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DETERMINATION OF PLASMA TESTOSTERONE BY MASS FRAGMENTOGRAPHY USING TESTOSTERONE-19-d_3 AS AN INTERNAL STANDARD

COMPARISON WITH RADIOIMMUNOASSAY

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SUMMARY

Analytical procedures for the measurement of testosterone by mass fragmentography (MF) using trideuterated testosterone (testosterone-19,19,19-d_3) are described. For the calculation of plasma testosterone, peak height ratios were measured by MF performed on the molecular ions of the TFA derivative of testosterone (*m/e* 480) and testosterone-19, 19,19-d_3 (*m/e* 483). The sensitivity of the method was judged from the lower limit of detection of the mass spectrometer which was at 10 pg. For the measurement of the precision, the inter- and intra-assay coefficients of variation (C.V.) were calculated by using a pooled plasma sample; they were 3.15% and 1.79%, respectively. The specificity was investigated by the use of 5α-dihydrotestosterone and the MF method was found to afford a highly selective technique. These results obtained by MF have been compared with the results obtained by a radioimmunoassay method.

INTRODUCTION

The assay of steroid hormones in body tissues or fluids has been greatly simplified by the introduction of analytical methods involving radioimmunoassay (RIA) [1—5], enzyme immunoassay [6—8], and the competitive protein binding technique [9—11]. There are, however, often serious restrictions to the use of these methods in that the preparation of the appropriate antisera or binding proteins is relatively complicated. Cross-reactivity with other steroids is also a serious problem in achieving acceptable specificity [12—14]. Moreover, these methods have the inherent drawback of the non-tracer technique in that the correction for losses of a particular substance in various biological samples in the extraction and purification procedure can not be easily made.

The usefulness of mass fragmentography (MF) in which gas chromatography—mass spectrometry with a multiple ion detector (GC—MS—MID) is

used in conjunction with stable isotope labeling has recently been of great interest in the measurement of trace amounts of substance in biological materials because of its high sensitivity and high selectivity [15—18]. In this technique stable isotope (SI)-labeled carriers serve as the ideal internal standard to correct for losses of a substance under study in the initial isolation procedures. This MF technique provides a sensitive, specific, and reliable method for biological assays.

The MF technique reported in this paper was developed from our need for a simple, reliable and rapid method for the determination of plasma testosterone levels in a biological sample. As the internal standard, we used testosterone-19,19,19-d_3 (testosterone-19-d_3), synthesized in our laboratory [19]. We compared the MF technique for the determination of male plasma testosterone with a radioimmunoassay method used in routine work.

MATERIALS AND METHODS

Chemicals

Testosterone-19,19,19-d_3 (testosterone-19-d_3) was synthesized in our laboratory as described previously [19]. The isotopic composition was 99.0 atom% deuterium (d_3, 97.8%; d_2, 2.2%; d_1, 0.0%). Non-labeled testosterone was purchased from Tokyo Kasei Kogyo, Tokyo, Japan (reagent grade) and was recrystallized from *n*-hexane—acetone before use.

Mass fragmentography

MF measurements were made with a Shimadzu LKB-9000B gas chromatograph—mass spectrometer equipped with a Shimadzu high-speed multiple ion detector—peak matcher 9060S. The electron energy was set to 20 eV and the trap current to 60 μA. The MID was focused on the ions at *m/e* 480 and *m/e* 483 and the peak height ratio was determined. GC was performed on a glass column (2 m × 3 mm I.D.) packed with 1.5% OV-1 on Shimalate W (80—100 mesh). The column temperature was 230° and the temperature of the flash heater and the separator was 250°. The temperature of the ion source was 270°. Helium carrier gas flow-rate was about 30 ml/min. The recordings were made on a Shimadzu two-pen recorder R-12M, the chart speed being 10 mm/min.

Preparation of calibration curve

To each of four standards containing 2—20 ng of testosterone in 20 μl of ethanol, 10 ng of testosterone-19-d_3 dissolved in 20 μl of ethanol were added. After evaporation of the solvent, each standard was dried in an evacuated desiccator. To each sample was added 200 μl of trifluoroacetic anhydride (reagent grade: Wako, Osaka, 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 50 μl of *n*-hexane. A 2—5 μl volume of the above *n*-hexane solution was analyzed by GC—MS.

