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Simultaneous determination of testosterone and androstadienone (sex attractant) in human plasma by gas chromatography–mass spectrometry with high-resolution selected-ion monitoring

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ABSTRACT

Androsta-4,16-dien-3-one (androstadienone) and androst-4-en-3-one-17β-ol (testosterone) in healthy human plasma were simultaneously determined under several experimental conditions by gas chromatography–mass spectrometry with high-resolution selected-ion monitoring. Internal standards were [2,2,4,6,6-$^2H_5$]androstadienone and [2,2,4,6,6-$^2H_5$]testosterone. Samples were extracted with an Extrelut® column, purified using Lipidex® 5000 and converted into hydroxime-trimethylsilyl derivatives for determination. Physiological concentrations of androstadienone and testosterone found in eleven healthy men were 2.05 ± 0.74 and 18.6 ± 4.9 pmol/ml in plasma (mean ± S.D.), respectively. No correlation was observed between these steroid concentrations.

INTRODUCTION

Musk-smelling androsta-4,16-dien-3-one (androstadienone) is a 16-unsaturated C_{19} steroid thought to function as a sex attractant in male mammals [1]. Such a pheromone-like smelling steroid has been identified in urine and tissues such as testis and adrenal gland of mammals. However, the physiological levels, distribution and physiological roles of the steroid and the relationship with androgens in human organs have remained obscure. In testicular microsomes, androstadienone is produced from C_{21} steroids such as pregnenolone and progesterone [1–5]. On the other hand, Stylianou and co-workers reported the *in vitro* formation of androstadienone from testosterone in human liver [6] and rat testis [7]. To elucidate the *in vivo* conversion of testosterone into androstadienone, the concentrations of these steroids need to be determined in plasma, urine and tissues. The presence of small amounts of androstadienone in human plasma was initially demonstrated by Brooksbank *et al.* [8] using gas chromatography. For further

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studies on androstadienone, the development of a more sensitive and reproducible method was required.

Gas chromatography–mass spectrometry (GC–MS) is being increasingly applied as a very sensitive and highly specific method for determining many biomedical substances, including steroids [9–13]. This paper describes a method using GC–MS with high-resolution selected-ion monitoring (HR-SIM) for the simultaneous determination of physiological levels of androstadienone and testosterone.

EXPERIMENTAL

Chemicals and reagents

Unlabelled androst-5-en-17-one-3β-ol (dehydroepiandrosterone, DHA) and testosterone were obtained from Sigma (St. Louis, MO, U.S.A.). Deuterated methanol (>99.5 atom-%, CH_3O^2H), 20% deuterium chloride (>99 atom-%, ^2HCl, 6 *M* in ^2H_2O) and sodium deuteroxide (>99.5 atom-%, NaO^2H) were purchased from Aldrich (Milwaukee, WI, U.S.A.). Reagents for derivatization were obtained from Wako (Osaka, Japan). Extrelut® columns and Kieselgel 60 F_{254} plates for thin-layer chromatography (TLC) were purchased from Merck (Rahway, NJ, U.S.A.), and Lipidex® 5000 from Packard-Becker (Groningen, The Netherlands). All other reagents and solvents were of analytical-reagent grade from Nacalai Tesque (Kyoto, Japan) and were used without further purification.

Preparation of standard steroids and deuterated isomers

Unlabelled testosterone was recrystallized from acetone. Androstadienone was synthesized from androsta-5,16-dien-3β-ol (andien-β) by Oppenauer oxidation. The precursor andien-β was produced from DHA according to the method of Barton *et al.* [14]. Briefly, DHA acetate was treated with hydrazine using triethylamine as the catalyst. The hydrazone derivative of DHA was then oxidized by iodine in triethylamine–tetrahydrofuran, resulting in the formation of the vinyl iodide derivative. Subsequent reduction of the derivative with sodium methylate afforded andien-β, which was oxidized to androstadienone by aluminium isopropoxide in cyclohexanone solvent. This crude product was purified by filtration through an alumina column with benzene–ethyl acetate (5:1, v/v) and by TLC [15] on a Kieselgel 60 F_{254} plate with benzene–ethyl acetate (1:1, v/v) as solvent. The gel zone with R_F = 0.52 was removed and androstadienone was eluted with benzene. A high-purity product was obtained after recrystallization twice from absolute ethanol. The melting point of the product was in the range 133–135°C.

