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Identification and quantification of metabolites common to 17α-methyltestosterone and mestanolone in horse urine

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Abstract

Anabolic steroids with the 17α-methyl,17β-hydroxyl group, which were developed as oral formulations for therapeutic purposes, have been abused in the field of human sports. These anabolic steroids are also used to enhance racing performance in racehorses. In humans, structurally related 17α-methyltestosterone (MTS) and mestanolone (MSL), which are anabolic steroids with the 17α-methyl,17β-hydroxyl group, have metabolites in common. The purpose of this study was to determine metabolites common to these two steroids in horses, which may serve as readily available screening targets for the doping test of these steroids in racehorses.

Urine sample collected after administering MTS and MSL to horses was treated to obtain unconjugated steroid, glucuronide, and sulfate fractions. The fractions were subjected to gas chromatography/mass spectrometry (GC/MS), and 17α-methyl-5α-androstan-3β,17β-diol, 17α-hydroxymethyl-5α-androstan-3β,17β-diol, 17α-methyl-5α-androstan-3β,16β,17β-triol, and 17α-methyl-5α-androstan-3β,16α,17β-triol were detected as the common metabolites by comparison with synthesized reference standards. The urinary concentrations of these metabolites after dosing were determined by GC/MS. 17α-Methyl-5α-androstan-3β,16β,17β-triol was mainly detected in the sulfate fractions of urine samples after administration. This compound was consistently detected for the longest time in the urine samples after dosing with both steroids. The results suggest that 17α-methyl-5α-androstan-3β,16β,17β-triol is a very useful screening target for the doping test of MTS and MSL in racehorses.
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Keywords: 17α-Methyltestosterone; Mestanolone; Equine; Metabolite; Anabolic-androgenic steroid; GC/MS

1. Introduction

Anabolic steroids with the 17α-methyl,17β-hydroxyl group were mainly developed for oral administration, and 17α-methyltestosterone (MTS) and mestanolone (MSL) are commercially available in Japan. In comparison with intravenously administered steroids that tend to have a long excretion time, orally administered steroids have short-term effects and a short excretion time. Anabolic steroids were developed primarily for therapeutic purposes. However, they have been abused to improve physical performance in human sports and horseracing. Hence, the use of anabolic steroids is now forbidden in athletes and racehorses. Drug testing laboratories have been requested to develop methods for the detection of misused anabolic steroids. In general, doping acts are confirmed based on the detection and identification of presumed drugs and their metabolites. However, most 17α-methyl,17β-hydroxy steroids are extensively metabolized, and the parent steroids persist only for a short time after administration. Therefore, knowledge of the metabolism of these steroids is vital to establishing an appropriate method for the doping test. Studies have shown that structurally related MTS and MSL yield metabolites in common in human [1,2]. There are also reports of the urinary metabolites of MTS in horse; Stanley et al. [3] and Dumasia [4] used GC/MS to identify 17-hydroxymethyl metabolites, and McKinney et al. examined the stereochemistry of urinary metabolites by comparing with authentic reference standards [5]. The main steps in the metabolism of MTS in horses are as follows: reduction of A-ring 3-oxo and 4-ene groups; oxidation at C-6, C-15, and C-16; and epimerization at C-17 during phase I biotransformation, followed by sulfate and glucuronide conjugation during phase II biotransformation [3,4]. However, we could not find

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any reports of the urinary metabolites of MSL and their relation to the metabolites of MTS. To establish an appropriate method for the doping test, it is important to know the major metabolites and the length of time for which they are detectable. In this study, we investigated the urinary metabolites of MTS and MSL to establish an appropriate method for the doping test in racehorses. Using the MS data of urinary metabolites after MTS and MSL administration in horse, we determined the metabolites in common. Then, we synthesized those metabolites and used them as reference standards for identification and quantification. Based on the analytical data, we determined the target substance for the doping test of MTS and MSL in racehorses.

2. Experimental (materials and methods)

2.1. Steroids

Authentic MTS and MSL were purchased from Sigma–Aldrich Japan (Tokyo, Japan). MTS and MSL tablets were obtained from Asuka Pharmaceutical (Tokyo, Japan) and Teikokuzouki (Tokyo, Japan), respectively. [16,16,17-D_3]-5α-Androstane-3α,17β-diol used as internal standard was synthesized in our laboratory according to the report of Dehennin et al. [6]. Androsterone sodium sulfate (ADS), nandrolone (NAD), testosterone, 17α-methyl-5α-androstan-3α,17β-diol, and 5α-androstan-3β-ol-17-one were purchased from Steraloids (Newport, RI, USA). 17α-Methyl-5β-androstan-3α,16β,17β-triol, 17α-hydroxymethyl-5α-androstan-3β,17β-diol, 17α-methyl-5α-androstan-3β,16α,17β-triol, and 17α-methyl-5α-androstan-3β,16β,17β-triol were synthesized in our laboratory.

2.2. Chemicals

N-Methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA), trimethylchlorosilane (TMCS), and hexamethyldisilazane (HMDS) were purchased from GL Sciences (Tokyo, Japan). Sep-Pak Plus C_{18} 360 mg/cartridge (Nihon Waters, Tokyo, Japan) was used as SPE column. β-Glucuronidase derived from *Pomacea canaliculata*, Type A-I, 22,000 Fishman units/ml, was purchased from Nihon Biotest (Tokyo, Japan), and 1 M hydrogen chloride–methanol solution was obtained from Kokusan Kagaku (Tokyo, Japan). All other reagents and solvents were of analytical or HPLC grade (purchased from Wako Pure Chemical Industries, Tokyo, Japan or Kanto Kagaku Reagent Division, Tokyo, Japan).

