Journal of Chromatography, 525 (1990) 15–23
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5036

SIMULTANEOUS MEASUREMENTS OF ENDOGENOUS AND DEUTERIUM-LABELLED TRACER VARIANTS OF ANDROSTENEDIONE AND TESTOSTERONE BY CAPILLARY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

TAKASHI FURUTA, KOHTARO KUSANO and YASUJI KASUYA*

Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03 (Japan)

(First received July 18th, 1989; revised manuscript received September 19th, 1989)

SUMMARY

A capillary gas chromatographic–mass spectrometric method for the simultaneous determination of androstenedione and testosterone in human plasma using [19,19,19-$^2H_3$]androstenedione and [19,19,19-$^2H_3$]testosterone as internal standards is described. For calculation of plasma androstenedione and testosterone, peak heights were measured by selected-ion monitoring of the molecular ions of the heptafluorobutyryl derivatives of androstenedione and [$^2H_3$]androstenedione (m/z 482 and 485) and of testosterone and [$^2H_3$]testosterone (m/z 680 and 683). The isotope dilution method needed no complex corrections for contributions and provides a sensitive and reliable technique with good accuracy, precision and reproducibility.

INTRODUCTION

The metabolism of androstenedione to testosterone in vivo can be investigated by treating human subjects with stable isotopically labelled androstenedione. In our attempt to investigate 17-ketosteroid reductase deficiency in the metabolic conversion of androstenedione into testosterone in male pseudohermaphroditism, we have developed a gas chromatographic–mass spectrometric (GC–MS) isotope dilution method for the simultaneous measurement of endogenous and deuterium-labelled tracer variants of androstenedione and testosterone.

For detecting low concentrations of endogenous androstenedione and testosterone and their deuterium-labelled variants separately but simultaneously

0378-4347/90/$03.50

by GC–MS with selected-ion monitoring (SIM), the choice of suitable derivatives and appropriately labelled molecules of the steroids was of primary importance. This paper describes analytical procedures that permit sensitive and reliable measurement of deuterium-labelled and unlabelled androstenedione and testosterone in human plasma by GC–MS–SIM.

EXPERIMENTAL

Chemicals and reagents

[19,19,19-$^2H_3$]Testosterone (testosterone-19-d_3) was synthesized in our laboratory as described previously [1]. [19,19,19-$^2H_3$]Androstenedione (androstenedione-19-d_3) was prepared by Jones oxidation of testosterone-19-d_3. The atomic composition was 99.0 atom% deuterium for both compounds. Unlabelled androstenedione and testosterone (reagent grade) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Heptafluorobutyric anhydride (HFBA) (reagent grade) was purchased from Gasukuro Kogyo (Tokyo, Japan). [4-^{14}C]Testosterone (2.11 GBq/mmol) and [4-^{14}C]androstenedione (2.18 GBq/mmol) were products of Amersham International (Buckinghamshire, U.K.). All other chemicals and reagents were of analytical-reagent grade and were used without purification.

GC–MS–SIM

GC–MS–SIM measurements were made with a Shimadzu QP2000 GC–MS system equipped with a data-processing system. GC was performed on an SPB-1 fused-silica capillary column (20 m × 0.5 mm I.D.) with a 0.25-μm thin film (Supelco, Bellefonte, PA, U.S.A.). Helium was used as the carrier gas at a column head-pressure of 0.5 kg/cm^2. The splitless injector was used with a septum purge flow-rate of 10 ml/min and a split flow-rate of 60 ml/min. The purge activation time was 2 min. The initial column temperature was set at 100°C, and 2 min after the sample injection increased at 30°C/min to 240°C; this temperature was maintained for 1 min. Thereafter the column temperature was increased at 8°C/min to 280°C. The temperature of the injector was 260°C. The electron energy was set at 70 eV and the ion source temperature was 280°C. The multiple-ion detector was focused on the molecular ions (M^+) at m/z 482 for the monoheptafluorobutyryl (mono-HFB) derivative of unlabelled androstenedione, m/z 485 for the mono-HFB derivative of androstenedione-19-d_3, m/z 680 for the di-HFB derivative of unlabelled testosterone and m/z 683 for the di-HFB derivative of testosterone-19-d_3.

