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Measurement of plasma testosterone by gas chromatography–negative-ion mass spectrometry using pentafluoropropionic derivatives

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Abstract

Plasma testosterone was measured by gas chromatography–negative-ion chemical ionisation mass spectrometry (GC–MS). The testosterone was extracted from plasma using home-made Extrelut columns and diethyl ether elution. It was quantified as the pentafluoropropionate (PFP) derivative by selected-ion monitoring at m/z 560 (testosterone) and 563 (d_3-testosterone), accounting for about 34% of the total ion. The characteristics of the method were: extraction recovery about 95%; linearity over the range 1.7–71.5 nmol l^{-1} with linear regression equation $y = 1.41x + 0.0217$, $r = 0.999$; detection limit 3.5 fmol injected with a signal-to-noise ratio of 7.4; within-day variation, 3% for GC–MS, and 5.8% for the whole process; day-to-day coefficient of variation, 6.6–11%, depending on the concentrations. There was a good correlation between the results obtained by GC–MS and RIA ($r = 0.994$), but the GC–MS values were significantly lower ($p < 0.05$) than those obtained by RIA.

1. Introduction

The plasma testosterone concentration is clinically important for evaluating the testicular function in man [1], hyperandrogenic disorders in women [2,3] and puberty problems in children and teenagers [4]. Radioimmunoassay (RIA) is currently considered to be the method of choice for routine measurement of testosterone [5,6]. The method is sufficiently sensitive, many samples can be assayed in one series and only small sample volumes are required. However, there are restrictions to its use; cross-reactivity with other steroids can reduce the specificity, and the hormone concentrations measured in a given sample may vary considerably depending on the antibody used [7]. Isotope dilution with gas chromatography–mass spectrometry (GC–MS) is widely recognized as the most reliable technique for quantifying organic analytes, particularly steroids of clinical significance [8,9].

This report describes a GC–MS method for determining testosterone in plasma at concentrations that make the method useful for physicians. Testosterone was derivatized with PFPA, and quantified by negative ionization. A solid-phase extraction improved the recovery of testosterone and shortened the handling time.

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2. Experimental

2.1. Chemicals and reagents

Testosterone and [19,19,19-$^2H_3$]testosterone, used as internal standard, were purchased from Sigma (St. Louis, MO, USA). Pentafluoropropionic anhydride (PFPA) was obtained from Pierce Chemical Company (Rockford, IL, USA). All solvents were HPLC grade and were supplied by Merck (Darmstadt, Germany). Analytical grade chemicals were obtained from Sigma (St. Quentin Fallavier, France).

2.2. Apparatus

The GC–MS system consisted of a Hewlett-Packard 5988 A (Palo Alto, CA, USA) gas chromatograph–mass spectrometer system equipped with a fused-silica capillary column (SPB1, 30 m × 0.32 mm I.D., Supelco, St. Germain en Laye, France). Helium, the carrier gas, was at a column head pressure of $2.8 \cdot 10^4$ Pa. 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 0.75 min. The initial column temperature was set at 50°C for 0.75 min after the sample injection, and then increased at 30°/min to 230°C, then at 10°/min to 290°C and maintained at 290°C for 3 min. The temperatures of the injector, transfer line and source were 250°C, 270°C and 200°C, respectively.

Negative-ion chemical ionization (NCI) was chosen for selected-ion monitoring (SIM). Methane was used as reagent gas, the ion volume pressure was about 93 Pa. The ionization current was 300 μA and the ionization voltage was 70 eV. Fragments of the derivatives of testosterone (*m/z* 560) and d_3-testosterone (*m/z* 563) were monitored. Testosterone was determined from peak-area ratios with respect to the corresponding deuterium-labelled compound.

2.3. Blood collection

Blood samples collected with lithium heparinate were supplied by the RIA department (Chemistry Laboratory A, CHRU Nancy, France). Samples were obtained from both healthy and sick male adults (age ranging from 20 to 60 years). Serum was separated by centrifugation for 15 min at 3000 *g* at room temperature. Aliquots were stored at −20°C until assayed.

2.4. Procedure

Extraction and derivatization

A 50-μl volume of d_3-testosterone in ethanol (17.4 nmol l^{-1}) and 0.5 ml of 1 *M* phosphate buffer pH 8.5 were added to 0.5 ml of plasma. The mixture was loaded onto an Extrelut column (Merck) and the testosterone was extracted with 2 × 5 ml of diethyl ether. The organic phases were collected and evaporated to dryness under a gentle stream of nitrogen. The dry residue was dissolved in 2 × 1 ml of diethyl ether, transferred to a mini reactivial (Pierce) and evaporated to dryness. The PFP derivatives were prepared by incubation at 30°C for 1 h with 150 μl of PFPA. Excess PFPA was removed by evaporation and the dry residue was dissolved in 50 μl of ethyl acetate. A 1-μl aliquot of the extract was injected onto the GC–MS system.

