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Development and validation of a simple reversed-phase high-performance liquid chromatography method for the determination of testosterone in serum of males

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Abstract

An isocratic high-performance liquid chromatographic method with detection at 234 nm was developed, optimized and validated for the determination of testosterone in human serum. Propylparaben was used as internal standard. A Hypersil BDS RP-C_{18} column (150 mm × 4.6 mm, 5 μm), was equilibrated with a mobile phase composed of acetonitrile and water (35:65, v/v) and having a flow rate of 1 ml/min. The elution time for testosterone and internal standard was approximately 11.6 and 9.9 min, respectively. Calibration curves of testosterone in serum were linear in the concentration range of 1–20 ng/ml. Limits of detection and quantification in serum were 0.4 and 1.1 ng/ml, respectively. Recovery was greater than 92%. Intra- and inter-day relative standard deviation for testosterone in serum was less than 2.1 and 3.9%, respectively. This method was applied to the determination of testosterone serum levels of 12 healthy males and data were correlated with data obtained using a radioimmunoassay method.

Keywords: Testosterone; RP-HPLC; Serum; Liquid–liquid extraction (LLE)

1. Introduction

Testosterone (17β-hydroxy-4-androstene-3-one), a C_{19} steroid (Fig. 1A), is the most potent naturally secreted androgen. Serum testosterone levels substantially augment the clinical evaluation of a number of very common endocrine disorders. The measurement of circulating testosterone is clinically relevant in the investigation of androgen disorders in humans.

For measurements of total testosterone, commercial radio- and non radio-labelled immunoassay kits [1] as well as automated platform immunoassays that mostly use chemiluminescent detection are available [2–4]. However, although they are economical and rapid, they have limited published validation data raising questions about accuracy and/or specificity [1,2,4]. Recently, comparison of commonly used automated immunoassay instruments with measurements performed by liquid chromatography–tandem mass spectrometry showed that although immunoassay methods could distinguish eugonadal from hypogonadal males, the male reference ranges need to be established for each individual laboratory as they show substantial variability [4]. The reasons for the lack of agreement among immunoassays are the matrix effect (immunoassays are performed without extraction steps) [1,2] and the cross-reactivity of the antibody used [1].

Based on the above data, the need to develop alternative methods is essential. Various chromatographic methods using liquid chromatography–tandem mass spectrometry [5], gas chromatography–mass spectrometry [6] and other sophisticated techniques have been proposed [7] which, although precise and accurate, they are not readily available in many laboratories. Testosterone has also been determined in biological samples by high-performance liquid chromatography (HPLC) [8–15]. However, these methods involve complicated instrumentation, e.g. special collector and/or HPLC coupled with radioimmunossay [8,9,12,15], gradient elution [9,13], and/or time-consuming experimental procedures including complicated solid phase extraction steps [10,11] and increased total elution times [9,10,13].

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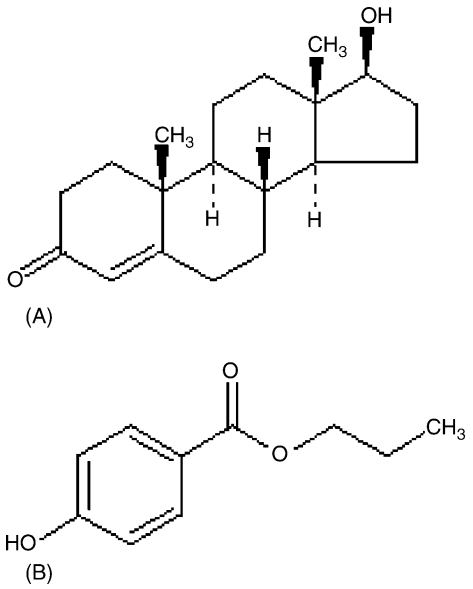

Fig. 1. Structure of testosterone (A) and propylparaben (B).

Our aim was the development of an optimized simple isocratic reversed-phase HPLC method with ultraviolet spectrometric detection for the determination of testosterone in serum samples of males. Apart from its usefulness to clinical laboratories in the evaluation of biological activity of the testis and in the monitoring of patients with metastatic prostate [16], such method can also provide an easy tool for the evaluation of bias of immunoassays [1,2,4].