2.3. Drug administration and sample collection

MTS and MSL tablets were administered at a dose of 1.0 mg/kg to 3 gelding thoroughbreds (12–15-year-old, 450–600 kg) through a nasogastric tube. The interval between administrations of each drug to the same horse was more than 1 month.

Urine samples were collected prior to administration and at 6, 12, 24, 48, 72, 96, 120, 144, and 168 h post-administration from each horse. All urine samples were stored at temperatures below −40 °C until analysis.

2.4. Sample preparation for structure elucidation of metabolites

2.4.1. Unconjugated steroid fraction

The pH of the urine sample (5 ml) was adjusted to 5.0 with acetate buffer, and the sample was loaded onto a Sep-Pak Plus C_{18} column preconditioned with methanol (5 ml) and deionized water (5 ml). The column was rinsed with deionized water (8 ml) and dried under vacuum, and the unconjugated steroid fraction was eluted with diethyl ether (8 ml). The eluate was evaporated under nitrogen at temperatures below 40 °C, and the residue was dissolved in diethyl ether (5 ml). Next, the solution was washed first with 1 M hydrochloric acid (2 ml) and then with 1 M sodium hydroxide (2 ml). The solvents were evaporated under nitrogen at temperatures below 40 °C. The residue was dissolved in MSTFA/TMCS/pyridine (20:2:78) mixture and incubated for 30 min at 80 °C. After removal of excess reagent, the dried residue was reconstituted with 0.1 ml of *n*-undecane/HMDS (99:1, v/v), and 1 μl of this reconstituted solution was subjected to gas chromatography/mass spectrometry (GC/MS) as the unconjugated steroid fraction.

2.4.2. Glucuronide fraction

After collecting the unconjugated steroid fraction, the glucuronide conjugates that remained in the column were eluted with 8 ml of ethyl acetate/methanol (4:1). After evaporating the eluate, the residue was dissolved in 5 ml of 0.2 M acetate buffer (pH 5.0) and hydrolyzed with β-glucuronidase (derived from *P. canaliculata*, 5500 Fishman units) for 2 h at 60 °C. This solution was loaded onto another preconditioned Sep-Pak Plus C_{18} column. The column was rinsed with deionized water (8 ml), and the unconjugated steroids derived from glucuronides were eluted with diethyl ether (8 ml). The eluate was evaporated under nitrogen at temperatures below 40 °C, and the residue was dissolved in diethyl ether (5 ml). Next, the solution was washed first with 1 M hydrochloric acid (2 ml) and then with 1 M sodium hydroxide (2 ml); subsequently, the solvents were evaporated under nitrogen at temperatures below 40 °C. The residue was treated with the same method as that for the unconjugated steroid fraction for TMS derivatization, and analyzed with GC/MS as the glucuronide fraction.

2.4.3. Sulfate fraction

After collecting the glucuronide fraction, sulfate conjugates that remained in the column were eluted with 8 ml of ethyl acetate/methanol (4:1). After evaporating the eluate, the residue was dissolved in 1 M anhydrous hydrogen chloride–methanol solution (0.5 ml) and incubated for 10 min at 60 °C. The reaction mixture was evaporated under nitrogen at temperatures below 40 °C, and the residue was dissolved in diethyl ether (5 ml). Next, the solution was washed first with 1 M hydrochloric acid (2 ml) and then with 1 M sodium hydroxide (2 ml). The solvents were evaporated under nitrogen at temperatures below 40 °C, and the residue was treated with the same method as that for the unconjugated steroid fraction for trimethylsilyl (TMS) derivatization, and analyzed with GC/MS as the sulfate fraction.

2.5. Sample preparation for quantification of metabolites

2.5.1. Calibration curves

The calibration curves for quantification were constructed as follows. Blank urine was spiked with authentic reference standards and internal standards and quantified. The concentration of spiked reference standards ranged from 0.05 to 5 μg/ml.

2.5.2. Unconjugated steroids and glucuronides (U + G fraction)

Internal standards were added to 1 ml of urine sample (IS: NAD and ADS, 1 μg each) and pH was adjusted to 5.0 with 1.0 ml of 0.2 M acetate buffer. After hydrolysis with β-glucuronidase from *P. canaliculata* (4400 Fishman units) for 2 h at 60 °C, the reaction mixture was loaded onto a Sep-Pak Plus C_{18} column (pretreated with 5 ml of methanol and 5 ml of deionized water). The column was rinsed with 10 ml of deionized water and dried under vacuum. Then, the column was eluted with 10 ml of diethyl ether. The eluate was evaporated and reconstituted with 5 ml of diethyl ether. The solution was purified twice by liquid–liquid extraction with 2 ml of 1 M sodium hydroxide. After evaporating the solvent, the residue was dissolved in 100 μl of NH_4I/dithioerythritol/MSTFA (2 mg/4 mg/1 ml) and the reaction was allowed to proceed at 80 °C for 30 min, and 1 μl of this solution was injected into GC/MS as the U + G fraction.

2.5.3. Sulfates (S fraction)

After collecting the U + G fraction, the Sep-Pak Plus C_{18} column was eluted with 8 ml of ethyl acetate/methanol (4:1, v/v). After evaporating the eluate, the residue was dissolved in 1 M anhydrous hydrogen chloride–methanol solution (0.5 ml) and incubated for 10 min at 60 °C. Then, the reaction mixture was evaporated under nitrogen at temperatures below 40 °C. The residue was dissolved in 5 ml of diethyl ether, and the solution was purified twice by liquid-liquid extraction with 2 ml of 1 M sodium hydroxide. After evaporating the solvent, the residue was dissolved in 100 μl of NH_4I/dithioerythritol/MSTFA (2 mg/4 mg/1 ml) and the reaction was allowed to proceed at 80 °C for 30 min, and 1 μl of this solution was injected into GC/MS as the S fraction.

