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Biotransformation of 17-alkyl steroids in the equine: high-performance liquid chromatography–mass spectrometric and gas chromatography–mass spectrometric analysis of fluoxymesterone metabolites in urine samples

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Abstract

In this study the equine metabolism of fluoxymesterone (9α-fluoro-11β-17β-dihydroxy-17α-methylandrost-4-ene-3-one) given orally has been investigated. The parent material was not detected, but two major 16-hydroxy metabolites which corresponded to a mono- and a di-hydroxylation product were evident. One of the hydroxylation positions was identified as C-16. Phase II metabolism in the form of glucuronide formation was also common. These steroids will provide target compounds for confirming abuse of this drug in the horse. © 1997 Elsevier Science B.V.

Keywords: Steroids; Fluoxymesterone

1. Introduction

Anabolic steroids that are presently available all have, to some degree, an androgenic side effect which stimulates aggression and the so-called 'competitive spirit' [1]. However, in the case of fluoxymesterone (FM) where the anabolic:androgenic ratio is twice that of testosterone [2] it enhances muscle building properties, but is less likely to cause the horse to become fractious. In addition it has been reported [3] to stimulate the secretion of eythropoietin in humans which leads to increases in the hematocrit. In certain circumstances this may enhance stamina. These effects have resulted in the abuse of this anabolic steroid by those who believe it will give their racehorse an advantage over untreated animals in the race.

Very little has been published regarding the fate of 17-alkylated anabolic steroids after administration to the horse. This is despite the fact that there are a large variety of proprietary medicines sold internationally. In particular, there has been little documented on the metabolism of FM in the horse, while mention has been made of a 6-hydroxy fluoxymesterone metabolite in humans [4]. FM is a steroid that can be given orally because of the protection from first pass inactivation that is afforded by the 17α-alkyl moiety. This is a consequence of steric hindrance inhibiting access of metabolic enzymes to the C-17 hydroxyl group. This functionality also influences other biotransformation pathways. For example, previous studies [5–10] of 17α-methyl

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anabolic steroids administered to horses have shown that the metabolism of these drugs results in a complex mixture of products with very little of the unchanged parent material being excreted in the urine. This differs considerably from metabolism of their analogues that are not alkylated at the 17 position.

We report the results of an investigation of the metabolism of fluoxymesterone in equines using both high-performance liquid chromatography (HPLC)–mass spectrometry (MS) and gas chromatography (GC)–mass spectrometry. Five steroids (I–V) were detected, of which two were established as 16 hydroxy metabolites.

2. Experimental

2.1. Materials

Methanol, ethyl acetate, chloroform, 2-propanol, dichloromethane and hexane B and J brand HPLC grade solvents were obtained from Baxter (Muskegon, MI, USA). Diethyl ether was distilled from commercial grade (Illovo Merebank, Johannesburg, South Africa) after NaOH addition. Acetic acid of >99.8% purity and dodecane were supplied by Riedel-de Haën (Seelze, Germany). Associated Chemical Enterprises (Johannesburg, South Africa) supplied sulphuric acid (>98.8%). Methoxyamine·HCl, trimethylsilylimidazole, methylsilyl trifluoroacetamide (MSTFA), *E. coli* β-glucuronidase and Sephadex LH-20 were obtained from Sigma (St. Louis, MO, USA). Phosphorous pentoxide, KOH and NaOH were purchased from Saarchem (Krugersdorp, South Africa). Bis(trimethylsilyl)acetamide and pyridine (dried GR) were from Merck (Darmstadt, Germany). BDH (Poole, UK) supplied trimethylchlorosilane and ethoxamine·HCl. N-Methylboronic acid was obtained from Fluka (Buchs, Switzerland). N-Butylboronic acid was obtained from Tokyo Kasei (Tokyo, Japan). Fluoxymesterone was purchased from Steraloids (Wilton, NH, USA).

2.2. Animal administration

A 480-kg mare was administered 500 mg of fluoxymesterone (Halotestin) via a nasogastric tube. Urine samples were obtained using a catheter and frozen within twenty min of collection. Storage was at −20°C.