Two deuterium-labelled internal standards, [2,2,4,6,6-$^2H_5$]androstadienone (androstadienone-d_5) and [2,2,4,6,6-$^2H_5$]testosterone (testosterone-d_5), were pre-

pared as follows [12]: after dissolution of the steroids in [^{2}H]methanol, the mixture was acidified with ^{2}HCl and incubated in a heating block at 65°C for 36 h. Following neutralization with NaO^{2}H, the solvent was evaporated and the residue dissolved in dichloromethane. The mixture was immediately washed with distilled water and evaporated. The internal standards thus prepared were then purified by TLC and by recrystallization as described above. The $^2H_5$ isotopic purity of each labelled compound as checked by GC–MS was >99.8%.

GC–MS-HR-SIM

GC–MS was carried out on a JMS-D300 double-focusing mass spectrometer with a JMA-2000S data system (JEOL) connected to an HP 5710A gas chromatograph (Hewlett-Packard) with a fused-silica large-bore capillary column (DB-1) (15 m × 0.542 mm I.D., 0.1 μm film thickness) (J&W, Rancho Cordova, CA, U.S.A.) and a splitless injector. The GC–MS conditions were as follows: carrier gas, helium at 1.0 kg/cm^2; oven temperature, programmed from 220 to 280°C at 8°C/min; mode electron-impact ionization (EI) or positive- and negative-ion chemical ionization (PCI and NCI); ion source temperature, 230°C for EI or 180°C for CI; electron energy, 24 eV for EI or 180 eV for CI; trap current, 300 μA; scanning mass range, *m/z* 50–800; CI reagent gas, isobutane at $1.5 \cdot 10^{-5}$ mmHg. HR-SIM was effected in the EI mode at a switching rate of 0.1 s. The MS resolving power was 5000 and the apparatus was focused on molecular ions of the derivatives of androstadienone (*m/z* 357.249), androstadienone-d_5 (362.280), testosterone (447.299) and testosterone-d_5 (452.330). Steroids were identified by HR-SIM and were determined from peak-area ratios with respect to the corresponding deuterium-labelled compounds.

Sample preparation for GC–MS-HR-SIM

Plasma samples extracted from eleven healthy male volunteers (age range 21–31 years; mean 22 years) were stored at −20°C until used. Following the addition of 100 μl of 1 nmol/ml deuterated steroid solution in methanol to 1.0 ml of plasma and 2.0 ml of phosphate buffer (pH 8.5), the steroids were extracted with ethyl acetate using an Extrelut column (20 cm × 1.0 cm I.D.) [12]. After evaporation, 3-keto steroid extracts were treated with hydroxylamine hydrochloride to give 3-hydroxime-steroids. The derivatives were then purified by filtration through a Lipidex 5000 column with methanol–water–chloroform–acetic acid (95:5:2:0.5, v/v) [9,12]. Following evaporation, these products were treated with pyridine–hexamethyldisilazane–trimethylchlorosilane (100:50:10, v/v) at room temperature for 1 h. The thus prepared hydroxime (HO)-trimethylsilyl (TMS) derivatives were dried with nitrogen and dissolved in ethyl acetate for GC–MS-HR-SIM analysis.