Radioimmunoassay

Plasma testosterone was determined by RIA using a commercial kit from Immunotech International (Marseille, France). The detection limit was 0.17 nmol l^{-1}, and the day-to-day coefficient of variation was 6.9–15%, depending on the concentration measured.

2.5. Statistical analysis

The results are expressed as mean ± S.D.. The data were compared using Student's paired t-test. The GC–MS and RIA methods were compared by regression analysis and Pearson's coefficient of correlation (*r*).

3. Result and discussion

3.1. Choice of derivative

Several derivatization methods have been used to quantify testosterone in blood and urine.

Furuta et al. [10] described a sensitive, reliable technique using a heptafluorobutyryl (HFB) derivative that gives accurate, precise and reproducible results. Testosterone reacts with HFBA to give either the mono-HFB or the di-HFB derivative [11], depending on the reaction conditions employed. Fukushima et al. [12] used hydroxylamine hydrochloride, and trimethysilyl (TMS) derivatives to simultaneously determine testosterone, androstenedione, but the relative intensities of the molecular ions were low (< 6%) in the EI mode. Thus, the methoxime derivative does not provide high sensitivity in GC–MS analysis. Baba et al. [13] used trifluoroacetate (TFA) derivatization and EI detection. Under these conditions, the lowest quantity detected was 35 fmol injected. A pentafluorobenzylcarboxymethoxime-trimethylsilyl derivative and NCI detection were used by Bagnati and Fanelli [14], but this derivatization gave rise to the formation of two isomers.

We tested several derivatization agents (TMS, TFA, HFBA, PFPA). In our hands the best results were obtained with PFPA derivatives. Both testosterone and d_3-testosterone gave a single peak on the total-ion chromatogram. The di-PFP derivatives obtained produced ions at m/z 560 for testosterone and at 563 [M − 19] for d_3-testosterone. In the mass spectrum the relative abundance of m/z 560 was about 30% (Fig. 1). These ions therefore provided good sensitivity for SIM. The high mass of the peak monitored for testosterone assay (m/z 560, 563) reduced the risk of interference from other products co-extracted from plasma. No other peak was seen with plasma extract at the retention times for testosterone and d_3-testosterone (Fig. 2). The derivatives prepared as described were stable for several days at room temperature. The derivatization method was reproducible and the retention time of plasma testosterone was measured on two different days. The data obtained on the first day were plasma testosterone $8.782 \pm 9.5 \cdot 10^{-6}$ min ($n = 6$), d_3-testosterone $8.775 \pm 5.6 \cdot 10^{-6}$ min ($n = 6$), and on the second day the plasma testosterone was $8.791 \pm 15 \cdot 10^{-6}$ min ($n = 6$), and d_3-testosterone was $8.784 \pm 12.8 \cdot 10^{-6}$ min ($n = 6$). The difference between the results was not significant. PFP derivatives are known to be moisture sensitive and all traces of moisture were carefully avoided during their preparation.

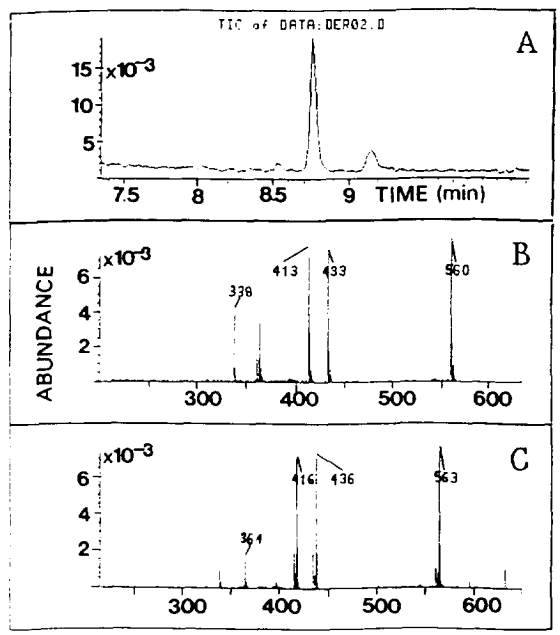

Fig. 1. Total-ion chromatogram (A) and mass spectra of testosterone (B) and d_3-testosterone (C).

