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RAPID DETERMINATION OF METHANDROSTENOLONE IN EQUINE URINE BY ISOTOPE DILUTION LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY

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SUMMARY

Urine samples were spiked with [17-methyl-$^2H_3$]methandrostenolone as internal standard and extracted with a mixture of dichloromethane and cyclohexane. The organic phase was concentrated and injected onto a short octyl-silica column (30 mm×4.6 mm I.D.) for separation of methandrostenolone and 17-epimethandrostenolone. The effluent from the column was connected to a Sciex TAGA 6000E triple quadrupole mass spectrometer equipped with an atmospheric pressure ion source for sampling of ions generated by a heated pneumatic nebulizer with corona discharge ionization. This ion source produced abundant $[M+H]^+$ ions and a weak fragment ion due to loss of water. The protonated molecular ions at m/z 301 and 304 for methandrostenolone, 17-epimethandrostenolone and the internal standard were transmitted to the second quadrupole for collision-induced dissociation. Quantification was obtained by selected reaction monitoring of three daughter ions. Methandrostenolone and 17-epimethandrostenolone were separated by liquid chromatography, but gave identical mass spectra. The method detection limit by injection of a urine extract corresponding to 2.8 ml urine was 180 pg/ml at the 99% confidence level. The precision (relative standard deviation) was 3% at the 16 ng/ml level and the linear dynamic range was at least 3 orders of magnitude. Screening for unknown metabolites in urine

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after administration of methandrostenolone to horses and humans was accomplished by a parent ion scan of m/z 121, a fragment corresponding to the intact A-ring of the steroids.

INTRODUCTION

Tandem mass spectrometry (MS–MS) has developed into a powerful technique for the analysis of complex samples and rapid determination of target compounds [1]. Even though early reports demonstrated the capability of MS–MS to separate analytes from complex samples after direct insertion of crude samples into the ion source [2–4], later work has been directed towards the use of off-line [5] or on-line [1] chromatographic separations to eliminate matrix effects during the ionization process and to include a different separation principle for separation of isomers with equal mass and similar MS–MS spectra.

The metabolism of methandrostenolone in man has been well characterized and monitoring its presence in urine has been reported [6–10]. Most methods described for the determination of methandrostenolone involve one or two derivatization steps which is time-consuming and imparts possible sources of error. The use of coupled-column liquid chromatography (LC) for separation of methandrostenolone, 17-epimethandrostenolone and methandrostenolone sulfate was described in a previous work where MS–MS was used for confirmation and identification of unknown metabolites [6]. In the present work we have used the speed and selectivity of MS–MS on-line with LC on a short LC column for fast quantification and confirmation of methandrostenolone and 17-epimethandrostenolone in crude equine and human urine extracts.

EXPERIMENTAL

Chemicals and reagents

17α-Methyl-17β-hydroxyandrosta-1,4-dien-3-one (methandrostenolone) was purchased from Steraloids (Wilton, NH, U.S.A.). 17α-Hydroxy-17β-methylandrosta-1,4-dien-3-one was synthesized from methandrostenolone sulfate as described previously [6]. Dichloromethane, cyclohexane and HPLC-grade methanol were purchased from Fisher Scientific (Rochester, NY, U.S.A.). C^2H_3I, magnesium turnings, aluminum *tert.*-butoxide, dichlorodicyanoquinone, acetone, anhydrous benzene and dioxane were purchased from Aldrich (Milwaukee, WI, U.S.A.).

Synthesis of internal standard

The method described by Bjorkhem et al. [7] was used to synthesize [17-methyl-$^2H_3$]methandrostenolone. The purity of the product was checked by thin-layer chromatography and LC. The product contained about 4% of the

17-C^2H_3 epimer, but no other impurities. The internal standard was diluted in methanol to a concentration of 1 μg/ml.

Sample preparation

Equine and human urine samples (10 ml) were spiked with the internal standard (100 ng) and extracted with 8 ml of 20% (v/v) dichloromethane in cyclohexane by rotation in test-tubes at low speed for 15 min. The samples were centrifuged and 5 ml of the organic phase were pipetted into a new tube for evaporation under 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 column for HPLC analysis.

Unconjugated metabolites which might remain unaltered on the A-ring were extracted in the same manner with a more polar solvent (ethyl acetate) to assure high recoveries of the unknown polar analytes.