TABLE I

GC–MS PROPERTIES OF ANDROSTADIENONE, TESTOSTERONE AND THEIR DERIVATIVES

Derivative		Mode	Retention time (min)	Molecular mass	Significant ions, *m/z* value [relative intensity (%) in parentheses or brackets]
Androstadienone					
Free		EI	2.00	270	270(M^+,3.2),255(3.6),228(2.5),147(5.7),146(6.0),124(3.3)
3-MO		EI	2.17	299	299(M^+,10.2),284(2.0),268(4.0),153(5.0),137(4.6),125(5.4)
3-HO-TMS		EI	2.00	362	362(M^+,10.0),347(6.4),273(1.4),216(4.2)
3-enol-HFB[a]	(30%)	EI	1.04	466	466(M^+,8.1),451(1.5),317(1.3),147(3.6),107(5.4),94(12.7)
	(70%)		1.53	466	466(M^+,11.3),451(3.6),369(1.4),146(13.1),119(3.2),93(4.0)
3-PFBO[a]	(70%)	EI	5.07	465	465(M^+,11.7),450(2.0),319(2.4),303(2.6),291(3.0),268(7.3)
	(30%)		5.30	465	465(M^+,11.2),450(2.3),319(2.6),303(3.5),291(3.5),268(8.0)
Free		PCI	2.00	270	271[$(M+H)^+$,65.0]
3-MO		PCI	2.17	299	300[$(M+H)^+$,50.4],356[$(M+C_4H_9)^+$,3.5],268(9.8)
3-enol-HFB[a]	(30%)	NCI	1.40	466	197[$(C_3F_7CO)^-$,93.7]
	(70%)		1.53	466	197[$(C_3F_7CO)^-$,94.1]
3-PFBO[a]	(60%)	NCI	5.07	465	445[$(M-HF)^-$,15.0],415(4.5),197(8.6),181(32.6)
	(40%)		5.30	465	445[$(M-HF)^-$,13.4],415(5.5),197(7.1),181(25.9)
Testosterone					
Free		EI	3.83	288	288(M^+,3.9),246(5.0),288(1.5),203(2.4),147(3.7),124(8.1)
17-TMS		EI	3.87	360	360(M^+,4.4),345(1.7),304(1.5),270(4.7),147(3.6),129(10.1)
3-MO-17-TMS		EI	4.07	389	389(M^+,10.6),358(3.0),268(3.8),153(3.4),125(4.8)
3-HO-TMS-17-TMS		EI	3.95	452	452(M^+,12.0),437(5.0),363(1.4),216(5.5)
3-enol-17-bis-HFB[a]	(30%)	EI	2.40	680	680(M^+,4.7),451(2.1),318(6.2),147(5.2),104(5.8)
	(70%)		2.60	680	680(M^+,11.3),665(2.4),465(2.2),320(5.4),133(4.7),119(4.2)
3-PFBO-17-TMS[a]	(70%)	EI	7.50	555	555(M^+,9.2),358(4.5),268(4.7),129(4.7)
	(30%)		7.77	555	555(M^+,10.8),358(4.9),268(5.6),129(5.1)
17-TMS		PCI	3.87	360	361[$(M+H)^+$,56.2],271(5.6)
3-MO-17-TMS		PCI	4.07	389	390[$(M+H)^+$,42.2],447[$(M+C_4H_9)^+$,4.1]
3-enol-17-bis-HFB[a]	(30%)	NCI	2.40	680	197[$(C_3F_7CO)^-$,91.8]
	(70%)		2.60	680	197[$(C_3F_7CO)^-$,87.6]
3-PFBO-17-TMS[a]	(70%)	NCI	7.50	555	535[$(M-HF)^-$,13.0],505(4.6),377(1.9),285(3.1),197(4.9),181(39.2)
	(30%)		7.77	555	535[$(M-HF)^-$,10.9],505(5.8),377(1.3),285(6.6),197(4.0),181(28.5)

[a] Geometric isomers of the *syn* and *anti* type were formed; their proportions (%) are given in that order in parentheses.

RESULTS

Mass spectra of androstadienone and testosterone derivatives

When testosterone is injected into the GC–MS system without derivatization for rapid determination, the hydroxyl group at C-17 might adhere to the column so that less than 100 pg of the steroid would be undetectable even when a large-bore column is utilized. Therefore, several reagents for the derivatization of the steroid were investigated (Table I).

When TMS or methoxyloxime (MO)-TMS derivatives of androstadienone and testosterone were determined in the EI mode, the relative intensities of molecular ions were too low (<6%). In contrast, when these derivatives were analysed in the PCI mode, the quasi-molecular ion intensities were 40–60%. When heptafluorobutyl (HFB) ester or pentafluorobenzyl oxime (PFBO) derivatives were analysed in the NCI mode, ions from the derivatization reagents appeared intensively, interfering with the detection of steroid ions. Moreover, 3-keto steroid formed geometric isomers of the *syn* and *anti* type with these derivatization reagents [15], resulting in complex chromatograms. The saturation phenomenon