3.2. Extraction procedure

The specificity and accuracy of mass-spectrometry data for analytes in a biological matrix

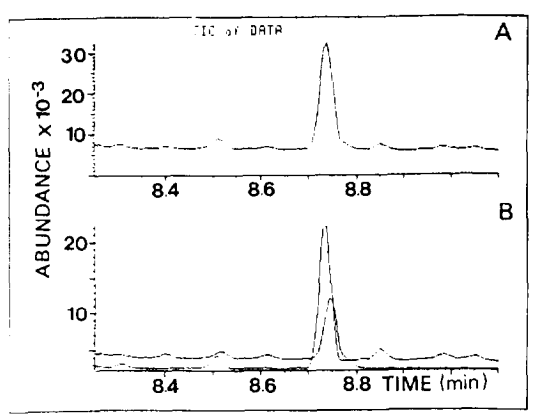

Fig. 2. Chromatograms for 0.5-ml of human plasma processed as described in the text. (A) Total-ion current, (B) m/z 560 for plasma testosterone, m/z 563 for d_3-testosterone (20 nmol l^{-1}).

Table 1
Recovery of testosterone from pooled plasma

	Concentration (nmol l^{-1})		Recovery (%)
	Theoretical	Found	
Plasma ($n = 5$)	15.3 ± 0.52		
Testosterone added			
14 nmol l^{-1} ($n = 8$)	29.3	28.2 ± 1.56	96.2
24.3 nmol l^{-1} ($n = 8$)	39.6	37.9 ± 2.06	95

Sample volume 0.5 ml. Samples contained 17.4 nmol l^{-1} d_3-testosterone.

depends greatly on the purity of the extracts used for analysis [15]. The lipids in plasma must be eliminated, because they interfere with steroid GC–MS analysis [13]. Several methods have been described for extracting steroids. Organic solvents such as methylene chloride, dichloromethane–isooctane (1:2, v/v), diethyl ether or ethyl acetate are widely used [16]. However, solvent extraction must be followed by a purification procedure by thin-layer chromatography or gel chromatography on Sephadex 2H-20 mini-columns [16] to provide samples clean enough for GC–MS analysis. These techniques are laborious and time-consuming. We therefore used a one-step solid-phase extraction with Extrelut mini-columns for routine testosterone assay because it is simple, fast and gave adequate recovery. The best recovery was obtained with diethyl ether elution. The plasma samples were adjusted to pH 8.5 with 1 *M* phosphate buffer to stabilize the deuterated internal standard [17]. Recoveries (Table 1) were similar to those obtained by others. Extraction assays were first performed with 1-ml samples. This volume was often limiting for testosterone determination in clinical samples. We therefore compared the extraction recoveries with different volumes (Table 2). As the recoveries were not significantly different, a sample volume of 0.25 ml was used for routine assay.

The main problem with solid-phase extraction are the costs. These were reduced by preparing the columns with Extrelut resin (ref. no. 13076, Merck) in-house. For 0.25-ml samples columns of 54 × 8 mm were filled with about 0.7 g dry resin packed by gentle tapping and used in the same way as the commercial columns. Larger columns (54 × 14 mm) containing about 2 g dry resin were used for samples of 0.5 or 1 ml.

3.3. Evaluation of the method

Specificity

Testosterone was identified in SIM by its retention time, the almost simultaneous appearance of d_3-testosterone and the high mass m/z. Other androgens such as nortestosterone, dihydrotestosterone, dehydroepiandrosterone (DHA), and Δ4-androstenedione were not interfering. The retention times (t_R) and main masses (MM) were different for each steroid (epitestosterone $t_R = 8.45$ min, MM 560–413; nortestos-

Table 2
Percentage recovery of testosterone from pools of male plasma according to sample volume

Sample volume (ml)		Testosterone concentration (nmol l^{-1})	Recovery (%)
1	Pool 1 ($n = 8$)	6.2 ± 0.25	100
	Pool 2 ($n = 8$)	17.2 ± 0.82	100
0.5	Pool 1 ($n = 8$)	6 ± 0.18	96.7
	Pool 2 ($n = 8$)	16.9 ± 0.94	98
0.25	Pool 1 ($n = 8$)	5.9 ± 0.28	95
	Pool 2 ($n = 8$)	17 ± 1.03	99

The percentage recovery was compared to results obtained with 1 ml plasma (100%).

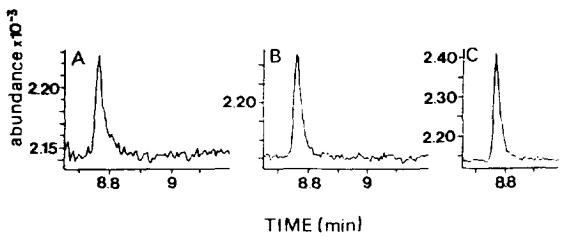

Fig. 3. Detection threshold for standard solution. (A) 3.5 fmol injected, signal-to-noise ratio 7.4; (B) 7 fmol injected, signal-to-noise ratio 21.9; (C) 35 fmol injected, signal-to-noise ratio 33.9.

terone $t_R = 8.63$ min, MM 546–399; dihydrotestosterone $t_R = 9.36$ min, MM 416; DHA + Δ4-androstenedione $t_R = 9.56$ min, MM 278–390). This method did not separate DHA and Δ4-androstenedione which have the same retention time and the same main masses.