2.3. Urine extraction

Samples of urine (20 ml) collected 4 h post fluoxymesterone administration were thawed and where the F and G fractions were separated, the free steroids were first extracted into 2×20 ml of CH_2Cl_2–2-propanol (9:1, v/v). Nitrogen was bubbled through the remaining aqueous phase until all the residual solvent was removed. The aqueous phase was adjusted to pH 6.8 and incubated overnight at 37°C with 4000 Fishman units of *E.coli* β-glucuronidase. Previously glucuronide bound steroids were then extracted with the solvent mixture mentioned above. Both organic phases were dried with oxygen-free-nitrogen (OFN) before being reconstituted in 100 μl methanol and retained for analysis.

The aqueous phase was centrifuged (2000 *g* for 15 min) after which the sulphate bound steroids were isolated on a C_{18} cartridge (Varian, Harbor City, CA, USA) preconditioned with 5 ml of methanol and 3 ml of water. After washing with 10 ml of water the sulphate bound steroids were eluted with 10 ml of solvolysis mix (ethyl acetate–methanol, 9:1, with 100 μl of sulphuric acid per 100 ml) and hydrolysed at 50°C for 2 h. This was evaporated to dryness and further purification was then performed using Bond Elut Certify II cartridges (Varian). The extracted material was reconstituted in 100 μl of methanol plus 5 ml of 0.2 *M* acetate buffer (pH 3) (AB, 100 ml water plus 1140 μl acetic acid and pH adjusted to 3.0 with KOH). This was then applied to Bond Elut cartridges preconditioned with 5 ml of methanol and 5 ml AB. After washing with 5 ml of AB and 10 ml of 5% methanol in water, the steroids were eluted with 5 ml ethyl acetate. The solvent was removed using oxygen-free-nitrogen (OFN) and the residue made up in 100 μl of methanol for analysis.

2.4. APCI-MS analysis of extracts

A Thermo Separation Products (Fremont, CA, USA) 3500/3200 MS binary pumping system connected to a Finnigan MAT (San Jose, CA, USA)

SSQ 7000 via an atmospheric pressure chemical ionisation (APCI) mass spectrometry interface was used in the positive ion full scan mode. Vaporizor temperature 270°C, corona discharge voltage +4.28 kV, heated capillary 150°C, sheath gas 275 kPa and auxiliary gas 10, were the settings used for APCI MS. Collision induced dissociation was achieved at +60 V. Loop injections were performed using a Themo Separation Products AS 3000 autosampler with a 0.25 ml/min flow-rate of methanol–1% aqueous acetic acid (99:1). HPLC separations were performed on a Phenomenex (Torrance, CA, USA) C_{18} (200×2 mm, 5 μm) column at a flow-rate of 0.25 ml/min. The initial composition was methanol–1% aqueous acetic acid (1:1). This was changed on a linear gradient to 100:0 at 15 min.

2.5. Preparative HPLC fractionation of steroid metabolites

The extracts were reconstituted in 75 μl of methanol and injected (3×25 μl) onto a Hewlett-Packard 1090L HPLC system fitted with a Prepex C_{18} (250×10 mm, 40 μm) column from Phenomenex and coupled to a microfraction collector. The oven temperature was set at 40°C and the flow-rate was 1 ml/min. The starting mixture was methanol–water (5:95), with steroid elution achieved by changing the composition to 95:5 over 35 min. The final composition was maintained for 15 min. Fractions were collected every 0.5 min between 10 and 50 min during the run. The fractions purified by HPLC were dried, reconstituted in 0.5 ml of methanol and analysed by loop injection of 1 μl into the APCI-MS system. Fractions were pooled together on the basis of their common characteristics and aliquots were derivatised.

2.6. Derivatisations

2.6.1. Methoxyamine (MO) and ethoxyamine (EO) derivatisation of keto groups

Extracts were reconstituted in 50 μl of an 8% solution of either methoxyamine·HCl or ethoxyamine·HCl in pyridine and heated at 80°C for 30 min. The pyridine was removed by evaporation with OFN and the residue was trimethyl silyl (TMS) derivatised.