Preparation of sample for MF

To 1.0 ml of male plasma was added 10 ng of testosterone-19-d_3 dissolved

in 20 μl ethanol and the plasma was allowed to stand for 30 min at room temperature. After adding 40 μl of 3 *N* NaOH, the plasma sample was extracted with 3 × 3 ml of diethyl ether. The diethyl ether phase was collected, washed with 1 ml of 5% acetic acid and then with 1 ml of water, and dried over anhydrous sodium sulphate. After evaporating the solvent, the residue of the diethyl ether extract was dissolved in 5 ml of 70% aqueous methanol and stored at −15° for 1 h. After centrifugation to remove plasma lipids, the upper layer was decanted and evaporated to dryness. The residue was then dissolved in chloroform and subjected to thin-layer chromatography (TLC) on Kieselgel $60F_{254}$ plates (0.25 mm thickness; Merck, Darmstadt, G.F.R.). The TLC plate was developed with chloroform—ethyl acetate (4:1, v/v) and the UV positive zone corresponding to standard testosterone with an R_F value of 0.3 was scraped off. Testosterone was eluted with 5 ml of chloroform and 5 ml of ethyl acetate in turn and the organic solvents were evaporated. The residue was dried in an evacuated desiccator. The trifluoroacetate (TFA) derivative was formed by reacting the residue with trifluoroacetic anhydride as described above and 2—5 μl of the sample was subjected to GC—MS.

Determination of accuracy

Testosterone in amounts of 1.93, 4.83 and 9.66 ng dissolved in 20 μl of ethanol was added to 1.0 ml aliquots of pooled male plasma. The testosterone content of the pooled plasma was 5.86 ± 0.02 (S.D.) ng/ml in triplicate determinations. After preparation of the sample for MF as described above, the observed peak height ratio of *m/e* 480 to *m/e* 483 was determined in triplicate.

Radioimmunoassay

Plasma testosterone was determined by an RIA method using a commercial kit from Cea Ire Sorin (Gif-Sur-Yvette, France). For the extraction of testosterone from plasma samples, 0.05 ml of plasma was used for each sample preparation.

RESULTS AND DISCUSSION

The availability of MF and of testosterone-19-d_3 synthesized in our laboratory enabled us to develop a method for measuring human plasma testosterone.

Considerations for MF analysis

Preparation of TFA derivative. Fig. 1 shows the mass spectra of testosterone-19-d_3 and testosterone-19-d_3-diTFA. In the mass spectrum of testosterone-19-d_3 (Fig. 1A) the relative abundance of the molecular ion (*m/e* 291) was about 6.5%. In the mass spectrum of testosterone-19-d_3-diTFA (Fig. 1B), on the other hand, the relative abundance of the molecular ion (*m/e* 483) was as high as 33%. Therefore it would be an advantage to make the TFA derivative for the MF analysis to obtain higher sensitivity. The preparation of testosterone-diTFA requires only a simple derivatization step. There was only a single peak derived from the TFA derivative and no other peaks were seen in the gas chromatogram. Furthermore as pointed out by Vestergaard, et al. [20], the specificity of the assay for testosterone was such that TFA deriva-

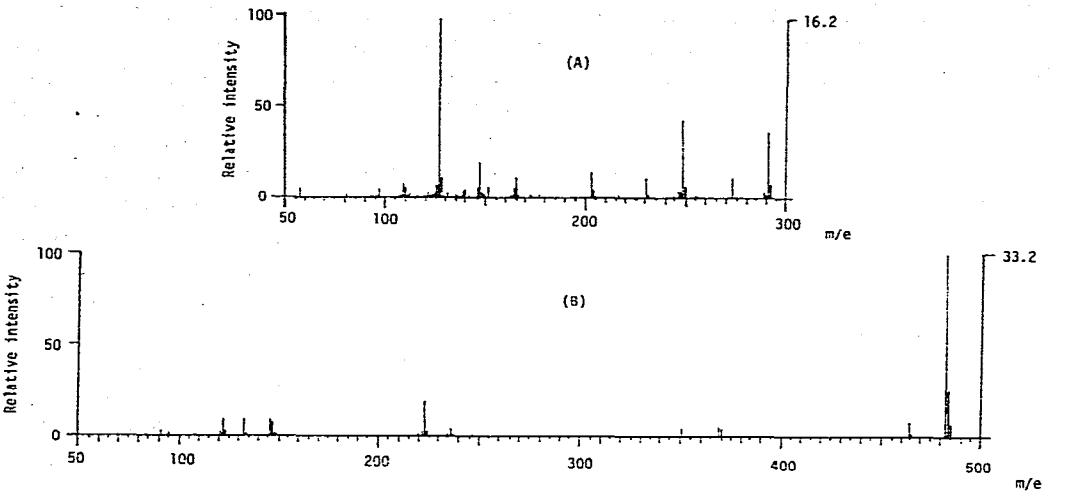

Fig. 1. Mass spectra of: (A) testosterone-19-d_3 ; (B) diTFA derivative of testosterone-19-d_3 .

tives of possible interfering steroids such as 17-oxo steroids, dihydrotestosterone, and epitestosterone did not interfere in the assay under the conditions of the present assay.

