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Stable isotope dilution analysis of human urinary metabolites of 17α-methyltestosterone

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Abstract

A method based on gas chromatography–mass spectrometry–selected-ion monitoring was developed to measure the main metabolites of 17α-methyltestosterone, 17α-methyl-5α-androstan-3α,17β-diol and 17α-methyl-5β-androstan-3α,17β-diol, in human urine. 17α-Methyl-[$^2H_3$]-5α-androstan-3α,17β-diol and 17α-methyl-[$^2H_3$]-5β-androstan-3α,17β-diol were used as internal standards. The methods involved purification using a Sep-Pak C_{18} cartridge, hydrolysis by β-glucuronidase from *Ampullaria* and derivatization with *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide/dithioerythriol/ammonium iodide. Quantitation was achieved by selected-ion monitoring of the characteristic fragment ions ([(M+H)−2×TMSOH]$^+$) of the di-TMS derivatives on the chemical ionization mode. The method provides a specific, sensitive and reliable technique to determine the urine levels of 17α-methyl-5α-androstan-3α,17β-diol and 17α-methyl-5β-androstan-3α,17β-diol, and can be applied to pharmacokinetic studies of 17α-methyltestosterone.

Keywords: Steroids; 17α-Methyltestosterone

1. Introduction

17α-Methyltestosterone (17β-hydroxy-17α-methyl-4-androstan-3-one, Fig. 1) is a synthetic androgen which has been used to treat patients with androgen deficiency and infertility. It is an orally effective hormone analogue that has considerably higher activity than testosterone [1–4]. Rongone et al. have shown extensive metabolism of radioactive 17α-methyltestosterone administered to man [5]. Two thirds of the radioactivity was present in the stools and one third in the urine. The glucuronides of 17α-methyl-5α-androstan-3α,17β-diol and 17α-methyl-5β-androstan-3α,17β-diol were found to be the main metabolites excreted in the urine [6,7].

17α-Methyltestosterone has also been encountered

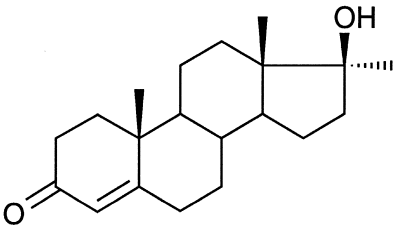

Fig. 1. Structure of 17α-methyltestosterone.

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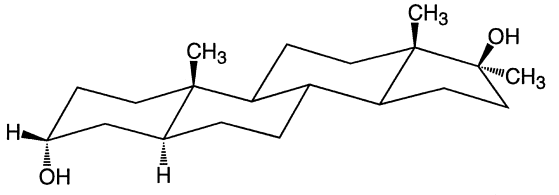
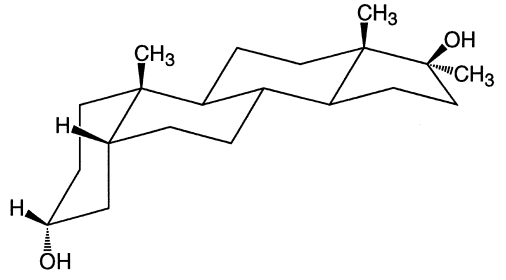
as a doping agent in sports. The use of 17α-methyltestosterone is banned by the International Olympic Committee and other organizations responsible for anti-doping control. This ban is controlled by the analysis of urine samples obtained from athletes, where the main metabolites, 17α-methyl-5α-androstan-3α,17β-diol and 17α-methyl-5β-androstan-3α,17β-diol, are identified by gas chromatography–mass spectrometry (GC–MS) [8–11]. However, very little has been published regarding the pharmacokinetic characteristics of the metabolites after administration of 17α-methyltestosterone.

The use of GC–MS and stable isotope-labeled substance as diluents has found broad application in pharmacological studies. In a previous study, we have reported the determination of serum 17α-methyltestosterone by GC–MS–selected ion monitoring (SIM) using deuterated 17α-methyltestosterone as an internal standard [12]. Furthermore, we applied this technique to follow the time course of serum 17α-methyltestosterone in normal men after oral administration of 10 mg of 17α-methyltestosterone [13].

