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DETERMINATION OF TESTOSTERONE PROPIONATE IN HUMAN PLASMA BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A specific, sensitive and accurate quantitative analysis of testosterone propionate in human plasma was developed using gas chromatography—mass spectrometry—selected-ion monitoring. For the calculation of testosterone propionate in plasma, peak height ratios were measured by selected-ion monitoring performed on the molecular ions of the trifluoroacetyl derivative of testosterone propionate (*m/z* 440) and testosterone propionate-19,19,19-d_3 (*m/z* 443). The sensitivity of the method was judged from the lower limit of the detection of the mass spectrometer which was at 20 pg. The inter-assay coefficients of variation and relative error at a concentration of 1.31 ng/ml of plasma were 5.47% and —2.3%, respectively. The method described was applied to the determination of plasma concentrations of testosterone propionate-19,19,19-d_3 following an intramuscular dose of testosterone propionate-19,19,19-d_3 in a healthy male volunteer.

INTRODUCTION

Testosterone propionate is considered to be a short-acting parenteral form of testosterone used primarily in the treatment of hypogonadism, oligospermia and impotence. Although the clinical applications of testosterone propionate have been investigated, pharmacokinetic studies have been limited by the lack of a specific and sensitive assay. Testosterone propionate in pharmaceutical preparations has been measured by several methods involving gas chromatography (GC) [1—3], high-performance liquid chromatography (HPLC) [4], high-performance thin-layer chromatography (HPTLC) [5], infrared spectrophotometry [6] and colorimetry [7]. However, all these methods were not successful in measuring testosterone propionate in biological fluids because of low sensitivity and low selectivity. In an attempt to examine the disposition of testosterone propionate, radioimmunoassay (RIA) [8—12] or competitive protein-binding assay (CPBA) [13, 14] have been employed to detect variations of unesterified testosterone concentration in plasma after

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intramuscular administration of testosterone propionate. However, no direct evidence is available regarding the pharmacokinetic analysis of intramuscular-administered testosterone propionate. RIA and CPBA techniques cannot address this problem, since these types of assay cannot differentiate between endogenous testosterone and testosterone derived from administered testosterone propionate.

We have initiated studies designed to characterize pharmacokinetic properties of intramuscular administered testosterone propionate and to clarify the influence of testosterone propionate on endogenous testosterone levels. We have already reported the determination of plasma testosterone levels by gas chromatography—mass spectrometry—selected-ion monitoring (GC—MS—SIM) using deuterated testosterone as an internal standard [15] and the time course of deuterated testosterone levels after oral administration of the labelled testosterone [16, 17]. In the present study, a sensitive and specific GC—MS—SIM technique was developed for the quantitation of testosterone propionate in biological fluids. The method was applied to determine the plasma levels of deuterated testosterone propionate after the intramuscular administration of labelled testosterone propionate to a healthy male volunteer.

MATERIALS AND METHODS

Chemicals

Testosterone-19,19,19-d_3 (testosterone-19-d_3) was synthesized in our laboratory as described previously [18]. The isotopic composition was 99.0% deuterium atoms (d_3, 97.8%; d_2, 2.2%; d_1, 0.0%). Non-labelled testosterone propionate (reagent grade) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and was recrystallized from *n*-hexane before use. Testosterone propionate-19-d_3 intramuscular preparation was prepared by dissolving 25 mg of testosterone propionate-19-d_3 in sesame oil containing 20% benzyl benzoic acid. The preparation was sterilely filtered through the membrane filter (HA, pore size 0.45 μm; Millipore, Bedford, MA, U.S.A.). All other chemicals and solvents were analytical grade and used without further purification.