Sample preparation for GC–MS–SIM

To 1.0 ml of plasma were added 19.536 ng of androstenedione-19-d_3 and 20.000 ng of testosterone-19-d_3, each dissolved in ethanol, and 3 ml of 0.1 *M* citric acid solution to acidify the plasma sample (pH ca. 3). The plasma sample

was applied to a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.). The cartridge was washed twice with 5 ml of distilled water and then eluted with 2 ml of diethyl ether. The eluate was evaporated under a stream of nitrogen, the residue was dissolved in 100 μl of ethanol and the sample was applied to a Kieselgel 60 F_{254} thin-layer chromatography (TLC) plate (Merck, Darmstadt, F.R.G.). The TLC plate was developed with chloroform–diethyl ether (9:1). The zones corresponding to testosterone (R_F 0.15) and androstenedione (R_F 0.30) were scraped off and eluted twice with 2 ml of acetone. After evaporation under a stream of nitrogen, 50 μl of HFBA and 200 μl of acetone were added to the residue. The reaction mixture was then vortex-mixed for 10 s and left at room temperature for 1 h. The excess of reagent was removed under a stream of nitrogen, and the residue was dissolved in 10 μl of dichloromethane. A 0.5-μl portion of the solution was subjected to GC–MS.

Absolute recovery

To 1.0 ml of plasma were added 74 Bq of [4-^{14}C]testosterone or 37 Bq of [4-^{14}C]androstenedione and 3 ml of 0.1 *M* citric acid buffer solution. The plasma was applied to a Sep-Pak C_{18} cartridge followed by TLC as described above.

Preparation of calibration curve

To each of five standards containing a known nanogram ratio of androstenedione to testosterone (0.0995:0.101, 0.398:0.404, 1.998:2.020, 4.995:5.050 and 19.980:20.200) dissolved in ethanol, 19.536 ng of androstenedione-19-d_3 and 20.000 ng of testosterone-19-d_3, each dissolved in 10 μl of ethanol, were added. After evaporation of the solvent, the sample was derivatized as described above. A 0.5-μl portion of a dichloromethane solution (10 μl) was analysed by GC–MS and the peak-height ratios (m/z 482/485 and m/z 680/683) were measured.

Determination of accuracy

Androstenedione and testosterone in nanogram amounts of 0.498:4.040 and 1.998:8.080, dissolved in 10 μl of ethanol, were added to 1.0-ml aliquots of pooled male plasma. The androstenedione and testosterone contents (mean $\pm$ S.D.) of the pooled plasma were 2.475 $\pm$ 0.192 and 10.492 $\pm$ 0.194 ng/ml, respectively. After preparation of the sample for GC–MS as described above, the observed peak-height ratios of m/z 482 to 485 and m/z 680 to 683 were determined in triplicate.

RESULTS AND DISCUSSION

Preparation of HFB derivatives

For the simultaneous assay of androstenedione and testosterone, solvent extraction and purification procedures followed by derivatization of the residue should preferably be the same for both steroids.

Derivatization procedures commonly used in GC–MS assays are in general the same as those used in conventional GC. For testosterone, these include fluoroacylation [2–6], trimethylsilylation [7,8], etc. For instance, we have successfully used the trifluoroacetyl (TFA) derivative for the determination of plasma testosterone by GC–MS–SIM using testosterone-19-d_3 as the internal standard [5]. Our attempt to prepare a TFA derivative of androstenedione, however, led to significant difficulty in the formation of a single thermally stable compound for GC–MS analysis. For androstenedione, methoxime formation at the C-3 and C-17 carbonyl groups [4] and the 3-enol-*tert.*-butyldimethylsilyl (3-enol-TBDMS) ether [9,10] were used in GC or GC–MS analysis. The methoxime derivatization, however, leads to formation of *syn–anti* isomers, producing two peaks on the gas chromatogram. Thus, the methoxime derivative is not suitable for obtaining high sensitivity by GC–MS analysis.