In the present work, we have developed a stable isotope dilution GC–MS–SIM method for the measurement of urinary main metabolites, 17α-methyl-5α-androstan-3α,17β-diol and 17α-methyl-5β-androstan-3α,17β-diol (Fig. 2). The method was applied to determine the urinary levels of 17α-methyl-5α-androstan-3α,17β-diol and 17α-methyl-5β-androstan-3α,17β-diol after oral administration of 17α-methyltestosterone in healthy male volunteers.



17α–methyl-5α-androstan-3α,17β-diol



17α–methyl-5β-androstan-3α,17β-diol

Fig. 2. Structures of metabolites of 17α-methyltestosterone.

2. Experimental

2.1. Chemicals and reagents

[$^2H_3$]Methyl iodide (99.5 atom% ^2H) was purchased from Merck (Montreal, Canada). Methyl iodide was purchased from Kanto (Tokyo, Japan). Androsterone (3α-hydroxy-5α-androstan-17-one) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Ethiocholan-3α-ol-17-one (3α-hydroxy-5β-androstan-17-one) was purchased from Sigma (St. Louis, MO, USA). β-Glucuronidase from *Ampullaria*, ammonium iodide, dithioerythriol and 17α-methyltestosterone were purchased from Wako (Osaka, Japan). *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide was purchased from Pierce (Rockford, IL, USA). All other chemicals and solvents were of analytical-reagent grade and were used without further purification.

2.2. Synthesis

2.2.1. 17α-Methyl-[$^2H_3$]-5α-androstan-3α,17β-diol

The deuterium-labeled Grignard reagent (0.81 *M* C[$^2H_3$]MgI in dry diethyl ether solution) was prepared by using [$^2H_3$]methyl iodide. To 20 ml (16.3 mmol) of the above Grignard reagent was added dropwise, over 10 min, a solution of 1.0 g (3.4 mmol) of androsterone in 90 ml of dry diethyl ether. The solution was refluxed for 8 h. After cooling, 100 ml of 1.5 *M* ammonium chloride solution was added dropwise. Dilution with H_2O, extraction with diethyl ether, washing with 3 *M* HCl, 5% $NaHCO_3$, 10% $Na_2S_2O_3$ and H_2O, followed by drying over $MgSO_4$ and evaporation gave 1.05 g of crystalline residue.

After silica gel column chromatography of the residue using benzene–AcOEt (1:1) as an eluting solvent, the purified product (640 mg, 60% yield) was obtained as colorless crystals following evaporation of the solvent under reduced pressure. The crystals were recrystallized from *n*-hexane–AcOEt (1:1), m.p. 205°C. NMR[$\delta_H(C^2HCl_3)$]: 0.80 (3H, s, 10-CH_3), 0.85 (3H, s, 13-CH_3), 4.04 (1H, br, 3-H). [$\delta_C(C^2HCl_3)$]: 24.63–25.23 (17α-C). MS[EI]: m/z 309 (M^+). Calculated for $C_{20}H^2_{31}H_3O_2$: C, 77.61; H(^{2}H), 11.07. Found: C, 77.69; H(^{2}H), 11.13.

2.2.2. 17α-Methyl-[$^2H_3$]-5β-androstan-3α,17β-diol

To 7 ml (5.7 mmol) of the above Grignard reagent (in Section 2.2.1) was added, dropwise over 10 min, a solution of 300 mg (1.0 mmol) of etiocholan-3α-ol-17-one in 30 ml of dry diethyl ether. The solution was refluxed for 8 h. After cooling, 50 ml of 1.5 *M* ammonium chloride solution was added dropwise. Dilution with H_2O, extraction with diethyl ether, washing with 3 *M* HCl, 5% $NaHCO_3$, 10% $Na_2S_2O_3$ and H_2O, followed by drying over $MgSO_4$ and evaporation gave 303 mg of crystalline residue. After silica gel column chromatography of the residue using benzene–AcOEt (1:1) as an eluting solvent, the purified product (233 mg, 73% yield) was obtained as colorless crystals following evaporation of the solvent under reduced pressure. The crystals were recrystallized from *n*-hexane–AcOEt (1:1), m.p. 165°C. NMR[$\delta_H(C^2HCl_3)$]: 0.84 (3H, s, 13-CH_3), 0.94 (3H, s, 10-CH_3), 3.64 (1H, br, 3-H). [$\delta_C(C^2HCl_3)$]: 24.63–25.24 (17α-C). MS[EI]: m/z 309 (M^+). Calculated for $C_{20}H_{31}{}^2H_3O_2$: C, 77.61; H(^{2}H), 11.07. Found: C, 77.64; H(^{2}H), 11.05.

