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DETERMINATION OF METHANDROSTENOLONE AND ITS METABOLITES IN EQUINE PLASMA AND URINE BY COUPLED-COLUMN LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET DETECTION AND CONFIRMATION BY TANDEM MASS SPECTROMETRY

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SUMMARY

Monitoring steroid use requires an understanding of the metabolism in the species in question and development of sensitive methods for screening of the steroid or its metabolites in urine. Qualitative information for confirmation of methandrostenolone and identification of its metabolites was primarily obtained by coupled-column high-performance liquid chromatography–tandem mass spectrometry. The steroids and a sulphuric acid conjugate were isolated and identified by their daughter ion mass spectra in the urine of both man and the horse following administration of methandrostenolone. Spontaneous hydrolysis of methandrostenolone sulphate gave 17-epimethandrostenolone and several dehydration products. This reaction had a half-life of 16 min in equine urine at 27°C. Mono- and dihydroxylated metabolites were also identified. Several screening methods were evaluated for detection and confirmation of methandrostenolone use including thin-layer chromatography and high-performance liquid chromatography. Coupled-column liquid chromatography was used for automated clean-up of analytes difficult to isolate by manual methods. The recovery of methandrostenolone was 101 ± 3.3% (mean ± S.D.) at 6.5 ng/ml and both methandrostenolone and 17-epimethandrostenolone were quantified in urine by ultraviolet detection up to six days after a 250-mg intramuscular dose to a horse. The utility of on-line tandem mass spectrometry for confirmation of suspected metabolites is also shown.

INTRODUCTION

The use of anabolic steroids to improve athletic performance is an increasing problem in both equine and human sport events. Although steroid use in humans

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is generally traced to the second World War, the first report of their use in horses was that of Vigre [1]. Following treatment of some 63 horses suffering from a variety of ailments with methandienone (17α-methyl-17β-hydroxy-1,4-androstadien-3-one; methandrostenolone; Dianabol; compound I), he noted that they "showed a dramatic improvement in their performance in training on the race track". Unfortunately, no data were presented to directly link the improvement of the ailing horses to steroid use. Vigre also reported "appreciable improvement in appetite and general tonus, well known effects of anabolic steroids". Recent studies in healthy humans indicate that although increases in muscle mass are associated with steroid use, no improvement in stamina or performance in several sports could be documented [2, 3]. Concern about the untoward side-effects of steroids, such as adenocarcinoma [4] and hepatic damage, has resulted in their ban in both human and equine athletes. Nevertheless, the use of steroids in sports continues to increase, presumably due to narcissan stimuli and the increase in aggressiveness and euphoric feelings associated with anabolic steroid ingestion.

Monitoring steroid use in athletes requires an understanding of the metabolism of the compound in the species in question and the development of analytical methods capable of accurately monitoring the steroid or its metabolites in urine. For example, earlier work in this laboratory identified the major equine urinary metabolite of boldenone (17β-hydroxy-1,4-androstadien-3-one) as the 17-sulphate [5]. The sulphate was identified in urine up to 42 days after a single intramuscular injection of boldenone undecylenate.

The metabolism of I in man has been relatively well studied and methods for monitoring its presence in urine have been reported [6–10]. It appears there is very little unchanged I excreted in the human urine. The major metabolic products reported involve epimerization at the 17-position to yield 17α-hydroxy-17β-methylandrostadien-3-one (17-epi-I) and hydroxylation at the 6β-position (6β-hydroxy-I). The mechanism of this epimer formation has been of great interest and debate. Other identified products include migration of a methyl group combined with dehydration or dehydrogenation [10]. Conjugate identification of the parent compound or its metabolites has not been well documented although most analytical procedures incorporate a glucuronidase and sulphatase hydrolysis in the sample preparation.

An attempt to study the mechanism of the epimerization reaction in the rabbit was unsuccessful [11]. Essentially, no 17-epi-I was detected in the urine of rabbits. The major metabolites were 6β,16β-dihydroxy-I, 6β-hydroxy-I and several other compounds which were hydroxylated on the D-ring or reduced in the A-ring. Although the rabbit may be a suitable metabolic model for the horse, preliminary studies in this laboratory indicated significant amounts of 17-epi-I in the horse urine. A study of I metabolism in the horse is therefore required to establish an equine urine steroid testing regimen.

This work describes the metabolism of I in the horse and possible mechanisms for formation of several urinary excretion products reported for other species. In the present work, I and its metabolites were isolated from equine urine and plasma by liquid–liquid extraction and separated from the remaining matrix components on a liquid chromatographic (LC) system comprised of three reversed-phase col-

umns. Large differences in selectivity and efficient enrichment between the columns were obtained when phenyl- and octyl-silica were used in the first and last column, respectively.

EXPERIMENTAL

Chemicals and reagents

Methandrostenolone and methandrostenolone sodium sulphate (I-SO_4) were purchased from Steraloids (Wilton, NH, U.S.A.). Dichloromethane, 2-butanol, cyclohexane and HPLC-grade methanol were purchased from Fisher Scientific (Rochester, NY, U.S.A.). Tetrabutylammonium hydrogensulfate ($TBAHSO_4$) was obtained from Eastman Kodak (Rochester, NY, U.S.A.). All other reagents were of analytical-grade quality. Water was purified by an in-house Barnstead Nanopure II system (Boston, MA, U.S.A.).

Synthesis of deuterated I

17α-Deuteramethyl-17β-hydroxy-1,4-androstadien-3-one (17-CD_3-I) was synthesized using a modification of the method reported by Bjorkhem et al. [9]. Reagents were obtained from Aldrich (Milwaukee, WI, U.S.A.).