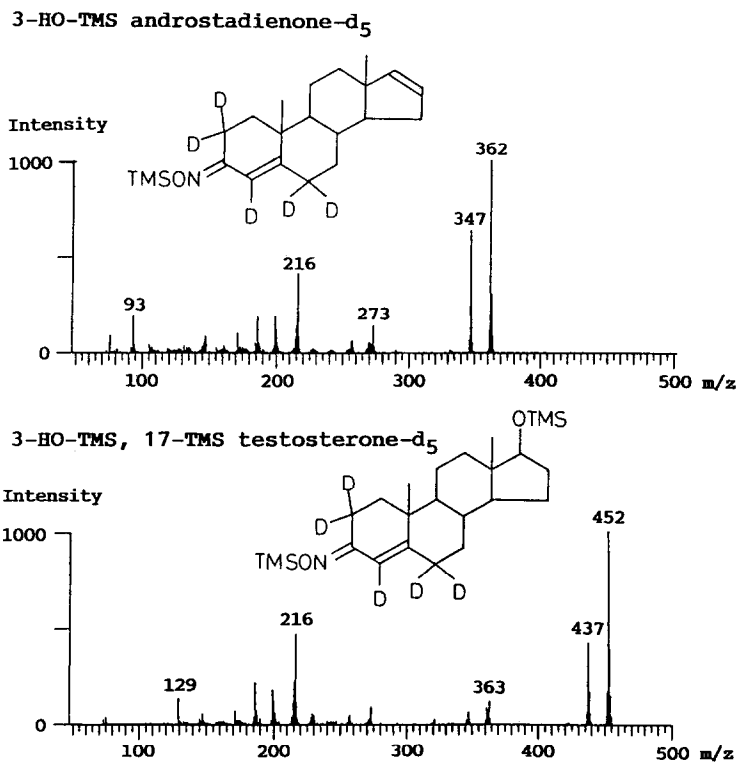

Fig. 1. EI mass spectra of 3-HO-TMS androstadienone-d_5 (top) and 3-HO-TMS-17-TMS testosterone-d_5 (bottom).

of negative ions [16] was not observed in this study. However, the absolute ion intensity in the PCI or NCI mode varied enormously with changes in ion source temperature. Although this variation could be corrected with the use of appropriate internal standards, the determination should have been performed in the more stable and equally sensitive EI mode.

Consequently, these steroids were determined as HO-TMS derivatives (Figs. 1 and 2) by GC–MS-HR-SIM in the EI mode.

Identification of plasma androstadienone

Following extraction from 200 ml of human plasma with the Extrelut column and subsequent purification using TLC, the plasma extract was analysed by GC–MS without derivatization. The major ions of *m/z* 270 (molecular ion), 255, 228 and 146 found in the mass spectrum and the GC retention time of this plasma extract were the same as those for the authentic androstadienone. This extract was therefore considered to be identical with androstadienone.

Stability of deuterium-labelled internal standards

The effect of pH on the stability of the C–^{2}H bond during analytical procedures was investigated as follows: 100 pmol of androstadienone-d_5 were added to