2.2.3. 17α-Methyl-5α-androstan-3α,17β-diol

17α-Methyl-5α-androstan-3α,17β-diol was synthesized from 2.2 ml (2.3 mmol) of 1.1 *M* Grignard reagent (CH_3MgI) and 107 mg (0.37 mmol) of androsterone by the same manner as described in Section 2.2.1. The purified product (93 mg, 83% yield) was obtained as colorless crystals, m.p. 208°C. NMR[$\delta_H(C^2HCl_3)$]: 0.80 (3H, s, 10-CH_3), 0.85 (3H, s, 13-CH_3), 1.21 (3H, s, 17α-CH_3), 4.04 (1H, br, 3-H). [$\delta_C(C^2HCl_3)$]: 25.84 (17α-C). MS[EI]: m/z 306 (M^+). Calculated for $C_{20}H_{34}O_2$: C, 78.38; H, 11.18. Found: C, 78.31; H, 11.16.

2.2.4. 17α-Methyl-5β-androstan-3α,17β-diol

17α-Methyl-5β-androstan-3α,17β-diol was synthesized from 2.2 ml (2.3 mmol) of 1.1 *M* Grignard reagent (CH_3MgI) and 94 mg (0.32 mmol) of etiocholan-3α-ol-17-one by the same manner as described in Section 2.2.2. The purified product (81 mg, 82% yield) was obtained as colorless crystals, m.p. 166°C. NMR[$\delta_H(C^2HCl_3)$]: 0.84 (3H, s, 13-CH_3), 0.94 (3H, s, 10-CH_3), 1.22 (3H, s, 17α-CH_3), 3.64 (1H, br, 3-H). [$\delta_C(C^2HCl_3)$]: 25.83 (17α-C). MS[EI]: m/z 306 (M^+). Calculated for $C_{20}H_{34}O_2$: C, 78.38; H, 11.18. Found: C, 78.51; H, 11.18.

2.3. Stock solutions

Stock solutions of the analytes (10 mg per 100 ml) were prepared in ethanol. Storage of these solutions at 4°C did not result in any detectable decomposition for more than six months. All analyses were performed by diluting the stock solutions with ethanol.

2.4. Gas chromatography–mass spectrometry–selected-ion monitoring (GC–MS–SIM)

GC–MS–SIM measurements were made with a Shimadzu (Kyoto, Japan) QP-1000EX quadrupole gas chromatograph–mass spectrometer equipped with a data processing system. A methylsilicone bonded-phase fused-silica capillary column SPB-1 (10 m×0.25 mm I.D.) with 0.25-μm film thickness (Supelco, Bellefonte, PA, USA) was connected directly to the ion source. Helium was used as the carrier gas at a column head pressure of 0.8 kg/cm^2. A split–splitless injection system Shimadzu SPL-G9 operating in the splitless mode was used with a septum purge flow-rate of 1.0 ml/min and split vent flow-rate of 30 ml/min. The purge activation time was 2 min after injection. The initial column temperature was set at 120°C. After the sample injection, it was maintained for 2 min and was increased at 40°C/min to 195°C, then increased at 1°C/min to 205°C, and maintained there for 10 min. The temperature of the injector was 280°C. The mass spectrometer was operated in chemical ionization mode with isobutane as the reagent gas at a pressure of $2\times10^{-5}-5\times10^{-5}$ Torr. The ionization voltage and ionization current were 200 eV and 150 mA, respec-

tively. The ion source temperature was 280°C. Selected-ion monitoring was performed on the fragment ions ($[(M+H)-2\times TMSOH]^+$) at m/z 274 (labeled steroids) and 271 (unlabeled steroids).

