Journal of Chromatography, 582 (1992) 7–12
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6515

Urinary testosterone measurement by gas chromatography after solid-phase extraction and high-performance liquid chromatography

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(First received February 18th, 1992; revised manuscript received July 8th, 1992)

ABSTRACT

A method was developed for the rapid determination of testosterone in urine. The procedure consists of solid-phase extraction (SPE) followed by high-performance liquid chromatographic (HPLC) clean-up before gas chromatographic determination. Recovery was evaluated by adding [^{3}H]testosterone (10^4 cpm) to urine samples; the mean recovery of radioactivity after SPE and HPLC was 82%. Precision was estimated by repeated measurement of testosterone in four different urine samples: the coefficient of variation was 7.9% (95% confidence limits 6.1–11.4%). Accuracy was evaluated by standard addition and dilution assays; a linear relationship was found between the expected and observed values ($r^2 = 0.982$). The method is rapid, effective and suitable for routine analysis.

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0378-4347/92/$05.00

INTRODUCTION

Fluctuations of plasma testosterone values have been observed during the day [1,2] and throughout the menstrual cycle [3]. Thus testosterone levels in blood reflect variations in episodic and diurnal secretion. In contrast, the 24-h urinary excretion of testosterone glucuronide represents a suitable estimate of daily endogenous production of this hormone [4]. Measurement in urine may therefore be used as a marker of androgenicity.

Different chromatographic and immunological methods have been developed to determine urinary testosterone concentrations [5–10] and show comparable ranges of precision and sensitivity. However, the immunological assay does not discriminate between substances that evoke similar immunological responses and chromatographic techniques are sometimes more specific. Both immunological and chromatographic methods need the same preliminary purification steps and are, therefore, equally time-consuming.

In our laboratory, urinary testosterone is determined by gas chromatography (GC) according to the method of Mauvais-Jarvis *et al.* [7], with minor modifications. Although highly effective, this method is tedious and time-consuming. This work was aimed at improving the method of measuring urinary testosterone levels by combined solid-phase extraction (SPE), high-performance liquid chromatography (HPLC) and GC and assessing the performance in terms of precision and accuracy.

EXPERIMENTAL

Standards

Testosterone and 4-pregnen-20α-ol-3-one (20α-hydroxyprogesterone) were purchased from Sigma (St. Louis, MO, USA).

Reagents and chemicals

Acetonitrile, water (both of HPLC grade), ethanol and methanol were obtained from Merck (Darmstadt, Germany). *Helix pomatia* juice with enzymatic activities of β-glucuronidase 10^5 Fishman U/ml and sulphatase 10^6 Roy U/ml was obtained from IBF Biotechnics (Villeneuve-la-Garenne, France). A Sylon BFT kit containing N,O-(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) was obtained from Supelco (Bellefonte, PA, USA).

Samples

Urine samples were collected over 12 or 24 h and stored at −30°C until analysis.

Enzymatic hydrolysis

A urine sample was adjusted to pH 5.2 with glacial acetic acid, mixed with 10% acetate buffer then added to the *Helix Pomatia* juice (1 ml per 100 ml of sample). This mixture was incubated at 45°C for 22 h, followed by centrifugation and SPE.

Solid-phase extraction

SPE was performed using Adsorbex RP-18 (400 mg) and Adsorbex-NH_2 (100 mg) cartridges (Merck) in a Vac Elut vacuum chamber (Schilling, Milan, Italy) [11,12]. The columns were prewashed according to the manufacturer's instructions. The RP-18 sorbent cartridge was loaded with an aliquot (50 ml) of urine and, after aspiration, washed with 10% aqueous methanol. The Adsorbex-NH_2 column was coupled to the Adsorbex RP-18 and connected to the vacuum chamber. The androgens were eluted by passing 3 ml of acetonitrile through the column assembly. The eluent was collected and evaporated on a water-bath at 60°C under a stream of dry nitrogen. The dried residue was dissolved in 200 μl of 60% aqueous ethanol and passed through a syringe filter. Finally 100 μl were injected into the HPLC system [13].

High-performance liquid chromatography

The HPLC system was equipped with a Model 1050 variable-wavelength UV detector, a Model 1050 gradient pump and a chromatographic data station (Hewlett-Packard, Palo Alto, CA, USA). The column (250 mm × 4 mm I.D.) was packed with 5-μm Spherisorb ODS-2 (Hewlett-Packard) and coupled with a LiChrospher 100 RP-18 (5

μm) guard column (4 mm × 4 mm I.D.) (Hewlett-Packard). The initial mobile phase was acetonitrile–water (10:90) at a flow-rate of 1.0 ml/min. The acetonitrile content was increased to 100% in 25 min and maintained at 100% for 5 min. Sample absorbance was monitored at 254 nm and recorded by the chromatographic data station.