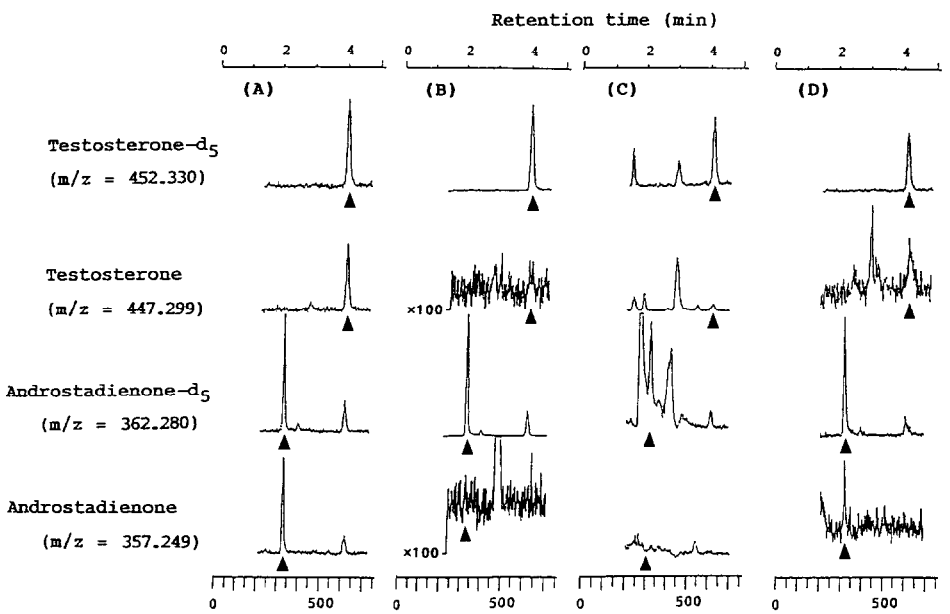

Fig. 2. HR-SIM chromatograms of 3-HO-TMS-17-TMS testosterone, 3-HO-TMS-androstadienone and internal standards (I.S.). (A) Aliquot containing 100 pmol of the standard steroids and I.S.; (B) aliquot including I.S.; (C) and (D) I.S.-added human plasma samples. The MS resolving power was 5000 in (A), (B) and (D) and 1000 in (C).

human plasma which had been adjusted to pH 5.0, 7.0 or 8.5 by the addition of an equal volume of phosphate buffer. The steroids were then extracted from the plasma sample 5, 10, 30 and 60 min later, and determined as described above. As shown in Fig. 3, androstadienone-d_5 in the weakly alkaline plasma was sufficiently stable, whereas deuterium–hydrogen exchange had evidently occurred in the acidified sample (pH 5.0).

Effects of elution solvent on the Extrelut column extraction

Ethyl acetate was the most suitable of several organic solvents tested for the extraction of androstadienone and testosterone using an Extrelut column (Table II). Although diethyl ether gave the highest recoveries, unknown compounds were simultaneously extracted from the Extrelut that interfered in the determination.

As the pH of the sample did not affect the elution with ethyl acetate (Table II), plasma samples were adjusted to pH 8.5 with phosphate buffer to stabilize the deuterated internal standards.

Effect of Lipidex 5000 reversed-phase column

To eliminate large amounts of lipids and non-polar phospholipids from samples, Extrelut extracts were filtered through a Lipidex 5000 column. Modifying the method of Axelson and Sjövall [9], methanol–water–chloroform–acetic acid (95:5:2:0.5, v/v) was used as the elution solvent; a high recovery of testosterone was achieved by the method of Axelson and Sjövall [methanol–water–chloroform (9:1:2, v/v)], whereas less polar androstadienone co-eluted with non-polar impurities. To increase the polarity of androstadienone, the ketone group at C-3 was

TABLE II

EFFECT OF ELUTION SOLVENT ON THE RECOVERIES OF THE STEROIDS FROM THE EXTRELUT COLUMN

Solvent	Recovery (%) (mean ± S.D., n = 6)[a]	
	Androstadienone	Testosterone
Benzene	65.3 ± 3.0 (4.6)	87.5 ± 1.1 (1.3)
Ethyl acetate (pH 5.0)	78.3 ± 2.2 (2.8)	91.2 ± 1.7 (1.9)
Ethyl acetate (pH 7.0)	79.8 ± 4.7 (5.9)	91.6 ± 2.5 (2.7)
Ethyl acetate (pH 8.5)	78.5 ± 1.8 (2.3)	93.1 ± 2.7 (2.9)
Hexane	36.2 ± 6.2 (17.1)	37.6 ± 9.8 (26.1)
Trichloroethylene	60.4 ± 7.2 (11.9)	83.9 ± 2.1 (2.5)
Dichloromethane	61.2 ± 1.8 (2.9)	86.6 ± 1.1 (1.3)
Diethyl ether	77.5 ± 12.4 (16.0)	72.3 ± 9.4 (13.0)

[a] R.S.D.s (%) in parentheses.