2.5. Sample preparation for GC–MS–SIM

2.5.1. Extraction

Frozen urine samples were thawed at room temperature. To a PTFE-lined screw-cap culture tube (100×16 mm I.D.) were added 1.0 ml of urine and 1 μg of 17α-methyl-[$^2H_3$]-5α-androstan-3α,17β-diol and 1 μg of 17α-methyl-[$^2H_3$]-5β-androstan-3α,17β-diol as the internal standards dissolved in 0.1 ml of ethanol. The urine sample was applied to a Sep-Pak C_{18} cartridge, which was prewashed with 5 ml of methanol and 5 ml of H_2O. The cartridge was washed with 5 ml of H_2O and then eluted with 5 ml of methanol into a conical centrifuge tube (100×13 mm I.D.). After evaporation to dryness at 40°C under a stream of nitrogen, the residue was dissolved in 1 ml of 0.1 *M* acetate buffer (pH 5.0) and 20 μl (440 units) of β-glucuronidase from *Ampullaria*. The resulting mixture was incubated for 1 h at 60°C and cooled at room temperature. Following addition of 40 μl of 3 *M* sodium hydroxide, the steroids were extracted with diethyl ether (two 3-ml portions). The extract was washed with 1 ml of 5% acetic acid and then 1 ml of H_2O. The solution was evaporated to dryness at room temperature under a stream of nitrogen.

2.5.2. Derivatization

To the dried residue were added 50 μl of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide/dithioerythriol/ammonium iodide (1000:2:4, v/w/w). The resulting mixture was heated at 60°C for 15 min. After evaporating the excess reagent under a stream of nitrogen at room temperature, the residue was dissolved in 3 ml of *n*-hexane and 1 ml of H_2O. After vortex-mixing and centrifuging for 5 min at 3000 rpm, the upper organic layer was transferred to a conical centrifuge tube (100×13 mm I.D.) and evaporated to dryness under a stream of nitrogen at room temperature. The residue was dissolved in 100 μl of *n*-hexane. A 0.2–2.0 μl volume of the solution was subjected to GC–MS–SIM.

2.6. Calibration curves and quantitation

To each of a series of standards containing known amounts of 17α-methyl-5α-androstan-3α,17β-diol (0, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 μg) or 17α-methyl-5β-androstan-3α,17β-diol (0, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 μg) dissolved in ethanol, 1 μg of 17α-methyl-[$^2H_3$]-5α-androstan-3α,17β-diol and 1 μg of 17α-methyl-[$^2H_3$]-5β-androstan-3α,17β-diol dissolved in ethanol were added. Each sample was prepared in triplicate. After evaporation of the solvent to dryness, the samples were derivatized and analyzed as described above. The peak-area ratios of m/z 271 (unlabeled steroid) versus m/z 274 (labeled steroid) were determined and plotted against mixed molar ratios. Because of the wide dynamic range covered by the calibration curve, it was necessary to calculate weighted least-squares linear regression analyses [14] for each of the standard curves. Urine concentrations were calculated by comparing the peak-area ratios obtained from the unknown samples with those obtained from the standard mixtures.

2.7. Accuracy

Accuracy was determined by assaying five preparations of 1.0-ml portions of human blank urine spiked with 17α-methyl-5α-androstan-3α,17β-diol (0.1, 0.5, 1, 5 and 10 μg) and 17α-methyl-5β-androstan-3α,17β-diol (0.1, 0.5, 1, 5 and 10 μg). After preparation of the sample for GC–MS–SIM as described above, the peak-area ratios were measured.

2.8. Drug administration

Four healthy adult male volunteers participated in the pharmacokinetic study. The subjects had to refrain from all medications for 7 days prior to the study day and 3 days following drug administration. Informed consent was obtained from all subjects. After an overnight fast, each subject was orally administered 500 mg of 17α-methyltestosterone dissolved in 50 ml of 5% ethanol. Complete collections of urine were made 0–4, 4–8, 8–12, 12–16, 16–20, 20–24, 24–32, 32–40, 40–48, 48–60 and 60–72 h after dosing. The urine volume was measured, and

the samples were stored frozen in polyethylene bottles at −20°C until analysis.

