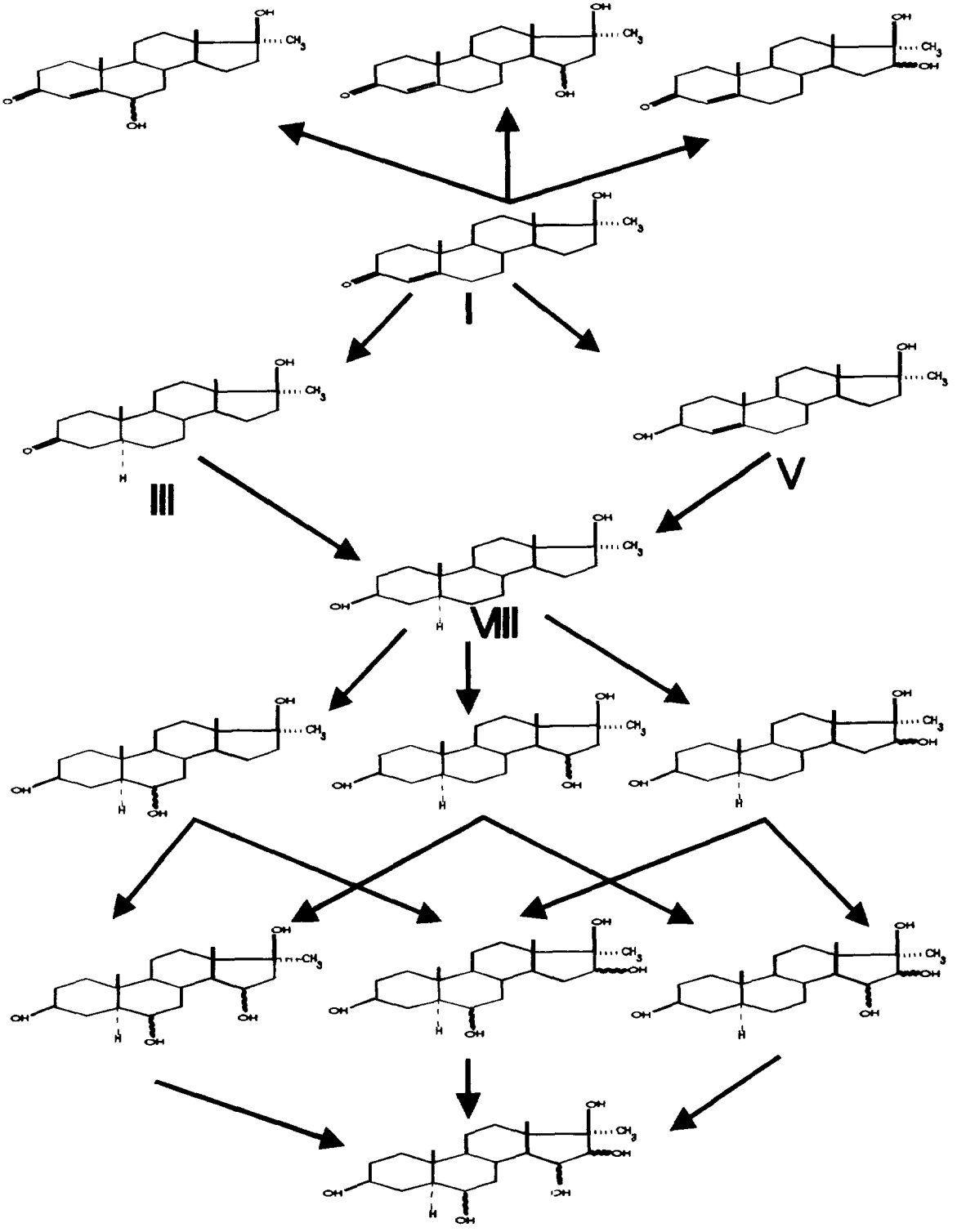

Fig. 3. Metabolism of MT in the equine: proposed major pathway.

3.8. Monohydroxylation of IV, V, VI [TMS $M^{+\cdot}$, m/z 536] and VII, VIII, IX [TMS $M^{+\cdot}$, m/z 538]

These steroids are mostly excreted as sulphate bound phase II metabolites. There are three major S fraction metabolites (Fig. 4) and they all posses a molecular ion *m/z* 538 characteristic of the fully reduced 3-oxo and 4-ene group of MT plus the addition of a single hydroxyl group. Two of these were identified as having a 16 hydroxyl group on the basis of the *m/z* 218+231 ion combination.