Liquid chromatography

The liquid chromatograph consisted of two Model 510 pumps, a 60-μl Model U6K loop injector and a Model 680 system controller (Waters, Milford, MA, U.S.A.). Fast separations during selected reaction monitoring (SRM) were performed under isocratic conditions on a short octyl-silica column packed with 3-μm particles (Perkin Elmer, Norwalk, CT, U.S.A.) with a mobile phase consisting of 60% (v/v) methanol in water at a flow-rate of 1 ml/min. Metabolite screening was performed with a linear gradient from 40% methanol–60% water to 80% methanol–20% water during 8 min at a flow-rate of 0.4 ml/min on a longer (100 mm × 2 mm I.D.) column, packed with 3-μm Spherisorb octyl-silica (Phase Separations, Queensferry, U.K.).

Tandem mass spectrometry

The effluent from the HPLC column was connected to a Sciex TAGA 6000E triple quadrupole mass spectrometer (Sciex, Thornhill, Canada) equipped with an atmospheric pressure ion source. Ions were generated by a heated pneumatic nebulizer with corona discharge ionization. The nebulizer probe temperature was optimized for maximum sensitivity to be 165 and 275 °C at a mobile phase flow-rate of 0.4 and 1 ml/min, respectively, resulting in an eluent vapour temperature of 150 °C at both flow-rates. The protonated molecular ions at m/z 301 and 304 of methandrostenolone, 17-epimethandrostenolone and the internal standard were transmitted to the second quadrupole for collision-induced dissiciation (CID). Argon was used for CID in the second quadrupole at $3 \cdot 10^{14}$ atoms per cm^2 target gas thickness.

The parent ion and three daughter ions located at m/z 283, 149 and 121 were monitored for confirmation and the peak-height ratio of the analytes and the internal standard at m/z 121 were used for construction of calibration curves and quantification. A dwell time of 300 ms was used during SRM and a scan-

rate of 1 and 3 s per scan was used during parent and daughter ion scans, respectively.

RESULTS AND DISCUSSION

Metabolite screening

In the previous work [6] it was found that very little unchanged methandrostenolone was excreted to the urine while sulfate conjugation and hydroxylation were found to be a common route of metabolism in the horse and man. The sulfate was very unstable and hydrolyzed spontaneously in water to give 17-epimethandrostenolone. Elimination of the sulfate group gave several dehydration products via a stable carbonium ion at the 17-position. In the present work LC–MS–MS with a parent ion scan of m/z 121 and a daughter ion originating from the intact A-ring of methandrostenolone was used to screen

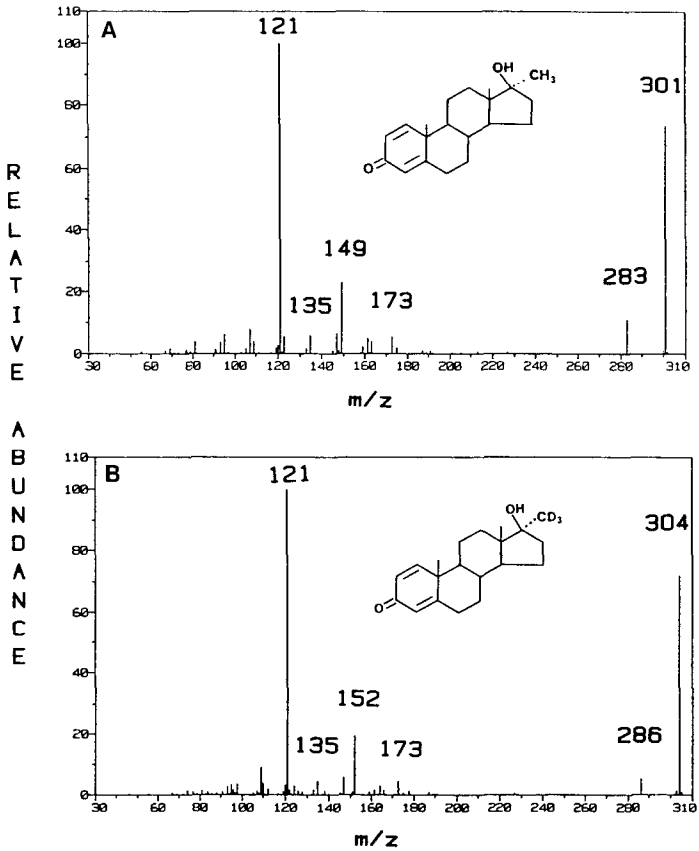

Fig. 1. LC-MS-MS daughter ion mass spectra of methandrostenolone (A) and [17-methyl-$^2H_3$]methandrostenolone (B).

