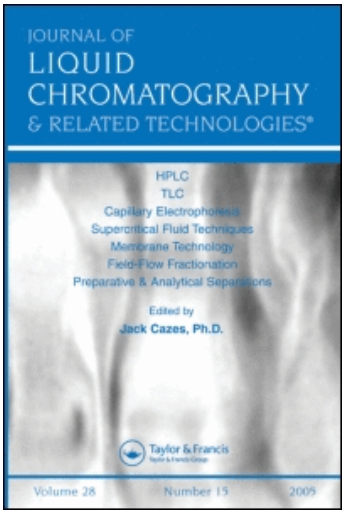

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Simultaneous Determination of Testosterone and its Major Metabolite Epitestosterone in Biological Fluids by HPLC

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Abstract: A reversed-phase high performance liquid chromatographic (HPLC) method is developed and validated for the simultaneous determination of anabolic steroids: testosterone and its major metabolite, epitestosterone. The analytical column, Inertsil ODS-2, 5 μm, 250 × 4 mm, was operated at ambient temperature. Isocratic elution was performed using 35% of A = buffer solution 0.11% CH_3COOH-CH_3COONa 7.5 mmol/L, pH = 4 and 65% of B = CH_3CN, at a flow rate of 0.8 mL/min. Inlet pressure was 155 bar. UV detection was performed at 236 nm.

The limit of detection was 0.02 ng per 20 μL injection volume, while linearity held up to 2 ng/μL. p-Cresol was used as internal standard at a concentration of 2 ng/μL. Validation of the method was performed in terms of accuracy and precision: intra-day assay (n = 6) and inter-day assay (n = 3 × 5) and was found to be satisfactory, with high accuracy and precision results.

The method was successfully applied to biological fluids after solid phase extraction (SPE) on Nexus cartridges. Recovery rates from blood plasma ranged between 92.0% and 107.5% for testosterone and between 82.5% and 98.8% for epitestosterone, while the analysis of urine provided recovery rates from 85.0% to 108.0% for testosterone and from 85.5% to 103.0% for epitestosterone. The developed method was applied to the analysis of urine samples of one female and four male volunteers.

Keywords: Testosterone, Epitestosterone, Biological fluids, Plasma, Urine, HPLC

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INTRODUCTION

Steroid hormones share cholesterol (Fig. 1a) as their common precursor and can be divided into three main physiological groups: the gonadal steroids produced in the ovary (estrogens and progestins) and testis (androgens), the adrenal steroids produced in the adrenal cortex (glucocorticoids and mineralcorticoids) and the calcium regulating sterols (calciferols). Testosterone is the most important gonadal steroid, responsible for the secondary sex characteristics in the male and supports spermatogenesis in synergy with follicle stimulating hormone (FSH).

Since 1930, many groups of workers have synthesized steroid hormone analogues to obtain synthetic hormones with improved therapeutic properties for the control of pituitary function, for substitution therapy in deficiency syndromes, such as hypogonadism or after the menopause, and particularly for the control of ovulation (contraceptive steroids). In general, the pharmacological activity of synthetic gonadal steroids is never limited to interaction with a particular type of receptor (i.e., estrogenic, androgenic, progestagenic).

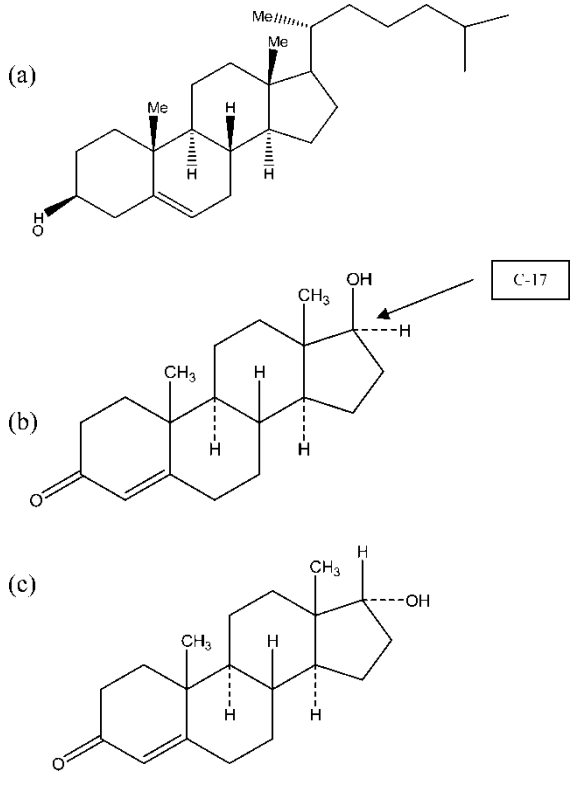

Figure 1. Chemical structures of: (a) Cholesterol, (b) Testosterone, (c) Epitestosterone.

