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DETERMINATION OF 20α-HYDROXY-9β,10α-PREGNA-4,6-DIEN-3-ONE IN PLASMA BY SELECTED ION MONITORING

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SUMMARY

A selected ion monitoring (SIM) method has been devised for the determination of metabolites of dydrogesterone, 20α-hydroxy-9β,10α-pregna-4,6-dien-3-one (DHD) and DHD glucuronide, in plasma. Using testosterone as an internal standard (IS), DHD and IS were extracted with *n*-hexane and were purified by means of magnesium oxide column chromatography. The purified DHD and IS were converted to their diheptafluorobutyryl derivatives (DHD diHFB and testosterone diHFB) with heptafluorobutyric anhydride in acetone for analysis by SIM.

SIM was carried out with a 2% OV-17 column (1 m) at 230°C by monitoring the molecular ions of the derivatives (*m/z* 706 for DHD diHFB, *m/z* 680 for testosterone diHFB). DHD was determined from a calibration curve using a peak area method. The determination limit of the devised method was about 5 ng DHD per ml of plasma and the reproducibility was within ± 6% of the coefficient of variation for 30 ng of DHD per ml of plasma or above.

INTRODUCTION

Dydrogesterone (6-dehydro-9β,10α-progesterone) is a progestationally active steroid hormone synthesized in 1960 [1]. It is a retrosteroid characterized by the β-position of the C-9 hydrogen atom and the α-position of the C-10 methyl group.

The absorption, metabolism and excretion of dydrogesterone have been studied in detail by use of the ^{3}H-labelled drug. It has been revealed that dydrogesterone is mainly metabolized to 20α-hydroxy-9β,10α-pregna-4,6-dien-3-one (DHD) and further to DHD glucuronide, as shown in Fig. 1 [2]. In addition, it has been reported that the plasma level of dydrogesterone in female subjects who received orally 10 mg of [^{3}H]dydrogesterone was 1 ng/ml of plasma or below, and that of DHD was about 10 ng/ml of plasma [3].

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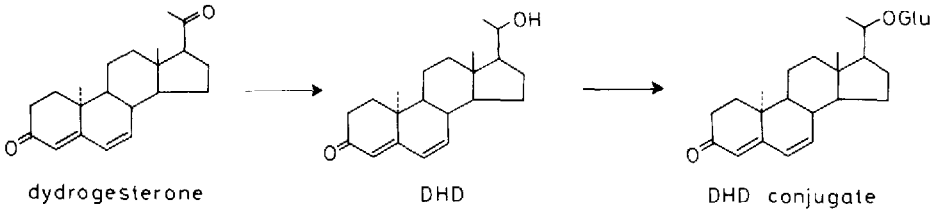


Fig. 1. Metabolic pathway of dydrogesterone.

On the other hand, no chemical methods for the determination of dydrogesterone and the metabolites have been presented. This would be due to the difficulty of measuring dydrogesterone and/or DHD because of their very low plasma levels. In order to examine the pharmacokinetics of dydrogesterone in man after oral administration, we devised a convenient and sensitive selected ion monitoring (SIM) method for the determination of DHD and DHD glucuronide in plasma. This paper describes the method in detail and its application to the pharmacokinetic study of DHD and DHD glucuronide in man.

EXPERIMENTAL

Chemicals and reagents

DHD was provided by Philips Duphar (Amsterdam, The Netherlands). Testosterone of reagent grade was purchased from Daiichi Pure Chemicals (Tokyo, Japan). Heptafluorobutyric anhydride (HFBA) of reagent grade was purchased from Tokyo-Kasei Kogyo (Tokyo, Japan). β-Glucuronidase/arylsulfatase (from *Helix pomatia*, 5.2 U/ml for β-glucuronidase and 2.6 U/ml for arylsulfatase) was purchased from Boehringer Mannheim (Mannheim, G.F.R.). All other chemicals of reagent grade were used without further purification.

A stock DHD solution was prepared by dissolving DHD in ethyl acetate at a concentration of 1.0 μg/ml. An internal standard (IS) solution was prepared by dissolving testosterone in ethyl acetate at a concentration of 2.0 μg/ml. These solutions were stored at 5°C protected from light. The magnesium oxide (MgO) column was a 2.7 cm × 0.5 cm I.D. silanized glass tube. MgO of reagent grade was used after drying in an oven at 300°C overnight and then grinding in a mortar.