2.6. GC/MS analysis

GC/MS was performed on an HP 5973 Mass Selective Detector (Agilent Technologies, Tokyo, Japan) connected to an HP 6890 series GC. The fused-silica capillary columns used were DB-1, DB-5ms, and DB-17 (J&W Scientific, CA, USA), each having 15 m × 0.25 mm diameter and 0.25 μm film thickness. Helium was used as the carrier gas (1.0 ml/min) and the column oven temperature was programmed to increase from 150 to 210 °C at 20 °C/min, then to 300 °C at 10 °C/min, and maintained at 300 °C for 1 min. The injector temperature was set at 250 °C; the MS transfer line temperature, at 275 °C; and ionization energy, at 70 eV.

Structure elucidation of metabolites was carried out in the full-scan mode with *m/z* 50–650, and the capillary column used was DB-5ms. Identification of urinary metabolites by comparison with synthesized reference standards was carried out in the full-scan mode with *m/z* 50–550, and the capillary columns used were DB-1, DB-5ms, and DB-17. Quantification was carried out in the SIM mode using the characteristic ion of each metabolite, and the capillary column used was DB-1.

2.7. Synthesis of reference standards

2.7.1. 17α-Methyl-5β-androstan-3α,16β,17β-triol

The synthesis of 17α-methyl-5β-androstan-3α,16β,17β-triol from 4-androstene-17β-ol-3-one (testosterone) is shown in Fig. 1 The product obtained after seven steps was recrystallized repeatedly from ethyl acetate and methanol to obtain crystals having mp 232–235 °C, and the yield of the entire procedure was approximately 11%.

2.7.2. 17α-Hydroxymethyl-5α-androstan-3β,17β-diol

The synthesis of 17α-hydroxymethyl-5α-androstan-3β,17β-diol was carried out using the method of Takeda et al. [7]. Fig. 2 shows the synthesis that used 5α-androstan-3β-ol-17-one as the starting material. The product obtained after 2 steps was recrystallized from methanol to obtain crystals having mp 210–212 °C, and the yield of the entire procedure was approximately 41%.

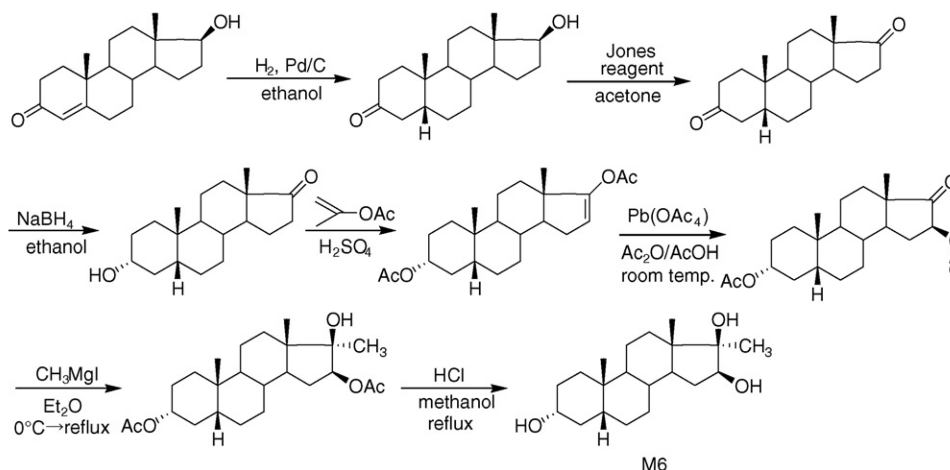

Fig. 1. Synthesis of 17α-methyl-5β-androstan-3α,16β,17-triol (**M6**).

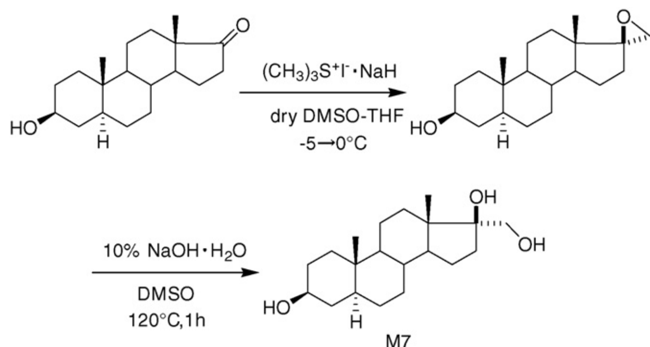

Fig. 2. Synthesis of 17α-hydroxymethyl-5α-androstan-3β,17-diol (**M7**).

2.7.3. 17α-Methyl-5α-androstan-3β,16α,17β-triol

The synthesis of 17α-methyl-5α-androstan-3β,16α,17β-triol from 5α-androstan-3β-ol-17-one is shown in Fig. 3. From GC/MS data, the product obtained at the final step of the Grignard reaction was presumed to be a mixture of 17α-methyl-5α-androstan-3β,16α,17β-triol and 17α-methyl-5α-androstan-3β,16α,17α-triol. The mixture was dissolved in acetone and *p*-toluenesulfonic acid monohydrate was added. Then, it was refluxed and only 17α-methyl-5α-androstan-3β,16α,17α-triol (*cis*-16,17-diol) was obtained as acetonide. After that, 17α-methyl-5α-androstan-3β,16α,17β-triol (*trans*-16,17-diol) was extracted with chloroform/methanol (4:1, v/v) and this was recrystallized repeatedly from ethyl acetate to obtain crystals having mp 205–208 °C. The yield of the entire procedure was approximately 4%.

