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Simultaneous determination of androstenedione, 11β-hydroxyandrostenedione, and testosterone in human plasma by stable isotope dilution mass spectrometry

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ABSTRACT

This study describes a GC–MS method for the simultaneous determination of androstenedione (AD), 11β-hydroxyandrostenedione (11β-OHAD), and testosterone (TS) in human plasma. [19,19,19-$^{2}H_3$]Androstenedione (AD-$^{2}H_3$), 11β-hydroxy-[1,2,4,19-$^{13}C_4$]androstenedione (11β-OHAD-$^{13}C_4$), and [1,16,16,17-$^{2}H_4$]testosterone (TS-$^{2}H_4$) were used as internal standards. Pentafluoropropionic (PFP) derivatization with good GC behavior was employed for the GC–MS analysis of the three steroids. The detection limit of the present GC–MS–SIM method was found to be 1 pg per injection for AD (S/N ratio = 4.5), 5 pg for 11β-OHAD (S/N ratio = 5.0), and 1 pg for TS (S/N ratio = 4.4), respectively. Calibration curves were linear from 0.22 to 2.80 ng/mL (r = 0.9998) for AD, from 0.56 to 3.19 ng/mL (r = 0.9996) for 11β-OHAD, and from 2.05 to 10.3 ng/mL (r = 0.9996) for TS. The intra- and inter-day assay reproducibilities in the amounts of the three androgens determined were in good agreement with the actual amounts added, the relative errors (R.E.) were −3.1 to 2.4%. The inter-assay relative standard deviation (R.S.D.) was less than 5.3%. The present method provides a sensitive and reliable technique for the simultaneous determination of AD, 11β-OHAD, and TS in plasma. The method can be applied to pharmacokinetic and metabolic studies of androgens with a particular interest in evaluating the conversion of AD to 11β-OHAD and the interconversion of AD and TS in humans.

1. Introduction

Measurement of adrenal androgen production is important in identifying the adrenal or ovarian excessive androgen secretion in patients with hyperandrogenism such as the polycystic ovary syndrome and androgen-secreting tumors [1,2]. It is difficult to determine the source of increased androgen production in women with hyperandrogenism. 11β-Hydroxyandrostenedione (11β-Hydroxy-4-androstene-3,17-dione; 11β-OHAD) has been demonstrated to be a valuable marker of adrenal androgen production in women with hyperandrogenism, because 11β-OHAD is thought to be an exclusive product derived from the intra-adrenal 11β-hydroxylation of AD [3–8]. The production rate of 11β-OHAD (1.5 mg/day) has been reported to be slightly less than that of AD (2.3–3.3 mg/day) [9]. AD produced in the adrenal glands and the gonads then circulates in the blood in a protein-bound and an unbound fraction. Carmina et al. [7] reported that the plasma ratio of AD: 11β-OHAD may better distinguish between patients who predominantly have adrenal or ovarian sources of hyperandrogenism.

Dexamethasone suppression test can be used to distinguish between adrenal and ovarian excessive androgen secretion [10]. An accurate estimation of adrenal androgen production using peripheral markers could be useful in identifying these patients without requiring a dexamethasone suppression test [7]. Androstenedione (AD) is converted to 11β-OHAD by 11β-hydroxylase in the adrenal zona reticularis and also is inter-converted to testosterone (TS) by 17β-hydroxysteroid dehydrogenase in peripheral tissues [11]. Measurements of AD, 11β-OHAD, and TS in plasma might provide another possible marker of adrenal androgen excess in women. However, it is unclear whether the plasma ratio (AD + TS): 11β-OHAD is an accurate indicator to distinguish between adrenal and ovarian excessive androgen secretion. Recently, Shackleton et al. [12] reported that 17-hydroxylase/C17,20-lyase is not the enzyme responsible for the side-chain cleavage of cortisol and its metabolites. In our study, only 0.3% of cortisol was converted to 11β-hydroxyandrosterone (reductive metabolite of 11β-OHAD in urine) by administrating stable isotope-labelled cortisol to a healthy volunteer (unpublished data). It is reasonable to assume that 11β-OHAD is almost exclusively converted from

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AD by 11β-hydroxylase in the adrenal zona reticularis and AD is inter-converted in peripheral tissues to TS by 17β-hydroxysteroid dehydrogenase.