Sample preparation

To 1.0 ml of plasma samples in 10-ml glass tubes was added 1 ml of pH 7.0 Michaelis phosphate buffer solution. Subsequently, to these solutions were added exactly 0.1 ml of IS solution (corresponding to 200 ng of testosterone) and 5 ml of *n*-hexane. The tubes were shaken vigorously for 15 min and centrifuged for 5 min at ca. 1000 *g*. The aqueous layer was frozen in a dry-ice–acetone bath, and then the organic layer was transferred to a 10-ml glass tube and evaporated in vacuo to dryness. The residue was dissolved in 0.1 ml of isooctane and passed through the MgO column [4]. The column was washed with 2 ml of isooctane and 2 ml of isooctane–ethyl acetate (20:1) mixture to remove contaminating plasma components; then DHD and IS were eluted with

8 ml of isooctane—ethyl acetate (3:1) mixture. The eluate was evaporated in vacuo to dryness. The residue was reacted with 10 μl of HFBA in 100 μl of acetone at room temperature for 1 h. Then the reaction mixture was evaporated in vacuo to dryness. The residue was dissolved in 50 μl of acetone to give the sample solution. The sample solutions were analyzed by SIM.

In the case of the determination of total DHD including the glucuronide, enzymatic hydrolysis was carried out before the extraction procedure as follows. To 1.0 ml of plasma sample were added 1 ml of pH 4.5 Michaelis acetate buffer solution, 50 μl of β-glucuronidase and 10 μl of toluene, and these solutions were incubated at 37°C for 48 h. Then the solutions were purified in the same manner as described above to prepare the sample solutions for SIM.

Instrumentation

A JEOL JMS-D300 mass spectrometer equipped with a JMA 2000 data processing system was used. A 1 m × 2 mm I.D. silanized glass column packed with 2% OV-17 coated on 80—100 mesh Gas-Chrom Q was used. The column and the injection port were maintained at 230°C and 250°C, respectively. Helium was used as a carrier gas at a flow-rate of 90 ml/min. SIM chromatograms were obtained by monitoring the molecular ions at *m/z* 706 for DHD diHFB and *m/z* 680 for testosterone diHFB at an ionizing energy of 70 eV.

Calculation

The concentration of DHD in plasma samples was determined from a calibration curve obtained using the peak area ratio of the peak at *m/z* 706 against that at *m/z* 680. The standard samples were prepared by adding DHD stock solution in a concentration of 0—140 ng/ml and IS solution (corresponding to 200 ng of testosterone) to 1 ml of blank plasma, and then the calibration curve was obtained from the standard samples treated in the same manner as the plasma samples. The concentration of DHD glucuronide in plasma samples was determined from the difference between the concentration of DHD plus DHD glucuronide and that of DHD alone.

RESULTS AND DISCUSSION

Derivatization, mass spectra and gas chromatographic behaviour

The mass spectrum of underivatized DHD gave intensive peaks at *m/z* 161 and *m/z* 296 as shown in Table I. But the attempt to use these ions for SIM failed in sensitive detection because DHD gave the broad gas chromatographic (GC) peak caused by adsorption on the GC column, and many fragment peaks due to thermal decomposition.

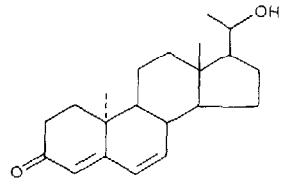
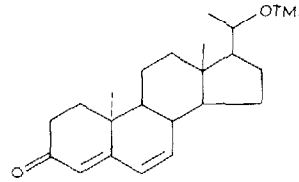
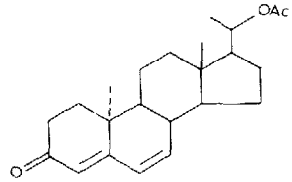
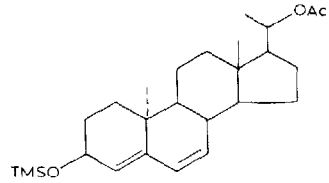
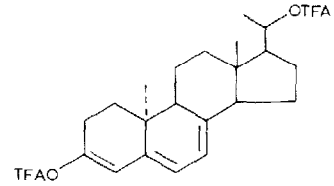
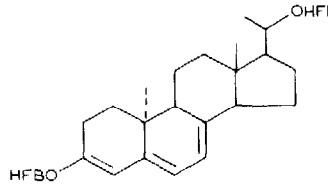
We examined the various derivatives of DHD favourable for SIM measurement, such as 20-trimethylsilyl (TMS), 20-acetyl (Ac), 3-TMS-20-Ac, ditrifluoroacetyl (diTFA) and diheptafluorobutyryl (diHFB) derivatives. The major intense peaks characteristic of the mass spectra of these derivatives are shown in Table I.