Preparation of 17-epi-I

I-SO_4 was hydrolyzed by dissolving 0.5 g of the salt in 50 ml of methanol–water (20:80, v/v). The hydrolysis products precipitated and were recovered by filtering and drying after two days of spontaneous hydrolysis. 17-epi-I was isolated from at least three other hydrolysis products in the filtrate by preparative liquid chromatography on a cyanopropyl-silica column (100 mm × 10 mm I.D.) packed with 5-μm Spherisorb particles by Keystone Scientific (State College, PA, U.S.A.). 17-epi-I eluted with a capacity factor of 4.7 with a mixture of hexane–dichloromethane–methanol (80:19:1). Approximately twenty consecutive separations gave 95 mg pure crystalline 17α-hydroxy-17β-methyl-1,4-androstadien-3-one upon evaporation of the mobile phase. The identity was confirmed by mass spectrometry (MS) and ^{1}H NMR.

The non-retained dehydration products were collected, the fractions evaporated and separated with a weaker eluent containing hexane–dichloromethane–methanol (95:4.75:0.25) which gave three fractions. The first fraction was identified as 18-nor-17-dimethyl-1,4,13(14)-androstatrien-3-one by MS and NMR. The second fraction contained several dehydration products and the third fraction was identified as the methanolysis product, 17α-methoxy-17β-methyl-1,4-androstadien-3-one.

Liquid–liquid extraction

Compounds I and 17-epi-I were isolated from plasma (5 ml) or urine (10 ml) by extraction with 8 ml of 20% (v/v) dichloromethane in cyclohexane by rotation in 20-ml test tubes at low speed for 15 min. The samples were centrifuged and 5 ml of the organic phase were pipetted to a new tube for evaporation under a dry nitrogen flow at 50°C. The residue was reconstituted in 150 μl of 35% (v/v)

methanol in water and 60 μl were injected onto the first LC column for analysis. Hydroxylated metabolites were isolated in the same manner with ethyl acetate as the organic solvent.

I-SO_4 was extracted as an ion pair with tetrabutylammonium (TBA) by addition of 0.5 ml of 0.5 *M* $TBAHSO_4$ to 4 ml of urine or plasma and extraction with 5 ml of 20% (v/v) 2-butanol in cyclohexane, and the residue was reconstituted in 200 μl of 35% (v/v) methanol–water. Of this solution 40 μl were injected for analysis of the sample.

Thin-layer chromatographic (TLC) screening

17-epi-I was isolated from urine as discussed above, plus an additional wash of the organic phase (5 ml) with 2 ml of 1 *M* sodium hydroxide prior to evaporation. The sample residue was dissolved in 20 μl of ethyl acetate and spotted on 10 cm × 20 cm Kieselgel $60F_{254}$ TLC plates of 0.25 mm layer thickness (E.M. Science, Cherry Hill, NJ, U.S.A.). Each plate was also spotted with 500 ng of 17-epi-I and developed 5 cm with chloroform–ethyl acetate–methanol (50:45:5) which produced an R_F value of 0.64 for 17-epi-I. The steroid was observed under short-wavelength UV light for fluorescence quenching.

Liquid chromatography

The liquid chromatograph consisted of two pumps (Model 510), a system controller (Model 680), a six-port column-switching accessory (Model 60057) and a fixed-wavelength (254 nm) Model 440 UV detector (Waters, Milford, MA, U.S.A.). Samples were injected with a 50-μl loop injector (Model 7010, Rheodyne, Cotati, CA, U.S.A.). The first column was packed with 3-μm Spherisorb phenyl-silica and the last column with 3-μm Spherisorb octyl-silica (Phase Separations, Queensferry, U.K). Heart-cut fractions from the first column were trapped on a small column, 10 mm × 2.6 mm I.D. (Keystone Scientific, State College, PA, U.S.A.) slurry-packed with 5-μm octadecyl-silica particles. A schematic drawing of the column-switching system is presented in Fig. 1. The dimensions of the phenyl column were 50 mm × 4.6 mm I.D. for analysis of I and 17-epi-I and 100 mm × 4.6 mm I.D. for determination of I-SO_4, while the octyl column was 100 mm × 4.6 mm I.D. for all separations. UV detection at 254 nm was used for screening and pharmacokinetic studies. Columns with the same length but 2.1 mm I.D. were also used for the LC–MS and LC–MS–MS studies. The mobile phases for the two columns consisted of methanol–water mixtures during separation of I, 17-epi-I and hydroxylated metabolites (Table I). The flow-rate on both columns was 1 ml/min for 4.6 mm I.D. columns and 0.3 ml/min for 2.1 mm I.D. columns. The mobile phase for separation of I-SO_4 on the phenyl column contained 3 m*M* $TBAHSO_4$ and 3 m*M* sulphuric acid in a methanol–water mixture (40:60). The mobile phase for the octyl column contained 12 m*M* ammonium hydrogensulphate in methanol–water mixture (45:55). The fractions collected from the first column by automated column switching for continued separation on the last column are given in Table I.