A fraction of eluent was collected between 15.9 and 16.6 min, then evaporated on a water-bath at 60°C under a stream of dry nitrogen. The dried eluent was derivatized with 50 μl of BSTFA + 1% TMCS for 50 min at 60°C [14], after addition of 20α-hydroxyprogesterone (1 μg) as internal standard (I.S.) for GC. 20α-Hydroxyprogesterone is a natural product of progesterone, especially in the luteal phase. This hormone was chosen as the I.S. for GC after accurate control that the HPLC system could remove all endogenous 20α-hydroxyprogesterone (retention time 19.4 min) from the collection window (15.9–16.6 min).

A 1-μl sample aliquot was injected into the GC apparatus.

Gas chromatography

The gas chromatograph was a Dani (Monza, Italy) Model 6500, equipped with a programmed temperature vaporizer (PTV) injection system and flame ionization detection (FID) system. The sample was introduced into the capillary column using a splitless technique [15].

The analytical conditions were as follows: column, BP1 (25 m × 0.22 mm I.D.) with film thickness of 0.25 μm, purchased from SGE (Ringwood, Australia); carrier gas, helium at 40 cm/s, air at 1.5 bar, hydrogen at 0.5 bar and nitrogen (make-up gas) at 0.5 bar; and oven temperature increased from 100 to 280°C at 3°C/min and maintained at 280°C for 6 min. The chromatogram was recorded and processed by computer-aided methods.

Gas chromatography–mass spectrometry (GC–MS).

A Model 5970 mass spectrometer linked to a Model 5890 gas chromatograph was supplied by Hewlett-Packard. The GC conditions were as follows: on-column injection; column, DB1 (30 m × 0.25 mm I.D.) with a film thickness of 0.25 μm, purchased from J & W Scientific (Folsom, CA, USA); carrier gas, helium at 40 cm/s; oven temperature, increased from 50 to 100°C at 20°C/min and from 100 to 280°C at 3°C/min, then maintained at the final temperature for 5 min. The following MS conditions were used: transfer line temperature, 260°C; electron-impact energy, 70 eV; scan spectrum range, 70–700 u; and electron multiplier voltage, 1600 V. Mass spectra were recorded on an HP-9816 workstation.

Features of experiments carried out to assess method performance

Recovery. The yield of urinary testosterone from the extraction and clean-up procedures was determined using [^{3}H]testosterone. A known amount of labelled compound (10^4 cpm) was added to three urine samples before the SPE and HPLC analysis. The dried HPLC eluate was dissolved in 150 μl of ethanol; 50 μl were mixed with scintillation liquid and the activity (cpm) was determined with a β-counter Beckman (Irvine, CA, USA).

Calibration. Five calibration graphs were constructed to verify whether they passed through the origin. In this instance, a calibration graph for quantification of testosterone in urine samples might be drawn from the origin to the value of a known amount of standard testosterone (1 μg in 50 ml, which corresponded to a testosterone/I.S. concentration ratio of 0.5).

Calibration graphs were obtained by adding known amounts of testosterone (0.2, 0.4, 0.8 and 1.6 μg in 50 ml) to aliquots of acetate buffer. The testosterone concentration was determined by GC after SPE and HPLC. 20α-Hydroxyprogesterone (1 μg) was added as the I.S. after purification procedures. Graphs were drawn by plotting testosterone/I.S. peak-area ratios against testosterone/I.S. concentration ratios. With reference to the standard solution used for the calibration graph described above, the values for the concentration ratio were 0.1 0.2, 0.4 and 0.8, respectively.

Precision. Precision was determined by mea-

suring testosterone levels in six specimens of four different urines.

Accuracy. Accuracy was evaluated by standard addition and dilution assays. Testosterone levels were measured in 50-ml samples of pooled urine diluted 1:1 (four specimens), 1:2 (three specimens) and 1:4 (three specimens) with distilled water or spiked with standard testosterone (0.25, 0.5, 0.75 and 1 μg in 50 ml; three specimens in each instance).