It seemed to be convenient for the SIM analysis to choose the DHD derivatives giving intense ions at a higher mass range where little interference from

TABLE I

CHARACTERISTIC PEAKS FOR MASS SPECTRA OF DHD DERIVATIVES

The values in parentheses represent the relative intensity (%) and the underlines indicate the base peaks of respective spectra.

Derivatives		Peaks observed at 70 eV
DHD		314 (2, $M^{\cdot+}$), 296 (88) 281 (44), 161 (100)
DHD 20-TMS		386 (1, $M^{\cdot+}$), 371 (2) 296 (3), 271 (3), 117 (100)
DHD 20-Ac		356 (1, $M^{\cdot+}$), 296 (45), 281 (14), 161 (100)
DHD 3-TMS-20-Ac		430 (63, $M^{\cdot+}$), 415 (6), 370 (8), 340 (11), 75 (100)
DHD diTFA		506 (12, $M^{\cdot+}$), 491 (2) 392 (20), 377 (10), 79 (100)
DHD diHFB		706 (63, $M^{\cdot+}$), 691 (12) 370 (100), 357 (93), 355 (56)

plasma components was observed. Among the derivatives examined, DHD 3-TMS-20-Ac and DHD diHFB derivatives have intense peaks at a higher mass range such as the peak at *m/z* 430 for the former and that at *m/z* 706 for the

latter derivative. In addition to these mass spectral features, both the derivatives exhibited sharp and symmetric GC peaks. Of the two derivatives, we chose the DHD diHFB derivative in view of the simplicity of derivatization. The mass spectrum of DHD diHFB is shown in Fig. 2a.

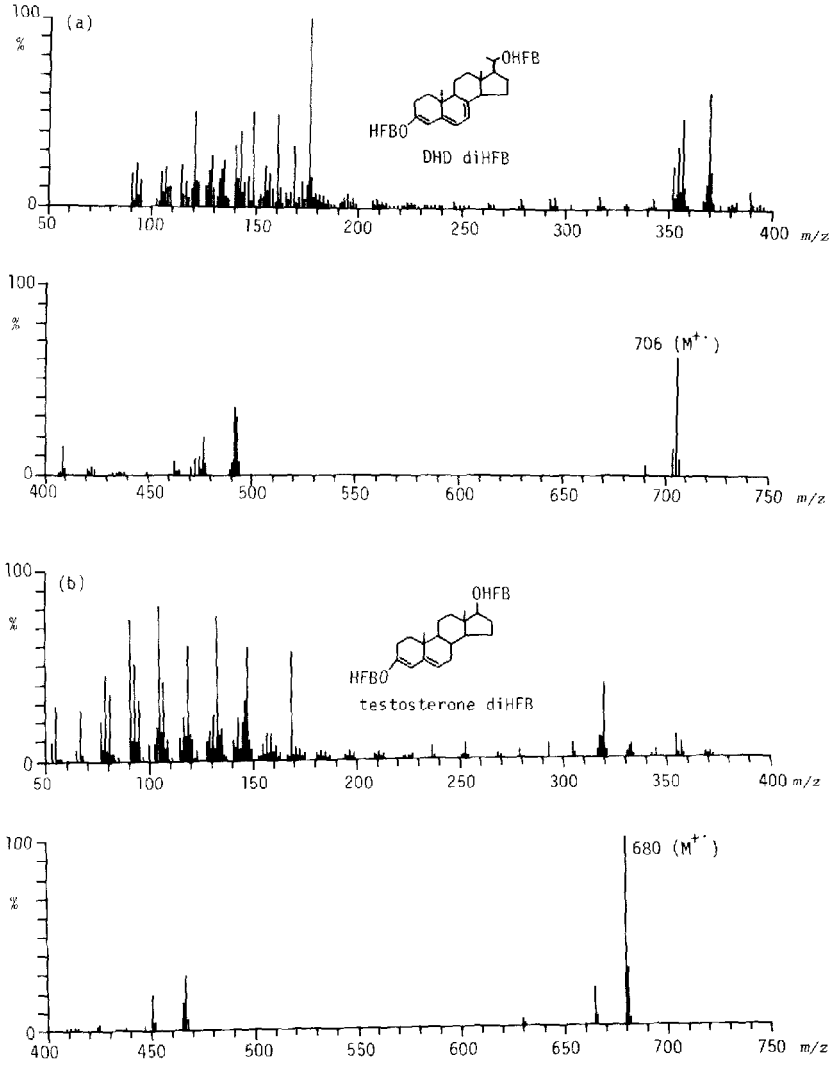


Fig. 2. Mass spectra of (a) DHD diHFB and (b) testosterone diHFB.