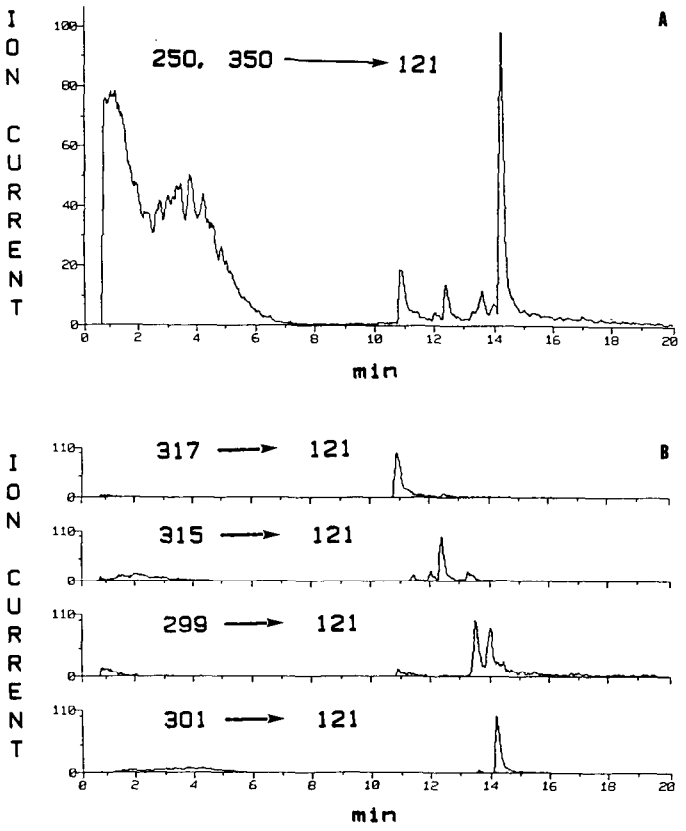

Fig. 2. Total and extracted ion current profiles obtained from an extract of a 20-h human urine after a 25-mg dose of methandrostenolone. Methanol gradient from 40 to 80% methanol in water during 8 min on an octyl-silica column. The total ion current during parent-ion scan of the daughter ion at m/z 121 is shown at the top (A) and extracted ion current profiles for the pseudomolecular ions of the most abundant metabolites are displayed at the bottom (B).

for metabolited formed by metabolic conversion, such as dehydrogenation and hydroxylation occurring in other parts of the molecule. The daughter ion spectra of methandrostenolone and [17-methyl-$^2H_3$]methandrostenolone are shown in Fig. 1. This technique with parent ion scan was used to compare the urinary metabolite pattern in man and the horse (Figs. 2 and 3). Parent ions corresponding to methandrostenolone and 17-epimethandrostenolone with an $[M+H]^+$ ion at m/z 301 and the monohydroxylation product with an $[M+H]^+$ ion at m/z 317 were detected in both equine and human urine. The dehydration product of methandrostenolone sulfate appeared as an $[M+H]^+$ ion at m/z 283 and was most abundant in equine urine (Fig. 3). The total ion current chromatograms showed two metabolites in the human urine with $[M+H]^+$ ions at m/z 299 and 315 which were not present in the equine urine.