Statistical methods

Calibration. A multiple regression model was fitted to the five calibration lines. The testosterone/I.S. peak-area ratio was the dependent variable and the independent variables were x = testosterone/I.S. concentration ratio, five dummy variables (d_i) [16] which specify each of the calibration graphs and five obtained by multipling x by d_i. The residual variance has ten degrees of freedom (d.f.).

Accuracy. To process, in one analysis only, the whole set of data obtained in the standard addition and dilution assays, the following regression model was fitted:

$$y_{ij} = \beta_0 + \beta_1 x_{1i} + \beta_2 x_{2i} + \varepsilon_{ij} \quad (1)$$

where y_{ij} = testosterone level in each specimen tested; x_{1i} = amount of testosterone added (0, 0.25, 0.5, 0.75, 1 μg in 50 ml); x_{2i} = dilution levels (1, 0.5, 0.25); ε_{ij} = random error component; i = 1, 2, ..., 7: number of points of the experimental design (x_{1i} = 0 and x_{2i} = 1 specify the original specimen of urine); j = 1, 2, ..., n_i: number order of replicates at each point.

If the method is accurate and the linear relationship holds in the tested ranges of the addition and dilution assays, then (i) the amount of testosterone added is exactly titred and, as the scale is additions arithmetic, β_1 is expected to be 1 and the null hypothesis H_0: $\beta_1 = 1$ is tested by Student's t-test; and (ii) let μ indicate the unknown "true" amount of testosterone; at the dilution level 1:k, $k^{-1}\mu$ is the testosterone expected to be determined. Hence, as $x_{2i} = k^{-1}$, β_2 (the estimate of β_2) is an estimate of μ; an alternative estimate of μ is given by $\bar{y}_1$, the mean of the determinations at the point $x_{1i} = 0$ and $x_{2i} = 1$. A good correlation between these estimates indicates that the method is accurate.

As the assumption of homoscedasticity was not tenable, the weighted least-squares method [16] was adopted to estimate the parameters of the model. Empirical weights inversely proportional to the "between replicates" variance were used.

RESULTS

Typical HPLC traces for standard testosterone and urine samples, after SPE extraction are shown in Figs. 1 and 2, respectively. It was not possible to determine which peak (Fig. 2) corresponds to testosterone. Consequently, fractions were collected between 15.9 and 16.6 min, based on the elution time of a standard compound (Fig. 1). The elution time of the standard compound was shown to be highly reproducible [n = 10, mean = 16.22, coefficient of variation (C.V.) = 0.27%]. Fig. 3 shows the GC resolution of the testosterone peak in the urine sample after SPE and HPLC steps. Testosterone identification was confirmed by GC–MS. The peak identified as testosterone in GC shows the characteristic fragmentations of testosterone [17]. The mass spectrum of TMS-testosterone obtained from the biological sample was pure compared with the

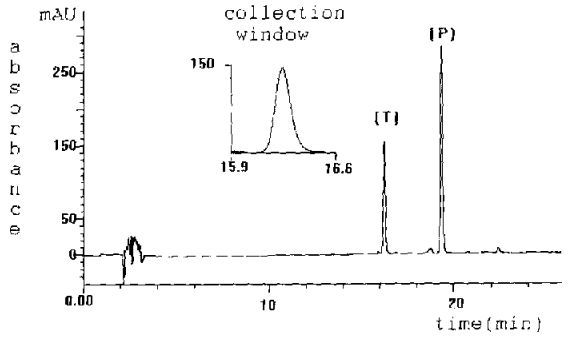

Fig. 1. Typical HPLC trace obtained from standard testosterone (T) and 20α-hydroxyprogesterone (P). Analytical conditions are given in the text.

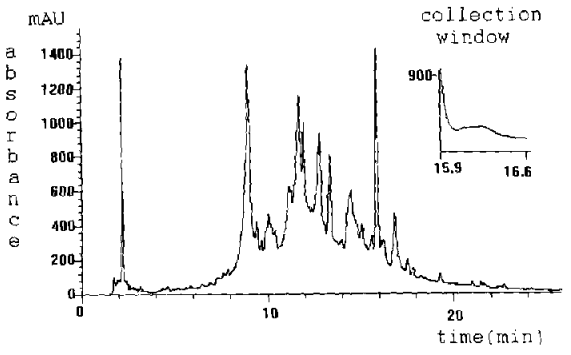

Fig. 2. Typical HPLC trace obtained from urine after SPE. Analytical conditions are given in the text.