The concentration of 11β-OHAD in plasma has been determined by various immunochemical techniques such as radioimmunoassay [3,13–15] and fluoroimmunoassay [16]. These methods are highly sensitive, but they lack selectivity for 11β-OHAD. On the other hand, stable isotope dilution MS analysis provides a useful technique for measuring trace amounts of substance in biological fluids because of its high sensitivity and high selectivity. However, mass spectrometric analyses of 11β-OHAD using GC–MS or LC–MS have not been reported. Furthermore, there have been no reports concerning stable isotope-labelled 11β-OHAD for use as internal standard.

We have previously developed a sensitive and reliable GC–MS method for the simultaneous determination of AD and TS in plasma using their stable isotope-labelled analogues as internal standards [17]. In the present study, we developed a stable isotope dilution MS method for the simultaneous determination of AD, 11β-OHAD, and TS in human plasma, using the stable isotope-labelled analogues [19,19,19-$^{2}H_3$]AD (AD-$^{2}H_3$), 11β-[1,2,4,19-$^{13}C_4$]OHAD (11β-OHAD-$^{13}C_4$), and [1,16,16,17-$^{2}H_4$]TS (TS-$^{2}H_4$) as internal standards.

2. Experimental

2.1. Chemicals and reagents

11β-Hydroxyandrostenedione (11β-OHAD) was purchased from Steraloids Inc. (Newport, USA). Androstenedione (AD) and testosterone (TS) were purchased from Tokyo Chemical Industry (Tokyo, Japan). [19,19,19-$^{2}H_3$]Androstenedione (AD-$^{2}H_3$), [1,16,16,17-$^{2}H_4$]testosterone (TS-$^{2}H_4$), and [1,2,4,19-$^{13}C_4$]cortisol (cortisol-$^{13}C_4$) were synthesized in our laboratory [17–19]. The isotopic compositions of the labelled compounds were >98 at.% (AD-$^{2}H_3$, cortisol-$^{13}C_4$) and 76 at.% (TS-$^{2}H_4$). Pentafluoropropionic anhydride (PFPA) was purchased from GL Science (Tokyo, Japan). Preparative TLC was performed on glass plates coated with a 0.5-mm layer of silica gel 60 F_{254} (Merck, Darmstadt, Germany). All other chemicals and solvents were of analytical-reagent grade (Kanto, Tokyo, Japan). Type 4A molecular sieves were used to dry dehydration solvent for derivatization.

2.2. Synthesis of 11β-hydroxy-[1,2,4,19-$^{13}C_4$]androstenedione (11β-OHAD-$^{13}C_4$)

Sodium bismuthate (27 mg) was added to a solution of cortisol-$^{13}C_4$ (1.5 mg) in 50% aqueous acetic acid (1.2 mL) and the suspension was stirred at room temperature for 20 h. The reaction mixture was then filtered and diluted with chloroform. The solution was treated with 10% $NaHCO_3$ (60 mL), washed with water, and the solvent was evaporated to dryness, resulting in the crude product. Purification of the residue by preparative TLC (R_F 0.54, $CHCl_3$–MeOH = 9:1, v/v) gave pure 11β-OHAD-$^{13}C_4$ in a 71.5% yield (0.89 mg). The diPFP derivative of 11β-OHAD-$^{13}C_4$ gave the molecular ion [$M^{+\bullet}$] at *m/z* 598. The isotopic compositions of the labelled compounds was >98 at.%.

2.3. GC–MS–selected ion monitoring (GC–MS–SIM)

Capillary GC–MS–SIM analysis was done on a Shimadzu QP2010 GC–MS equipped with a data-processing system. Sample injections were made by AOC-20i Automatic Sampler. GC–MS employed an SPB-1 fused-silica capillary column (15 m × 0.25 mm i.d.) with the stationary phase coated at a 0.25 μm film thickness (Supelco, Bellefonte, PA, USA). Helium was used as the carrier gas at a column head pressure of 50 kPa.

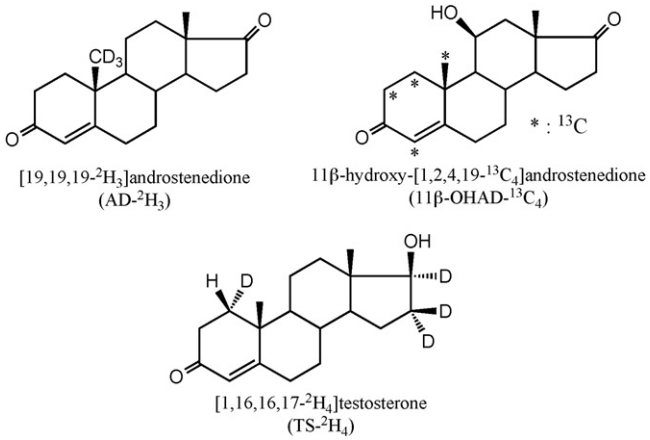

Fig. 1. Structures of stable isotope-labelled internal standards: [19,19,19-$^{2}H_3$]androstenedione (AD-$^{2}H_3$), 11β-hydroxy-[1,2,4,19-$^{13}C_4$]androstenedione (11β-OHAD-$^{13}C_4$), and [1,16,16,17-$^{2}H_4$]testosterone (TS-$^{2}H_4$).