2.7.4. 17α-Methyl-5α-androstan-3β,16β,17β-triol

The synthesis of 17α-methyl-5α-androstan-3β,16β,17β-triol from 5α-androstan-3β-ol-17-one is shown in Fig. 3. The product obtained in the final step of the Grignard reaction was separated on silica gel using chloroform/methanol (9:1, v/v), and was recrystallized from methanol to obtain crystals having mp 247–250 °C. The yield of the entire procedure was approximately 46%.

3. Results

3.1. Structure elucidation and identification of MTS and MSL metabolites

The main metabolites of MTS and MSL were detected from the total ion chromatograms of glucuronide and sulfate fractions (Figs. 4 and 5). The metabolites were tentatively labeled **M1**–**M9**, and their mass spectra are shown in Fig. 6. **M4**, **M7**, **M8**, and **M9** were detected in both glucuronide and sulfate fractions, and were common to MTS and MSL. In contrast, **M3**, **M5**, and **M6** were detected only in the glucuronide fraction after MTS administration. The electron impact (EI) mass spectrum of **M2** and **M4** showed a molecular ion peak at *m/z* 450, which is indicative of a di-TMS derivative, and fragment ions at *m/z* 143 and 130, which are indicative of D-ring cleavage of 17α-methyl,17β-hydroxy steroids. From the MS data, **M2** and **M4** were presumed to be the stereoisomers of 17-methyl-5-androstane-3,17-diol. In contrast, the EI mass spectrum of **M3** and **M7** showed a molecular ion peak at *m/z* 538, which is indicative of a tri-TMS derivative, and a fragment ion at *m/z* 435 due to an initial loss of 103 mass units, indicating oxidation of the 17-methyl function in the D ring. From these MS data, **M3** and **M7** were assumed to be 17-hydroxymethyl-5-androstane-3,17-diol. Meanwhile, the EI mass spectrum of **M5**, **M6**, **M8**, and **M9** showed a molecular ion peak at *m/z* 538, which is indicative of a tri-TMS derivative, and fragment ions at *m/z* 231 and 218, indicating C-16 hydroxylation of the D ring. From these MS data, **M5**, **M6**, **M8**, and **M9** were presumed to be the stereoisomers of 17-methyl-5-androstane-3,16,17-triol. One study has reported that the above-mentioned metabolites are stereoisomers of the equine metabolites of MTS [4]. Interestingly, **M3**, **M5**, and **M6** were derived from only MTS. On the other hand, the EI mass spectrum of **M1** showed

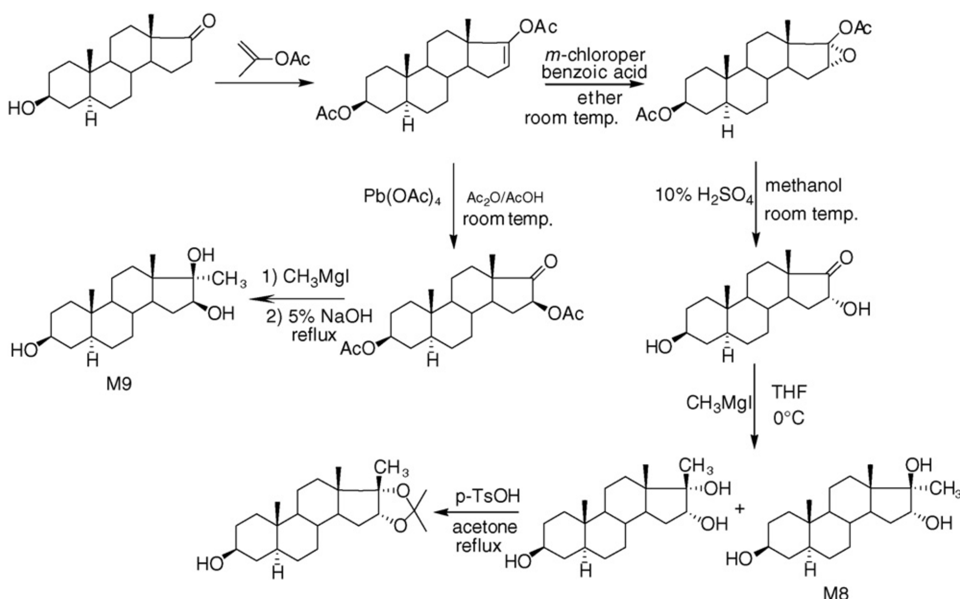

Fig. 3. Synthesis of 17α-methyl-5α-androstan-3β,16α,17β-triol (**M8**) and17α-methyl-5-androstan-3β,16,17-triol (**M9**).