For the quantitative analysis of testosterone in plasma, the MF analysis was performed using the TFA derivatives by measuring the abundant molecular ions of *m/e* 480 for testosterone-diTFA and of *m/e* 483 for testosterone-19-d_3 -diTFA. The peak height ratio was then measured.

Extraction and purification. In the extraction procedure of blood samples it was necessary to eliminate lipids present in plasma, since these interfered with the MF analysis. This required the storage of a 70% methanol solution of the ether extract at —15° for 1 h. Under the GC conditions employed, the retention time of cholesterol was about 30 min. A purification step by TLC was useful in eliminating cholesterol from the blood sample and it allowed us to reduce the MF analysis time.

Fig. 2 shows the mass fragmentogram from the plasma extract with recordings of the peak intensities of the molecular ions for testosterone-diTFA (*m/e* 480) and testosterone-19-d_3 -diTFA (*m/e* 483). The assay was performed by adding about 10 ng of the deuterated internal standard (testosterone-19-d_3) to the plasma sample. The retention times of testosterone-diTFA and testosterone-19-d_3 diTFA were the same (about 4 min) and there was no interference in the molecular ion peaks by contributions from other materials in the plasma extract at these masses.

Sensitivity. The sensitivity of the determination procedures described here was judged based on the signal-to-noise ratio. The lower limit of detection of the mass spectrometer was 10 pg for testosterone as shown in Fig. 3.

Calibration curve. Known mixtures of testosterone and testosterone-19-d_3 were prepared so that the sample size (2—5 μl) injected into the gas chromatograph—mass spectrometer covered the testosterone range of 200 pg—2 ng with a fixed amount (1 ng) of testosterone-19-d_3 . Each mixture was then analyzed as the TFA derivative, focussing on the molecular ions at *m/e* 480