The present work includes an extensive solvent optimization of the liquid–liquid step that is missing from other similar studies (e.g. [5,9,14]). Also, unlike previous methods [14], the proposed method uses a simple mobile phase (acetonitrile and water) without need of pH control. Finally, the proposed method was developed using testosterone-free serum [17,18]. Therefore, unlike previously developed HPLC-UV methods [14], corrections for endogenous testosterone (that becomes critical at low concentration levels) were not required.

2. Experimental

2.1. Instrumentation

The chromatographic system consisted of a Spectra System P1000 pump, a Spectra System UV 2000 absorbance detector and an autosampler AS 3000. The above system was controlled by a Specta System Controller SN 4000 and a software package Chromquest (Thermoquest Inc., San Jose, USA). A Hettich centrifuge Universal 32R (Tuttlingen, Germany) was utilized to centrifuge serum samples and a Techne Dri-Block® DB-3 (Cambridge, UK) sample concentrator for evaporation of samples.

2.2. Chemicals and reagents

All chemicals were of analytical purity grade. Acetonitrile, methanol, dichloromethane, iosooctane, isoamylic alcohol, pentane and hexane of HPLC grade were purchased from E. Merck (Darmstadt, Germany). Diethylether and isopropanol were purchased from Panreac Quimica SA (Barcelona, Spain). Testosterone was purchased from Sigma–Aldrich Inc. (St. Louis, USA). Propylparaben (4-hydroxybenzoic acid propyl ester, $C_{10}H_{12}O_3$,) was used as internal standard (IS) and was of analytical purity grade. Water purified with Labconco water pro ps system (Kansas City, Missouri, USA) was used in all procedures. Testosterone RIA DSL-4100 was from Diagnostics Systems Laboratories UK Ltd. (Oxon, UK).

2.3. Serum samples

Serum from healthy female dogs (testosterone-free serum) was used as blank; testosterone levels in serum of female dogs are less than 0.3 ng/ml [17,18]. The dogs are hosted at an animal facility that operates in the Laboratory of Biopharmaceutics and Pharmacokinetics (Faculty of Pharmacy, National and Kapodistrian University of Athens) according to European Union regulations for the maintenance and experimentation on animals and which has been approved by the Veterinary Directorate of the Municipality of Athens.

All blood samples were drawn into test tubes and centrifuged at 4000 rpm (1828 g) for 30 min. The serum was transferred into test tubes and frozen at −20 °C until analysis [19].

Serum samples were collected from 12 healthy human males (18–50 years of age). All subjects gave their written informed consent before blood venipuncture.

2.4. Chromatographic conditions

A reversed-phase Hypersil BDS-C_{18} column (150 mm × 4.6 mm, 5 μm particle size) equipped with a precolumn Hypersil BDS-C_{18} (10 mm × 4 mm, 5 μm particle size) was used. Mobile phase was composed of acetonitrile and water (35:65, v/v) and it was degassed for 10 min with helium at a degassing rate of 20 ml/min. Its flow rate was 1 ml/min. Injection volume was 50 μl. Experiments were performed at ambient temperature. Absorbance was measured at 234 nm, wavelength that was optimum for testosterone and satisfactory for IS. The elution times of testosterone and IS in serum samples were approximately 11.6 and 9.9 min, respectively.

2.5. Solution preparation

2.5.1. Stock solutions

A stock solution of testosterone (100 μg/ml) was prepared by dissolving 10 mg of this compound in 100 ml of methanol. A stock solution of IS (100 μg/ml) was prepared by dissolving 10 mg of propylparaben in 100 ml of methanol. Stock solutions were stored at 4 °C and were stable for at least 2 weeks.

2.5.2. Standard solutions

Testosterone working standard solutions were prepared in the concentration range of 1–20 ng/ml for construction of calibration curves, evaluation of the precision of the analytical method and estimation of limits of detection and quantification. All

dilutions to volume were performed with methanol. Calibration curves were performed either with solutions of standards in mobile phase or with spiked serum standards. Concentration of the working solution of IS was 1 μg/ml.