A split–splitless injection system was used with a total flow-rate of 30 mL/min and a column flow-rate of 1.9 mL/min and septum purge flow-rate of 6.0 mL/min and split ratio of 11.6. The purge activation time was 2 min. The initial column temperature was set to 50 °C. After the sample injection, the temperature was maintained for 2 min and was increased at 20 °C/min to 250 °C, then increased at 10 °C/min to 280 °C. The temperature of the injector was 260 °C. The mass spectrometer was operated in the electron-impact mode at an energy of 70 eV and the ion source temperature was set to 200 °C.

2.4. Preparation of standards

Stock solutions of AD (56.05 μg/mL), 11β-OHAD (39.9 μg/mL) TS (102.7 μg/mL), AD-$^{2}H_3$ (94.36 pg/μL), 11β-OHAD-$^{13}C_4$ (208.2 pg/μL), and TS-$^{2}H_4$ (571.8 pg/μL) were prepared in methanol. All analyses were performed by diluting the stock solution with methanol.

2.5. Sample preparation for GC–MS–SIM

2.5.1. Extraction

Three kinds of internal standards, i.e., 2.83 ng of AD-$^{2}H_3$ (30 μL), 6.28 ng of 11β-OHAD-$^{13}C_4$ (30 μL), and 14.3 ng of TS-$^{2}H_4$ (25 μL), dissolved in methanol were added to 1.0 mL of human plasma. The plasma sample was acidified with 0.1 M citric acid solution (3 mL) and purified with Sep-Pak C_{18} Plus short-body cartridge (Waters, Milford, MA, USA). The cartridge was washed with 8 mL of distilled water and then eluted with 4 mL of ethyl acetate into disposable culture tubes (13 mm × 100 mm). After evaporation to dryness at 60 °C under a stream of nitrogen, acetone (200 μL) was added to the residue and the sample was transferred into screw cap test tube (Maruemu, Osaka, Japan). The transfer procedure was repeated three times, and then evaporated to dryness at 60 °C under a stream of nitrogen.

2.5.2. Derivatization

Pentafluoropropionic anhydride (PFPA, 100 μL) was added to the residue dissolved in toluene (100 μL). The reaction mixture was vortexed for 1 min and then heated for 60 min at 80 °C. After evaporating the excess reagent under a stream of nitrogen at room temperature, the residue was dissolved with cyclohexane (20 μL). A 1.0-μL portion of the solution was subjected to GC–MS–SIM.