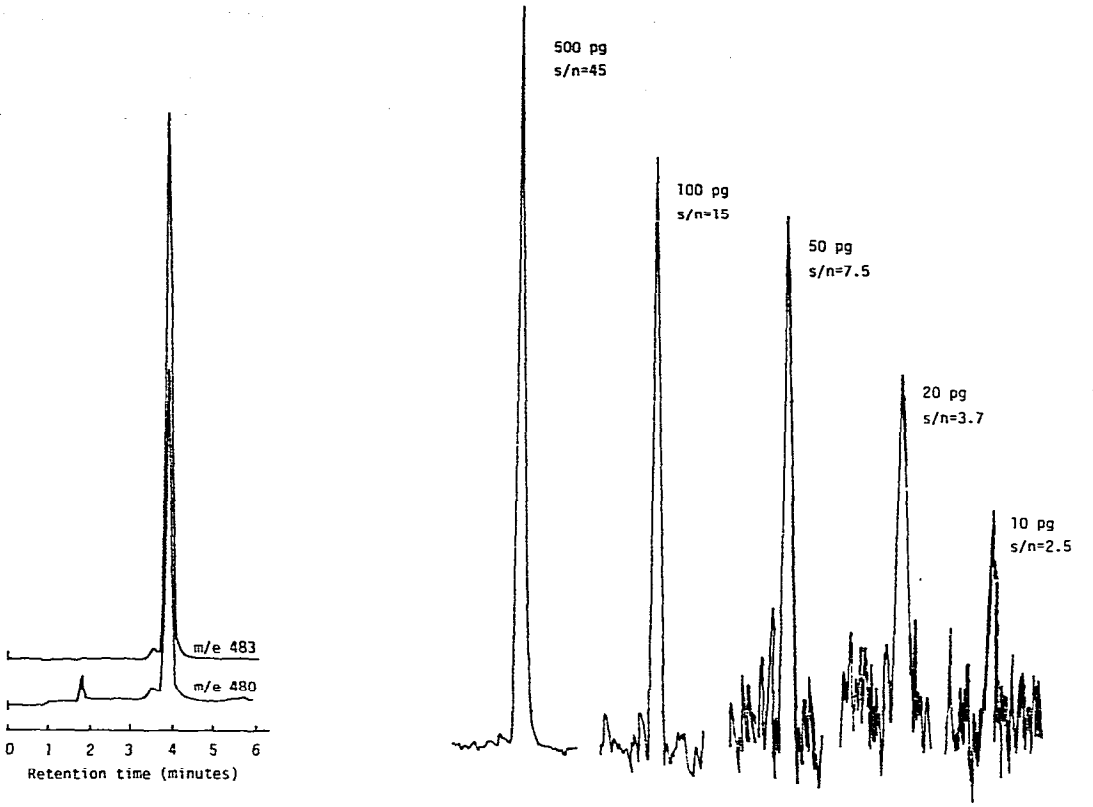

Fig. 2. Mass fragmentograms of testosterone-diTFA and testosterone-19-d_3-diTFA after processing from plasma sample.

Fig. 3. Sensitivity.

for testosterone and *m/e* 483 for testosterone-19-d_3. There was a good correlation between the mixed molar ratio and the observed peak height ratio as shown in Table I.

The peak height ratio was plotted against the molar ratio of testosterone to testosterone-19-d_3 and a calibration curve was obtained. The curve was rectilinear for molar ratios 0.2—2.0. Least-square analysis of the observed ratio gave a regression line with a slope coefficient of 0.9999.

As internal standards in the MF analysis of plasma or serum testosterone levels, isotopically-labeled testosterones such as deuterated testosterone prepared by the exchange reaction with deuterium oxide [21], testosterone-4-^{14}C [22], and testosterone-1,2,6,7-^{3}H [20] have been used. However, the presence of non-labeled testosterone in these labeled compounds requires relatively complicated correction techniques for the observed peak height ratio. The deuterated testosterone used in the present study, on the other hand, served as the ideal internal standard for quantitative determinations because of the absence of non-deuterated testosterone (the isotopic composition of testosterone-19-d_3 was 97.8% for d_3, 2.2% for d_2, and 0.0% for d_1). Therefore, the use of our deuterated testosterone has the advantage over the

deuterated internal standard with lower isotopic purity or the commercially available radioisotope-labeled testosterones.

The difference in mass number between the non-labeled and the labeled testosterone (testosterone-19-d_3) was three and this was appropriate for the precise assay of plasma testosterone.

Accuracy. Testosterone in amounts of 1.93, 4.83, and 9.66 ng was added to 1.0-ml aliquots of pooled male plasma. The amount of testosterone in these samples was then measured by the present method. The testosterone content in the pooled plasma measured in triplicate was 5.86 ± 0.02 ng/ml. The results presented in Table II show that the amounts of testosterone added were in good agreement with the amounts of testosterone measured, the relative error being less than 2.0%.

Comparison of results by MF and RIA

The reliability of the present MF method was determined by measuring the testosterone content in a pooled plasma sample divided into 17 aliquots of 1.0 ml. The results were then compared with those from an RIA method used in routine work, as shown in Table III. The mean testosterone concentrations of the pooled plasma from a healthy male were 6.22 ± 0.19 ng/ml for the MF method and 6.34 ± 0.84 ng/ml for the RIA method. These values were essentially identical and were within the reported "normal" range of plasma testosterone levels (3.6—11.7 ng/ml) [23]. The inter-assay coefficients of variation (C.V.) obtained with pooled plasma divided into 17 samples were

TABLE I

RELATIONSHIP BETWEEN MIXED MOLAR RATIO ADDED TO PLASMA SAMPLE AND OBSERVED RATIO DETERMINED BY MASS FRAGMENTOGRAPHY

Mixed ratio*	Observed ratio**
0.1954	0.1998 ± 0.0021
0.4886	0.4912 ± 0.0085
0.9772	0.9884 ± 0.0197
1.9544	1.9793 ± 0.0274

*Molar ratio of testosterone to testosterone-19-d_3.
**Mean ± S.D. of triplicate measurements.

TABLE II

ACCURACY OF MASS FRAGMENTOGRAPHY ANALYSIS OF TESTOSTERONE IN PLASMA

Added (ng)	Expected (ng)	Found (ng)				Relative error (%)
		Individual values*			Mean ± S.D.	
—	—	5.88	5.86	5.83	5.86 ± 0.02	—
1.93	7.79	7.63	7.76	7.57	7.65 ± 0.08	—1.84
4.83	10.69	10.78	10.35	10.48	10.53 ± 0.18	—1.49
9.66	15.52	15.19	15.51	15.72	15.47 ± 0.21	—0.32

*Each individual value represents the mean of triplicate measurements.