3. Results and discussion

The use of GC–MS–SIM has found broad application in the measurement of trace amounts of substances in biological materials because of its high sensitivity and high selectivity [15,16]. In this technique, stable isotopically labeled compound serve as the ideal internal standards to correct for losses of a substance under study in the initial isolation procedure. Successful in a stable isotope dilution, GC–MS–SIM is dependent upon the availability of compounds labeled at predesigned positions that are chemically inert. The choice of the 17-position for deuterium labeling was based primarily on a desire to introduce three deuterium atoms in the steroid molecule for the accurate, precise and selective mass spectrometer analysis. Introduction of three deuterium atoms was achieved by treatment of commercially available 17-keto steroids, androsterone or etiocholan-3α-ol-17-one, with a deuterium-labeled methyl magnesium iodide. The electron-ionization (EI) mass spectra showed that the molecular ions at m/z 309 were three mass units higher than those (m/z 306) of unlabeled compounds. The ^{1}H NMR spectra showed no proton signals at 17α-methyl for 17α-methyl-[$^{2}H_3$]-5α-androstan-3α,17β-diol (δ_H 1.21) and 17α-methyl-[$^{2}H_3$]-5β-androstan-3α,17β-diol (δ_H 1.22). The proton-decoupled ^{13}C NMR spectra gave complex signals at δ_C 24.63–25.23 and 24.63–25.24 corresponding to 17α-methyl in the 17α-methyl-[$^{2}H_3$]-5α-androstan-3α,17β-diol and 17α-methyl-[$^{2}H_3$]-5β-androstan-3α,17β-diol, respectively. The isotopic purities were estimated to be 99.5 atom% ^{2}H for 17α-methyl-[$^{2}H_3$]-5α-androstan-3α,17β-diol and 99.7 atom% ^{2}H for 17α-methyl-[$^{2}H_3$]-5β-androstan-3α,17β-diol, based on the ion intensities in the region of the molecular ion measured by EI–MS analysis.

Several derivatization methods have been used to measure anabolic steroids in blood and urine by GC–MS [17–19]. Numerous anti-doping laboratories use the reaction involving the mixture of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide, dithioerythriol and ammonium iodide [20]. This regent leads to the formation of 3,17 di-TMS-ether derivatives (m=450) of 17α-methyl-5α-androstan-3α,17β-diol and 17α-methyl-5β-androstan-3α,17β-diol. Fig. 3 shows the chemical ionization (CI) mass spectra of the TMS derivatives. The TMS derivatives of 17α-methyl-5α-androstan-3α,17β-diol and 17α-methyl-5β-andros-

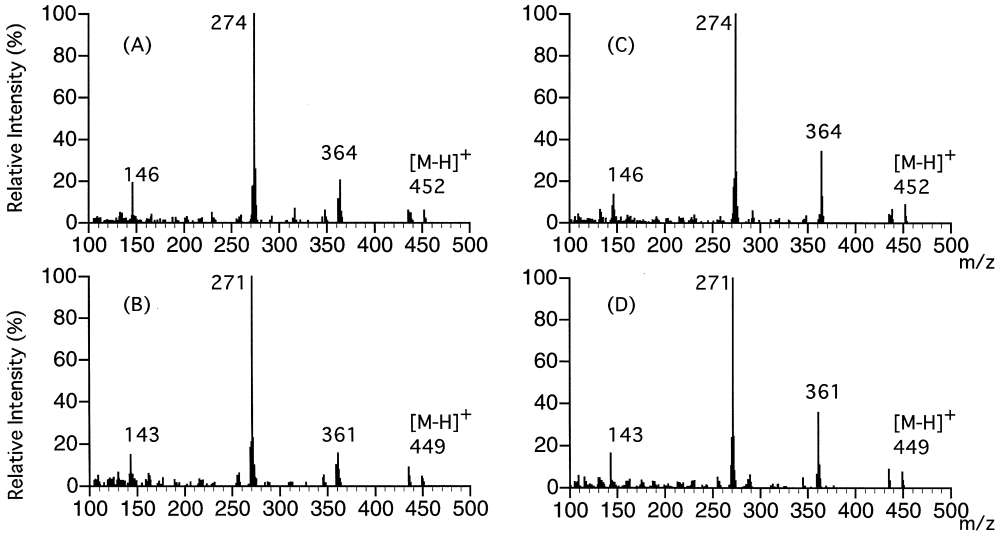


Fig. 3. Chemical-ionization mass spectra of di-TMS derivatives of 17α-methyl-[$^{2}H_3$]-5α-androstan-3α,17β-diol (A), 17α-methyl-5α-androstan-3α,17β-diol (B), 17α-methyl-[$^{2}H_3$]-5β-androstan-3α,17β-diol (C), and 17α-methyl-5β-androstan-3α,17β-diol (D).

tan-3α,17β-diol produced quite similar fragmentation patterns. Quasi molecular ions of low abundance are present in both steroids. Intense fragment ions corresponding to the neutral loss of two molecules of TMSOH ([(M+H)−2×90]) were found at *m/z* 274 and 271 in the mass spectra of labeled and unlabeled steroids, respectively. Exhibiting no loss of label, these ions were chosen for quantitation by the SIM method.