A *tert.*-butyldimethylsilyl (TBDMS) or trimethylsilyl (TMS) derivative may be chosen, but the presence of the two major isotopes of ^{28}Si, i.e., ^{29}Si and ^{30}Si, in the TBDMS or TMS group imposes certain restrictions on the choice of an internal standard for the GC–MS analysis using the isotope dilution tech-

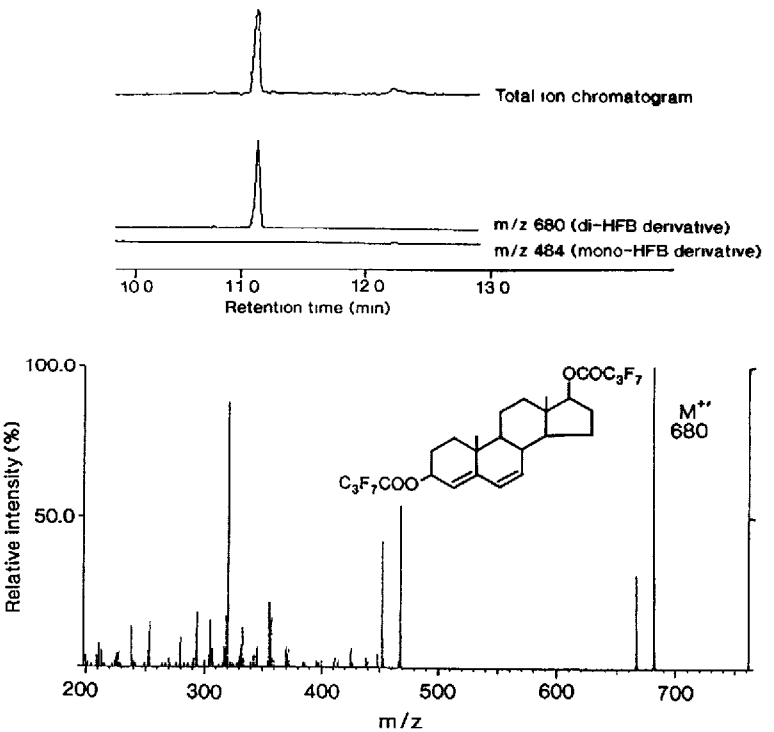

Fig. 1. Mass chromatogram (upper) and mass spectrum (lower) of di-HFB derivative of testosterone.

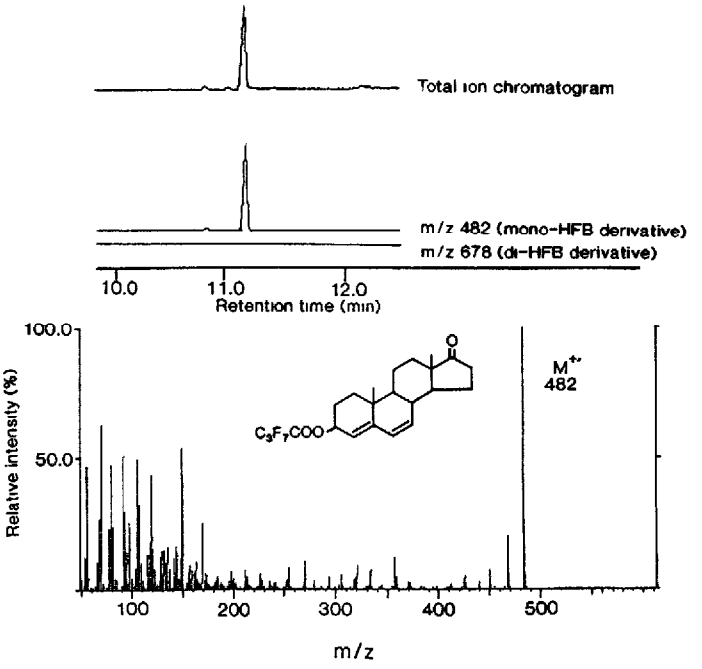

Fig. 2. Mass chromatogram (upper) and mass spectrum (lower) of mono-HFB derivative of androstenedione.