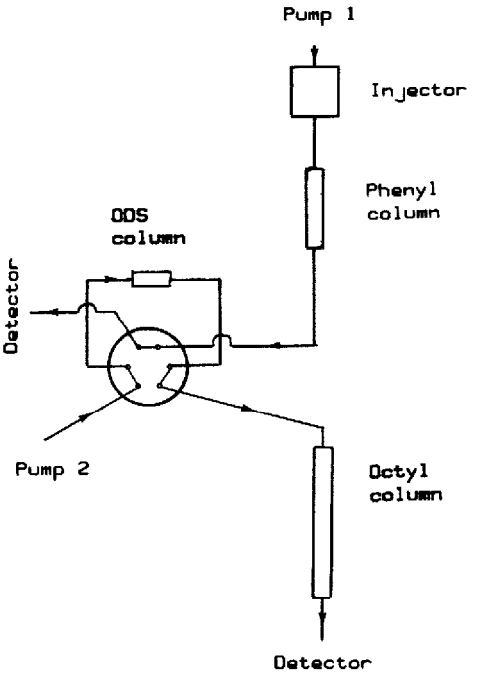

Fig. 1. Scheme of coupled-column HPLC system.

TABLE I

CHROMATOGRAPHIC CONDITIONS

Compound	Phenyl column	Heart cut (min)	Octyl column
I[a]	37% Methanol	2.90–3.65	60% Methanol
17-epi-I[a]	40% Methanol	2.30–3.00	65% Methanol
I-SO_4[b]	3 m*M* $TBAHSO_4$		
	3 m*M* H_2SO_4		12 m*M* NH_4HSO_4
	40% Methanol	3.75–4.65	45% Methanol
Monohydroxy-I[c]	38% Methanol	1.80–2.30	60% Methanol
Dihydroxy-I[c]	38% Methanol	1 20–2.00	60% Methanol
Dehydrated-I[c]	60% Methanol	3.00–4.00	80% Methanol

[a]Phenyl column: 50 mm × 4.6 mm I.D., octyl column: 100 mm × 4.6 mm I.D. Flow-rates were 1 ml/min on both columns.
[b]Phenyl column: 100 mm × 4.6 mm I.D.; octyl column: 100 mm × 4.6 mm I D. Flow-rates were 1 ml/min on both columns.
[c]Phenyl column: 50 mm × 2.1 mm I.D.; octyl column: 100 mm × 2.1 mm I.D. Flow-rates were 0.3 ml/min on both columns.

Coupled-column LC–MS and LC–MS–MS

The effluent from the last column was connected to a Sciex TAGA 6000E triple quadropole mass spectrometer (Sciex, Thornhill, Canada) equipped with an atmospheric pressure ion (API) source. Ions were generated by a heated pneumatic

nebulizer with corona discharge ionization. The nebulizer probe temperature was set to 165 or 265 °C for flow-rates of 0.3 and 1 ml/min, respectively. Cations were sampled from the heated nebulizer discharge. An ion spray LC–MS interface [12] was used to sample the anion of I-SO_4.

Argon was used in the second quadrupole for collision-induced dissociation (CID) experiments with approximately $300 \cdot 10^{12}$ atoms per cm^2 target gas thickness. Full-scan MS and MS–MS mass spectra were recorded with a scan-rate of 1 and 2.7 s/scan, respectively.

Animal experiments

Equine plasma and urine samples were obtained from experimental horses housed within the Cornell Equine Drug Testing and Research Program. A female standard bred horse (513 kg) was injected intramuscularly with 0.49 mg/kg body weight of I dissolved in a mixture of 1 ml of ethanol and 1 ml of glycerol. Urine samples were collected during 2-h intervals via an indwelling urethral catheter at 2, 4, 6, 8 and 24 h and 2, 3, 6, 13 and 24 days post-dose. The urine samples were immediately dispensed to several 50-ml polypropylene containers and frozen on dry ice. Whole blood (30 ml) was collected in heparinized tubes. The tubes were centrifuged and the plasma was frozen on dry ice. The plasma and urine samples were stored at −60 °C until time of analysis. A 300-ml 20-h human frozen urine sample following a 10-mg oral dose of I was supplied by Dr. Barry Sample, Indiana University, and stored frozen until time of analysis.

RESULTS AND DISCUSSION

Lipophilic steroids are frequently metabolized via hydroxylation and this has been reported for I in different species [9,11]. Excretion to the urine in the form of more hydrophilic conjugates of sulphuric and glucuronic acid is also frequently observed [13]. The 17-sulphate derivative of boldenone was identified as the major metabolite in the horse from previous work in this laboratory [5]. Enzymatic hydrolysis of this sulphate was slow and solvolysis at 50 °C was required to achieve hydrolysis. Similar results have been observed for other 17-hydroxysteroids such as testosterone and 19-nortestosterone. In contradistinction, the 17α-methyl steroids, such as I, have been shown to yield primarily an epimer derivative, which is an unusual metabolic reaction [10].

Detection of I-SO_4 in urine

Preliminary studies indicated that I-SO_4 was very unstable in urine, and thus rapid freezing of the samples was essential for identification of this metabolite. Some loss during sample collection was unavoidable due to hydrolysis in the bladder and the collection container.

A rapid extraction procedure was developed to isolate I-SO_4 from the urine to minimize hydrolysis during sample preparation. I-SO_4 was extracted as an ion pair with TBA ion into cyclohexane containing 20% 2-butanol. The ion-pair extraction constant was determined to be 170 M^{-1} with this solvent and the recovery from urine was measured to be $100.5 \pm 10\%$ ($n = 7$) when compensated for

phase transfer loss (3.5 ml of 5 ml organic phase). The ion pair was stable in organic solvents and evaporated samples could be stored in a freezer for several days without decomposition.

