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Short communication

Determination of plasma testosterone using a simple liquid chromatographic method

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Abstract

A simple and sensitive high-performance liquid chromatographic (HPLC) method using ultraviolet detection was developed for the determination of testosterone in human plasma. Testosterone and the internal standard, griseofulvin, were extracted from 0.50 ml plasma sample using a mixture of dichloromethane–2,2,4-trimethylpentane (3:2, v/v). The mobile phase, consisted of 0.02 *M* sodium dihydrogenphosphate–acetonitrile–methanol (51:47:2, v/v) adjusted to pH 3.1 and delivered to a C_{18} analytical column (150×4.6 mm I.D., 4 μm particles) at a flow-rate of 1 ml/min while the detection wavelength was set at 240 nm with a sensitivity range of 0.005 a.u.f.s. The method has a quantification limit of 1.6 ng/ml. Recoveries of testosterone were all greater than 92% over the linear concentration range of 1.6–400 ng/ml while that of griseofulvin was approximately 95%. The within- and between-day RSD values were all less than 8% while the accuracy values ranged from 96.0 to 106.0% over the concentration range studied. The method was applied to the analysis of early morning plasma testosterone levels of 12 healthy human male volunteers. The levels were found to range from 3.1 to 8.4 ng/ml, within the normal range reported in the literature.
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1. Introduction

Testosterone (17β-hydroxyandrost-4-en-3-one) is a steroid secreted by Leydig cells of the testes [1]. Being the principal androgen, testosterone has been widely employed in androgen replacement therapy for the treatment of male hypogonadism [2,3].

Various analytical methods based on radioimmunoassay (RIA), high-performance liquid chromatography (HPLC) as well as gas chromatography (GC), have been used in the quantification of testosterone in plasma or urine following oral, transdermal or buccal administration. Wilke and Utley [4] and Slaats et al. [5] have reported methods that could yield rapid measurements using RIA. However, the specificity of these RIA methods could be compromised due to cross-reactivity with other steroids, and the concentration values obtained may vary considerably depending on the antibodies used [6]. In a method using ultraviolet detection described by Pascucci and Yeager [7], an elaborate sample preparation procedure involving 15 min of continuous shaking was required. Suzuki et al. [8] reported an automated system using ultraviolet detection but the

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method required a long run time of more than 20 min and between-run flushing of 10 min. Although Wintersteiger and Sepulveda [9] reported a method with relatively good sensitivity (12.5 ng/ml) and recovery (85.7–96.9%) using electrochemical detection, the method was rather complex involving a solid-phase extraction (SPE) procedure, pre-column derivatization and column switching. Moreover, methods employing electrochemical detection often encounter problems of baseline stability. In addition, the method was not evaluated for its accuracy, while the intra- and inter-assay precision was carried out using single concentration values of 110 and 125 ng/ml, respectively. On the other hand, Legrand et al. [10] have reported a GC–negative ion mass spectrometry method, which entailed SPE and derivatization. Besides being tedious, such a detection system may not be readily available in most laboratories.

This paper describes an inexpensive, simple, sensitive and specific HPLC method using ultraviolet detection, for the determination of testosterone in human plasma. The method was also applied in the analysis of early morning plasma testosterone levels of 12 healthy human male volunteers.

2. Experimental

2.1. Materials

Sodium dihydrogenphosphate dihydrate, sodium hydroxide pellets, methanol, acetonitrile (ACN) and dichloromethane were of AR or HPLC grade, obtained from Merck (Darmstadt, Germany). 2,2,4-Trimethylpentane and hydrochloric acid 37% of AR grade were purchased from Mallinckrodt (Paris, KY, USA) while testosterone and the internal standard, griseofulvin, purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Standard solutions

Stock solutions of testosterone and the internal standard, griseofulvin were prepared separately in methanol at a concentration of 100 μg/ml each. The stock solutions were then diluted with mobile phase to yield working solutions of testosterone and griseofulvin internal standard at concentrations of 10 and 1 μg/ml, respectively. Calibration curves of testosterone in plasma at 400.0, 200.0, 100.0, 50.0, 25.0, 12.5, 6.3, 3.1 and 1.6 ng/ml were prepared by serial dilution with human blank plasma.