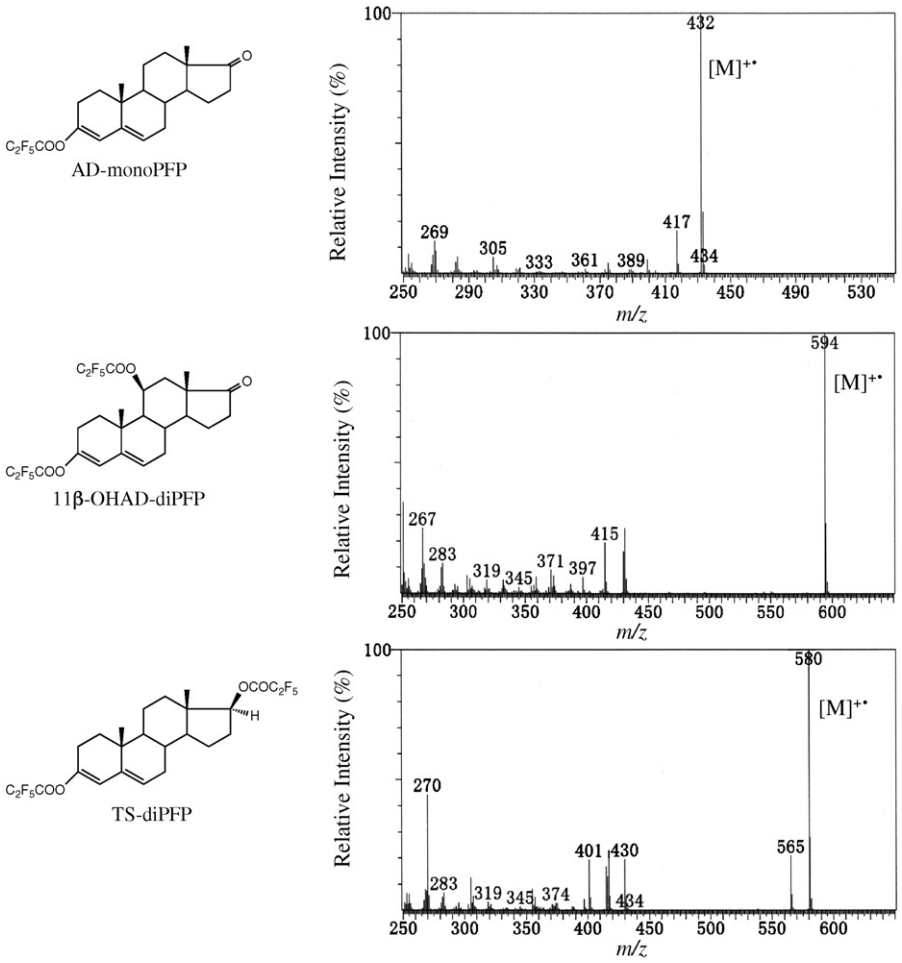

Fig. 2. Electron-impact mass spectra of pentafluoropropionic (PFP) derivatives of androstenedione (AD), 11β-hydroxyandrostenedione (11β-OHAD), and testosterone (TS).

2.6. Lower limit of detection (LLOD) and lower limit of quantification (LLOQ)

The LLOD was defined as the concentration with a signal-to-noise (S/N) ratio of 3 or greater. The LLOQ was defined as the lowest concentration of the analyte that could be determined with a signal-to-noise (S/N) ratio of 10 or greater.

2.7. Stability

Freeze–thaw stability and storage stability were carried out on three different concentrations, and measured at three times per concentration level ($n = 3$). The freeze–thaw cycle was repeated three times, frozen at −80 °C and thawed at room temperature. Storage stability samples were stored at 4 °C for 24 h (short-term storage stability) and for 1 week (long-term storage stability).

2.8. Recovery

The recoveries of AD, 11β-OHAD, and TS were determined at three different concentrations (2.83, 5.66, and 8.49 ng/mL for AD, 6.28, 12.55, and 18.83 ng/mL for 11β-OHAD, and 14.30, 28.59, and 42.89 ng/mL for TS), based upon the peak area ratios of the endogenous compounds *vs*. the corresponding internal standards before and after the extraction procedures.

2.9. Linearity

AD-$^{2}H_3$ (2.83 ng), 11β-OHAD-$^{13}C_4$ (6.28 ng), and TS-$^{2}H_4$ (14.3 ng) were added to each of five different levels for standards containing known amounts of AD (0.22, 0.56, 1.01, 1.79, and 2.80 ng), 11β-OHAD (0.56, 0.80, 1.20, 2.00, and 3.19 ng), and TS (2.05, 3.08, 5.14, 7.19, and 10.3 ng) dissolved in methanol. After evaporation of the solvent to dryness, the samples were derivatized as described above. A 1.0-μL portion of a cyclohexane solution (20 μL) was subjected to GC–MS. The peak-area ratios (at *m*/*z* 432 *vs.* at *m*/*z* 435 for AD, at *m*/*z* 594 *vs.* at *m*/*z* 598 for 11β-OHAD, and at *m*/*z* 580 *vs.* at *m*/*z* 584 for TS) were determined in triplicate. The calibration graphs were obtained by an unweighted least-squares linear fitting of the peak-area ratios *vs*. expected analyte concentrations of AD, 11β-OHAD, and TS on each analysis of the standard mixtures. The calibration graphs of AD, 11β-OHAD, and TS were performed on 20 sequential days. Mean and confidence interval of the slope, intercept, and correlation coefficient were calculated.