2.6.2. Trimethylsilyl (TMS)

Samples were incubated at 60°C for 48 h with 100 μl of a solution of trimethylsilyl imidazole–bis(trimethylsilyl)acetamide–trimethylchlorosilane (3:3:2, v/v/v). After drying under OFN, the sample was reconstituted in 0.5 ml of a solution of chloroform–hexane (1:1) and passed through a 2-cm bed of Sephadex LH-20 packed in a glass Pasteur pipette. The sample vial was washed with 500 μl of the chloroform–hexane solution and the column eluted with a further 3 ml of this solvent. The samples were dried under OFN and the residue reconstituted in 30 μl of dodecane for GC–MS analysis.

2.6.3. Methyl and butyl boronic acid derivatisation of vicinal diols

The sample was reconstituted in 50 μl of a 1 mg/ml solution of either methyl or butyl boronic acid in ethyl acetate. The sample was incubated at room temperature for 12 h before the solvent was removed with OFN followed by TMS derivatisation of the extract.

2.7. Gas chromatography–mass spectrometry

A Finnigan GCQ GC–MS–MS fitted with a 30-m DB5-MS (0.25 mm I.D., 0.25 μm film thickness) J&W Scientific column (Folsom, CA, USA) was used. The carrier gas was helium of 99.999% purity from Fedgas (Johannesburg, South Africa). The column which was at an initial temperature of 100°C was ramped at 40°C/min to 220°C and then 10°C/min to 310°C. The maximum temperature was held for 5 min. The injector and transfer line were at 290°C and 270°C respectively. The scan range in full scan MS mode was 100 to 800 u. (mass defect setting 50 m.m.u. (milli mass units)/100 u) and the range in MS—MS mode was from 100 u up to the parent ion selected. A notch width of the parent ion ±1 u and an energy setting of 1.2 were chosen for all MS–MS experiments.

3. Results

The steroid metabolites in the urine were initially separated into free (F), glucuronide (G) and sulphate (S) conjugated fractions. These isolates were subject-

ed to HPLC–MS analysis via an APCI interface and it was evident that the F and G fractions, which contained the bulk of the steroids of interest, were essentially very similar in composition and differed from the sulphate bound metabolites. Two steroids predominated and these were mainly excreted either free or as glucuronic acid conjugates. The major biotransformation product gave a spectrum with a dominant m/z 353 ion ($[M+H]^+$, Fig. 1) corresponding to a compound (I) produced by the monohydroxylation of fluoxymesterone. The other metabolite of significance gave a spectrum (Fig. 1) consistent with steroid of 368 u (parent material +32) and therefore corresponds to a di-hydroxy metabolite (II). It was notable that the reconstructed ion chromatogram corresponding to the pseudo-molecular cation of fluoxymesterone (m/z 337) did not show a peak at the retention time equivalent to that of our FM reference material. However, there was a response at an earlier retention time and this analyte (III) was postulated to be the 17β-methyl-17α-hydroxyl epimer of fluoxymesterone. This type of transformation has been encountered previously in other studies of similar 17-alkyl steroids in the horse (e.g. [5]). HPLC–MS analysis of the sulphate fraction showed that it contained a small amount of the II metabolite and a compound with a spectrum consistent with 3 keto reduced I.

The extracts were examined by GC–MS. Before analysis these steroids must be derivatised to prevent absorption to active sites on the fused-silica capillary GC column which causes a loss of resolution and sensitivity. The extracts were methoxyamine (MO) and trimethylsilyl (TMS) derivatised using a published double derivatisation method [11,12], but the expected MO-TMS products were not detected in significant quantities. It was suspected that the conditions employed were insufficiently rigorous for the conversion of the 11-hydroxy group to a TMS ether [13] and a different technique [14], reported to be effective with hindered hydroxyl groups, was adopted. A further aliquot of the 4 h urine collection was extracted but this time a modified procedure was employed to obtain a combined F+G fraction. The result (Fig. 2) was improved detectability of the analytes without any noticeable loss of information as the phase I metabolites appeared to be the same in both of these fractions. The dominant biotransformation product (Fig. 3, Table 1) was detected in the F+G fraction and had a molecular ion m/z 597 for the MO-TMS and m/z 611 for EO-TMS derivative. This was postulated to be the equivalent to I seen by HPLC–MS analysis. The D-ring fragment ion combination of m/z 218+231 from the rupture of C-13–C-17 and C-14–C-15 bonds was indicative of 16 hydroxylation [9], thereby establishing the position of the introduced hydroxyl group as C-16. This compound was examined further by MS–MS and the fragments ions found in its spectrum are shown in Table 2. It was possible to establish that the elimination of the m/z 231 and 218 ions from the structure were via different mechanisms. The production of the fragment ion m/z 231 directly from the intact molecule is in accordance with the pathway suggested by Schoene et al. [9] for TMS derivatised 16-hydroxylated methyltestosterone. The second compound of interest had a molecular ion (MO-TMS) at m/z 685 (Fig. 4, Tables 1 and 3) and thus corresponded to the derivatised II metabolite. There were prominent m/z 218 + m/z 231 ions suggesting that II is likely to be a product of the hydroxylation of I. The position of introduction for the second hydroxyl is unknown but could be at C-6 as this has been encountered before in studies of similar compounds [5+6] and in metabolism of FM in the human [4]. The I and II metabolites were isolated by preparative HPLC fractionation of a F+G extract. The two steroids identified were MO-TMS derivatised before GC–MS analysis. These results confirm that the materials detected by GC–MS correspond to those identified by HPLC–MS.