3.8.1. 17α-Methyl-3α,16α,17β-trihydroxy-5α-androstane

(X)

One of the minor metabolites (X) matched the RT and spectral characteristics of a 16 hydroxy standard that we have synthesised.

3.9. Dihydroxylation of I, II [MO-TMS $M^{+\cdot}$, m/z 579] and III [MO-TMS $M^{+\cdot}$, m/z 581]

The majority of these compounds were observed in the G fraction. The two most abundant steroids in this isolate had spectra consistent to a 16 hydroxylation coupled with attachment of a hydroxyl at another, as yet unidentified, position.

3.10. Dihydroxylation of IV, V, VI [TMS $M^{+\cdot}$, m/z 624] and VII, VIII, IX [TMS $M^{+\cdot}$, m/z 626]

The tetra-hydroxy steroids found in the G and free fractions appear to be of the same type, whereas the S fraction exhibited a different GC–MS profile. Many of the more abundant steroids also have *m/z* 218+231 ions, yet again confirming that 16 hydroxylation is a prominent step in the metabolism of MT.

The maximum scan range of the GC–MS system used prevented any of the penta TMS derivatives of pentol steroid metabolites being observed during this study. However, several compounds with either *m/z* 642+624 or 644+626 ($M^{+\cdot}$ and $[M-H_2O]^{+\cdot}$) ions were present in S and G fractions. Based on their water loss fragments ($[M^{+\cdot}-18$) these are postulated to be tetra TMS derivatised pentahydroxy steroids with an underivatised OH group. This is likely to be

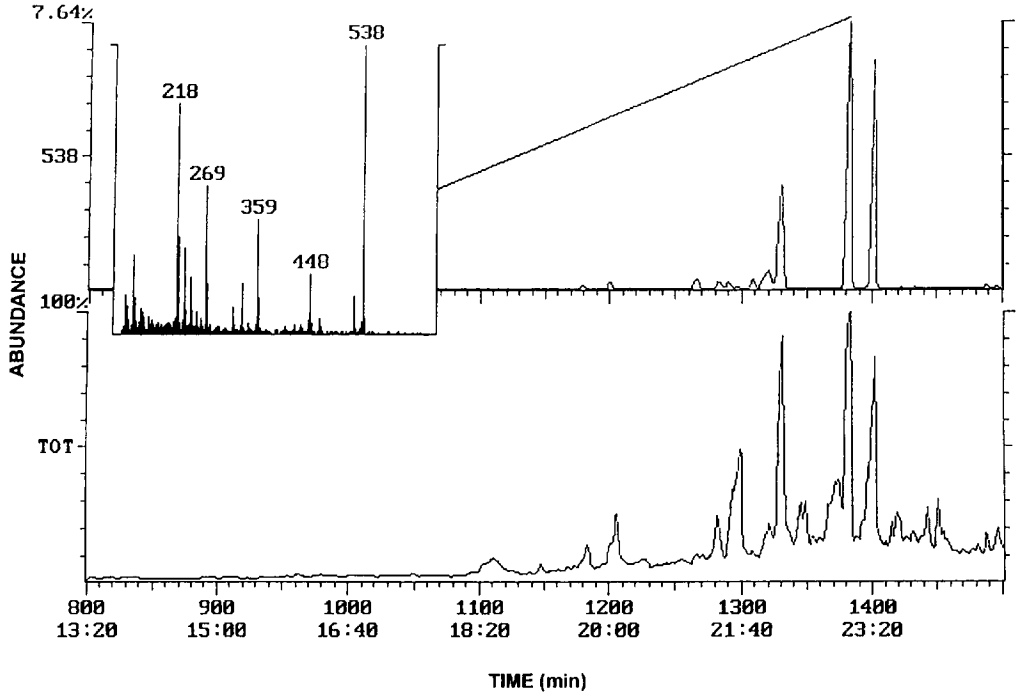

Fig. 4. Total ion chromatogram, spectrum and reconstructed ion (*m/z* 538) chromatogram from the MO-TMS derivatised S fraction of the 8-h sample.

a result of one of the hydroxy groups being located at a position that hinders TMS formation.

The majority of MT is metabolised following a phase I metabolic pathway involving the reduction of the 4-ene and 3-keto group and hydroxylation to produce predominately 3β-hydroxyl-5α-androstane containing polyhydroxylated steroids. These are excreted in equine urine predominantly as glucuronide and sulphate conjugated phase II products. The most likely candidates to be used in confirmatory analytical procedures for these anabolic steroids would be those shown in Fig. 3.

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