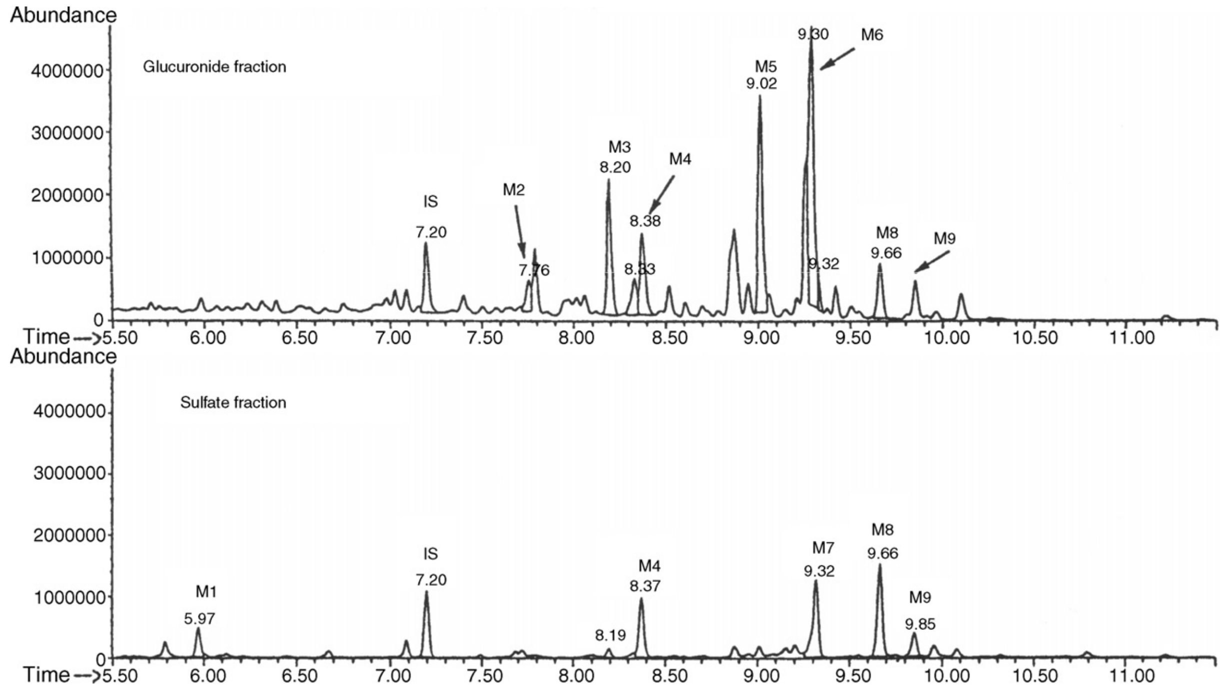

Fig. 4. Chromatograms of 17α-methyltestosterone metabolites.

a molecular ion peak at *m/z* 360 and a weak fragment ion at *m/z* 270 [M–90], but no characteristic ions (*m/z* 143 and 130) indicative of D-ring cleavage of 17α-methyl,17β-hydroxy steroids. It has been suggested that 17α-methyl-17β-sulfate conjugates decomposed to 17,17-dimethyl-18-norandrost-13-ene during epimerization [8]. From these results, **M1** was assumed to be 17,17-dimethyl-5-androst-13-en-3-ol. After MTS administration, a small amount of unmetabolized MTS was identified in the unconjugated steroid fraction by comparing with the reference standard. A peak with a similar MS pattern was also observed at an earlier retention time than that of MTS, and the metabolite corresponding to that peak was considered to be the 17β-epimer of MTS. In contrast, unmetabolized MSL was not detected in the unconjugated steroid fraction after MSL administration. Several peaks in the total ion chromatograms could not be assigned, such as the distinct peak appearing before the peak

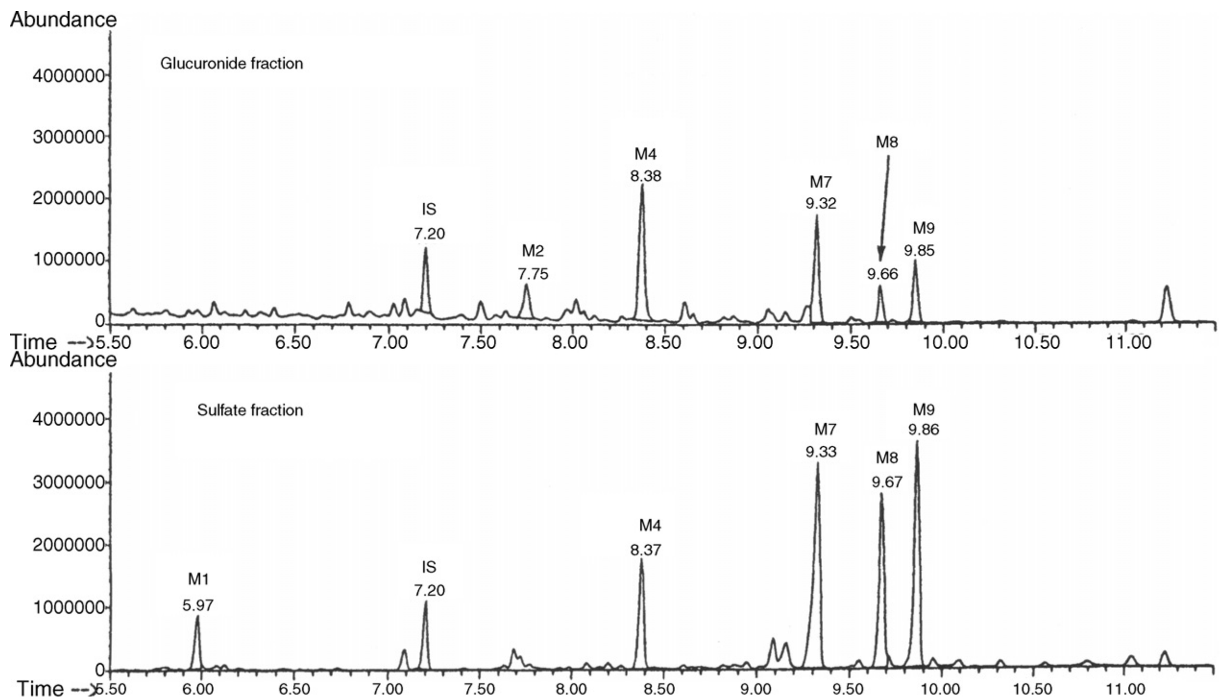

Fig. 5. Chromatograms of mestanolone metabolites.