2.10. Accuracy and precision

The intra- and inter-day accuracy and precision were determined by assaying 15 preparations of 1.0 mL portions of human plasma. Five blank plasma samples were prepared, and 5 plasma samples were spiked with 0.561 ng of AD, 0.399 ng of 11β-OHAD, and 2.05 ng of TS. Additionally, another 5 plasma samples were spiked with 1.12 ng of AD, 0.798 ng of 11β-OHAD, and 4.11 ng

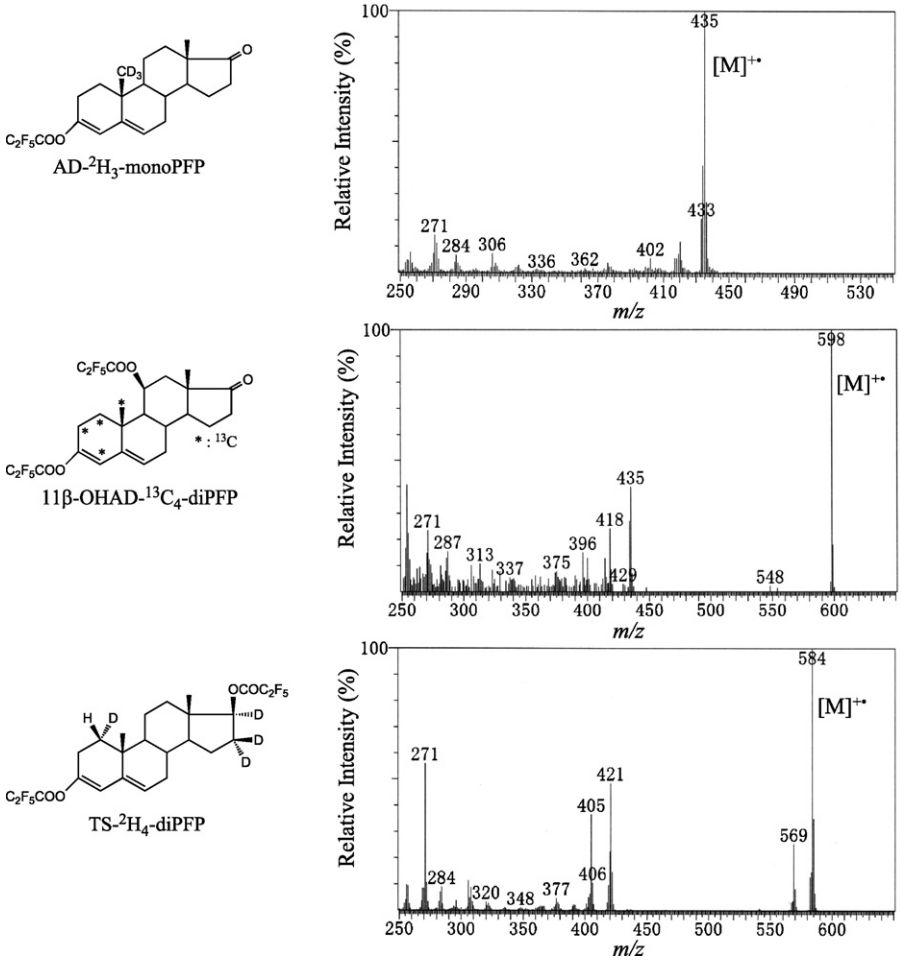

Fig. 3. Electron-impact mass spectra of pentafluoropropionic (PFP) derivatives of [19,19,19-$^{2}H_3$]androstenedione (AD-$^{2}H_3$), 11β-hydroxy-[1,2,4,19-$^{13}C_4$]androstenedione (11β-OHAD-$^{13}C_4$), and [1,16,16,17-$^{2}H_4$]testosterone (TS-$^{2}H_4$).

of TS. Three internal standard; i.e., 2.83 ng of AD-$^{2}H_3$, 6.28 ng of 11β-OHAD-$^{13}C_4$, and 14.3 ng of TS-$^{2}H_4$, were added to all plasma samples. After preparation of the sample for GC–MS–SIM as described above, the peak-area ratios were measured. The intra-day accuracy and precision of the assay were determined by preparing and measuring 5 replicate samples at three concentrations. The inter-day accuracy and precision were evaluated in 5 replicate runs (5 days). The relative standard deviation (R.S.D.) and the relative errors (R.E.) were used as a measure for intra- and inter-day accuracy and precision.

3. Results and discussion

The use of stable isotope-labelled internal standards for the GC–MS–SIM analysis offers the major advantage that they behave in almost identical manner to the analyte through all steps in the isolation and chromatographic procedures, thereby allowing procedural losses to be disregarded. Successful application of stable isotope dilution MS to the pharmacokinetic and metabolic investigations is dependent upon the availability of compounds labelled at predesigned positions that are chemically inert.