nique. The monitored ion cluster of the analyte may overlap with the monitored ion of its isotopically labelled internal standard, and corrections for contributions then have to be made. Therefore, the analyte and its isotopically labelled internal standard must differ by at least 5 mass units so that the monitored ion cluster of the unlabelled molecule does not overlap with the monitored ion of the isotopically labelled molecule and the calibration curve is rectilinear [11]. In this study, preparation of TBDMS or TMS derivatives was avoided because of the use of androstenedione-19-d_3 and testosterone-19-d_3 as internal standards.

In order to obtain compounds with suitable GC and MS properties, we attempted to prepare an HFB derivative of androstenedione according to the methods described for the HFB derivatization of testosterone [2–4,6]. The reaction of testosterone with HFBA has been reported to provide either the mono-HFB (at C-17) [2–4] or the di-HFB (at C-3 and C-17) derivative [2,6] depending on the reaction conditions employed. We found that the preparation of a single HFB derivative of di-HFB testosterone was successful by using the method of Moneti et al. [6]. All the other methods [2–4] produced a mixture of the mono- and di-HFB derivatives.

Fig. 1 shows the mass spectral data of the di-HFB derivative of testosterone prepared by reaction with HFBA for 1 h at room temperature in acetone as

solvent. There was only a single peak derived from the HFB derivative and no other peaks were seen on the mass chromatogram. The relative intensity of the molecular ion (M^+) at m/z 680 was prominent. Thus, the derivative chosen for the measurement of testosterone was considered to be suitable. In this regard, it was important to employ the HFB derivatization method for testosterone to prepare an HFB derivative of androstenedione.

When androstenedione was treated with HFBA under the same conditions employed for testosterone, a thermally stable mono-HFB derivative with good GC and MS characteristics was obtained as shown in Fig. 2. It should be pointed out that the HFB derivative of androstenedione was an excellent compound to be determined by GC–MS, since it had a very intense peak at m/z 482 corresponding to M^+. The HFB derivatives of androstenedione and testosterone were therefore chosen as suitable compounds for the actual assay for simultaneous measurements of these steroids in plasma.

Extraction and purification

Sep-Pak C_{18} cartridges provide a simple method for extracting steroids from biological fluids prior to GC–MS analysis [12,13]. We used these cartridges to extract dexamethasone [14] and cortisol [11] from plasma and obtained good recovery. In the present study, we adjusted the plasma pH to ca. 3 prior to application to the cartridge, and used methanol or diethyl ether as an eluting solvent.

The absolute recovery from human plasma were estimated by using [4-^{14}C]androstenedione and [4-^{14}C]testosterone. Elution with diethyl ether gave an absolute recovery of 86.6% for androstenedione and 95.9% for testosterone. The recovery was higher when eluting with methanol: 98.7% for androstenedione and 100.0% for testosterone.