The final residue after extraction contained some TBA which affected the separation when samples were injected on reversed-phase columns. The best separations were obtained when TBA was added to the mobile phase for ion-pair chromatography. The sample extracts were very complex due to co-extraction of acidic urine components and it was not possible to resolve the analyte from the urine components on a single column with UV detection. Furthermore, the addition of TBA to the mobile phase is known to impair the sensitivity for ion-spray LC–MS [14]. Coupled-column LC was used to solve both of these problems. This technique has become an important tool to increase the selectivity of chromatographic systems. Provided that different retention mechanisms are used on the columns and that enrichment effects are created between the columns, the total peak capacity equals the product of the peak capacity of the individual columns [15]. I-SO_4 was first separated as an ion pair with TBA on the phenyl column with a capacity factor of 2.5. The I-SO_4 fraction was collected onto a small octadecyl-silica column for enrichment (peak compression) by column switching. The ion pair was strongly retained on the ODS column and then eluted with reversed eluent flow direction onto an octyl-silica column with a mobile phase containing NH_4HSO_4 instead of TBA and a higher concentration of organic modifier. By this arrangement a steep gradient was created with decreasing TBA concentration on the octyl column due to rapid elution of the TBA adsorbed on the enrichment (ODS) column. This LC system gave a large difference in selectivity and efficient enrichment between the phenyl and octyl-columns (Fig. 2). The detection limit in equine urine was determined at the 99% confidence level according to ref. 16 and was 20 ng/ml using this method with UV detection at 254 nm. Human urine gave a cleaner blank and the detection limit was estimated to be 10 ng/ml.

When coupled-column high-performance liquid chromatography (HPLC) is combined with MS or tandem MS, very powerful separation systems can be constructed for trace analysis of chemically complex samples. The flexibility offered by coupled-column chromatography made it possible to choose a buffer for the last column that was more suitable for ion-spray LC–MS compared with the buffer on the first column. Ion-spray coupled-column LC–MS of I-SO_4 gave the molecular ion $[M-H]^-$ at m/z 378. CID of the molecular ion gave only the parent ion and a fragment corresponding to the sulphate group at m/z 97. This finding agrees with previous studies in which sulfate conjugates on the D-ring produce primarily HSO_4^- ions [17].

The retention times on the two columns and the MS–MS mass spectra were identical for I-SO_4 isolated from urine and authentic I-SO_4 which confirmed the presence of this metabolite in equine urine. It was possible to detect I-SO_4 in urine up to two days post-dose, but the measured concentrations are too low due to unavoidable hydrolysis during sample collection as discussed above. The human urine sample also contained I-SO_4 (21 ng/ml), but the hydrolysis during sample

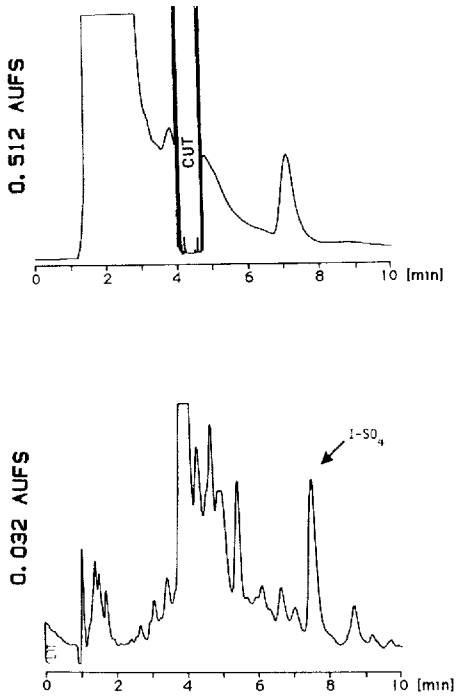

Fig. 2. Chromatograms obtained from an extract of a 6-h equine urine sample containing 307 ng/ml I-SO_4 quantified by UV detection at 254 nm. The cut made on the phenyl column (upper chromatogram) was collected by column switching and separated on an octyl column (lower chromatogram).

collection resulted in reduced levels of this metabolite. The major metabolite in this sample was 17-epi-I probably formed from I-SO_4 during sample handling.

Decomposition of I-SO_4

Loss of the 17-sulphate group of I can generate a tertiary carbonium ion at the 17-position. I-SO_4 was labile in aqueous media, and nucleophilic attack by water readily gave 17-epi-I. The hydrolysis of I-SO_4 was used for production of 17-epi-I. Pure 17-epi-I was isolated from the other dehydration products by preparative LC on a normal-phase system. The yield of 17-epi-I from urine fortified with I-SO_4 was 37%. The half-life of I-SO_4 in equine urine was 16 min at 37°C. The hydrolysis was much slower at room temperature and not affected by pH (see Table II). The sulphate was remarkably stable in equine plasma with a half-life longer than 3 h, presumably due to stabilization by binding to plasma proteins. The sulphate was quite stable in methanol and standards could be stored at 4°C for at least two days.

Further evidence for the instability of I-SO_4 can be found in the presence of other products in both urine and aqueous systems. Elimination of the sulphate group gave several dehydration products presumably via the tertiary carbonium ion. Rearrangement of the carbonium ion by migration of the methyl group at the 13-position gave 18-nor17,17-dimethyl-1,4,13(14)-androstatrien-3-one. This product has been identified by gas chromatography–MS in human urine after

TABLE II

HALF-LIFE OF I-SO_4 IN DIFFERENT SOLUTIONS

The samples were spiked with I-SO_4 (1 μg/ml) and extracted after different incubation times for quantification of I-SO_4 by coupled-column LC as described in Experimental.