Synthesis of testosterone propionate-19,19,19-d_3

To a solution of 0.258 g (0.887 mmol) of testosterone-19-d_3 in 4.5 ml of dry toluene were added 0.193 g (1.483 mmol) of anhydrous propionic acid and 0.321 g of dry pyridine. The mixture was then heated under reflux for 2.5 h. After cooling the mixture, chloroform and water were added, separated and the aqueous layer was re-extracted. The combined extract was washed with water and dried over anhydrous sodium sulphate. After evaporating the solvent, the residue was subjected to thin-layer chromatography (TLC) (Kieselgel 60 F_{254} plates, 0.25 mm thick; E. Merck, Darmstadt, F.R.G.) and the zone corresponding to testosterone propionate (R_F 0.45, chloroform—ethyl acetate, 4:1, as developing solvent) was scraped off. After extraction with chloroform, the product was obtained as colourless needle crystals which were recrystallized from *n*-hexane (0.220 g, 71.5%). m.p. 120—121°C. NMR: 0.84 (3H, s, 18-CH_3), 1.14 (3H, t, J = 7.0 Hz, —CO—CH_2—CH_3). MS: m/z 347 (M^+). Anal. calc. for $C_{22}H_{29}{}^2H_3O_3$: C, 76.03; H, 9.28. Found: C, 76.02; H, 9.33. The isotopic composition was 99.0% deuterium atoms (d_3, 97.8%; d_2, 2.2%; d_1, 0.0%).

Gas chromatography—mass spectrometry—selected-ion monitoring

GC—MS—SIM measurements were made with a Shimadzu LKB-9000B gas chromatograph—mass spectrometer equipped with Shimadzu high-speed multiple ion detector—peak matcher 9060S. The GC column was a glass column (2 m × 3 mm I.D.) packed with 1.5% OV-1 on Shimalate W (80—100 mesh). The injector, column, and ion source temperatures were 260°C, 230°C and 270°C, respectively. Helium was used as the carrier gas at a flow-rate of 30 ml/min. The electron energy was set to 20 eV and the trap current to 60 μA. The multiple-ion detector was focused on the ions at *m/z* 440 and 443 to obtain peak height ratios.

Preparation of calibration curve

To each of four standards containing 2.62, 6.56, 13.12 and 26.24 ng of testosterone propionate in 20 μl of ethanol, 11.84 ng of testosterone propionate-19-d_3 in 20 μl of ethanol were added. After evaporation of the solvent to dryness, to each sample were added 200 μl of trifluoroacetic anhydride (reagent grade; Nakarai Chemicals, Kyoto, Japan). After standing for 30 min at room temperature, excess trifluoroacetic anhydride was removed under a stream of nitrogen and the residue was dissolved in 20 μl of *n*-hexane. A 1—3 μl volume of the *n*-hexane solution was analysed by GC—MS.

Sample preparation for GC—MS—SIM

Frozen plasma samples were thawed at room temperature. To a PTFE-lined screw-cap culture tube (100 × 16 mm) were added 1.0 ml of plasma sample and 13.12 ng of testosterone propionate dissolved in 20 μl ethanol as an internal standard. The plasma sample was allowed to stand for 30 min at room temperature. The sample was cooled in an ice-bath. Immediately after adding 40 μl of 3 *M* sodium hydroxide the sample was extracted with 3 × 3 ml of *n*-hexane using a vortex mixer for a few seconds, followed by centrifugation at 1000 *g* for 5 min at 4°C. The organic phase was carefully pipetted out into a 10-ml conical centrifuge tube and washed with 1 ml of 5% acetic acid and then with 1 ml of water. After evaporating the solvent, the residue was dissolved in 5 ml of 70% aqueous methanol and stored at −15°C for 1 h. After centrifugation, the upper layer was decanted and evaporated to dryness in a 50°C water bath under vacuum. The trifluoroacetate (TFA) derivative was formed by reacting the residue with trifluoroacetic anhydride as described above and 1—3 μl of the sample were subjected to GC—MS.

Stability studies

The first stability study evaluated the effect of the pH and the temperature in the extraction. To 10 ml each of 0.04 *M* Britton—Robinson buffer pH 7.4 and 12.0 were added 118.4 ng of testosterone propionate-19-d_3 dissolved in 20 μl of ethanol. The samples were incubated at 4°C, 25°C and 37°C. An aliquot of the sample (1 ml each) was assayed at various time intervals up to 4 h.