2.3. Instrumentation

The HPLC system comprised a Jasco PU-980 pump (Jasco, Tokyo, Japan), a Gilson 119 UV–Vis detector (Gilson, Villiers-Le Bel, France), a Rheodyne 7125 sample injector fitted with a 50 μl sample loop (Rheodyne, CA, USA) and a Hitachi D-2500 chromato-integrator (Hitachi, Tokyo, Japan). A Genesis C_{18}, 150×4.6 mm I.D., 4 μm analytical column (Jones, CO, USA) was used for the chromatographic separation and was preceded by a refillable guard column (Upchurch Scientific, Oak Harbour, WA, USA) packed with Perisorb RP-18 (30–40 μm, pellicular). The mobile phase consisting of 0.02 *M* sodium dihydrogenphosphate–ACN–methanol (51:47:2, v/v), was adjusted to pH 3.1 using 5 *M* hydrochloric acid solution. The flow-rate was set at 1 ml/min and the detection wavelength was 240 nm with a sensitivity range of 0.005 a.u.f.s.

2.4. Sample preparation

A 0.5-ml aliquot of human plasma was accurately measured into a glass tube with a PTFE lined screw cap, followed by the addition of 100 μl of 1 μg/ml griseofulvin internal standard solution and 10 μl of 2 *M* sodium hydroxide solution. The mixture was vortex-mixed for 5 s, followed by the addition of 5 ml of dichloromethane–2,2,4-trimethylpentane (3:2, v/v) as the extraction solvent, and the mixture again vortex-mixed for 1 min, before being centrifuged at 2000 *g* for 15 min. The organic layer was transferred to a reactivial and evaporated to dryness at 35 °C under a gentle stream of nitrogen gas. The residue was reconstituted with 100 μl of mobile phase and 50 μl was injected into the HPLC system.

2.5. Extraction recovery, accuracy and precision

Samples were quantified using peak height ratios of testosterone versus the internal standard. Corrections were made for interference from endogenous

testosterone by subtracting the peak height ratio of endogenous testosterone over the internal standard obtained from blank plasma samples used in preparation of the calibration curves. Extraction recovery, within- and between-day precision and accuracy ($n=6$) of the method were determined using the testosterone plasma working standards, i.e., from dilutions different from those used for the calibration curves. The recovery of the extraction procedure for testosterone and the internal standard was calculated by comparing the peak height obtained from the spiked plasma samples with that of aqueous drug solutions of corresponding concentrations without extraction. The accuracy was reported as percentage of the measured concentration over that of the theoretical spiked value, whereas the precision was denoted using the relative standard deviation (RSD).

3. Results

Chromatograms obtained with blank plasma (in the presence of endogenous testosterone) and plasma spiked with 50 ng/ml testosterone and 1 μg/ml griseofulvin are shown in Fig. 1A and B, while Fig. 1C illustrates the chromatogram of a plasma sample obtained from a healthy human male volunteer. Except for the small peak attributable to the endogenous testosterone, the blank chromatogram was generally clean and devoid of interference from other endogenous compounds especially at the retention time of the internal standard.

Calibration curves ($n=6$), obtained by plotting the subtracted peak height ratio of testosterone to griseofulvin versus the plasma concentration of testosterone, were linear over the entire range of the calibration curve with a mean correlation coefficient of 0.99993, mean slope of 0.00442 and mean intercept of 0.00018. Regression data of these six calibration curves are shown in Table 1. The limit of quantification of this method was 1.6 ng/ml, being the lowest concentration used in the construction of the calibration curves.

The extraction recovery, within- and between-day precision and accuracy values of the assay method are presented in Table 2. It can be inferred from the table that the RSD values of both within- and between-day were all less than 8%, whereas the accuracy values ranged from 96.0 to 106.0% over the range of concentrations studied. The recovery of testosterone ranged between 92.2 and 102.5% while that of griseofulvin was approximately 94.7%.

The early morning plasma testosterone levels of 12 healthy human male volunteers were found to range from 3.1 to 8.4 ng/ml, being within the normal range reported in the literature [3,11].

4. Discussion

The presence of 2 parts (v/v) of methanol in the mobile phase facilitated a better separation between the griseofulvin internal standard and testosterone, with retention times of 6.46 and 7.96 min, respectively. In the absence of methanol in the mobile phase, the retention time difference between these two compounds was less than 1 min. On the other hand, as the consequence of the absence of any ionizable functional groups, the retention times of both testosterone and griseofulvin were unaffected by the increase in pH of the mobile phase from pH 3.0 to 6.5. However, when reducing the pH of the mobile phase to 3.1, the peak of the internal standard griseofulvin was found to be well resolved and free from interfering endogenous compounds. Above this pH value especially at about 3.5, an interference peak was observed.