Medium	pH	Temperature (°C)	Half-life (min)
Phosphate buffer	7.4	22	109
Phosphate buffer	3.0	22	96
0.1 *M* H_3PO_4	1	22	74
Equine urine	8	22	132
Equine urine	8	37	16
Equine urine	8	70	2
Equine plasma	7.5	37	> 180

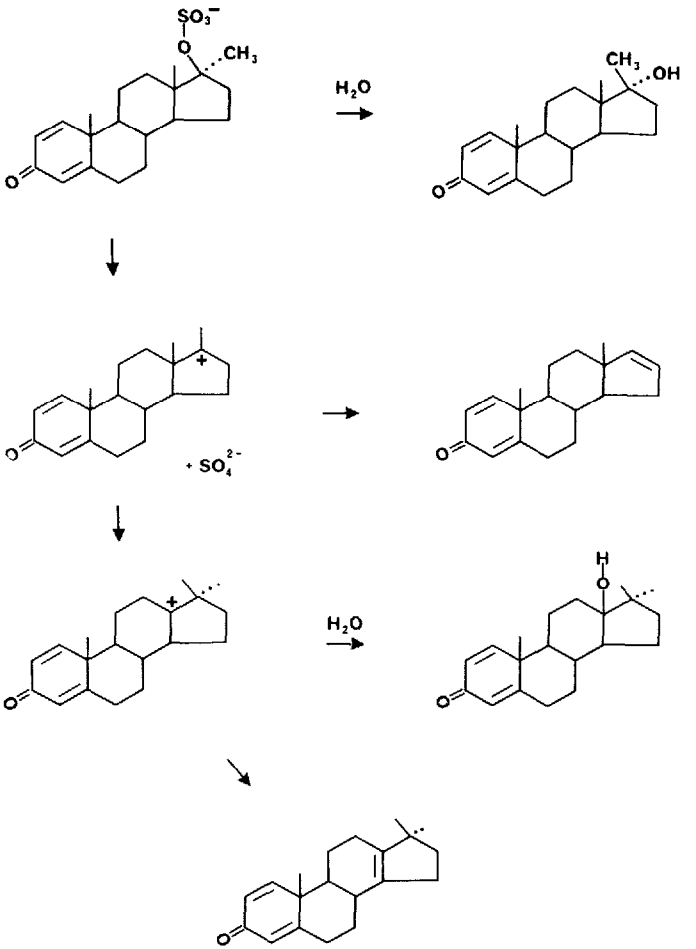

Fig. 3. Proposed hydrolysis products formed from I-SO_4 in urine.

TABLE III

CONCENTRATION OF I, 17-EPI-I AND I-SO_4 IN URINE AFTER A 250-mg INTRAMUSCULAR DOSE TO A HORSE

Time post-dose	Concentration (nmol/l)				
	I		17-epi-I		I-SO_4
	Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis	
2 h	4.1	22.6	28	227	418
4 h	15.4	42.5	227	567	688
6 h	12.1	32.5	173	397	764
8 h	101	154	367	510	56
24 h	12.1	27.1	61	153	58
2 days	9.1	25.7	117	143	90
3 days	7.5	N.M.[a]	107	N.M.[a]	N.D.[b]
6 days	22	33	80	117	N.D.[b]

[a]N.M. = not measured.
[b]N.D. = not detected.

administration of I to humans [10]. It was suggested that this product was formed from I in the stomach by acidic catalysis of the dehydration.

We observed that additional dehydration products were formed and attempts were made to purify them for identification by ^1H NMR. Several compounds were isolated by preparative LC, but evaporation of the sample fractions gave isomerization and/or conformational changes and mixed ^1H NMR spectra were obtained. A summary of the potential hydrolysis and elimination reactions of I-SO_4 is presented in Fig. 3. We were able to identify 17-epi-I, 13-hydroxy-17,17-dimethyl-1,4-androstadien-3-one and 18-nor-17,17-dimethyl-1,4,13(14)-androstatrien-3-one in both the aqueous hydrolysis solution and urine matrix.

Compounds I and 17-epi-I were determined before and after spontaneous hydrolysis of the urine overnight at room temperature (Table III). The concentration of both epimers increased after hydrolysis which indicates the presence of unstable conjugates. Accurate quantification of free and sulphate-conjugated I was not possible due to the instability of I-SO_4. Urine samples were collected during 2 h and the half-life of I-SO_4 increased from 16 min at 37°C (Table II) in the urine bladder to higher values at ambient temperature in the barn (5–10°C). Thus, most of the free 17-epi-I detected in urine originates from I-SO_4 which was also detected in urine (see Table III). Furthermore, the increase in 17-epi-I concentration after spontaneous hydrolysis of the 2–6 h samples could be accounted for by hydrolysis of I-SO_4, but some contribution from other conjugates cannot be excluded. Later samples appear to give a lower contribution to 17-epi-I from I-SO_4, but the precision for determination of I-SO_4 was lower at these levels and the instability of the sulphate could be the cause of this difference. The increase in I concentration is probably due to hydrolysis of 17-epi-I-SO_4, but contribution from I-SO_4 cannot be excluded.

Screening methods for I and 17-epi-I

Neutral steroids are difficult to isolate with high selectivity by manual methods. In this work, I and 17-epi-I were isolated by a simple liquid–liquid extraction followed by coupled-column chromatography for automated clean-up and final separation. Compounds I and 17-epi-I were isolated from urine and plasma by extraction with 20% dichloromethane in cyclohexane. The recovery of I in urine was $101 \pm 3.3\%$ at the 6.5 ng/ml level when compensated for phase transfer loss (5 ml of 8 ml organic solvent). Hexane can be substituted for cyclohexane to speed-up the evaporation of the extracts at the expense of a larger variation in recovery due to evaporation of solvent during extraction (recovery $= 101 \pm 11\%$, $n = 11$).

TLC can be used to screen for I. The detection limit of 17-epi-I in equine urine by TLC screening was about 40 ng/ml which was sufficient for positive identification up to six days after a 250-mg intramuscular dose to a horse.