The second stability study evaluated the effect of freezing, storage at −20°C and thawing on control plasma spiked with the reference compound. Seven spiked plasma samples (1 ml each) were prepared by mixing 1-ml aliquots of

drug-free pooled human plasma with 11.84 ng of testosterone propionate-19-d_3 dissolved in 20 μl ethanol. One sample was immediately analysed. The remaining six samples were frozen at −20° C and one sample was analysed after 7, 14, 21, 30, 60 and 90 days of storage.

Intramuscular administration of testosterone propionate-19-d_3

A healthy male volunteer (67 kg, 27 year) was intramuscularly administered a single dose of 25 mg of testosterone propionate-19-d_3. Heparinized blood samples (10 ml) were taken 5 min before and 0.5, 1, 2, 3, 4, 6, 10, 14, 24, 30, 36, 48, 54, 60, 72, 78, 84, and 96 h after dosing. Plasma was separated by centrifugation and kept in a frozen state at −20° C until analysis.

RESULTS AND DISCUSSION

Choice of derivatives and sample preparation

Fig. 1 shows the electron-impact mass spectra of testosterone propionate-19-d_3 and testosterone propionate-19-d_3-TFA. In the mass spectrum of testosterone propionate-19-d_3 (Fig. 1A), the relative abundance of the molecular ion (*m/z* 347) was about 7.3%. In the mass spectrum of testosterone propionate-19-d_3-TFA (Fig. 1B), on the other hand, the relative abundance of the molecular ion (*m/z* 443) was as high as 25%. Therefore it would be an advantage to make the TFA derivative for the SIM analysis by measuring the abundant molecular ions to obtain higher sensitivity. The preparation of testosterone propionate-TFA required only a simple derivatization step.

In the extraction procedure of plasma samples it was necessary to eliminate lipids present in plasma, since these interfered with the SIM. This required the

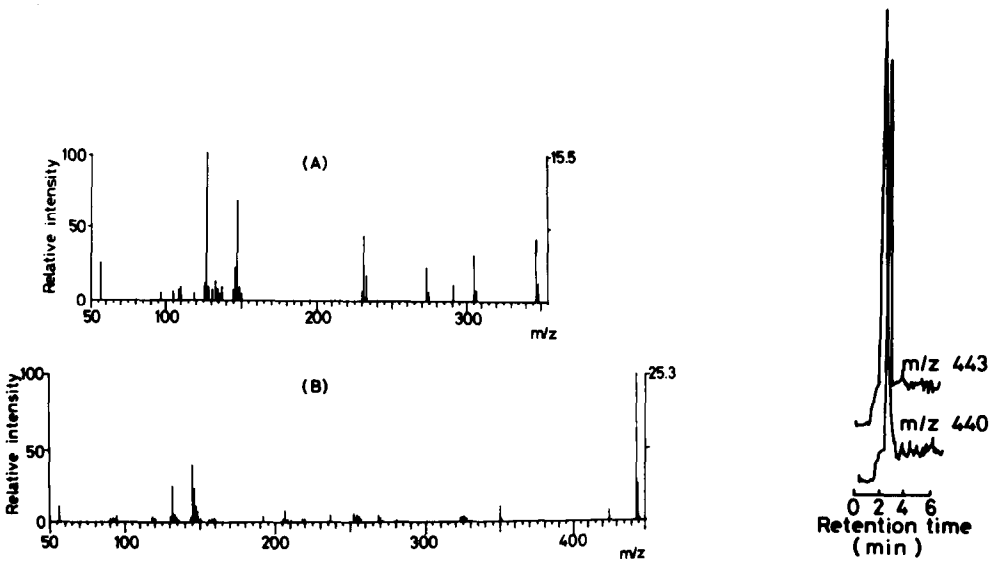

Fig. 1. Electron-impact mass spectra; (A) testosterone propionate-19-d_3; (B) TFA derivative of testosterone propionate-19-d_3.