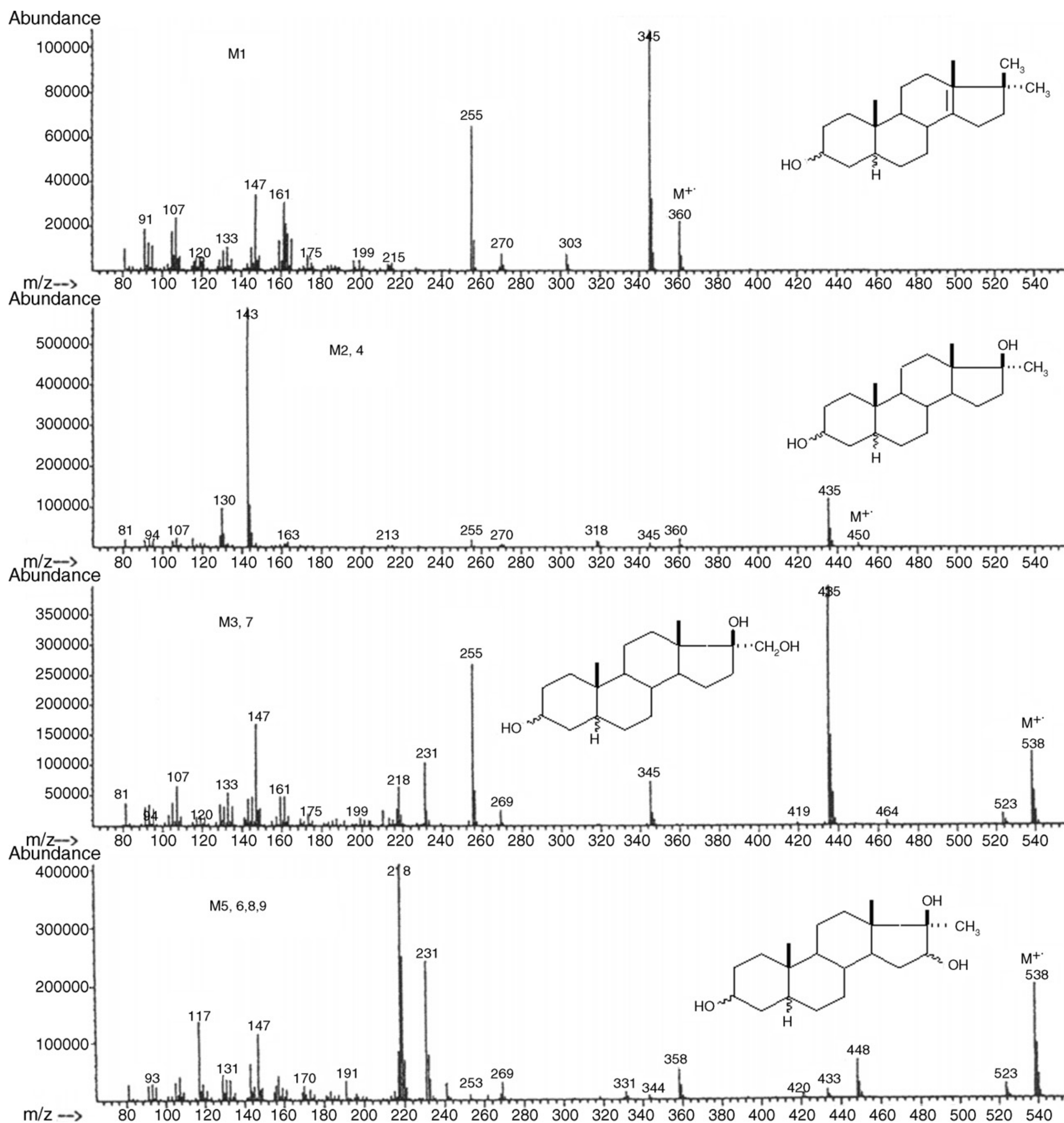

Fig. 6. Mass spectra of common metabolites.

assigned to **M5** in Fig. 4 or small peaks in Figs. 4 and 5. As these peaks also appeared in the chromatogram of the urine sample collected prior to MTS/MSL administration, it is presumed that these peaks originate in urinary components that are unrelated to MTS/MSL administration.

After the structures were elucidated, the metabolites were compared with synthesized reference standards in terms of GC/MS patterns and retention times on three capillary columns, DB-1, DB-5ms, and DB-17. Furthermore, McKinney et al. have reported that all the isomers of **M4**, **M6**, **M7**, **M8**, and **M9** have different GC retention data when derivatized with the combination of three kinds of derivatives, TMS, cyclic phenylboronate ester, and *tert*-butyldimethylsilyl ether [5]. Comparing our synthesized reference standards with the metabolites detected from horse urine in the report of McKinney et al., we found that they had the same retention times. Thus, we presumed that the identified metabolites of MSL and MTS were not isomers and **M4**, **M6**, **M7**, **M8**, and **M9** are 17α-methyl-5α-androstan-3α,17β-diol, 17α-methyl-5β-androstan-3α,16β,17β-triol, 17α-hydroxymethyl-5α-androstan-3β,17β-diol, 17α-methyl-5α- androstan-3β,16α,17β-triol, and 17α-methyl-5α-androstan-3β,16β,17β-triol, respectively.

3.2. Determination of urinary metabolites

We quantified **M4**, **M6**, **M7**, **M8**, and **M9**, and the unmetabolized MTS and MSL in horse urine samples after administration of MTS and MSL. Prior to the determination, calibration curves

Table 1
Recovery rates of metabolites

	ADS (IS)	NAD (IS)	MSL	MTS
U + G fraction		85.8 ± 3.6	78.8 ± 5.5	93.3 ± 3.2
S fraction[a]	114.9 ± 4.8	86.0 ± 3.7	72.0 ± 2.0	83.7 ± 6.3

	M4	**M6**	**M7**	**M8**	**M9**
U + G fraction	82.3 ± 7.5	80.5 ± 8.9	79.7 ± 2.9	94.4 ± 4.8	72.4 ± 6.3
S fraction[a]	70.8 ± 7.7	87.9 ± 4.6	101.3 ± 2.0	97.1 ± 6.4	86.2 ± 3.3

Spiked concentration: 1 μg/ml, mean ± CV (%), $n = 7$.
[a] Eluates from Sep-Pak Plus C_{18} were spiked with standards, except for NAD (IS).