HPLC was investigated as a rapid alternative to TLC. Attempts were made to inject centrifuged equine urine directly on short pre-columns packed with 3- or 5-μm reversed-phase particles, but the back-pressure soon became excessive, probably due to adsorption of polysaccharides present in the horse urine. The polysaccharides could be precipitated with perchloric acid and the supernatant neutralized with potassium hydroxide prior to injection, but liquid–liquid extrac-

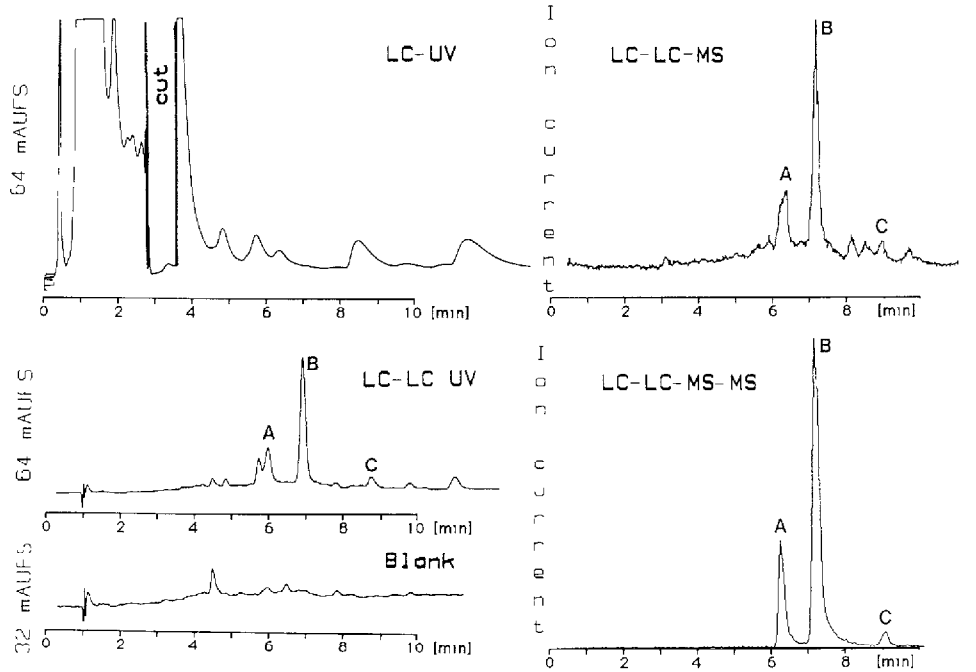

Fig. 4. Comparison of LC-UV and LC-MS chromatograms obtained with an extract of an 8-h urine sample containing 46 ng/ml I (peak B). The other peaks were identified as 13-hydroxy-17,17-dimethyl-1,4-androstadien-3-one (A) and 17-epi-I (C) unresolved from I on the first column The cut from the phenyl column (LC-UV) was collected by column switching and separated on the octyl column with UV detection (LC-LC-UV), detection by MS (LC-LC-MS) and tandem MS (LC-LC-MS-MS).

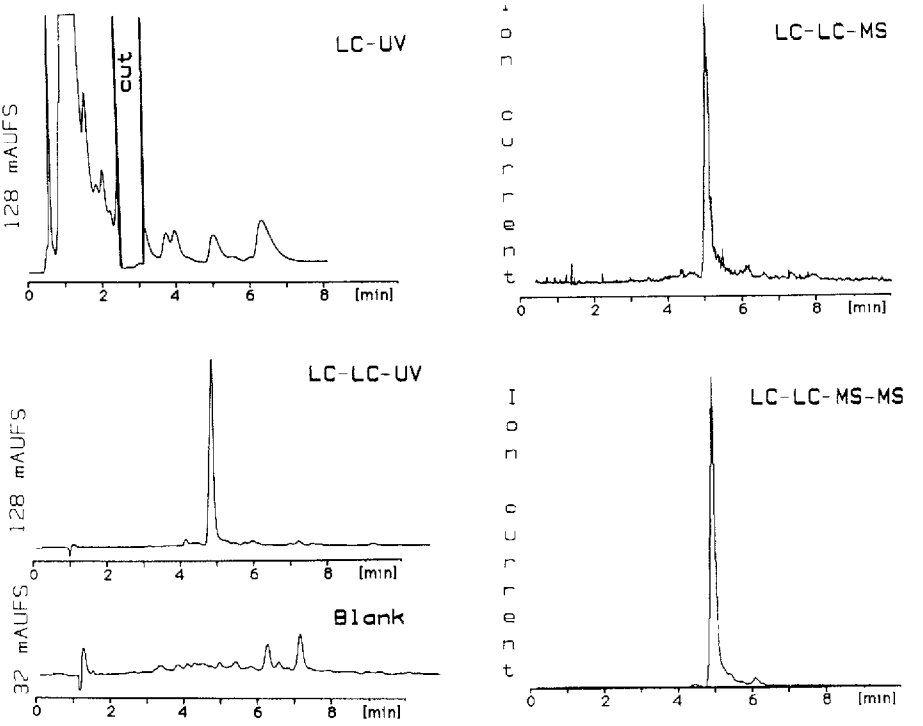

Fig. 5. Comparison of chromatograms obtained with an extract of an 8-h urine sample containing 153 ng/ml 17-epi-I. Chromatographic conditions were as described in Table I.

tion was preferred since large sample volumes (10 ml) usually are available for extraction.