In this study, three stable isotope-labelled androgens, AD-$^{2}H_3$, TS-$^{2}H_4$, and 11β-OHAD-$^{13}C_4$ were used as the internal standards for the simultaneous determination of these endogenous steroids in human plasma by GC–MS–SIM (Fig. 1). $^{13}C_4$-Labelled 11β-hydroxyandrostenedione (11β-OHAD-$^{13}C_4$) was synthesized in a 71.5% yield by oxidative cleavage of the C-17 side-chain of [1,2,4,19-$^{13}C_4$]cortisol [19] with sodium bismuthate in 50% aqueous acetic acid. The isotopic purity of 11β-OHAD-$^{13}C_4$ as [$^{13}C_4$]-form was estimated to be >98 at.%, based on the ion intensities in the region of the molecular ion of 3,11-di-pentatafluoropropionic (diPFP) derivatives measured by GC–MS–SIM. Labelled androstenedione and testosterone (AD-$^{2}H_3$ and TS-$^{2}H_4$) with 3 or 4 non-exchangeable deuterium atoms were previously synthesized in our laboratory [17,18]. In our previous study, we developed a sensitive and reliable GC–MS method for the simultaneous determination of AD and TS in plasma using their stable isotope-labelled analogues as internal standards [17]. However, there has been no stable isotope-labelled 11β-OHAD available for an internal standard. This is the first exam-

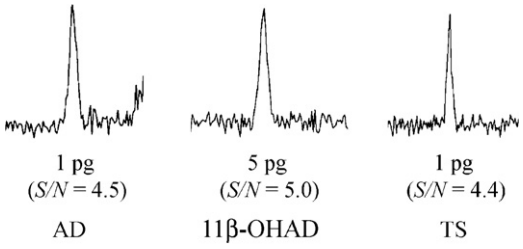

Fig. 4. Lower limit of detection (LLOD) for androstenedione (AD), 11β-hydroxyandrostenedione (11β-OHAD), and testosterone (TS). The LLOD was defined as the concentration (pg/injection) with a signal-to-noise (S/N) ratio of 3 or greater.

Table 1
Lower limits of quantification (LLOQ) of the method for androstenedione (AD), 11β-hydroxyandrostenedione (11β-OHAD), and testosterone (TS) ($n = 20$).

	LLOQ[a]		R.E.[b] (%)	R.S.D.[c] (%)	
	(pg/injection)	(ng/mL)			
AD	11	0.22	0.5	4.9	($n = 20$)
11β-OHAD	28	0.56	0.4	5.0	($n = 20$)
TS	103	2.05	−2.1	2.8	($n = 20$)

[a] Lower limit of quantification.
[b] Relative error.
[c] Relative standard deviation.

ple demonstrating stable isotope dilution analysis of 11β-OHAD in human plasma by GC–MS using [1,2,4,19-$^{13}C_4$]11β-OHAD.

A trimethylsilyl ether (TMS) derivatization has usually been employed for the GC–MS assay of AD and TS [20,21]. However, the presence of naturally occurring isotopes of Si in TMS derivative become a problem for overlap with the monitored ion of isotopically labelled and unlabelled molecule. In our previous study, AD and TS were derivatized with heptafluoropropionic anhydride (HFBA) in acetone for the simultaneous GC–MS measurement of AD and TS in plasma [17].

However, the use of acetone as a solvent for the HFB derivatization was shown to proceed with acid-catalyzed β-elimination of the hydroxyl group at C-11, forming the double bond at positions C-9 and C-11 or at C-11 and C-12 (m/z 430). The use of toluene instead of acetone as a solvent resulted in a 3,11-diheptafluoro-*n*-butyration (diHFB) derivative of 11β-OHAD (m/z 694). Furthermore, the employment of pentafluoropropionic anhydride (PFPA)/toluene instead of HFBA/toluene led to approximately fourfold increase in sensitivity. Therefore in this study, derivatization was performed with 100 μL of PFPA in toluene (100 μL) by heating of the samples at 80 °C for 60 min, providing a good GC behavior with high sensitivity.

Fig. 2 shows the electron-impact (EI) mass spectra of the PFP derivatives of unlabelled AD, 11β-OHAD, and TS. The PFP derivatives gave the molecular ions $[M]^{+\bullet}$ at m/z 432 for AD, at m/z 594 for 11β-OHAD, and at m/z 580 for TS. As shown in Fig. 3, the molecular ion of mono-PFP derivative of AD-$^2H_3$ (m/z 435) was three mass unit higher than the molecular ion (m/z 432) of unlabelled AD. In the mass spectra of the di-PFP derivatives of labelled 11β-OHAD-$^{13}C_4$ and TS-$^2H_4$, the molecular ions (m/z 598 and 584) were four mass units higher than those of unlabelled compounds (m/z 594 for 11β-OHAD and m/z 580 for TS). The molecular ions were chosen for the selected ion monitoring of the PFP derivatives.