2.6. Analysis of serum samples

Two millilitres of serum sample were transferred in a centrifuge tube and 50 μl of working solution of IS (1 μg/ml) were added. After vortexing for 30 s, 5 ml of diethylether containing 2% isopropanol were added and the new mixture was vortexed for 1 min. Then, the tubes were centrifuged for 10 min in 4000 rpm (1828 g) at 10 °C. The clear organic phase was evaporated to dryness under nitrogen stream at 40 °C. The residue was redissolved in 100 μl of methanol and vortexed for 1 min. Then, 100 μl of mobile phase were added and the solution was vortexed for 1 min. Finally, part of the last solution was injected into the HPLC system.

2.7. Method validation

2.7.1. Calibration curves

Aliquots of testosterone-free pooled canine serum were used for preparation of spiked serum standards. Blank (testosterone-free) serum (1950 μl) were transferred to a centrifuge tube and 50 μl of testosterone working solution were added to it. Afterwards, the procedure described above in Section 2.6 was followed with vortexing for 1 min. Calibration curves for testosterone were constructed in the concentration range of 1–20 ng/ml ($n = 8$, each point was the mean of three experimental measurements) by measuring peak-area ratios of testosterone standards to IS.

Regression equations were obtained through unweighted least-square linear regression analysis and applied to peak-area ratios as a function of testosterone concentration. Also, calibration curves of serum testosterone standards obtained in a very low concentration region (1–5 ng/ml) were constructed for the calculation of the limit of detection and the limit of quantification.

2.7.2. Recovery

Known amounts of testosterone and constant amounts of IS standard solutions were added to pooled serum from female dogs (i.e. to pooled testosterone-free serum) so that concentrations of 2, 6 and 20 ng/ml were obtained that were analyzed using the procedure described in Section 2.6 (i.e. actual injected testosterone concentrations were 20, 60 and 200 ng/ml). Each sample was prepared in triplicate. The ratios of peak-areas of testosterone to IS in serum were compared with the corresponding ratios in mobile phase.

2.7.3. Precision

The precision of the proposed HPLC method was studied by estimating intra- and inter-day relative standard deviation (R.S.D.) of values using standards in serum of female dogs (low, medium and high concentrations) and samples from male humans in triplicate.

2.7.4. Accuracy

Known amounts of testosterone and constant amounts of IS standard solutions were added to serum samples from human males that had already been analyzed so that the concentration of testosterone in serum samples were increased by 2, 4, 6, 10 and 20 ng/ml. Analysis of the resulting samples was performed using the procedure described in Section 2.6 and the percentage recovery of the added testosterone was calculated. Each sample was prepared in triplicate.

3. Results

3.1. Mobile phase

In order to optimize the elution of testosterone several eluent mixtures were tested. A mixture of acetonitrile–water (35:65, v/v) was the simplest and also the most appropriate mobile phase for the elution of testosterone from serum. The elution time of testosterone was approximately 12 min, i.e. quite reasonable for an analysis in serum. By increasing the amount of acetonitrile, the total elution time was decreasing but values of resolution of peaks became smaller because testosterone peak was moving closer to the serum background peaks. It was also noticed that the use of buffers or the adjustment of the pH of the mobile phase was not necessary because it did not improve any analytical parameter (e.g. resolution or elution time).

3.2. Choice of internal standard

Several substances were tested as internal standards. Among these, propylparaben (Fig. 1B) met all the typical requirements of a compound to be used as IS, i.e. it was stable during the analysis, it was easily available, it is not an endogenous substance, its elution time was shorter than that of testosterone, and its peak did not interfere with the matrix of serum samples. The extraction recovery value for the IS was greater than 95%.

3.3. Treatment of samples

For the isolation of testosterone from serum samples liquid–liquid extraction was used. Extraction conditions were optimized by using several solvents such as dichlomethane, hexane, pentane, diethylether, isopropanol, isooctane, isoamylic alcohol and combinations of them (Table 1). Among these, diethylether gave high recovery. The addition of 2% isopropanol to diethylether increased the extraction recovery and decreased the number of interfering endogenous peaks. Precipitation of serum proteins with the addition of sodium hydroxide, hyperchloric acid or hydrochloric acid, did not increase the recovery of testosterone. Therefore, diethylether containing 2% isopropanol was proved to be a simple (no need to add any mean of precipitation), rapid (diethylether is easy to evaporate) and efficient way (recovery not less than 92%) for extracting testosterone from serum. Also, it improved selectivity and sensitivity of the chromatographic assay whereas compared with other tested solvents (Table 1) it practically did not affect the life-time of column.