The urine extracts were first separated on a phenyl-silica column with methanol as the organic modifier and the fraction containing I or 17-epi-I was collected onto a short ODS column for enrichment (peak compression) in a similar manner as described for I-SO_4. The sample fraction was eluted from the enrichment column with reversed eluent flow direction with a mobile phase containing a higher concentration of methanol and finally separated on an octyl-silica column. The separation was optimized by a step-wise increase of the retention on the first column and repeated injections of spiked samples and blanks until a sufficiently low background was obtained to be separated on the last column. The separation on the last column was then adjusted to obtain complete separation from the components present in urine (Figs. 4 and 5). The retention of the analytes on the first column was checked each day to set the correct time window for fraction collection (heart cut).

The detection limit with UV detection was 1 ng/ml for I and 17-epi-I. High concentrations of 17-epi-I could be detected after separation on a single octyl-silica column, but the coupled-column system decreased the detection limit at least ten times and will decrease the risk for a false positive sample during screening.

Confirmation by LC–MS and LC–MS–MS

Two complimentary LC–MS interfaces were used for on-line LC–MS and MS–MS to confirm the identity of I and its metabolites in equine urine. The heated nebulizer LC–MS interface with corona discharge ionization generated solvent ions that resulted in API of neutral analytes in the gas phase. The ion-spray LC–MS interface combines some of the characteristics of electrospray and ion evaporation interfaces and is especially suited for thermolabile compounds that are ionic in solution, like I-SO_4 [12]. Both ionization techniques gave little or no fragmentation and CID was used to obtain fragmentation for structural confirmation.

LC–LC–MS of I and 17-epi-I gave simple positive-ion mass spectra showing the $[M+H]^+$ ion at m/z 301 and a weak fragment for loss of water at m/z 283. Their MS–MS mass spectra were identical with several characteristic daughter ions corresponding to loss of water to give m/z 283, a fragment involving the C- and D-ring at m/z 149, A-ring and part of B-ring at m/z 173 and the A-ring at m/z 135 and 121 (Fig. 6). The C- and D-ring fragment was verified by the MS–MS spectrum of 17-CD_3-I which gave a labeled fragment at m/z 152. The coupled-column LC–MS–MS confirmation of I in equine urine showed an unexpected

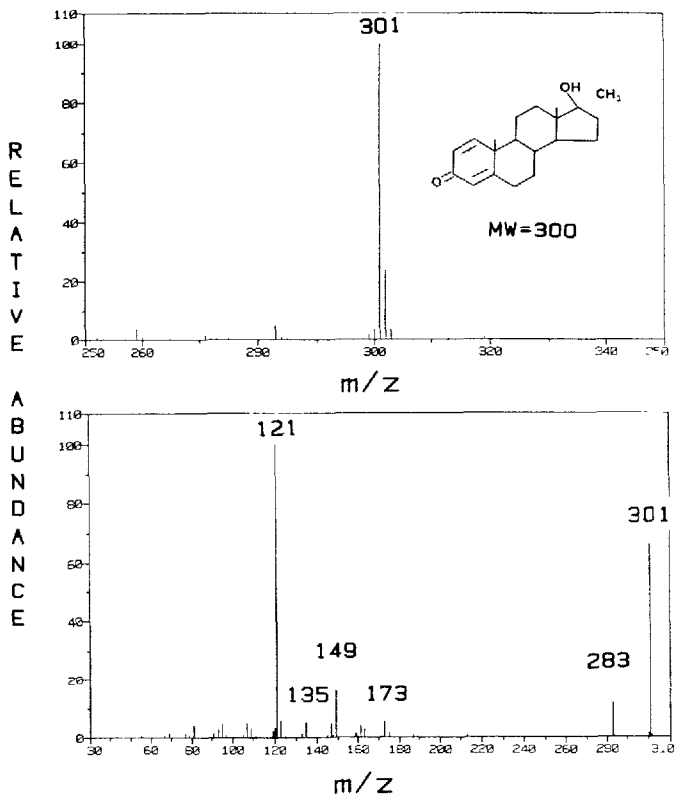

Fig. 6. HPLC–MS (top) and HPLC–MS–MS (bottom) mass spectra of peak B in Fig. 4. Peaks A, B and C in Fig. 4 and a pure standard of 17-epi-I and I gave identical spectra.