Linearity

Recovery calibration curves, without correction, were prepared using d_3-testosterone as internal standard. Linearity was tested by injecting increasing amounts of testosterone (1.7–71.5 nmol l^{-1}) and a constant quantity of d_3-testosterone (17.4 nmol). This range covered the physiological values found in men, women and children and most pathological values. If the values obtained did not fall within the standard curve, the sample volume extracted or volume injected onto the column was adjusted accordingly. A standard curve was established from the ratio of the peak areas testosterone/d3-testosterone, with the linear regression equation $y = 1.41x + 0.0217$, $r = 0.999$. The limit of detection measured with standard testosterone was 3.5 fmol injected, with a signal-to-noise ratio of 7.4 (Fig. 3).

Repeatability and reproducibility

The repeatability and reproducibility of the GC–MS analysis were evaluated from standard solutions and one plasma extract. Repeatability of the whole method was determined using pools of male plasma which were carried through the routine procedure: extraction, derivatization and CG–MS analysis (Table 3). Reproducibility was measured from three pools of plasma which were tested during routine analysis over a period of 16 days (Table 3).

3.4. Comparison of results obtained by GC–MS-SIM and RIA

The reliability of the GC–MS method was evaluated by comparing the results with those obtained by RIA for 73 plasma samples (Fig. 4). The mean ± S.D. was 8.31 ± 7.57 for GC–MS and 8.92 ± 7.68 nmol l^{-1} for RIA. The correlation between both methods was good ($r = 0.94$), however the values obtained by CG–MS were significantly lower than those obtained by RIA

Table 3
Repeatability and reproducibility of testosterone assays

Sample	Concentration (mean ± S.D.) (nmol l^{-1})	C.V. (%)	n
Repeatability			
Standard solution	15.5 ± 0.49	3	10
Plasma[a]	21.73 ± 0.60	2.76	10
Plasma[b]	20.12 ± 1.17	5.8	8
Reproducibility			
Plasma pool A	8.15 ± 0.91	1	16
Plasma pool B	14.82 ± 1.29	8.70	16
Plasma pool C	25.24 ± 1.68	6.6	16

[a] Same plasma pool extract chromatographed ten times.
[b] Plasma pool extracted 8 times the same day.

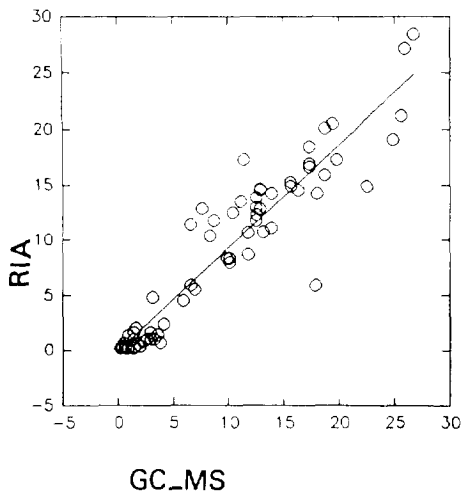

Fig. 4. Comparison of results obtained by RIA and GC–MS. Data are expressed as nmol testosterone per liter; $y = 0.931x - 0.0002$, $r = 0.943$.

($p < 0.05$) (Student's paired t-test). This difference may be due to: (1) the specificity of the methods; Pirke [18] found that the antitestosterone-sera used in RIA could cross-react with 5α-dihydrotestosterone (DHT). Cross-reactivity with 5α-DHT was 10% with the kit employed, and there may be cross-reactivity with other steroids also, especially with therapeutic agents. The GC–MS method identified the product to be tested by its retention time and a characteristic ion [M − 19], and is thus more specific. Other suitable ions can also be selected to improve the specificity if necessary. (2) In the direct testosterone radioimmunoassay that does not use extraction sex-hormone binding globulin may also interfere [6]. (3) The RIA involves pipetting small volumes which may give rise to errors. (4) The purification yield for each sample cannot be checked with the RIA method.

The advantage of the present GC–MS technique is that it is not necessary to correct for the purification yield, since d_3-testosterone was included as the internal standard before the extraction step.

To summarize, the described GC–MS method is a sensitive and highly specific technique for determining the concentration of plasma testosterone. The system can be left to operate unattended by employing the HP-7673 A autosampler and sample tray, controlled with the HP Series 200 data system and data processing with the MACRO program. This increases the number of samples processed per day.

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