Steroidal and non-steroidal receptor antagonists have also been developed. These competitively inhibit the receptor binding and the biological effects of natural androgens, estrogens, and progestins. The steroidal antiandrogen cyproterone acetate and the non-steroidal antiandrogen flutamide are of particular importance.[1]

Testosterone (17β-hydroxyandrost-4-en-3-one) (Fig. 1b) is a steroid secreted by Leydig cells of the testes and in hepatocytes. Testosterone is insoluble in water but can be diluted in organic fluids like alcohol or ether. This principal male hormone has many metabolites that are produced after an oxidation in site C-17. It is metabolized in-vivo in vital organs like prostate, and it is excreted in the form of glucoside or sulphide.[1,2]

The therapeutic uses of testosterone in men include the treatment of hypogonadism, eunuchoidism, and impotence; in women it is used for the treatment of metastatic breast cancer, postpartum breast engorgement, and vasomotor symptoms of the climacteric. When consumed in large doses, testosterone has numerous side effects in man, which include an increase in the retention of nitrogen, sodium, and water, oedema, increased vascularity of the skin, hypercalcaemia, impaired glucose tolerance, bone growth, and skeletal weight. Other effects include increased low density lipoprotein cholesterol, haematocrit, and increased risk of heart disease. Additionally large doses of testosterone in men suppress spermatogenesis and cause degenerative changes in seminiferous tubules. In women, the inhibitory action on the activity of the anterior pituitary results in suppression of ovarian activity and menstruation. Testosterone's commercial names are: androlin, malestrone, perandren, synadrol F, testoviron T, testryl.[1]

Epitestosterone (17α-hydroxy-4-androsten-3-one) (Fig. 1c) is a naturally occurring epimer of testosterone. It was reported for the first time in 1947 as an androgen metabolite on incubation with rabbit liver slices. Epitestosterone is also a naturally occurring fytosteroid, in the pollen of pine *Pinus Silvestris*. It is formed from testosterone and androstenedione in vitro by rabbit liver and kidney, dog liver, mare's ovary, ox and sheep blood, guinea pig liver, kidney, ovary, and testes, but not by adrenal slices. In humans, epitestosterone is excreted in the urine mainly as glucuronide, which has been reported to increase after intravenous administration of a very large amount of testosterone.

Urinary excretion of epitestosterone is slightly lower than that of testosterone, being 200–500 nmol per day in males and 80–500 nmol per day in females. Plasma concentrations of epitestosterone are age dependent and approximate an average of 2.5 nmol/L in adult men and 1.2 nmol/L in women.

The ratio of epitestosterone/testosterone in the blood circulation is high in childhood, epitestosterone being more abundant than testosterone before the age of 10 years. Later on, testosterone prevails. The plasma concentration of epitestosterone in women exhibits a peak, with a maximum around 20 years of age, followed by continuous decline up to menopause, and then a pronounced increase in postmenopausal women. In men, plasma concentrations

of epitestosterone exhibited a peak with a significant maximum around 35 years of age followed by continuous decrease later.

Epitestosterone in urine has attracted attention as a reference substance in the doping control of testosterone abuse. The almost constant ratio of urinary testosterone to epitestosterone ($1.1-1.5 \pm 1.0$, within a range of 0.03–4.9) in adults became the basis of the method of detection of exogenously administered testosterone, since epitestosterone does not originate from testosterone in significant amounts in humans. The maximum permissible testosterone to epitestosterone arbitrary ratio in the urine of normal men not suspected for doping has the critical value of 6. The International Olympic Committee adopted this ratio for its accredited laboratories as an arbitrary critical value, as the unique test for illicit testosterone self-administration. Since introduction of the testosterone to epitestosterone ratio in doping analysis, the parameters that may or may not influence this ratio and possibly lead to false positive results have been intensively debated. It has been believed that epitestosterone is virtually devoid of any biological activity, especially as no androgenic action could be demonstrated. A marginal note existed indicating that epitestosterone is an inhibitor of 5-reductase.[3]