Although reduction is one of the major transformations that generally occurs in the equine metabolism of 3-keto and 4-ene steroids (e.g. testosterone [15]) very little material that corresponded to a reduced FM metabolite was detected in either the HPLC–MS or the GC–MS chromatograms. Only one minor metabolite (IV) observed in the GC–MS analysis gave data (Table 1) consistent with a 3-keto reduced product.

Several other metabolites were identified from the HPLC–MS and GC–MS data. The steroid designated V (Table 1) was postulated to be a structural isomer of I arising from the hydroxylation of FM at the second (undetermined) site of attachment. This was based on the fact that there were m/z 597 and

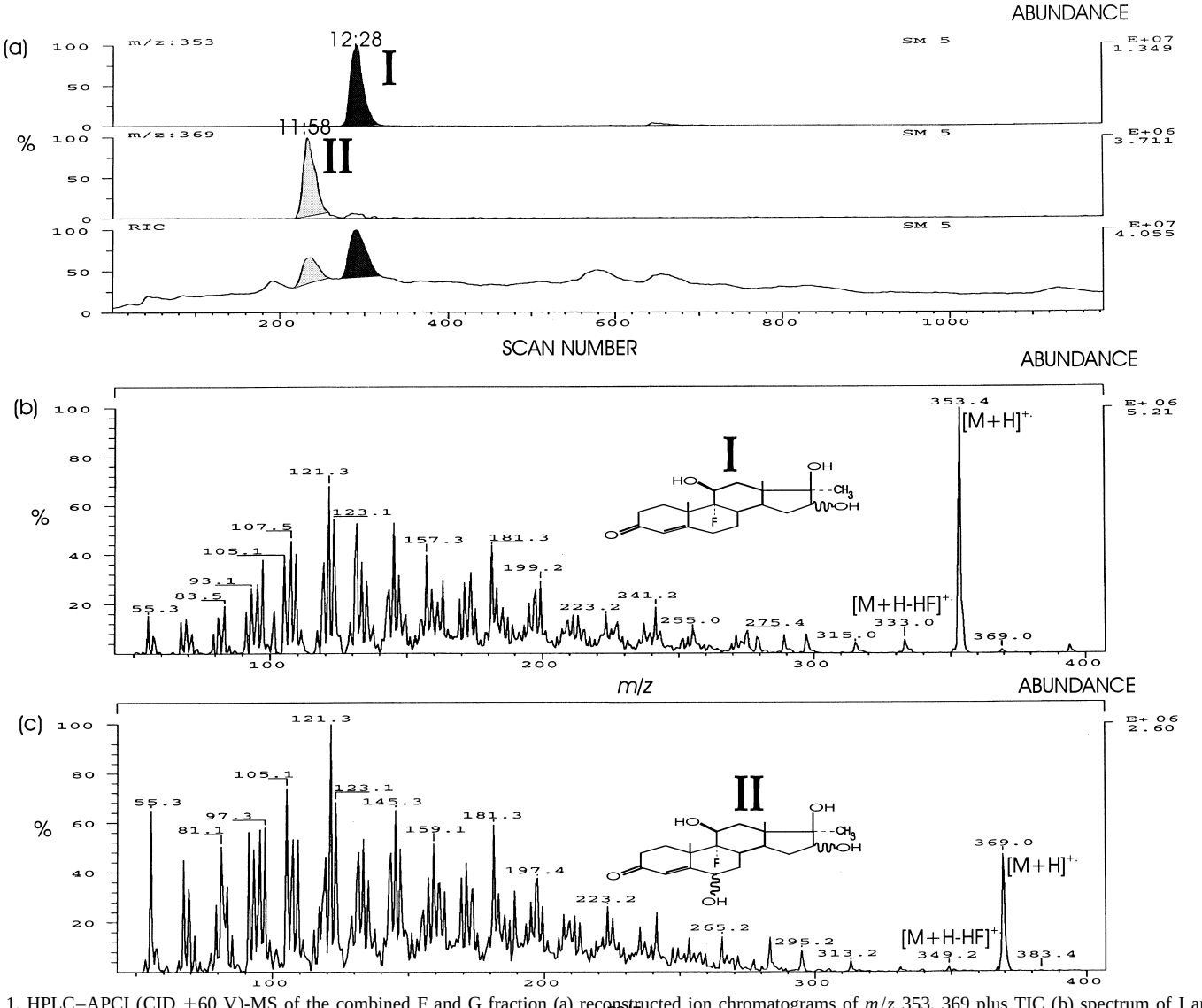

Fig. 1. HPLC–APCI (CID +60 V)-MS of the combined F and G fraction (a) reconstructed ion chromatograms of *m/z* 353, 369 plus TIC (b) spectrum of I and (c) spectrum of II.