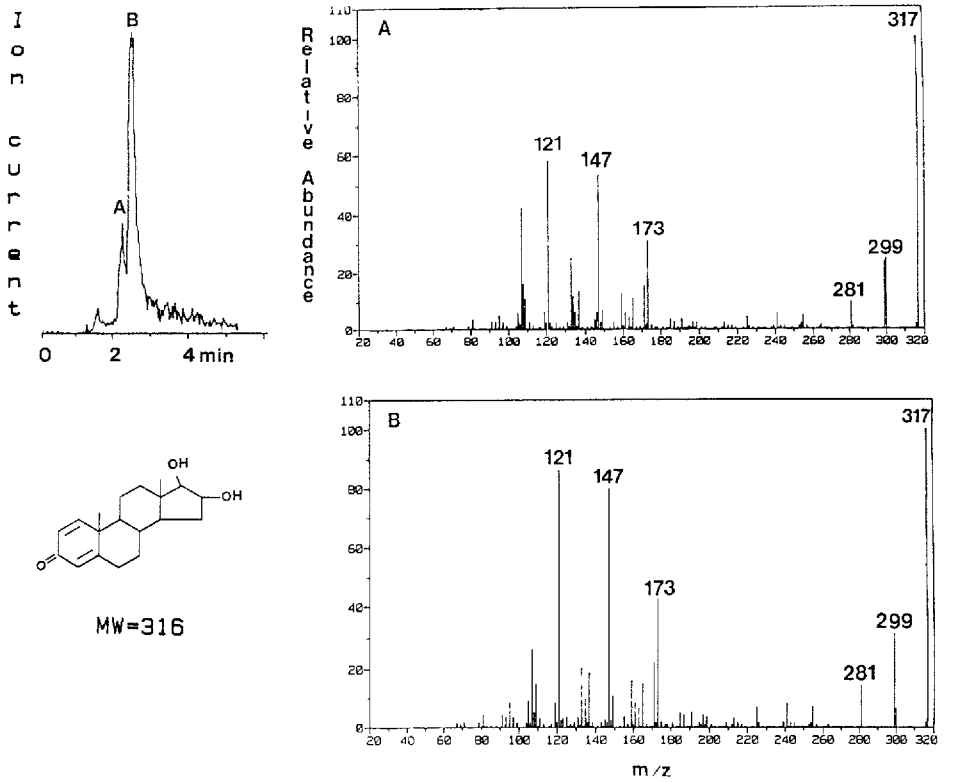

Fig. 7. HPLC–MS–MS spectra of a monohydroxylated I metabolite in equine urine. The proposed structure of the compound in peak B is shown Chromatographic conditions were as described in Table I.

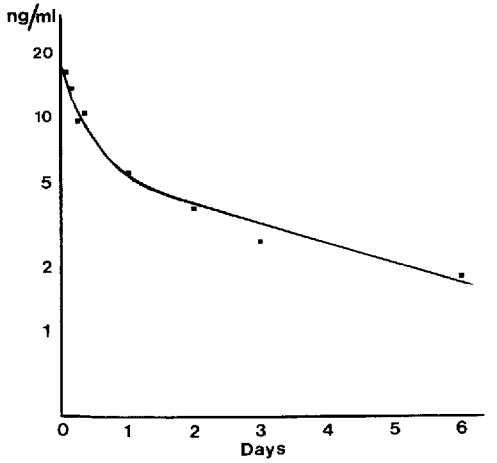

Fig. 8. Plasma concentration–time profile of I after a 250-mg intramuscular dose to a horse. The plasma concentrations were fit to a two-compartment model according to the equation $C = 13.5e^{-0.15t} + 5.96e^{-0.0089t}$ where t is the number of hours.

product with identical MS–MS spectrum but different retention time on the octyl-column (peak A in Fig. 4). This isomer of I appears to form from the rearranged carbonium ion related to I-SO_4 to form 13-hydroxy-17,17-dimethyl-1,4-androstadien-3-one.

Hydroxylation is a common route for metabolism of steroids and has been reported for I in different species [10, 11]. The retention time of the hydroxylated metabolites on the first column was estimated by selected-ion monitoring (SIM) LC–MS of the expected molecular ions at m/z 317 and 333, respectively. When the retention time on the first column was known, the coupled-column experiment with tandem MS was set up using the heated pneumatic nebulizer (SIM) LC–MS interface. Two monohydroxylated metabolites were found as shown in Fig. 7. The mass spectrum alone is not sufficient to determine the position of the hydroxyl group, but the fragment ion at m/z 147 suggests that the hydroxyl group is positioned at the 16-position or possibly the 12-position. The isomers detected may be due to hydroxylation of I and 17-epi-I, respectively. The primary hydroxylation of the D-ring is contradicted by the fact that the monohydroxylated I obtained from human urine gave the same mass spectra and Dürbeck and Buker [10] suggested that hydroxylation occurred primarily at the 6-position. It is likely that both 6- and 16-hydroxylation occurs since a dihydroxylated metabolite was also identified. Dihydroxylated I gave daughter ions at m/z 147 and 171 due to loss of water from the C + D-ring and A + B-ring fragments, respectively. One of the sulphate elimination products of I-SO_4, 18-nor-17,17-dimethyl-1,4,13(14)-androstriene-3-one, was also identified in urine after spontaneous hydrolysis.

Pharmacokinetics

The plasma concentration of I versus time post-dose was fitted to a two-compartment model by the exponential stripping program ESTRIP [18]. The intramuscular injection of I in ethanol–glycerol gave a rapid absorption and a distribution phase that lasted for about two days and a final elimination phase with a half-life of 78 h (Fig. 8). Plasma 17-epi-I was below the detection limit of the method (1 ng/ml) but I-SO_4 was detected in three plasma samples with a peak concentration of 9.7 ng/ml at 4 h post-dose.

CONCLUSIONS

A study of the metabolism of I in the horse indicates that the major products found in urine are epi-I, I and 6-hydroxylated I. As in man, epi-I is the most useful compound to use as a urine screen for use of I. We have shown that the origin of the epi-I is not an enzymatic epimerization of I, as suggested by others, but rather the product of a nucleophilic attack by water on the labile sulfate conjugate. The observation of I-SO_4 in both serum and urine under appropriate collection conditions supports the finding for the presence of epi-I and other decomposition products in a solution of I-SO_4 as a function of time. In addition to the production of I-SO_4, a number of mono- and dihydroxylated metabolites were identified in urine using coupled-column LC–MS–MS. This finding is in agreement with the work of Dürbeck and Büker [10].

Coupled-column chromatography on phenyl- and octyl-silica columns gave high selectivity for screening and quantification of I and its epimer in urine. The heated pneumatic nebulizer LC–MS interface with CID was well suited for ionization of neutral steroids and tandem MS with CID gave useful mass spectra for confirmation of I and its metabolites. The ion-spray LC–MS interface allowed direct monitoring of the labile I-SO_4, indicating the analytical potential of this technique for metabolic studies.

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