Various analytical techniques have been applied to the determination of anabolic steroids and several analytical methods can be found in the literature for the analysis of biological matrices, as well as pharmaceuticals and dietary supplements. Most of the reported methods involve RP-HPLC with UV detection and isocratic or gradient elution. High performance liquid chromatography coupled to mass spectrometry was also applied to the determination of testosterone and its metabolite epitestosterone at ppb levels in human biofluids (urine, plasma, plasma, and whole blood), as well as other tissues and fluids.[4–12]

Liquid chromatography-electrospray ionization-ion trap- Mass Spectrometry (LC-MS-MS) has been used for the determination of metabolites from multiple cytochrome P450 probe substrates.[11]

Other chromatographic techniques used for testosterone and epitestosterone determination include gas liquid chromatography, as well as GC/MS.[13,14]

GC-MS methods have been reported for the quantitative determination of the anabolic steroids in nutritional supplements.[14] Testosterone and epitestosterone conjugates in urine samples have been analyzed by LC linear ion trap mass spectrometry.[15]

Sample preparation techniques used for testosterone and epitestosterone determination include SPE in plasma and urine, LLE,[2,4] enzymatic hydrolysis,[4] extraction with ethyl acetate in cell microsomes,[9] incubation of microsomes with testosterone.[12] Finally, for the extraction of steroids hormones in human urine aqueous two-phase systems of ionic liquid and salt have been used.[16]

The aim of the present study was to develop a rapid, accurate, and sensitive method for the simultaneous determination of testosterone and its major metabolite epitestosterone in biological fluids: blood plasma and urine.

EXPERIMENTAL

Instrumentation and Chromatography

A Shimadzu (Kyoto, Japan) LC-10AD pump was used to deliver the mobile phase to the analytical column, Inertsil ODS-2 (5 μm, 250 × 4 mm by MZ Analytical, Mainz, Germany). Sample injection was performed via a Rheodyne 7125 injection valve (Rheodyne, Cotati California, U.S.A) with a 20 μL loop. Detection was achieved by an SSI 500 UV-vis detector (SSI, State College, PA, U.S.A.) at a wavelength of 236 nm and a sensitivity setting of 0.002 AUFS. Data acquisition was performed using software designed for chromatography, developed by Professor P. Nikitas (Laboratory of Physical Chemistry, Chemistry Department of University of Thessaloniki). A glass vacuum filtration apparatus obtained from Alltech Associates was employed for the filtration of the buffer solution (0.11% CH_3COOH-CH_3COONa 7.5 mmol/L, pH = 4) using 0.2 μm membrane filters (Schleicher and Schuell, Dassel, Germany). Degassing of solvents was achieved by helium sparging before use. Dissolution of compounds was enhanced by sonication in a Transonic 460/H Ultrasonic bath (Elma, Germany). A Glass-col, Terre Haute, IN 47802 small vortexer and a Hermle centrifuge, model Z 230 (B. Hermle, Gosheim, Germany) were employed for sample pretreatment. All evaporations were performed with a 9-port Reacti-Vap™ evaporator Pierce, Model 18780 (Rockford, IL, USA). The UV spectra of testosterone and epitestosterone for selecting the working wavelength of detection were taken using a Varian DMS 100S UV–Vis double-beam spectrophotometer (Varian, Inc. Corporate Headquarters, Palo Alto, CA, USA).

Mobile phase was a 35:65 v/v mixture of buffer solution 0.11% CH_3COOH–CH_3COONa 7.5 mmol/L, pH = 4 and acetonitrile. Inlet pressure observed at a flow rate of 0.8 mL/min was 155 bar. The injection volume was 20 μL. p-Cresol was used as internal standard at a concentration of 2 ng/μL.

Samples, Chemicals, and Reagents

Testosterone was supplied by Riedel-de-Haen (Seelze, Germany), while epitestosterone and p-Cresol were from Sigma-Aldrich, (St. Louis, MO, USA).