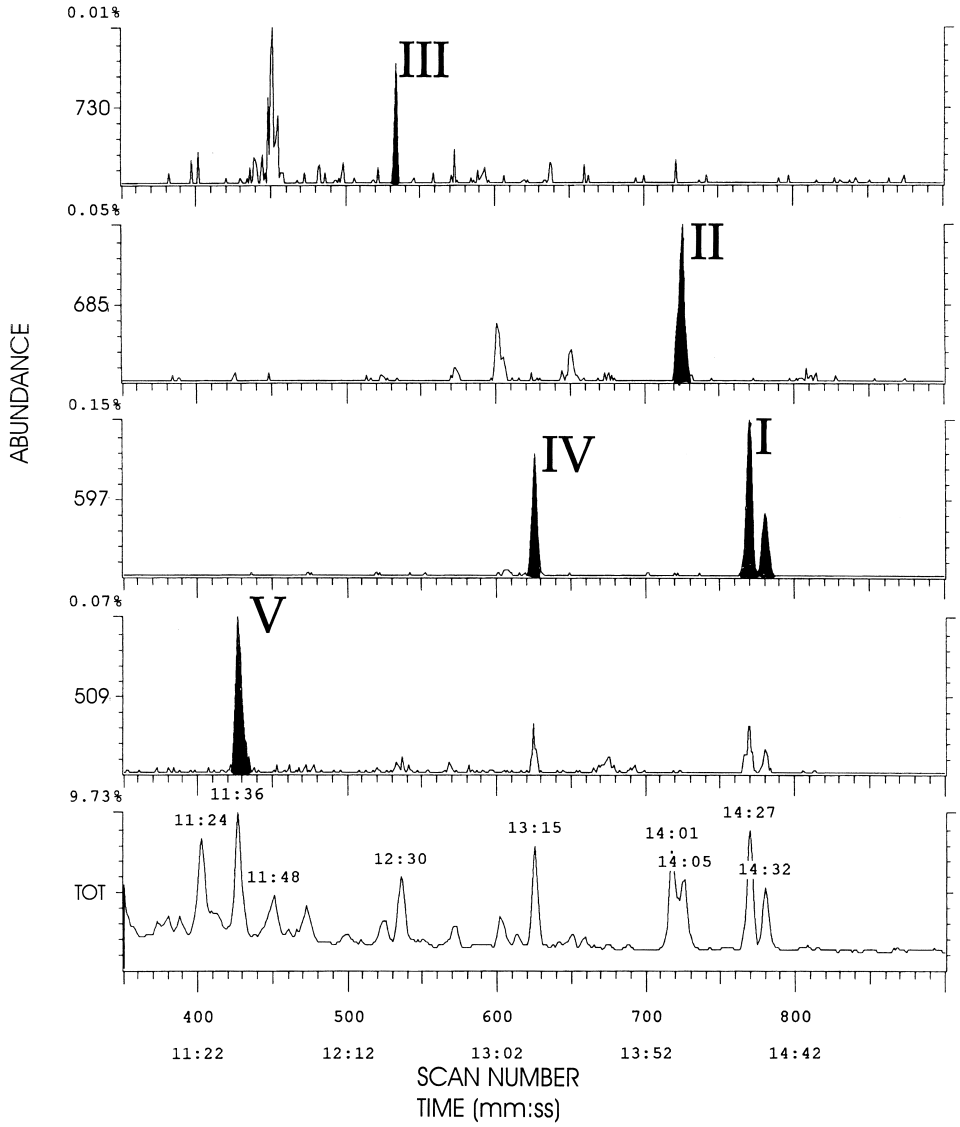

Fig. 2. GC–MS extracted ion chromatograms m/z 730, 685, 597, 509 and TIC from the MO-TMS derivatised F+G fractions of the 4 h extract.

611 molecular ions for MO-TMS and EO-TMS respectively, which establishes the molecular mass to be the same as I. However, the m/z 143 fragment ion plus the lack of the m/z 218+231 ion combination is indicative of an α,β-unsaturated ketone arising from an unaltered D-ring [9,16]. Thus the position of hydroxyl attachment in IV is neither C-15 nor C-16.

The presence of a steroid (III) with a APCI MS spectrum identical to FM, but exhibiting different HPLC behaviour from