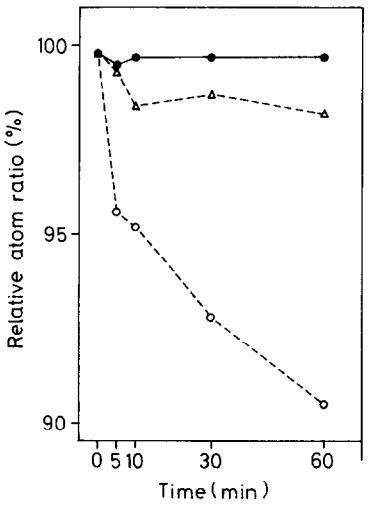

Fig. 3. Effect of plasma pH on the stability of deuterium-labelled internal standards. (●) pH 8.5; (△) pH 7.0; (○) pH 5.0.

derivatized to a hydroxime group before the filtration. Further, the proportion of water in the elution solvent was decreased. As a result of these modifications, the steroids were effectively recovered whereas plasma lipids and cholesterol were retained in the column.

Accuracy and precision of steroid determinations

The simultaneous determination of TMS derivatives of plasma androstadienone and testosterone by HR-SIM with a resolving power of 5000 gave clear chromatograms without interference from other compounds (Fig. 2). When the resolving power was 1000, plasma steroids could not be detected.

Calibration graphs for the determination of androstadienone and testosterone were obtained by HR-SIM analysis of plasma samples, from which steroids had first been removed with diethyl ether, and to which unlabelled steroids and constant amounts of internal standards had subsequently been added. Calibration graphs for the determination of androstadienone and testosterone were sufficiently linear for steroid concentrations in the range 0.1–100 pmol per tube. The correlation coefficients were 0.99696 and 0.97973, respectively.

The accuracy and precision of the determination were estimated from the relative standard deviations when plasma samples containing appropriate amounts of androstadienone and testosterone were analysed (Table III). The simultaneous determination of these steroids was sufficiently accurate. The limits of determination were 0.05 and 0.10 ng per tube, respectively.

The levels of androstadienone and testosterone in eleven human plasma sam-

TABLE III

ACCURACY AND PRECISION OF STEROID DETERMINATION BY GC–MS-HR-SIM

Amount added (ng)	*n*	Value determined (mean ± S.D.) (ng)	R.S.D. (%)
Androstadienone			
13.50	5	11.48 ± 0.757	6.6
2.70	6	2.756 ± 0.369	13.4
Testosterone			
14.40	6	15.07 ± 1.149	7.6
2.88	5	2.939 ± 0.494	16.8

ples were 2.05 ± 0.74 and 18.6 ± 4.9 pmol/ml (mean ± S.D.), respectively. The correlation coefficient between these concentrations was 0.197748.

DISCUSSION

The pheromone-like function of such 16-unsaturated C_{19} steroids such as androstadienone in humans and animals is interesting. They are produced in male tissues and may act in female mammals. However, the physiological levels and roles of the steroids in human organs have remained obscure. The presence of the steroids in human urine has been known for a long time [1,17]. In contrast, detection in human plasma has seldom been reported because of the low concentrations [8]. As one of a few examples, the presence of 5α-androst-16-en-3α-ol, a metabolite of adrostadienone, was detectable in blood from a woman with a virilizing adrenal carcinoma [18], which may have increased the production of 16-unsaturated C_{19} steroids. The analytical techniques for androstadienone will be also applied to micro-determinations of the other 16-unsaturated C_{19} steroids.

Androstadienone is thought to be produced in the adrenal gland or testis from pregnenolone or progesterone [1,2]. Pregnenolone is first converted into andien-β by andien-β synthetase [4] and subsequently into androstadienone. On the other hand, androstadienone synthetase catalyses the reaction from progesterone to androstadienone [5]. These synthesizing enzymes are located in microsomes and their characteristics have been investigated. As the third pathway, biosynthesis of androstadienone from testosterone was observed *in vitro* in human liver and rat testicular tissues [6,7]. To elucidate *in vivo* synthesis via this possible pathway, human plasma androstadienone and testosterone were simultaneously determined in this study. The mean concentration of androstadienone in eleven human plasma samples was 2.05 pmol/ml, which was similar to that reported by Brooksbank *et al.* [8]. However, no correlation was observed between these steroid levels,

suggesting that androstadienone may be dominantly produced from C_{21} steroids. Further studies using other human tissues should be performed with the sensitive and reproducible analysis for androstadienone using GC–MS-HR-SIM as described here.

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