Methanol and acetonitrile of HPLC grade were purchased from Carlo Erba (Rodano, Italy). Acetic acid (99.8%) and sodium acetate were supplied by Riedel-de-Haen (Seelze, Germany). Ultrapure water obtained by a Milli-Q® purification system (Millipore, Bedford, MA, USA) was used throughout the study. Two types of SPE cartridges were used for sample preparation: Nexus Abselut (30 mg/cm^3) by Varian (Harbor City, CA, USA) and C_{18} (500 mg/ 3 mL) Discovery by Supelco, (Bellefonte, PA, USA).

Plasma samples were kindly provided from the Blood Donation Unity of a State Hospital, while urine samples were provided by healthy volunteers.

Stock standard solutions (100.0 ng/μL) were prepared in methanol. Working methanolic standards were prepared by the appropriate dilution at 0.02, 0.05, 0.5, 1.0, 2.0 ng/μL. These solutions when kept in −18°C were found to be stable for 2 months.

A methanolic solution of p-Cresol at a concentration of 2 ng/μL was selected as the most suitable internal standard.

Validation of the Method

Method validation was performed in terms of selectivity, linearity, repeatability, intermediate precision, accuracy, and sensitivity.

The linearity response was assessed in the range of 0.02–2 ng/μL using standard solutions and control plasma and urine samples. Peak areas were measured versus peak area of the internal standard. Method validation regarding accuracy and precision was achieved by replicate injections of spiked biological samples at three concentration levels: 0.5, 1.0, and 1.5 ng/mL for urine and 0.5, 1.0, and 2.0 ng/mL for plasma. Within-day repeatability was checked by six replicates of spiked biological samples. Intermediate precision study was conducted during routine operation of the system over a period of five consecutive days. Accuracy was assessed in terms of recovery.

The sensitivity of the developed method was checked in terms of limits of detection (LOD) and quantitation (LOQ). The LOD was defined as the compound concentration that produced a signal-to-noise ratio greater than three. The limit of quantitation of the assay was evaluated as the concentration equal to ten times the value of the signal to- noise ratio. This was considered as the lowest concentration in the calibration range.

Sample Preparation

Solid Phase Extraction (SPE)-Sample Extraction Procedure

Two different sorbents were assayed for the extraction of the investigated steroids: C_{18} (500 mg) Discovery by Supelco and Nexus by Varian. Different extraction protocols were assayed using various washing and eluting solvents. Optimum extraction protocol in terms of highest extraction efficiency involves conditioning with 2 mL of methanol and 2 mL of water. No washing step was applied since a significant analytes' loss was noticed. Elution was performed by 2 mL of methanol. An optimal extraction protocol was then applied to standard solutions of testosterone and epitestosterone in biological fluids.

Blood Plasma and Urine

Aliquots of 50 μL human blood plasma were spiked with 200 μL of androgens solution at different concentration levels and treated with 200 μL

of CH_3CN in order to precipitate proteins. The sample was centrifuged at 3500 rpm for 15 min and the supernatant was evaporated, at 45°C, under a gentle nitrogen stream, to remove organic solvent. Subsequently, the sample was quantitatively transferred and slowly applied to the solid phase cartridge (Nexus, Varian), which was preconditioned with methanol and water. The sample was subsequently treated according to the procedure described under solid phase extraction paragraph.

Urine Sample Preparation

Spiked urine samples were analyzed after solid phase extraction. Aliquots of 200 μL urine samples were spiked with 200 μL of androgens solutions.

Calibration curves were constructed using spiked plasma and urine samples at concentration levels in the range from 0.2 to 2.0 ng/μL for both testosterone and epitestosterone.

RESULTS AND DISCUSSION

Chromatography

A typical chromatogram obtained using the developed methods conditions is illustrated in Fig. 2a. Retention times revealed were 4.6 min for testosterone, 5.3 min for epitestosterone, and 3.6 min for p-Cresol. The resolution factor between testosterone and the internal standard was 2.7 and between testosterone and epitestosterone was 1.5.

Method Validation

Linearity and Sensitivity

Calibration curves were obtained by least squares linear regression analysis of the peak areas ratio of testosterone or epitestosterone to internal standard versus absolute amount. The method was linear up to 2.0 ng/μL with a correlation coefficient of 0.997 for testosterone and 0.994 for epitestosterone.

The LOD for both compounds was defined as the compound concentration that produced a signal-to-noise ratio greater than three and it was found to be 0.02 ng. The limit of quantitation of the assay was evaluated as the concentration equal to ten times the value of the signal to noise ratio and it was found to be 0.05 ng.