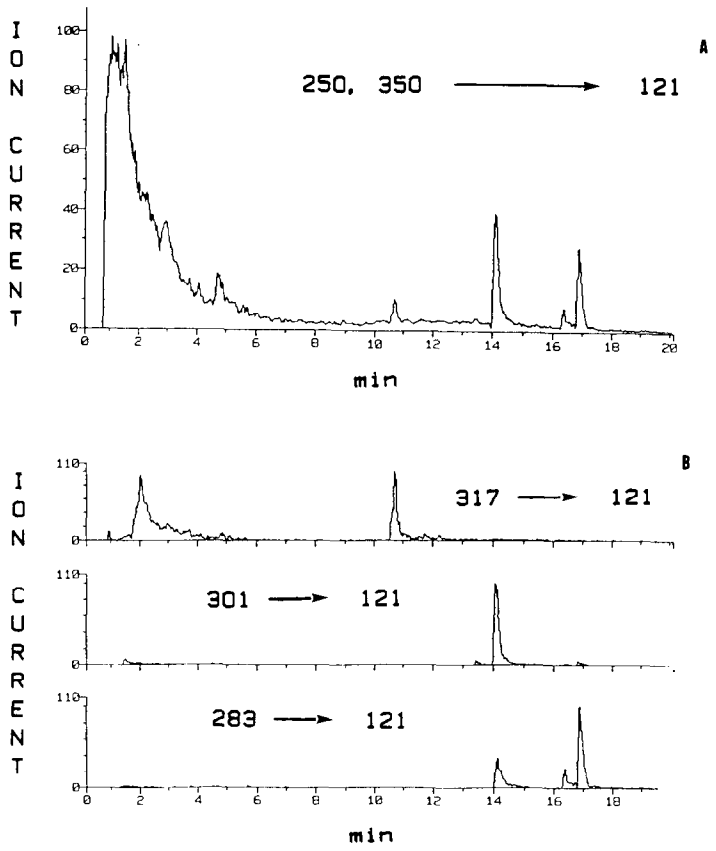

Fig. 3. Total (A) and extracted (B) ion current chromatogram obtained from an extract of an 8-h equine urine after a 250-mg intramuscular dose of methandrostenolone to a horse. The experimental conditions were the same as in Fig. 1.

The $[M+H]^+$ ion at m/z 299 is probably the 6,7-dehydrogenation product reported by Durbeck and Buker [10] using gas chromatography–MS. Hydroxylation and sulfate conjugation followed by elimination of bisulfate ion would give m/z 299, but this is less likely since conjugates of secondary alcohols are normally stable and require acid and heat for hydrolysis. An experiment with a daughter ion scan of the parent ion at m/z 315 showed several isomers (Fig. 4) that may have formed by hydroxylation of the dehydrogenation product giving a molecular weight of 314. A suggested structure for the major peak of these isomers is shown in Fig. 4 together with its MS–MS spectrum. These results demonstrate the utility of LC–MS–MS for screening of drug metabolites in biological samples, by a combination of experiments which include parent and daughter ion scans.

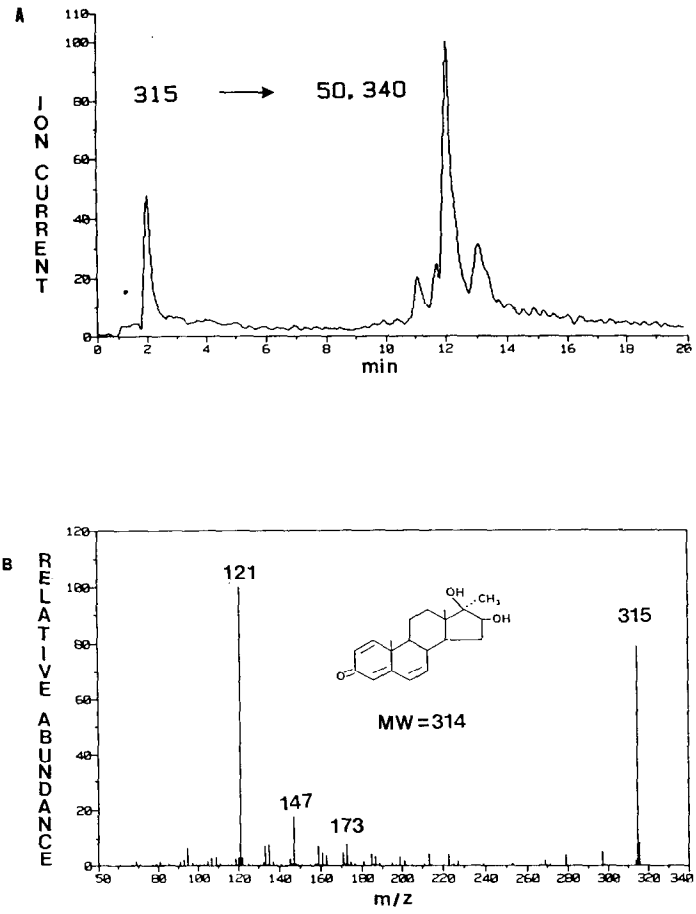

Fig. 4. (A) Total ion current profile (m/z 50–340) for a metabolite with an $[M+H]^+$ ion at m/z 315 in the human urine extract by daughter ion scan of this ion. (B) MS–MS mass spectrum for the major peak is shown together with a proposed structure of this metabolite.