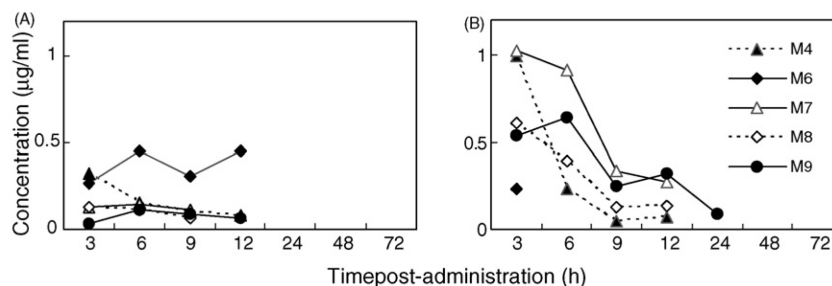

Fig. 7. Concentration of 17α-methyltestosterone metabolites in horse urine: (A) U + G fraction of 17α-methyltestosterone and (B) S fraction of 17α-methyltestosterone.

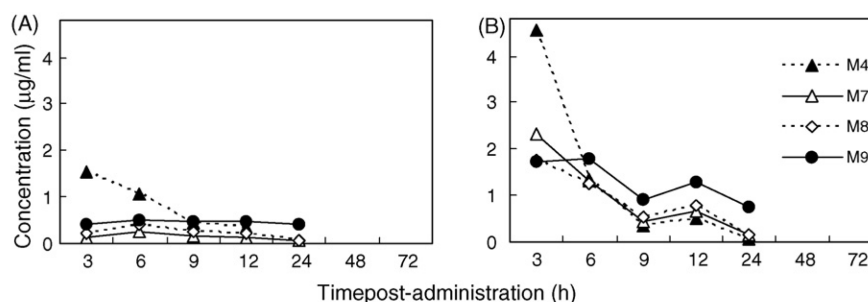

Fig. 8. Concentration of mestanolone metabolites in horse urine: (A) U + G fraction of mestanolone and (B) S fraction of mestanolone.

were prepared over the concentration range of 0.05–5 μg/ml. The correlation coefficient was higher than 0.993, and the limit of quantification (LOQ) was 0.05 μg/ml. The recovery rates of the metabolites ranged from 72.4% to 94.4% ($n = 7$) in U + G fraction and 70.8% to 101.3% ($n = 7$) in S fraction, and their coefficients of variation were from 2.9% to 8.9% and 2.0% to 7.7%, respectivery. Table 1 shows the recovery rates, and examples of determination results are shown in Figs. 7 and 8. Further, intra-day accuracy and precision of the quantification method, which were assessed by analyzing quality control samples spiked with authentic reference standards (1.0 μg/ml), are given in Table 2. Intra-day accuracy varied from −13.6% to 10.7% in U + G frac-

Table 2
Intra-day accuracy and precision of quantification method

	Nominal (μg/ml)	U + G fraction			S fraction[a]		
		Actual (μg/ml)	Accuracy (%)	Precision (CV, %)	Actual (μg/ml)	Accuracy (%)	Precision (CV, %)
MSL	1	1.01	1.0	10.7	0.88	−13.6	10.3
MTS	1	0.93	−7.5	2.4	0.99	−1.0	10.3
M4	1	0.88	−13.6	5.5	1.04	3.8	14.8
M6	1	0.88	−13.6	2.3	0.87	−14.9	7.8
M7	1	1.05	4.8	14.4	0.98	−2.1	8.6
M8	1	0.94	−6.4	7.1	0.91	−9.9	6.7
M9	1	1.12	10.7	4.8	0.90	−11.1	7.4

$n = 3$, accuracy = (nominal − actual)/nominal.
[a] Eluates from Sep-Pak Plus C_{18} were spiked with standards.

tion and −14.9% to 3.8% in S fraction, and their precision were between 2.4–14.4% and 6.7–14.8%, respectively.

3.2.1. MTS

M4 and **M6** were detected mainly at 3 h in the U + G fraction. The concentration of **M4** decreased with time, while that of **M6** was almost unchanged up to 12 h. The concentrations of **M7**, **M8**, and **M9** were lower than 0.2 μg/ml from 3 to 12 h in the U + G fraction. The concentrations of **M4**, **M7**, **M8**, and **M9** in the S fraction were higher than those in the U + G fraction. Whereas **M6** was the major metabolite detected in the U + G fraction, it was detected only at 3 h in the S fraction, and all samples contained metabolites having concentrations lower than LOQ (0.05 μg/ml) after 6 h. Among the 5 metabolites in the S fraction, **M7** and **M9** were detected as the major metabolites, and they could be detected up to 24 or 48 h post-administration. The detection time of **M9** was longer than that of **M7**. Unmetabolized MTS was detected in the U + G fraction, but its concentration was lower than LOQ.

3.2.2. MSL

M4, **M7**, **M8**, and **M9** were detected in both U + G and S fractions. Among them, **M4** was detected mainly at 3 h in the U + G fraction, and the concentrations of **M7**, **M8**, and **M9** were lower than 0.5 μg/ml from 3 to 24 h in this fraction. In the S fraction, whereas **M4** was the major metabolite detected at 3 h post-administration, the concentrations of **M4**, **M7**, and **M8** were almost the same from 6 to 24 h. **M9** was mainly detected after 6 h, and it was detectable up to 24 h post-administration. The long detection time of **M9** was similar to the results for MTS. Unmetabolized MSL and **M6** were not detected in all the U + G and S fractions from urine sampled from horses administered MSL. The urinary concentrations of MSL metabolites tended to be higher than those of MTS metabolites.