During the assay development, a number of interfering endogenous peaks were obtained in the chromatograms of the blank plasma at the wavelength of 240 nm used for the quantification of testosterone. It was found that these peaks could be removed by adding 10 μl of 2 *M* sodium hydroxide solution into the plasma prior to adding the extraction solvent, resulting in a cleaner chromatogram as well as preventing rapid clogging of the guard column. However, the concentration of sodium hydroxide added was critical with respect to the recovery of these two compounds, especially griseofulvin. Increasing the volume of 2 *M* sodium hydroxide solution added, from 10 to 20 μl during the extraction process, gave rise to a more than 10% decrease in the recovery of griseofulvin. A 10 μl aliquot of 2 *M* sodium hydroxide solution was found optimal and was thus used in the sample preparation.

The total run time for each sample was set at 12.5

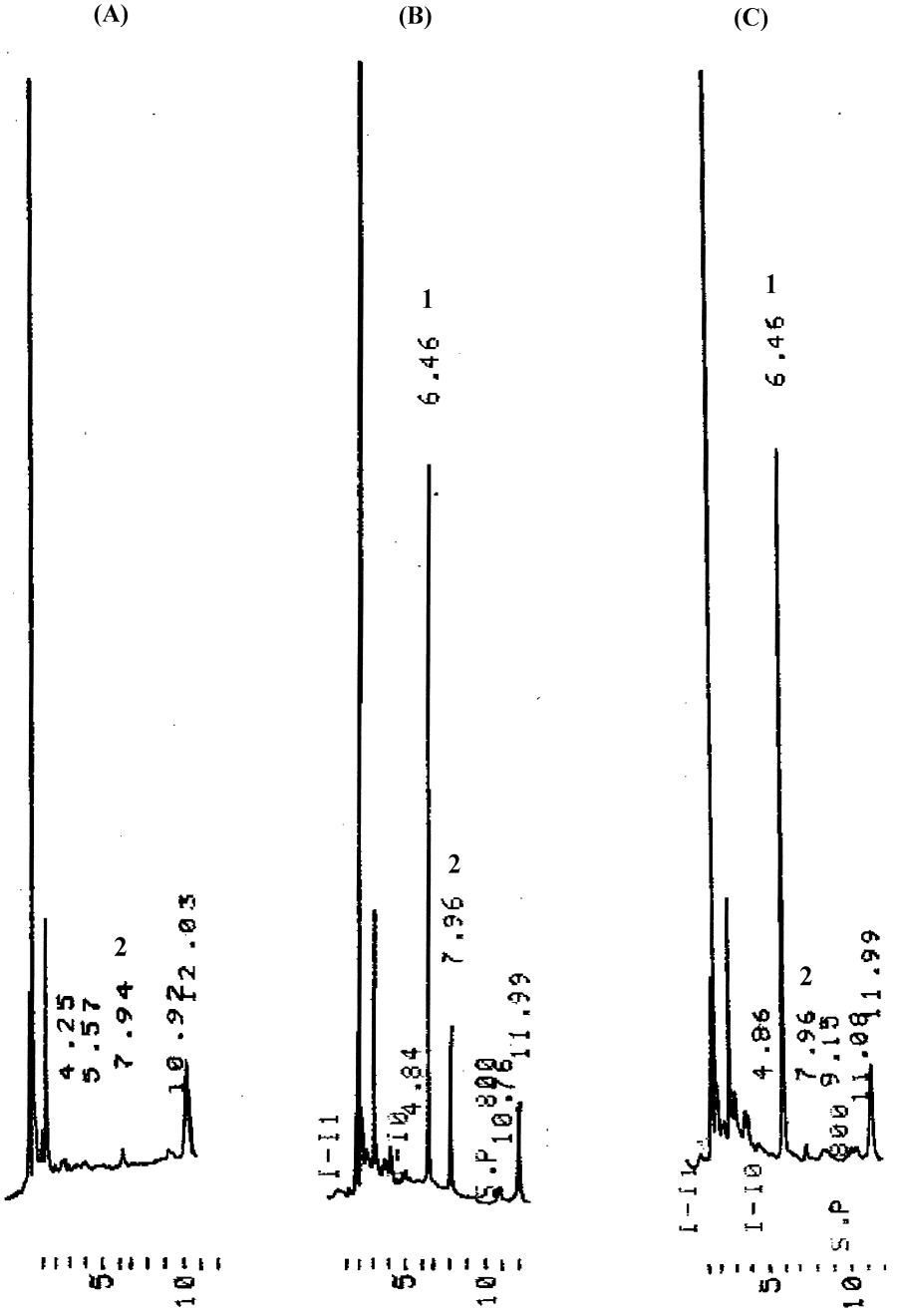

Fig. 1. Chromatograms for the analysis of testosterone in (A) blank plasma, (B) blank plasma spiked with 50 ng/ml testosterone and 1 μg/ml griseofulvin, (C) plasma of a healthy human male volunteer obtained in early morning containing 5.0 ng/ml testosterone (*y*-axis, attenuation 6; *x*-axis, chart speed 2.5 mm/min). Peaks: 1=griseofulvin, 2=testosterone.

Table 1
Regression parameters and statistics for calibration curves (n=6)

Calibration curve (n)	Correlation coefficient	Slope	Intercept
1	0.99986	0.00440	0.00014
2	0.99993	0.00444	0.00016
3	0.99993	0.00448	0.00016
4	0.99993	0.00444	0.00033
5	0.99998	0.00443	0.00017
6	0.99993	0.00434	0.00010
Mean	0.99993	0.00442	0.00018
SD	$3.98\cdot10^{-5}$	$4.81\cdot10^{-5}$	$8.12\cdot10^{-5}$
RSD (%)	$3.98\cdot10^{-3}$	1.09	45.7

Table 2
Extraction recovery, within- and between-day precision and accuracy (n=6)

Concentration (ng/ml)	Recovery		Within-day		Between-day	
	Mean (%)	RSD (%)	Precision (RSD, %)	Accuracy (%)	Precision (RSD, %)	Accuracy (%)
1.5625	102.5	6.4	7.8	96.0	7.2	99.9