When a signal-to-noise (S/N) ratio of 3 or greater was used as a criterion for a significant response, the lower limit of detection (LLOD) in present GC–MS–SIM method was found to be 1 pg per injection for AD (S/N = 4.5) and TS (S/N = 4.4), 5 pg per injection for 11β-OHAD (S/N = 5.0) (Fig. 4). The lower limit of quantification (LLOQ) was found to be 11 pg per injection for AD, 28 pg per injection for 11β-OHAD, and 103 pg per injection for TS (Table 1). The RE and RSD of LLOQ were less than 5.0%.

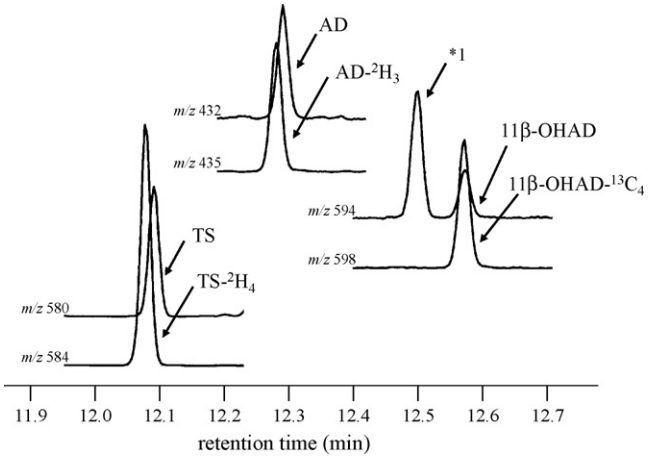

Fig. 5. Selected-ion recordings of pentafluoropropionic (PFP) derivatives of AD/AD-$^2H_3$ (m/z 432 and 435), 11β-OHAD/11β-OHAD-$^{13}C_4$ (m/z 594 and 598), and TS/TS-$^2H_4$ (m/z 580 and 584) after processing from plasma spiked with 2.83 ng of AD-$^2H_3$, 6.28 ng of 11β-OHAD-$^{13}C_4$ and 14.3 ng of TS-$^2H_4$. *1: an unknown endogenous substance, AD: androstenedione, 11β-OHAD: 11β-hydroxyandrostenedione, TS: testosterone.

The stability results are summarized in Table 2. The samples stored at −80 °C for 24 h were thawed at room temperature for freeze–thaw stability test. The freeze–thaw cycle was repeated three times. Storage stability was tested stored at 4 °C for 24 h (short-term storage stability) and for 1 week (long-term storage stability). The mean percentages of concentration to spiked value (%) were 76–96% (freeze–thaw stability), 82–115% (short-term storage stability), and 43–77% (long-term storage stability). The result indicated that these samples were stable at three freeze–thaw cycles and at a short-term storage at 4 °C for 24 h. However, the samples were not stable at 4 °C for 1 week. The GC–MS measurement should be performed immediately.

Therefore, the current method demonstrated the satisfactory sensitivity and stability for analyses of AD, 11β-OHAD and TS. The PFP derivatives of AD, 11β-OHAD and TS were chromatographically well separated from other endogenous steroids such as dehydroepiandrosterone, androstenediol, 11β-hydroxyandrosterone, 11β-hydroxyetiocholanolone, cortisol, cortisone, estradiol, estrone, etc. Fig. 5 shows the selected ion-recordings of the PFP derivatives of unlabelled and labelled AD, 11β-OHAD, and TS after processing from spiked plasma. The efficiencies for recovering AD, 11β-OHAD, and TS from plasma using Sep-Pak C_{18} cartridge were 89% ($n = 3$) for AD, 95% ($n = 3$) for 11β-OHAD and 99% ($n = 3$) for TS, respectively.

Calibration graphs were linear from 0.22 to 2.80 ng/mL for AD, from 0.56 to 3.19 ng/mL for 11β-OHAD, and from 2.05 to 10.3 ng/mL for TS. The mixture was analyzed as the PFP derivatives of AD, 11β-

Table 2
Stability of androstenedione (AD), 11β-hydroxyandrostenedione (11β-OHAD), and testosterone (TS) under various condition in three different concentrations ($n = 3$).