the reference material, was mentioned previously. This compound was also detected in the GC–MS chromatogram. Our observation has been that the 17α-hydroxyl-17β-methyl isomers of TMS derivatised steroids elute from the column faster than their corresponding epimers during GC analysis. The fact that this steroid had a shorter retention time than a FM standard and the close similarity between their spectra reinforces our

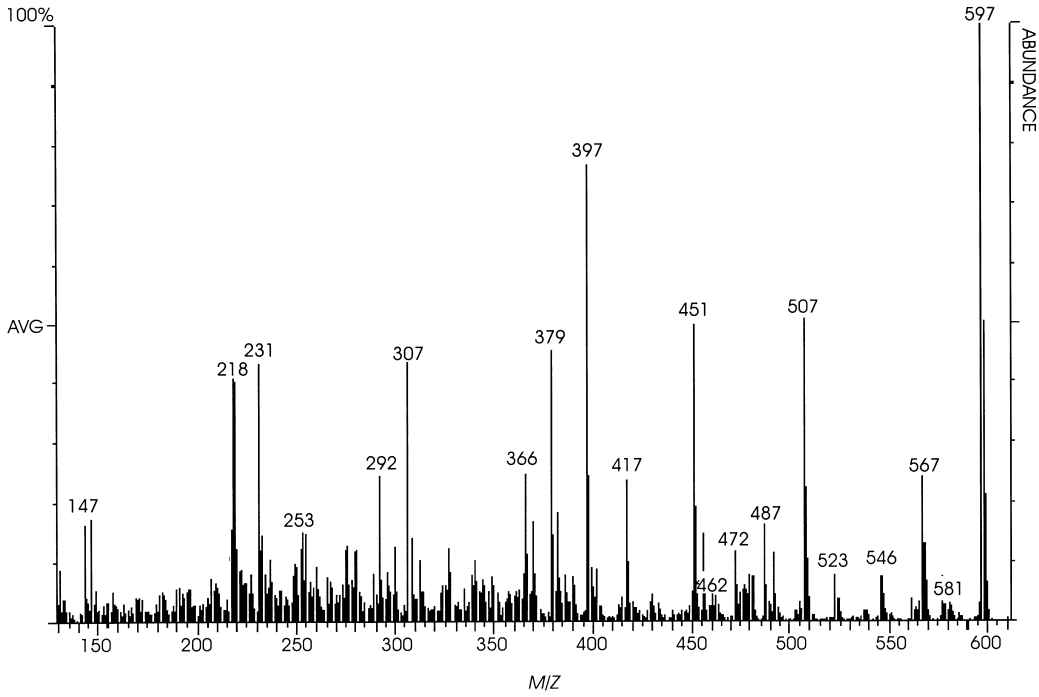

Fig. 3. GC–MS spectrum of the earlier eluting MO-TMS isomer of derivatised I from the F+G fraction of the 4 h sample.

assignment of this compound as an epimer of fluoxymesterone.