Table 1
Recovery data for the determination of testosterone in serum (10 ng/ml) using different extraction solvents

Solvent used for extraction	Mean% recovery ± S.D.[a]
Dichlomethane	60.9 ± 5.0
Dichloromethane-isooctane 60:40, v/v	68.1 ± 4.9
Isooctane	30.7 ± 6.9
Pentane	40.9 ± 5.9
Pentane:isoamylic alcohol 98:2, v/v	65.5 ± 3.2
Hexane	45.0 ± 7.2
Hexane-isoamylic alcohol 98:2, v/v	52.9 ± 4.3
Diethylether	80.9 ± 3.2
Diethylether–isooctane 50:50, v/v	85.7 ± 3.4
Diethylether-isoamylic alcohol 98:2, v/v	83.1 ± 2.1
Diethylether–isopropanol 98:2, v/v	92.3 ± 1.4

[a] S.D. is the standard deviation of the mean% recovery; standard solution was prepared and measured in triplicate.

Optimization of the volume of diethylether was also performed; 5 ml of diethylether containing 2% of isopropanol was the optimum volume for testosterone to be extracted from 2 ml serum. Higher diethylether volumes unreasonably increase the time of vaporization of samples.

The reconstitution step of the evaporated samples was also optimized. One hundred microlitres of methanol was the optimum volume for dissolving the amount of testosterone after the vaporization. Further, the addition of mobile phase ensured the compatibility of the injected samples with the "chromatographic system".

The extraction and reconstitution steps were very crucial because not only quantitative isolation of testosterone was accomplished but also preconcentration of injected solutions (by 10 times) was achieved.

3.4. Selectivity

Typical chromatograms of serum from a female dog (testosterone-free serum) and of serum of a female dog spiked with testosterone (3 ng/ml) and IS (25 ng/ml) are shown in Fig. 2A and B, respectively. Good resolution for the two peaks was assured by the R_s values, which were greater than 2. Also, both testosterone and IS peaks were symmetrical (asymmetry factors were between 1.05 and 1.10).

3.5. Method validation

3.5.1. Calibration curves

Linear calibration curves for testosterone were obtained throughout the concentration range studied. Regression analysis was performed for the ratios of peak-areas of testosterone to that of the IS (y), versus testosterone concentration (x). The results are presented in Table 2.

3.5.2. Recovery

The results are summarized in Table 3. Similar results were obtained when the recovery of the method was estimated by comparing the slopes of calibration curves in mobile phase and in serum (data not shown).