The diethyl ether or methanol eluates were further purified by TLC in order

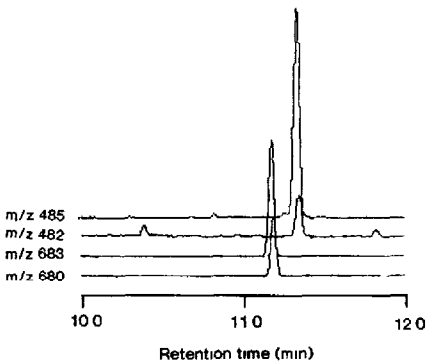

Fig. 3. Selected-ion recordings of di-HFB derivatives of testosterone (m/z 680) and testosterone-19-d_3 (m/z 683) and mono-HFB derivatives of androstenedione (m/z 482) and androstenedione-19-d_3 (m/z 485).

to provide sufficiently clean samples for GC–MS analysis. The loss of steroids during the TLC purification procedure was negligible. It was found that the methanol eluate gave a relatively large interfering peak at the retention time (t_R) of 15.5 min on the total ion chromatogram. Furthermore, evaporation of the methanol eluate was more time-consuming. We then decided to use diethyl ether as the eluting solvent for the Sep-Pak C_{18} cartridge.

Fig. 3 shows the selected-ion chromatograms of the mono-HFB derivatives of androstenedione (m/z 482) and androstenedione-19-d_3 (m/z 485) and of the di-HFB derivatives of testosterone (m/z 680) and testosterone-19-d_3 (m/z 683) for a processed plasma sample spiked with 20 ng each of androstenedione-19-d_3 and testosterone-19-d_3. Sharp and single peaks appeared at t_R of 11.1 min for testosterone and t_R of 11.3 min for androstenedione.

Calibration curve

Calibration curves were prepared without any correction for contributions by using androstenedione-19-d_3 and testosterone-19-d_3 as internal standards. The ratio between the relative intensities at m/z 482 and 485 and at m/z 680 and 683 was found to be linear with the various amounts (0.1–20 ng) of unlabelled androstenedione and testosterone added to the constant amount (20 ng) of deuterated androstenedione and testosterone.

Sensitivity and accuracy

The sensitivity of the capillary GC–MS–SIM assays was 1 pg per injection for the pure reference androstenedione and 10 pg per injection for the pure reference testosterone, with a signal-to-noise ratio of more than 2 (Fig. 4).

The accuracy of measurement was determined for androstenedione and tes-

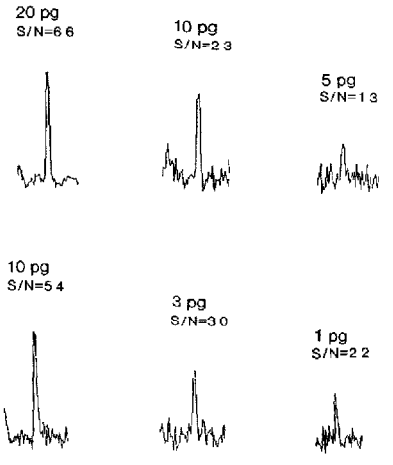

Fig. 4. Sensitivity of testosterone (upper) and androstenedione (lower).

TABLE I

ACCURACY OF GC–MS–SIM ANALYSIS OF ANDROSTENEDIONE IN PLASMA

Added (ng/ml)	Expected (ng/ml)	Found (ng/ml)					Relative error (%)	C.V. (%)
		Individual values				Mean ± S.D.		
–	–	2.578	2.347	2.281	2.684	2.475 ± 0.192	–	7.8
0 498	2.973	2.901	3.086	3.039	3.090	3.029 ± 0.088	+1 9	2.9
1.998	4.473	4.294	4.953	4.284	4.475	4.502 ± 0.314	+0.1	7.0

TABLE II

ACCURACY OF GC–MS–SIM ANALYSIS OF TESTOSTERONE IN PLASMA

Added (ng/ml)	Expected (ng/ml)	Found (ng/ml)					Relative error (%)	C.V. (%)
		Individual values				Mean ± S.D.		
–	–	10.723	10.263	10.552	10.430	10.492 ± 0.194	–	7 8
4.040	14.532	13.967	14.808	13.977	14.444	14.299 ± 0.406	−1.6	2.8
8.080	18.572	18.289	17.885	17.385	18.137	17.924 ± 0.396	−3.5	2.2

tosterone added to 1.0-ml aliquots of pooled plasma. The plasma sample contained 19.536 ng of androstenedione-19-d_3 and 20.000 ng of testosterone-19-d_3 and different amounts of androstenedione (0.498 and 1.998 ng) and testosterone (4.040 and 8.080 ng). The androstenedione and testosterone contents in the pooled plasma were 2.475 ± 0.192 and 10.485 ± 0.192 ng ($n=4$), respectively.