We attempted to establish the nature of the stereochemical arrangement at C-16 and C-17 in the two major metabolites I and II by derivatisation of the hydroxyl groups at these positions using the

Table 1
GC–MS analysis of the F+G combined fraction

Retention		Abundance		Metabolite[a]	Major ions[b]			
RT[c]	RRT[d]							
MO-TMS								
12.45	0.892	++	III	17-epi-FM MO bis-TMS	509	419	404	143
13.25	1.070	+	IV	6ξ,16ξ-dihydroxy-FM(+H_2) penta-TMS	730	640	231	218
11.63	1.179	+	V	6ξ-hydroxy-FM MO tris-TMS	597	507	417	143
14.08	1.181	+++	II	6ξ,16ξ-dihydroxy-FM-MO tetra-TMS	685	655	231	218
14.45+14.53	1.203+1.209	++++	I	6ξ-hydroxy-FM MO tris-TMS	597	507	231	218
EO-TMS								
10.40	0.911	++	III	17-epi-FM EO bis-TMS	523	433	418	143
12.22	1.070	+	IV	6ξ,16ξ-dihydroxy-FM(+H_2) penta-TMS	730	640	231	218
12.94	1.113	+	V	6ξ-hydroxy-FM EO tris-TMS	611	521	501	143
13.01	1.139	+++	II	6ξ,16ξ-dihydroxy-FM-EO tetra-TMS	699	684	231	218
13.18	1.154	++++	I	6ξ-hydroxy-FM EO tris-TMS	612	522	231	218

[a] Proposed structure.
[b] In descending numerical order.
[c] Retention time in min.
[d] Retention relative to FM.

Table 2
Fragment ions obtained by GC–MS–MS analysis of I (MO-TMS)

Parent[a]	Product ions[b]														
597	231(100)	232(27)	417(14)	143(12)	402(11)	365(11)	367(10)	451(8)	397((7)	507(7)	418(6)	379(6)	391(5)	439(5)	292(4)
567	477(100)	462(50)	334(28)	387(14)	349(11)	352(9)	372(9)	262(8)	442(8)	224(7)	242(7)	457(7)	421(6)	282(5)	-
507	292(100)	417(96)	472(87)	400(73)	456(64)	492(59)	366(50)	307(49)	382(49)	391(44)	476(40)	397(38)	281(32)	276(30)	402(29)
451	222(100)	237(88)	312(62)	242(51)	347(50)	197(41)	257(39)	327(20)	208(19)	217(19)	196(18)	228(18)	210(16)	236(16)	211(15)
417	292(100)	327(95)	386(85)	312(61)	296(47)	276(45)	366(37)	281(37)	307(35)	402(25)	362(14)	260(11)	329(10)	349(9)	382(9)
402	297(100)	312(80)	292(78)	382(35)	261(31)	260(24)	371(23)	281(37)	335(15)	310(14)	287(14)	352(13)	266(12)	280(12)	245(12)
397	382(100)	307(82)	366(53)	292(38)	275(28)	365(27)	276(22)	260(16)	349(13)	253(12)	252(10)	233(9)	266(9)	248(9)	281(8)
379	239(100)	259(54)	296(39)	348(36)	328(27)	221(23)	331(23)	288(18)	223(17)	365(15)	271(14)	206(14)	289(14)	323(14)	195(13)
365	274(100)	276(66)	351(58)	323(52)	260(42)	326(42)	348(34)	349(19)	335(30)	338(28)	224(25)	248(23)	258(21)	273(21)	245(15)
307	292(100)	260(64)	275(47)	276(35)	233(28)	248(17)	278(15)	252(32)	261(8)	239(7)	259(7)	265(6)	246(5)		
292	193(100)	246(70)	261(56)	235(54)	237(37)	262(31)	277(28)	219(27)	220(26)	221(24)	207(24)	245(24)	218(21)	260(18)	234(16)
233	218(100)	143(40)	203(20)	-	-	-	-	-	-	-	-	-	-	-	-
231	143(100)	215(7)	73(6)	216(2)	147(2)	-	-	-	-	-	-	-	-	-	-

[a] Parent ion±1 u.
[b] Daughters, with abundance shown in brackets, above 5% of the base peak arranged in order of decreasing intensity.