3.125	99.9	5.6	4.5	103.5	4.0	99.9
6.25	101.6	5.0	1.5	105.0	6.7	97.1
12.5	99.9	3.5	4.8	105.9	3.7	103.3
25	92.2	2.0	2.3	98.7	2.7	101.9
50	94.8	1.8	1.2	98.9	2.7	97.6
100	100.8	2.2	1.8	106.0	4.3	98.7
200	94.7	1.8	0.7	99.3	1.9	98.6
400	92.9	2.4	0.6	100.1	3.2	99.6

min although both testosterone and griseofulvin were eluted within 8 min. This was done because a peak attributable to a compound from the sample matrix was observed to elute at about 12 min, as shown in Fig. 1.

Table 3 shows the recovery values of both testosterone and griseofulvin obtained with different composition of the extraction solvent. When dichloromethane was used alone, the recovery of both compounds was relatively low, being less than 80% for testosterone and less than 70% for griseofulvin. It is apparent from Table 3 that the extraction efficiency was increased by the addition of 2,2,4-trimethylpentane. With dichloromethane–2,2,4-trimethylpentane (3:2, v/v), the extraction efficiency was increased to more than 90% for both com-

Table 3
Effect of changing the extraction solvent composition on the extraction recovery of testosterone and griseofulvin (n=6)

Extraction solvent composition	1 μg/ml testosterone in plasma			1 μg/ml griseofulvin in plasma		
	Mean recovery (%)	SD	RSD (%)	Mean recovery (%)	SD	RSD (%)
Dichloromethane	78.7	2.3	2.9	68.2	2.1	3.1
Dichloromethane–2,2,4-trimethylpentane (4:1, v/v)	82.1	4.2	5.2	76.8	4.4	5.8
Dichloromethane–2,2,4-trimethylpentane (3:2, v/v)	91.4	2.6	2.9	94.7	2.1	2.2

pounds. Moreover, as mentioned above, relatively clean chromatograms could be obtained by prior addition of sodium hydroxide to the plasma samples.

5. Conclusion

In summary, the HPLC method using ultraviolet detection described here is simple, sufficiently specific and sensitive for the determination of testosterone in plasma. Moreover, the assay method has good accuracy and precision over the entire range of concentrations evaluated.

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