A good agreement between found values and the amounts of androstenedione and testosterone added was observed as presented in Tables I and II, the relative error being less than 4%. The inter-assay coefficients of variation (C.V.) for androstenedione were 2.9% for plasma spiked with 0.498 ng and 7.0% for plasma spiked with 1.998 ng. The inter-assay C.V. for testosterone were 2.8% for plasma spiked with 4.040 ng and 2.2% for plasma spiked with 8.080 ng.

The present method provides a sensitive and reliable technique for determining simultaneously the nanogram concentration levels of androstenedione and testosterone in plasma. No corrections were necessary for the small contribution at m/z 482 and 680 from the deuterated internal standards or for the small contribution at m/z 485 and 683 from the undeuterated androstenedione and testosterone. The selective deuterium label at the C-19 angular methyl groups of androstenedione and testosterone used as the analytical internal standards in this study seems not to suffer from the serious problems due to isotope effect and loss of labelling atoms [15,16]. The stable isotope technique described here can be applied to study the metabolic conversion of androstenedione into testosterone by administering androstenedione-19-d_3 to human

subjects. The plasma levels of androstenedione-19-d_3 and testosterone-19-d_3 derived from the administered androstenedione-19-d_3 can be determined separately from endogenous androstenedione and testosterone by the double isotope dilution method. The application of androstenedione-19-d_3 as a metabolic tracer for investigating the enzymic defect of 17-ketosteroid reductase in male pseudohermaphroditism will be described in a separate paper.

REFERENCES

1 S. Baba, Y. Shinohara and Y. Kasuya, J. Labelled Compd. Radiopharm., 14 (1978) 783.
2 D Exley, Biochem. J., 107 (1968) 285
3 I.R. Sarda, P.E. Pochi, J.S. Straus and H.H. Wotiz, Steroids, 12 (1968) 607.
4 W.P. Collins, J.M. Sisterson, E.N. Koullapis, M.D. Mansfield and I.F. Sommerville, J. Chromatogr., 37 (1968) 33.
5 S. Baba, Y. Shinohara and Y. Kasuya, J. Chromatogr., 277 (1979) 314.
6 G. Moneti, M.G. Giovannini, A. Guarna, G. Forti, R. Salerno, A. Magini and M Serio, Steroid Biochem., 1 (1987) 53.
7 T.A. Baillie, C.J W. Brooks and B.S. Middleditch, Anal. Chem., 44 (1972) 30.
8 E.M.H. Finlay and S.J. Gaskell, Clin. Chem., 27 (1981) 1165.
9 S.H.G. Anderson and J. Sjövall, J. Chromatogr., 289 (1984) 195.
10 S.H G. Anderson and J. Sjövall, J. Steroid Biochem., 23 (1985) 469
11 N. Hirota, T. Furuta and Y. Kasuya, J. Chromatogr., 425 (1988) 237.
12 G.R. Cannell, J.P. Galligan, R.H. Mortimer and M.J. Thomas, Clin. Chim. Acta, 122 (1982) 419
13 M.W. Khalil and V. Lawson, Steroids, 41 (1983) 549.
14 Y. Kasuya, J.R. Althaus, J.P. Freeman, R.K. Mitchum and J.P. Skelly, J. Pharm. Sci., 73 (1984) 446.
15 S. Baba, Y. Shinohara and Y. Kasuya, J. Clin. Endocrinol. Metab., 50 (1980) 889.
16 Y. Shinohara, S. Baba and Y. Kasuya, J. Clin. Endocrinol. Metab., 51 (1980) 1459.