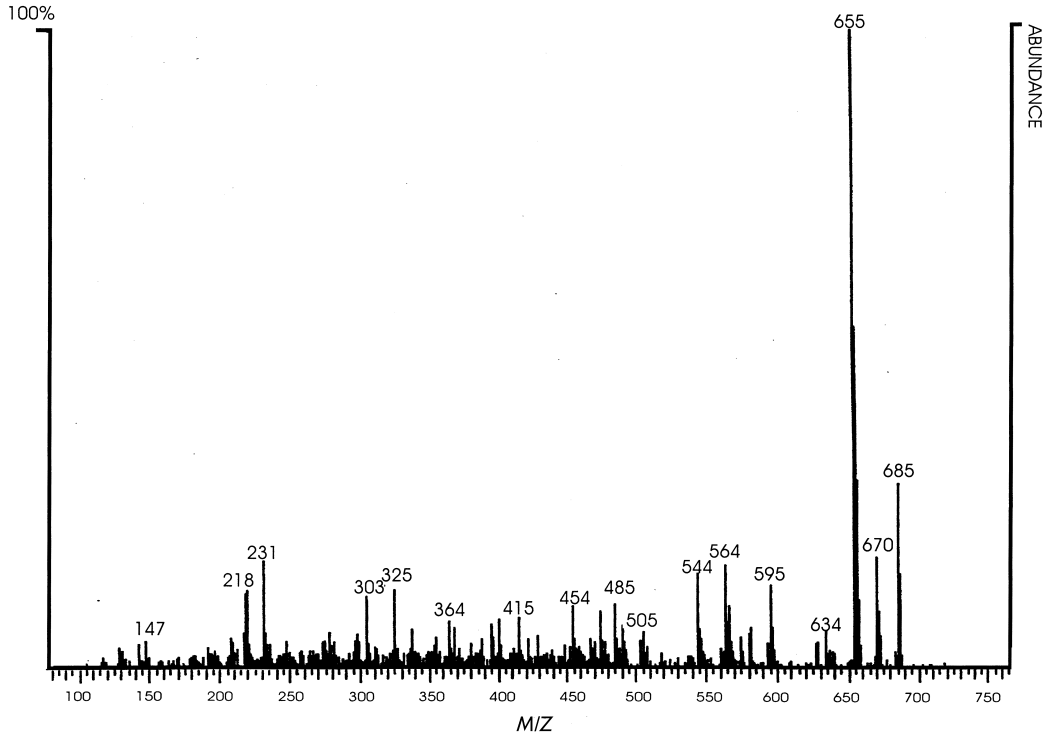

Fig. 4. GC–MS spectrum of II from the MO-TMS derivatised fraction of the 4 h sample.

bi-functional derivatising reagents methyl and butyl boronic acid. Despite our success with steroid standards having a 16β-17β-diol configuration we were unable to convert significant amounts of either I or II to the boronate product. In the absence of further data we are unable to determine whether this is due to the fact that the vicinal diol configuration is resistant to conversion or there is a simple failure to achieve adequate levels of derivatisation due to the matrix or other effects.

In conclusion, the metabolism of FM in equines appears to produce two major metabolites which result from hydroxylation of the steroid nucleus. These are predominantly found in equine urine either free or as glucuronic acid conjugates. The parent material was not detected in significant amounts and this is consistent with the results from previous metabolic studies of 17-alkyl containing anabolic steroids. However, unlike equine metabolism of other steroids which contain the 3-keto and/or 4-ene functionalities there was only limited reduction to 3-hydroxy and 5-androstane products. This may be a result of an inhibitory influence of the 9α-fluorine group on the equines' reductive enzymes.

Table 3
Fragment ions obtained by GC–MS–MS analysis of II (MO-TMS)

Parent[a]	Product ions[b]						
685	654(100)	231(29)	564(17)	422(10)	299(5)	634(5)	492(5)

[a] Parent ion±1 u.
[b] Daughters, with abundance shown in brackets, above 5% of the base peak arranged in order of decreasing intensity.

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