Purification of plasma samples

In order to purify DHD from the plasma components, organic solvent extraction using *n*-hexane, diethyl ether and *n*-heptane was examined. Among these solvents, *n*-hexane was found to be appropriate in view of the removal of the plasma components and higher recovery of DHD (about 90%). But the purification could not be achieved by *n*-hexane extraction alone. It was necessary to make a further purification for the more sensitive measurement of DHD in

plasma by the SIM method. Therefore the column chromatographic purification of *n*-hexane extracts was examined.

Consequently MgO column chromatography, which was reported to be effective for the purification of steroid hormones [4], was found to be very effective for the purification of DHD in plasma. This purification method was simple and rapid and was readily applicable to routine analysis of the plasma samples. On the other hand, silica gel column chromatography was inadequate to separate DHD from the plasma components. Use of Ameberlite XAD-2 and Sephadex LH-20 column chromatography was tedious for the treatment of many samples.

In the MgO column chromatographic method, nonpolar components in plasma were eluted in the isooctane and isooctane—ethyl acetate (20:1) fractions and DHD was eluted in the isooctane—ethyl acetate (3:1) fraction.

Choice of internal standard

As recognized in general, DHD labelled with a stable isotope seemed to be the most suitable IS for SIM analysis. Therefore we tried the synthesis of deuterium-labelled DHD by several methods, but these attempts failed because of the long synthetic route. The isotopic purity of deuterated DHD synthesized was too poor to use for an IS. So we examined the use of testosterone, methyltestosterone and progesterone because of their structural resemblance to DHD. Among these compounds, testosterone was suitable for the IS from both its chromatographic and mass spectral behaviour. Testosterone showed a similar behaviour to DHD during the clean-up of plasma samples. The retention time of testosterone diHFB in GC was close to that of DHD diHFB and the mass spectrum of testosterone diHFB gave the base peak at *m/z* 680 (M^+) as shown in Fig. 2b. It has been reported that testosterone is an endogenous steroid presenting at the maximum plasma level of a few nanograms per ml in male subjects [5]. But the effect of trace amounts of endogenous testosterone was considered to be not important for this SIM method, because testosterone was used as IS in higher amounts such as 200 ng/ml of plasma.

SIM chromatograms, calibration curve and precision

The SIM chromatograms of DHD in plasma are shown in Fig. 3. DHD diHFB and testosterone diHFB exhibit retention times of about 2 min and 1 min, respectively, and there is no interference from plasma components.

Known amounts of DHD (0—140 ng) were added to plasma containing a fixed amount (200 ng) of IS and these samples were analyzed by the devised SIM method. The calibration curve obtained by the peak area method showed good linearity ($r \geqslant 0.993$), and the determination limit seemed to be 5 ng of DHD per ml of plasma. The reproducibility of this method is shown in Table II. DHD in plasma could be determined within ± 6% of the coefficient of variation (C.V.) at the concentration of 30 ng DHD per ml plasma or above, although the C.V. at the concentration of 5 ng DHD per ml plasma was about ± 17%. It was considered that such sensitivity and precision would be acceptable for the pharmacokinetic studies of DHD and DHD glucuronide in plasma.