4. Discussion

MTS and MSL were orally administered to horses, and their metabolites were investigated in urine samples. These two steroids are closely related in terms of chemical structure, and the existence of metabolites common to them has been reported in humans [1,2]. As a result of the structure elucidation of metabolites, we confirmed that **M4**, **M7**, **M8**, and **M9** were the common metabolites mainly excreted in horse urine after administration of MTS and MSL. The metabolic pathway of MTS and MSL as presumed from the results of this study is shown in Fig. 9. MTS was metabolized to **M4** by reduction of the 3-keto group and the C-4, C-5 double bond in the A ring. Subsequently, **M4** was metabolized to **M7**, **M8**, and **M9** by hydroxylation of the 17α-methyl group, 16α-hydroxylation, and 16β-hydoxylation, respectively. MTS, but not MSL, was metabolized to **M6** after administration. Since MSL is a 5α-H structural steroid, all of its metabolites have only the 5α-H structure, and no 5β-H structural metabolites such as **M6** were detected. MS data indicated that **M3** and **M5** are isomers of 17-hydroxymethylandrostane-3,17-diol and 17-methylandrostane-3,16,17-triol, respectively, and we presumed that they have 5β-H structures because they were not detected in the urine samples after administration of MSL. Therefore, **M3** may be a 3-isomer of **M7**, and **M5** may be 3- or 16-isomer of **M8** and **M9**. The results of investigation of urinary metabolites after administration indicated that there were less metabolites in the urine samples after MSL administration than after MTS administration. Therefore, we assumed that the urinary concentrations of the common metabolites were

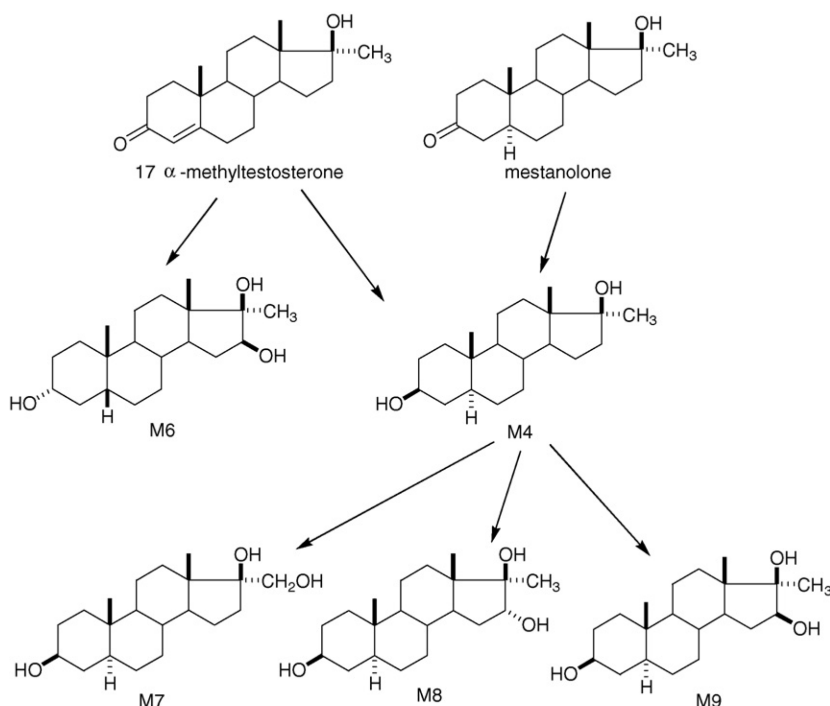

Fig. 9. Metabolism of 17α-methyltestosterone and mestanolone (proposed pathway).

higher after MSL administration than after MTS administration.

To quantify unconjugated metabolites in the U + G fraction, we subjected the non-hydrolyzed U + G fraction to GC/MS, and found that unconjugated **M4**, **M6**, **M7**, **M8**, and **M9** were hardly detected from the urine samples after MTS and MSL administration. From these results, we presumed that unconjugated **M4**, **M6**, **M7**, **M8**, and **M9** were hardly present in the U + G fraction.

It is known that 3β-hydroxy metabolites are excreted as sulfates while 3α-hydroxy metabolites are excreted as glucuronides [2]. Furthermore, it has been reported that 3β-hydroxy compounds are not metabolites of 17α-methyl,17β-hydroxy steroids in human [1,2]. Interestingly, in horse urine, **M4**, **M7**, **M8**, and **M9**, which are 3β-hydroxy metabolites, and **M6**, a 3α-hydroxy metabolite, were mainly excreted as sulfates and glucuronides, respectively. The results indicate that the conjugation of 3-hydroxy metabolites is the same in horse and human, but hydroxylation of the 3-keto group in the A ring is very different between the two species.

Our quantification results showed that **M9** could be detected for the longest time. In addition, we investigated the detection times of **M1**, **M2**, **M3**, and **M5**, whose urinary concentrations could not be determined, and found that none of them had a detection time longer than **M9**. Thus, **M9** may be a very useful screening target in the doping test for MTS and MSL in racehorses.

It has been reported that anabolic steroids with the 17α-methyl,17β-hydroxyl group, such as MTS, MSL, methanedienone, and oxymetholone, have metabolites in common in human urine [9]. Therefore, it is necessary to investigate further whether **M4**, **M6**, **M7**, **M8**, or **M9** could be detected from horse urine after the administration of structurally related steroids with the 17α-methyl,17β-hydroxyl group.

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