The simultaneous determination of the two metabolites by GC–MS–SIM requires the GC separation because of their identical mass numbers. Previous GC–MS methods failed to resolve the TMS derivatives of 17α-methyl-5α-androstan-3α,17β-diol and 17α-methyl-5β-androstan-3α,17β-diol [21]. After optimizing the GC conditions, good separation was achieved (Fig. 4). The retention times of 17α-methyl-5α-androstan-3α,17β-diol and 17α-methyl-5β-androstan-3α,17β-diol were about 16.0 and 16.3 min, respectively.

Calibration curves were prepared from a series of samples containing various amounts of 17α-methyl-5α-androstan-3α,17β-diol (0.05–20 μg) or 17α-methyl-5β-androstan-3α,17β-diol (0.05–20 μg) and a constant amount of 17α-methyl-[$^2H_3$]-5α-androstan-3α,17β-diol (1 μg) and 17α-methyl-[$^2H_3$]-5β-androstan-3α,17β-diol (1 μg). Each sample was assayed as the TMS derivative by monitoring the ion intensities at *m/z* 271 and 274. Because this constituted a large concentration range, the calibration standards were divided to cover two standard curves, 0.05 to 1.0 and 1.0 to 20.0 μg/ml. This led to an improvement in the accuracy and precision at the lower concentration range and was used throughout the study. When the peak-area ratios were plotted against the mixed molar ratios of unlabeled steroids to the corresponding deuterium-labeled steroids, a good correlation was found between the observed peak-area ratios and the mixed molar ratios.

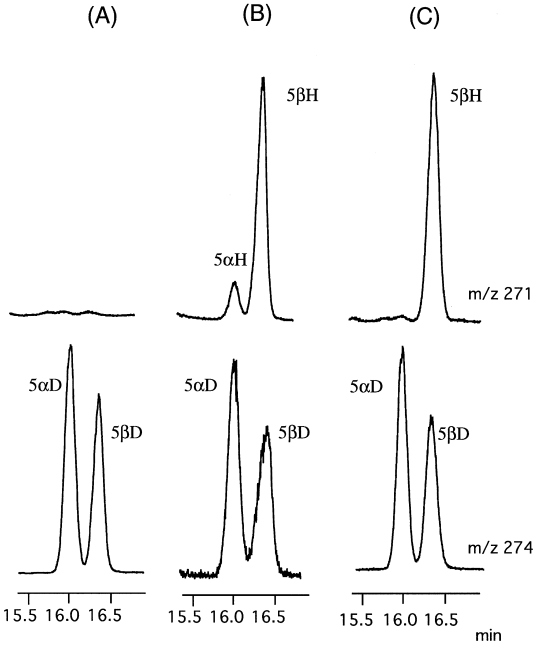


Fig. 4. Selected-ion monitoring profiles of blank human urine sample (2 μl injected) (A), human urine sample at 8–12 h (0.2 μl injected) (B) and at 60–72 h (1 μl injected) (C) after a single oral dose of 500 mg of 17α-methyltestosterone.

Sep-Pak C_{18} cartridges provide a simple method for extracting steroids from biological fluids prior to GC–MS analysis. Both conjugated and unconjugated metabolites of anabolic steroids are quantitatively absorbed from urine and recovered by elution with 5 ml of methanol [22]. Steroid glucuronides in urine have been usually hydrolyzed enzymatically and the released steroid has been quantified consecutively [23,24]. Hydrolysis is usually performed using the enzyme β-glucuronidase from *Helix pomatia*. However, this step is time consuming. Under normal hydrolysis condition, the buffered urinary extract is incubated at 37°C for 12–24 h. Tsukada et al. reported that hydrolysis of urinary steroid conjugates was completed by an incubation with β-glucuronidase from *Ampullaria* at 60°C for 1 h [25,26]. *Ampullaria* β-glucuronidase is suitable for hydrolysis of urinary steroid conjugates because of its higher affinity for 17-oxo steroid and the thermal stability.