Quantification by LC–MS–MS

The crude extracts obtained after liquid–liquid extraction of 10 ml equine urine were chemically complex, so coupled-column chromatography was used in a previous work to isolate the analytes for detection by UV at 254 nm [6]. In the present work a short reversed-phase column was used to separate methandrostenolone and 17-epimethandrostenolone from each other and most of the sample matrix. The first quadrupole mass analyzer was then used to separate the analytes from the remaining matrix components and to separate methandrostenolone from the internal standard. Very fast separations were possible due to the specificity of the MS detection as can be seen in Fig. 5 where methandrostenolone and 17-epimethandrostenolone were quantified and con-

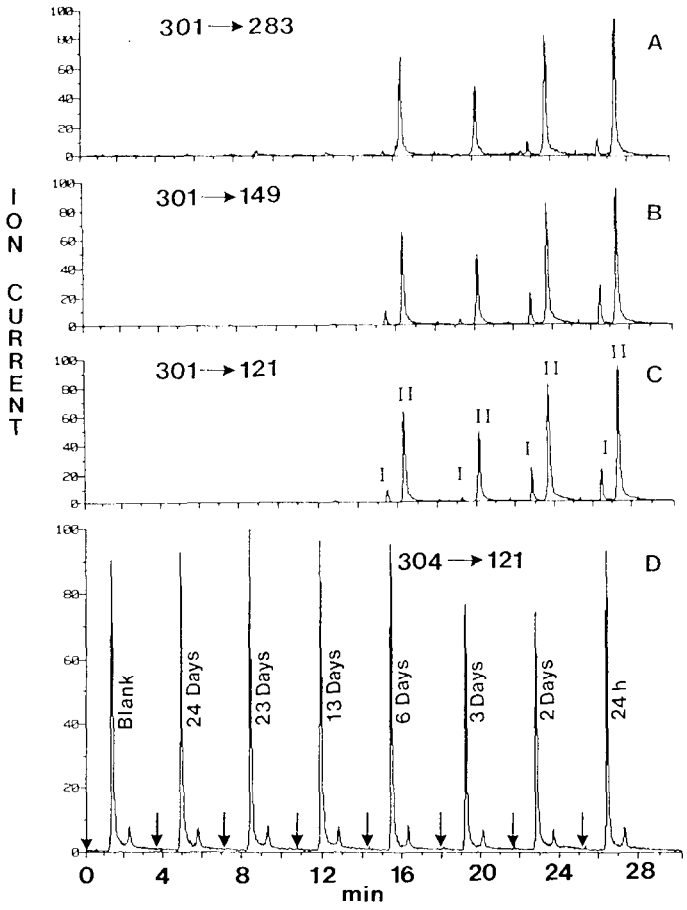

Fig. 5. Ion current chromatograms obtained from a series of equine urine samples with a blank and decreasing time from 24 days to 24 h post-dose. The samples were injected onto a short octylsilica column (30 mm×4.6 mm) at an interval of approximately 3.5 min. The SRM ion current profiles of methandrostenolone (I) and 17-epimethandrostenolone (II) are shown at the top (A, B and C) and the internal standard at the bottom (D).

firmed within 3.5 min. Attempts were made to inject the samples by a higher frequency but overlapping separations were not possible because the ionization of the analytes was quenched by the solvent front from the following injection, which restricted the analysis time to 3.5 min.

Calibration curves were linear in the range used for quantification (0.5–30 ng/ml) ($y=a+bx$; $r=0.999$) and no isotope contribution was obtained from the internal standard as can be seen in the blank injection in Fig. 5. The detection limit at the 99% confidence level, determined according to ref. 11, was 180 pg/ml with injection of a sample corresponding to 2.8 ml urine. The detec-

tion limit can be decreased further by injection of a larger sample volume. The precision was 2.9 and 4.6% (relative standard deviation, $n=8$) at 16.4 ng/ml methandrostenolone and 12.5 ng/ml 17-epimethandrostenolone, respectively. This sensitivity was sufficient to detect 17-epimethandrostenolone in urine up to thirteen days after a 250-mg intramuscular dose to a horse and provides the sensitivity required for pharmacokinetics of methandrostenolone in large animals.

CONCLUSIONS

A comparison of the metabolite pattern of methandrostenolone in man and the horse by LC–MS–MS with parent and daughter ion scans confirmed that 17-epimethandrostenolone is the target compound for screening of methandrostenolone use. A metabolite involving dehydrogenation and hydroxylation of methandrostenolone was also detected in human urine.

Ionization at atmospheric pressure with a heated nebulizer and a corona discharge needle gave high sensitivity for determination of methandrostenolone and its epimer. The selectivity of MS–MS made it possible to determine methandrostenolone and 17-epimethandrostenolone in urine at a rate of fifteen samples per hour.

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