TABLE I

PRECISION OF SPE–HPLC–GC MEASUREMENTS OF TESTOSTERONE IN URINE

Urine sample	n^a	Concentration (mean ± S.D.) (μg per 50 ml)	C.V. (%)
A	6	1.648 ± 0.137	8.3
B	6	0.858 ± 0.042	4.9
C	6	0.220 ± 0.017	7.7
D	6	0.218 ± 0.020	9.2

[a] Number of determinations.

standard compound. No supplementary m/z fragments were found and the same m/z 360/345, 360/270 and 360/129 fragment ratios were observed.

Recovery

After the addition of [^{3}H]testosterone (10^4 cpm) to three urine samples, the mean recovery of radioactivity, after the SPE and HPLC procedures, was 82%.

Calibration

After fitting the regression model the residual variance was $s_e^2 = 0.00192$ (10 d.f.). The sums of squares due to the five dummies were 0.01091; thus the $F_{5,10}$ statistic was (0.01090/5)/0.00192 = 1.13, which is far from significance. Further, the r^2 corresponding to the reduced model is 0.99140. This confirms that all the calibration graphs pass through the origin.

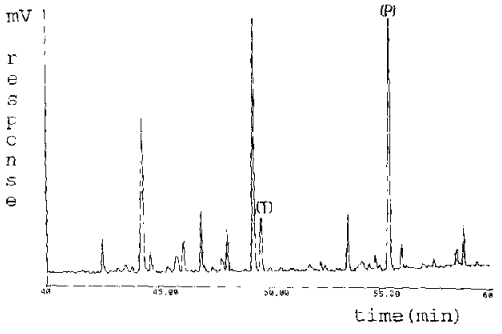

Fig. 3. Typical GC trace of urine after SPE–HPLC clean-up, spiked with 20α-hydroxyprogesterone (20 mg/μl) as internal standard. Peaks: T = testosterone; P = 20α-hydroxyprogesterone.

Precision

Table I gives the results in terms of mean, standard deviation and coefficient of variation. The variances in the four urine samples appear to be heteroscedastic (Bartlett's test: $\chi_3^2 = 24.3$). An approximately linear relationship between standard deviations and averages was found; this suggests that the coefficient of variation is the appropriate measure of precision. A pooled estimate of the latter was obtained by performing a one-way analysis of variance (ANOVA) after logarithmic transformation of the data. It has been shown [18] that $\sigma(\log x) = \sigma(x)/E(x)$. Therefore, the pooled "within" mean square in the above ANOVA is the square of the proper estimate of the coefficient of variation, *i.e.*, C.V. ≃ 7.9%, with 95% confidence limits of 6.1–11.4%.

Accuracy

The model expressed by eqn. 1 accounts for more than 98% of the total variability (r^2 = 0.982) and the pattern of weighted residuals (not given here) showed that the fit was satisfactory. The estimate of the coefficients, their standard errors and the result of the t-test for H_0: $\beta_1 = 1$ are given in Table II.

The estimate $\hat{\beta}_1$ differs from 1 by $6 \cdot 10^{-3}$ U only (t-test with 19. d.f. is far from significance); moreover, $\hat{\beta}_2$ is approximately equal to $\bar{y}_1 = 0.17125$. These statistical values indicate that the proposed method is accurate.

TABLE II

ACCURACY OF SPE–HPLC–GC MEASUREMENTS OF TESTOSTERONE IN URINE: RESULTS OF WEIGHTED MULTIPLE REGRESSION ANALYSIS

Parameter	Estimate	Standard error of estimate	t for H_0: parameter = 1	p value
Intercept	$\beta_0 = 0.0054$	0.0068		
Addition	$\beta_1 = 1.0060$	0.0415	0.1456	0.8858
Dilution	$\beta_2 = 0.1701$	0.0135		

DISCUSSION

Recent reports [19,20] indicate that SPE coupled with HPLC of the eluate makes a rapid and effective system for the extraction and purification of steroids from different biological fluids. The aim of this study was to validate a method for urinary testosterone measurement which combined an initial SPE–HPLC procedure with subsequent GC determination of the hormone. The results indicate that the extraction and purification of urinary testosterone by the SPE–HPLC technique is highly effective, requires small volume of solvent, allows high recoveries, is not time-consuming and consequently may be considered a suitable procedure.

The precision and accuracy of the proposed method were carefully checked by appropriate statistical analyses and found to be good.

In conclusion, our findings suggest that this method may be usefully employed for the routine measurement of urinary testosterone.

ACKNOWLEDGEMENT

The authors thank Dr. H. L. Ball (Italfarmaco, Italy) for his assistance in preparing the manuscript.

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