The accuracy of the assay was determined by spiking 1.0-ml aliquots of blank human urine with multiple standard solution of 17α-methyl-5α-androstan-3α,17β-diol and 17α-methyl-5β-androstan-3α,17β-diol in the concentration range 0.1–10 μg/ml. The results are presented in Table 1. The estimated amounts were in good agreement with the actual amounts added. The intra-day precision of the assay were less than 8% for each steroids at all concentrations. Inter-day precision of the assay was determined for a period of six working days by

Table 1
Intra- and inter-day accuracy and precision of 17α-methyl-5α-androstan-3α,17β-diol and 17α-methyl-5β-androstan-3α,17β-diol in human urine

Analyte	Spiked amount (μg)	Intra-day (n=3)			Inter-day (n=6)		
		Found amount (μg) (mean±SD)	RSD (%)	Relative error (%)	Found amount (μg) (mean±SD)	RSD (%)	Relative error (%)
17-α-methyl-5α-androstan-3α,17β-diol	10.2	10.110±0.227	2.2	0.90	9.973±0.179	1.8	−0.47
	5.01	4.963±0.072	1.5	−0.93	4.974±0.125	2.5	−0.72
	1.002	1.004±0.005	0.5	0.21	1.013±0.014	1.4	1.05
	0.501	0.501±0.002	0.4	0.02	0.492±0.007	1.5	−1.80
	0.100	0.102±0.003	2.5	1.40	0.103±0.002	2.0	2.50
17α-methyl-5β-androstan-3α,17β-diol	10.82	11.017±0.386	3.5	1.82	10.825±0.114	1.1	0.05
	5.41	5.595±0.060	1.1	3.43	5.478±0.087	1.6	1.26
	1.082	1.079±0.021	1.9	−0.30	1.089±0.005	0.5	0.60
	0.541	0.545±0.008	1.4	0.74	0.530±0.009	1.7	−2.00
	0.108	0.108±0.008	7.8	−0.55	0.111±0.003	2.3	2.87

performing triplicate analyses on urine samples. The results demonstrated an excellent reproducibility. The lower limit of quantitation (LOQ) was around 2 ng/ml urine for each steroid.

The present GC–MS–SIM method was applied for the quantitation of urine concentration of 17α-methyl-5α-androstan-3α,17β-diol and 17α-methyl-5β-androstan-3α,17β-diol after oral administration of 500 mg of 17α-methyltestosterone. Representative SIM profiles of urine samples are shown in Fig. 4. There was no interference from endogenous compounds in the vicinity of the peaks of analyses in the SIM. Urinary concentrations of total (conjugated+unconjugated) 17α-methyl-5α-androstan-3α,17β-diol and 17α-methyl-5β-androstan-3α,17β-diol could be followed up to 72 h (Fig. 5). A pharmacokinetic study of 17α-methyltestosterone is now in progress and will be described in details elsewhere.

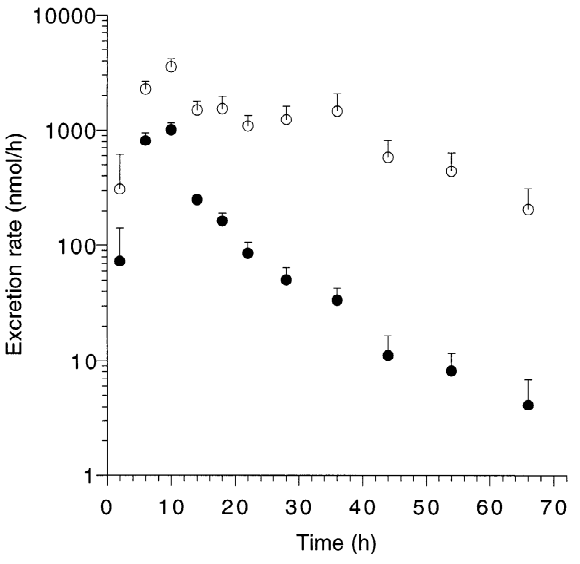


Fig. 5. Urinary excretion rate of total (conjugated+unconjugated) 17α-methyl-5α-androstan-3α,17β-diol (●) and 17α-methyl-5β-androstan-3α,17β-diol (○) versus time profiles in normal adult male volunteers after a single oral dose of 500 mg of 17α-methyltestosterone. Error bars are expressed as mean±SEM (n=4).

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