Fig. 2. Selected-ion monitoring of testosterone propionate-TFA and testosterone propionate-19-d_3-TFA after processing from plasma sample.

treatment of a 70% methanol solution of the hexane extract at −15°C for 1 h. The total absolute recovery of testosterone propionate from plasma was about 65%.

Fig. 2 shows the selected-ion monitoring for blank plasma containing 13.12 ng/ml testosterone propionate and 11.84 ng/ml testosterone propionate-19-d_3. The retention times of testosterone propionate-TFA and testosterone propionate-19-d_3-TFA were the same (about 2.0 min) and there was no interference in the molecular-ion peaks by contributions from other materials in the plasma extract at these masses.

Stability

It has been reported that testosterone propionate is hydrolysed to testosterone by endogenous esterase [19] or by heating with catalytic amounts of base in aqueous solution [20]. In the present work, the testosterone propionate was extracted under alkaline conditions (pH 10—12) since many endogenous substances which interfere with the SIM analysis are acidic and not extractable at a higher pH. Therefore, the stability of testosterone propionate was studied at pH 12.0 and pH 7.4 (physiological pH). The results (Fig. 3A) show that testosterone propionate is stable at physiological pH. On the other hand, at pH 12.0 the stability of testosterone propionate was temperature-dependent (Fig. 3B). However, at 4°C testosterone propionate was stable for 4 h, so that plasma samples should be extracted at 4°C.

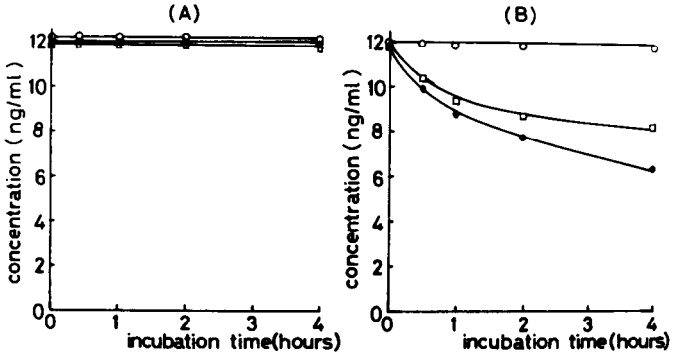

Fig. 3. Stability of testosterone propionate in Britton—Robinson buffer (A, pH 7.4; B, pH 12.0). (○) 4°C; (□) 25°C; (•) 37°C.

Secondly the effect of freezing, storage at −20°C and thawing on control plasma spiked with testosterone propionate was investigated. The data (Table I) showed that freezing and thawing do not have a significant effect on the stability of testosterone propionate and plasma samples could be stored at −20°C for 90 days without significant change.

Sensitivity

The sensitivity of the determination procedures described here was judged on the basis of the signal-to-noise ratio (S/N). The lower limit of detection of the mass spectrometer was 20 pg for testosterone propionate as shown in Fig. 4.

TABLE I

STABILITY OF TESTOSTERONE PROPIONATE IN PLASMA AT −20° C

Day	Plasma concentration (ng/ml)
0	12.34
7	12.08
14	12.24
21	11.72
30	11.65
60	11.53
90	11.45

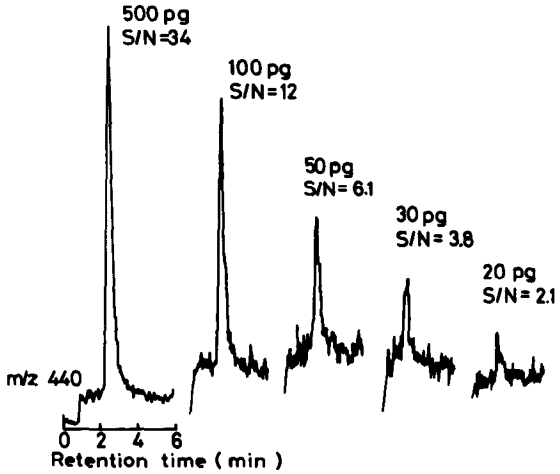

Fig. 4. Sensitivity of the determination on the basis of the signal-to-noise ratio (S/N).