TABLE III

MEASUREMENT OF TESTOSTERONE IN POOLED MALE PLASMA BY MF AND RIA

No.	MF* (ng/ml)	RIA** (ng/ml)
1	6.50 ± 0.28	5.22
2	6.07 ± 0.11	8.01
3	6.45 ± 0.06	7.44
4	5.99 ± 0.11	6.99
5	6.51 ± 0.08	5.70
6	6.14 ± 0.16	7.29
7	6.14 ± 0.03	6.93
8	6.12 ± 0.08	5.70
9	6.32 ± 0.12	6.72
10	6.06 ± 0.09	7.44
11	5.94 ± 0.07	6.54
12	6.02 ± 0.12	5.85
13	6.59 ± 0.13	5.43
14	6.13 ± 0.21	5.64
15	6.17 ± 0.09	5.37
16	6.42 ± 0.06	5.88
17	6.15 ± 0.02	5.70
Mean	6.22	6.34
inter-C.V. (%)	3.15	13.38

*Each value represents the mean ± S.D. of triplicate measurements: the mean intra-assay C.V. was 1.79% in 17 triplicate measurements.
**Each assay was made by duplicate measurements.

3.15% for the MF method and 13.38% for the RIA method. The intra-assay precision for the MF method, as calculated from triplicate analyses of each plasma sample, was excellent (C.V. = 1.79%). These results confirmed the assay reliability of the present MF method.

The inter-assay C.V. for the RIA method was about 10% higher than that for the MF method. The main reasons for this difference may be that in the RIA method: (1) a check on the purification yield for each sample can not be made; (2) errors involved in the more frequent pipetting are significant; and (3) the reproducibility of the curvilinear standard curves on repetitive assays is relatively poor.

The usefulness of the present MF techniques is that it was not necessary to take into account the correction for the purification yield, since deuterium-labeled testosterone was used as the internal standard. In addition, it was also advantageous that the calibration curve was linear and easily constructed. The reproducibility of the curve was excellent. Moreover, identity of the compound under study can be confirmed, not only by selecting the molecular ion, but also by selecting other suitable fragment ions.

The accuracy of analytical procedures must depend largely on the specificity of the assay. Anti-testosterone sera normally display a marked degree of crossreactivity toward 5α-dihydrotestosterone [2—4]. The specificity of the assay for testosterone was investigated by adding known amounts of 5α-dihy-

drotestosterone (0.5, 2.5, 5.0, 10.0 ng) to pooled plasma. The testosterone content in the plasma samples was then measured by the MF and RIA methods. The results are shown in Fig. 4. It is obvious that the MF method was highly specific in measuring plasma testosterone. In the RIA method, however, cross-reactivity toward 5α-dihydrotestosterone was significant.

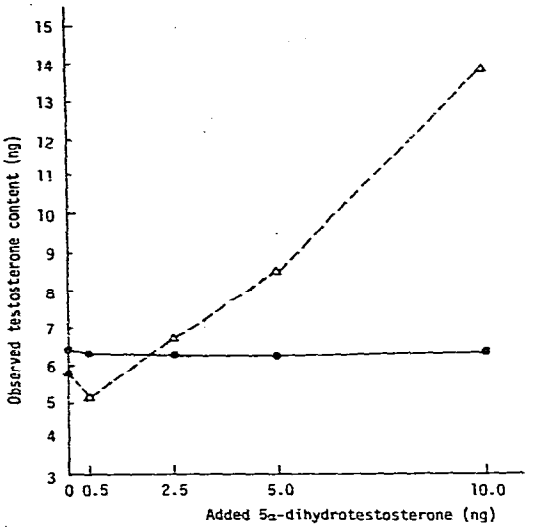

Fig. 4. Specificity of MF (•) and RIA (△). Each value represents the mean of triplicate measurements.

To summarize, the MF method described here afforded a sensitive and reliable technique to measure plasma testosterone. Specificity was high because the GC—MS system was used to separate and to detect specifically the compound in question. To analyze 20 plasma samples, the entire assay could be completed in 2½ working days, requiring one working day more than in the RIA method reported by Furuyama et al. [2]. However, pipetting is necessary only twice in the MF method described here and thus the practicability of this method is comparable to that of RIA.

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