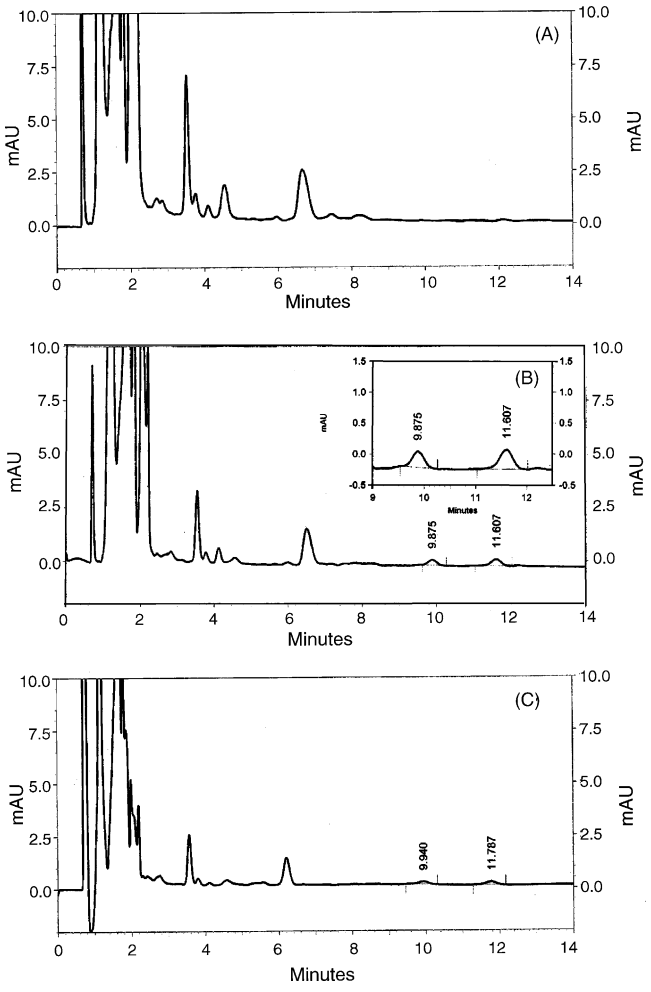

Fig. 2. Typical chromatograms of blank serum from a female dog (testosterone-free serum) (A), of serum from a female dog spiked with testosterone (3 ng/ml) and IS (25 ng/ml) (B), and of serum of a healthy male volunteer spiked with IS (25 ng/ml) (C). Retention times for IS and testosterone were 9.9 and 11.6 min, respectively. The chromatographic conditions were: BDS RP-C_{18} column (150 mm × 4.6 mm), mobile phase acetonitrile–water (35:65, v/v), flow rate 1 ml/min, detection wavelength 234 nm and room temperature.

Table 2
Analytical parameters of calibration curves of testosterone in serum of female dogs in the concentration range of 10–200 ng/ml of injected solution

Regression equation[a]		
Intercept, $a \pm$ S.D. ($\times 10^4$)	Slope, $b \pm$ S.D. ($\times 10^5$)	Correlation coefficient[b] (r)
486 ± 36	4983 ± 45	0.9998
424 ± 96	4904 ± 62	0.99991
523 ± 56	4926 ± 78	0.9997

Considering the 10 times preconcentration, the corresponding range in serum was 1–20 ng/ml.

[a] Linear unweighted regression analysis, with a regression equation $y = a + bx$, where y is the peak-area ratio of testosterone to IS and x is testosterone concentration in ng/ml. S.D. is the standard deviation of intercept and slope.

[b] The number of points in each calibration curve was eight and each point was the mean of three experimental measurements.

Table 3
Recovery data (% extraction yield) for the determination of testosterone in serum

Concentration (ng/ml)		Mean% recovery ± S.D.[a]
Serum	Injected	
2	20	98.2 ± 2.1
6	60	101.2 ± 1.5
20	200	99.3 ± 1.9

[a] S.D. is the standard deviation of the mean% recovery; standard solutions were prepared and measured in triplicate.

3.5.3. Precision and accuracy

Intra-day relative standard deviation for standards in serum from female dogs and for samples from serum of male humans were less than 2.1 and 3.8%, respectively, while the corresponding inter-day values were less than 3.9 and 4.2%, respectively.

Accuracy data are summarized in Table 4.

3.5.4. Robustness and ruggedness

Although the use of IS eliminates variations related to the liquid–liquid extraction procedure, robustness of the proposed method was assessed with respect to small alterations in several experimental parameters which were slightly different from day to day. Deliberate changes in mobile phase from acetonitrile–water (35:65, v/v) to (33:67, v/v) and (37:63, v/v) did not change the results for testosterone more than 1.8%. During these changes, the elution time (t_R), the asymmetry factor (a) and the resolution (R_s) remained statistically unchanged. Similar observations were made after changing the volume of diethylether used for liquid–liquid extraction from 5 ml to 4.5 and to 6 ml and the centrifugation time from 10 min to 9 and to 11 min. All these changes did not alter the resulting testosterone concentration more than 4%. This figure is within the R.S.D. range of the method.

Ruggedness of the developed method was assessed by the between-days precision because it included changes in reagents, chemicals and solvents. Moreover, using two different columns of the same type (Hypersil BDS RP-C_{18}), the parameters t_R, a and R_s of the chromatographic peaks remained statistically unchanged whereas the estimated testosterone concentrations varied by up to 3%.