Calibration curve

A calibration curve was prepared by adding known amounts of testosterone propionate (2.62—26.24 ng) to a fixed amount (11.84 ng) of testosterone propionate-19-d_3 and then assaying the mixture as the TFA derivative, monitoring the molecular ions at m/z 440 for testosterone propionate and m/z 443 for testosterone propionate-19-d_3. The peak height ratio was plotted against the molar ratio of testosterone propionate to testosterone propionate-19-d_3. The curve was linear for the molar ratio 0.2—2.2. A least-squares analysis gave a correlation coefficient of 0.999.

Accuracy

The accuracy of measurements was determined for testosterone propionate added to 1.0-ml aliquots of pooled plasma. The plasma samples contained 11.84 ng of testosterone propionate-19-d_3 and different amounts (1.31, 2.62, 6.56, 13.12 and 26.24 ng) of testosterone propionate. The amounts of testosterone propionate were measured by the present method. The amounts of testosterone propionate measured were in good agreement with the amounts of testosterone propionate spiked, the relative error being less than 3% (Table II).

TABLE II

ACCURACY OF SELECTED-ION MONITORING ANALYSIS OF TESTOSTERONE PROPIONATE IN PLASMA

Added (ng)	Found (ng)				C.V. (%)	Relative error (%)
	Individual values			Mean ± S.D.		
26.24	26.13	25.67	26.31	26.04 ± 0.33	1.27	−0.7
13.12	13.11	13.43	12.85	13.13 ± 0.24	1.83	0.7
6.56	6.44	6.51	6.48	6.48 ± 0.09	1.39	−1.2
2.62	2.48	2.67	2.66	2.60 ± 0.09	3.46	−0.8
1.31	1.36	1.29	1.19	1.28 ± 0.07	5.47	−2.3

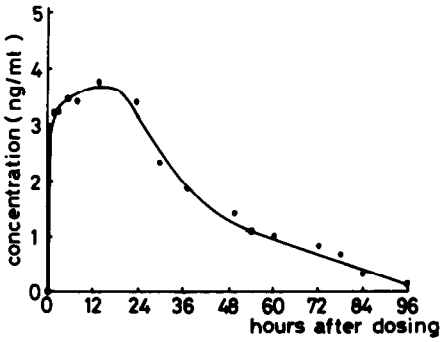

Fig. 5. Time course of testosterone propionate-19-d_3 after intramuscular administration of 25 mg of testosterone propionate-19-d_3 in a healthy male volunteer.

Intramuscular administration of testosterone propionate-19-d_3

The SIM method was applied to the determination of plasma levels of deuterated testosterone propionate in a healthy male volunteer after the intramuscular administration of the labelled testosterone propionate. Fig. 5 shows the time course of testosterone propionate-19-d_3. In the first blood samples, taken 30 min after administration of testosterone propionate-19-d_3, a plasma concentration of 2.94 ng/ml testosterone propionate-19-d_3 was detected. The plasma levels were kept constant (2—4 ng/ml) 24 h after administration and then decreased in monoexponential fashion. It became apparent that intramuscularly administered testosterone propionate-19-d_3 was gradually transferred from the site of injection in the muscle to the circulation.

CONCLUSION

In summary, the SIM method described here affords a specific, sensitive and accurate technique to determine testosterone propionate in plasma. The method makes it possible to examine the disposition of intramuscularly administered testosterone propionate by determining testosterone propionate in plasma. Pharmacokinetic and metabolic studies of testosterone propionate after the intramuscular administration of testosterone propionate are now in progress.

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