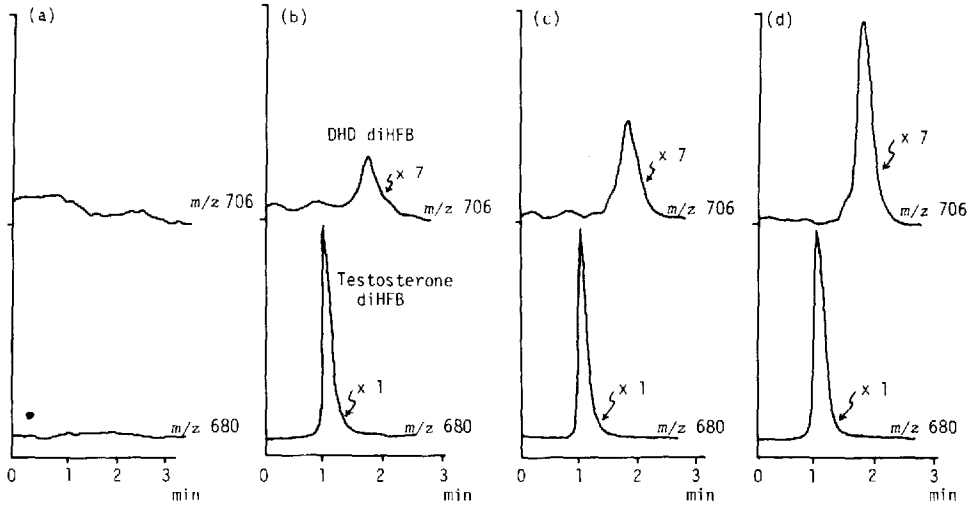


Fig. 3. Selected ion monitoring chromatograms of (a) blank plasma and standard plasma samples, (b) 20 ng DHD per ml of plasma, (c) 40 ng DHD per ml of plasma, and (d) 75 ng DHD per ml of plasma.

TABLE II

REPRODUCIBILITY OF THE METHOD

DHD concentration (ng/ml plasma)	DHD found (ng/ml, mean ± σ_n, $n = 7$)	C.V. (%)
5.0	4.1 ± 0.7	17.1
35.0	35.6 ± 2.1	6.35
110.0	112.4 ± 4.9	4.74

Application of the SIM method

The SIM method was applied to the determination of plasma levels of DHD and DHD glucuronide in five healthy male subjects who each received 10 mg of dydrogesterone in two tablets. Fig. 4 shows the time course of the concentrations of DHD and DHD glucuronide. It is apparent that dydrogesterone is rapidly absorbed into the blood and is readily metabolized to DHD and DHD glucuronide. There were some individual differences; however, the plasma concentrations of DHD and DHD glucuronide reached the maximum levels at 1 h after the single oral administration of 10 mg of dydrogesterone. In addition, the elimination process of DHD and DHD glucuronide could be interpreted in terms of a one-compartment model with elimination half-lives of 1.9 h for the former and 1.5 h for the latter compound. The elimination rate constants k_{el} were calculated by computer fitting as 0.39 h^{-1} and 0.48 h^{-1}, respectively.

This SIM method has been extensively and conveniently utilized for the routine analysis of DHD and DHD glucuronide in plasma.

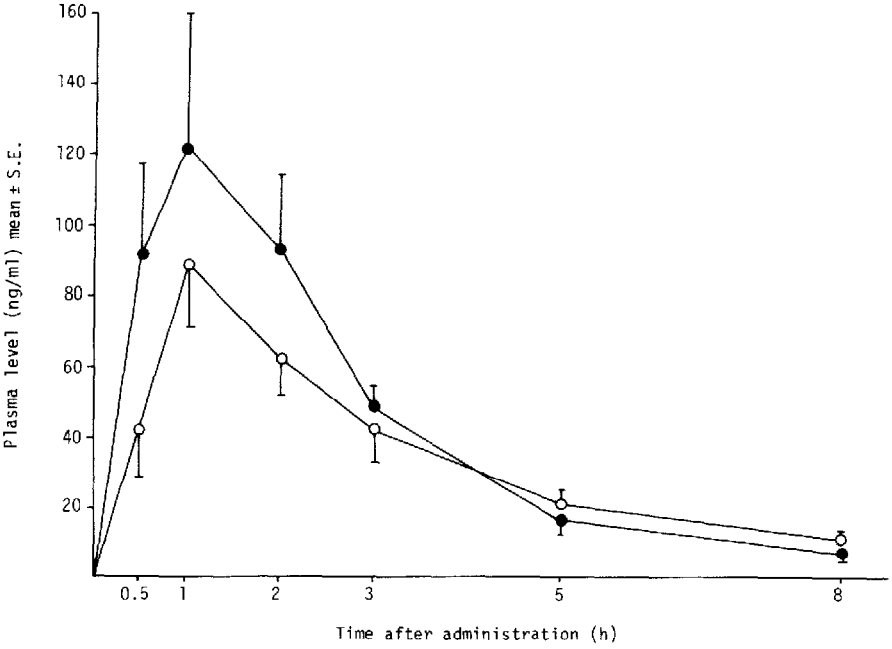


Fig. 4. Time course of DHD (○) and DHD glucuronide (●) in five healthy male subjects after the oral administration of 10 mg of dydrogesterone in the form of commercial tablets.

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