3.5.5. Limits of detection (LOD) and quantification (LOQ)

LOD was defined as the analyte concentration that gives a signal equal to $y_b + 3.3s_b$, where y_b is the signal of the blank and s_b is its standard deviation. Similarly, LOQ was defined as $y_b + 10s_b$. In an unweighted least-squares method is quite suitable in practice to use the statistic $s_{y/x}$ [20] instead of s_b and the value of the calculated intercept a instead of y_b. Values of $s_{y/x}$ and b were calculated from calibration curves of serum testosterone standards obtained in a very low concentration region in serum (1–5 ng/ml). Thus,

$$\mathrm{LOD} = \frac{3.3s_{y/x}}{b} \quad \text{and} \quad \mathrm{LOQ} = \frac{10s_{y/x}}{b}$$

where b is the slope of the regression line.

Based on the above equations, the calculated LOD value was 0.4 ng/ml while the LOQ was 1.1 ng/ml in serum.

Table 4
Accuracy data for the determination of testosterone in serum of human males

Concentration[a] (ng/ml)	Mean% recovery ± S.D.[b]
2	97.6 ± 4.1
4	99.7 ± 1.3
6	102.6 ± 3.2
10	99.3 ± 2.3
20	100.4 ± 1.1

[a] These concentrations were achieved by standard additions of testosterone into samples with known testosterone levels.

[b] S.D. is the standard deviation of the mean% recovery; standard solutions were prepared and measured in triplicate.

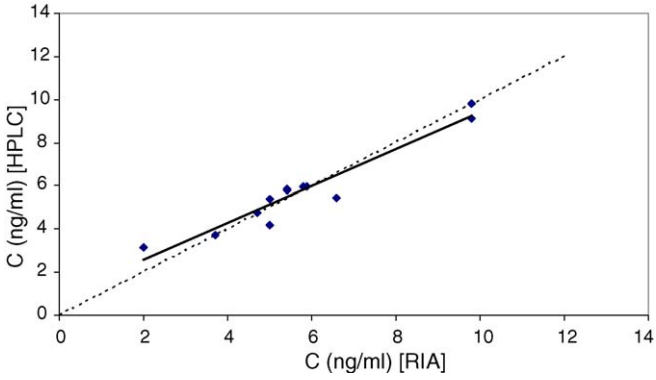

Fig. 3. Correlation between serum testosterone levels of 12 healthy male volunteers measured with the HPLC-UV method developed in this study and the testosterone levels measured with the DSL-RIA kit (slope of regression line: 0.85; r^2: 0.93, $n = 12$).

3.6. Application to human male serum samples

Concentrations of testosterone in male sera ranged between 3 and 10 ng/ml. A typical chromatogram of serum of a healthy male volunteer spiked with IS (25 ng/ml) obtained under the optimized conditions is shown in Fig. 2C.

4. Discussion

Advantages and novelty of the proposed isocratic method relatively to existing methods are its simplicity in instrumentation [9,12] and in the experimental procedures (i.e. the single liquid–liquid extraction step procedure for shorter sample preparation periods [10,11], short elution time of testosterone [9,10,13], addition of a proper internal standard for increased accuracy and precision and lower limits of detection [14]).

It is known that commonly used automated immunoassay techniques may lead to over- or under-estimation of actual serum testosterone levels [1,4]. The developed HPLC method can, therefore, serve as a cheap and easy tool for the evaluation of the bias of immunoassay methods used for the determination of testosterone levels in males. Fig. 3 shows a preliminary attempt for the evaluation of bias of the DSL-RIA kit. Although confirmation using more samples is required, the slope of the regression line in Fig. 3 suggests that the DSL-RIA kit

may slightly underestimate actual testosterone levels of healthy humans (slope: $0.85 < 1$).

5. Conclusion

A simple, fast and reliable reversed-phase isocratic HPLC method with UV spectrometric detection has been optimized and validated for the determination of testosterone in human male serum. Compared to previously developed HPLC methods, the advantages of the proposed method include simplified single-step liquid–liquid extraction procedure (i.e. better recovery and shorter sample preparation periods), use of IS (i.e. improved precision and accuracy) and low quantification limits (due to the preconcentration of injected solutions).

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