Storage period and storage condition	Spiked concentration (pg/injection)			Percentage of concentration to spiked value (%)			
	AD	11β-OHAD	TS	AD	11β-OHAD	TS	
Three freeze/thaw cycles (−80 °C to room temperature)	11	28	103	76	93	82	($n = 3$)
	50	60	257	83	96	87	($n = 3$)
	140	160	514	79	91	82	($n = 3$)
Short-term storage stability (4 °C, 24 h)	11	28	103	115	94	99	($n = 3$)
	50	60	257	102	84	82	($n = 3$)
	140	160	514	99	85	84	($n = 3$)
Long-term storage stability (4 °C, 1 week)	11	28	103	57	69	77	($n = 3$)
	50	60	257	43	56	61	($n = 3$)
	140	160	514	50	72	73	($n = 3$)

Table 3
Linearity data of androstenedione (AD), 11β-hydroxyandrostenedione (11β-OHAD), and testosterone (TS). The calibration graphs were made on 20 different days. Mean and confidence interval of the slope, intercept, and correlation coefficient were calculated.

Compound	Regression equation	Correlation coefficient	Concentration range (ng/mL)	Standard deviation		
				Slope	Intercept	Correlation coefficient
AD	$Y = 0.3726X + 0.0002$	0.9998	0.22–2.80	0.0116	0.0076	0.00043
11β-OHAD	$Y = 0.1641X + 0.0073$	0.9996	0.56–3.19	0.0100	0.0080	0.00038
TS	$Y = 0.0784X - 0.0058$	0.9996	2.05–10.3	0.0032	0.0071	0.00058

Table 4
Intra- and inter-day accuracy (R.E.) and precision (R.S.D.) of GC–MS determination of androstenedione (AD), 11β-hydroxyandrostenedione (11β-OHAD), and testosterone (TS) in human plasma ($n = 5$).

Added (ng/mL)	Expected (ng/mL)	Concentration[a] (ng/mL) (mean ± S.D.)	R.E.[b] (%)	R.S.D.[c] (%)
Intra-day ($n = 5$)				
AD				
0		0.67 ± 0.01		1.7
0.56	1.23	1.20 ± 0.03	−2.1	2.4
1.12	1.79	1.83 ± 0.04	2.4	2.3
11β-OHAD				
0		1.32 ± 0.02		1.8
0.40	1.71	1.71 ± 0.05	0.0	3.2
0.80	2.11	2.09 ± 0.03	−1.0	1.5
TS				
0		3.40 ± 0.10		2.9
2.05	5.46	5.29 ± 0.06	−3.1	1.2
4.11	7.51	7.28 ± 0.29	−3.1	4.0
Inter-day ($n = 5$)				
AD				
0		0.70 ± 0.03		4.4
0.56	1.26	1.26 ± 0.06	0.5	4.9
1.12	1.82	1.82 ± 0.08	−0.1	4.2
11β-OHAD				
0		2.45 ± 0.12		4.7
0.40	2.85	2.80 ± 0.15	−2.0	5.3
0.80	3.25	3.20 ± 0.12	−1.5	3.6
TS				
0		3.32 ± 0.10		3.1
2.05	5.38	5.22 ± 0.17	−2.8	3.2
4.11	7.43	7.20 ± 0.16	−3.1	2.2

[a] Each individual value is the mean of triplicate measurements.
[b] Relative error.
[c] Relative standard deviation.

OHAD and TS by monitoring $[M]^{+\bullet}$ ion intensities at m/z 432 (AD), m/z 435 (AD-$^{2}H_3$), m/z 594 (11β-OHAD), m/z 598 (11β-OHAD-$^{13}C_4$), m/z 580 (TS) and m/z 584 (TS-$^{2}H_4$). The calibration graphs of AD, 11β-OHAD, and TS were made on 20 different days. Mean and confidence interval of the calibration graphs were shown in Table 3. The calibration graphs of the method were linear up of AD, 11β-OHAD, and TS with the high correlation coefficients between the observed peak-area ratio (Y) and the expected analyte concentrations (X).

The intra- and inter-day reproducibility in which the amounts of AD, 11β-OHAD and TS determined were in good agreement with the actual amounts added. The precisions and accuracy results are summarized in Table 4. The intra- and inter-day precisions were less than 5.3%, accuracy in the relative errors was −3.1 to 2.4%.

4. Conclusions

The present method provides a sensitive and reliable technique for the simultaneous determination of AD, 11β-OHAD, and TS in plasma by using stable isotope-labelled internal standards. The method can be applied to pharmacokinetic and metabolic studies of androgens with a particular interest in evaluating the conversion of AD to 11β-OHAD and the interconversion of AD and TS in humans.

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