Table 1 summarizes all calibration and sensitivity data.

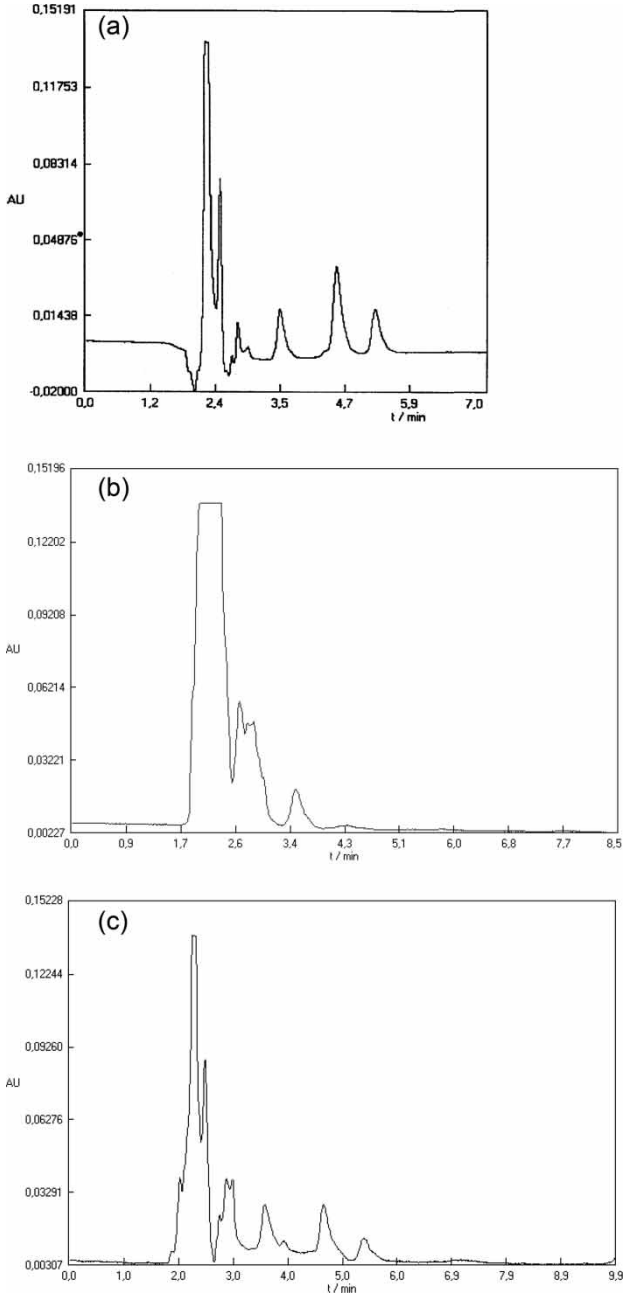

Figure 2. A. Chromatogram of testosterone (4.6 min) and epitestosterone (5.3 min) in the presence of p-Cresol as internal standard (3.6 min). B. Chromatogram of blank plasma. C. Chromatogram of spiked plasma: testosterone (4.6 min) and epitestosterone (5.3 min) determination in the presence of p-Cresol (3.6 min).

Table 1. Calibration data for simultaneous determination of testosterone and epitestosterone in standard solutions, blood plasma and urine. Internal standard: p-Cresole (2 ng/μL)

Analyte	Slope	Intercept	R^2
Standard solutions			
Testosterone	0.0631 ± 0.0597	0.0679 ± 0.0598	0.997
Epitestosterone	0.0571 ± 0.0410	0.0416 ± 0.0386	0.994
Blood plasma			
Testosterone	0.0675 ± 0.0039	0.0242 ± 0.0047	0.990
Epitestosterone	0.0532 ± 0.0019	0.0088 ± 0.023	0.995
Urine			
Testosterone	0.0621 ± 0.0013	0.0274 ± 0.0014	0.999
Epitestosterone	0.0513 ± 0.0032	0.0109 ± 0.0035	0.996
LOD (ng)	0.4		
LOQ (ng)	1.0		
Upper limit (ng/μL)	2.0		

Solid Phase Extraction

SPE protocol development was executed prior to the application of the method to biological fluids. After investigating two SPE sorbents and various elution systems, the optimum conditions were found involving the use of Nexus cartridges preconditioned with 2 mL of methanol, 2 mL of water, and methanol as elution solvent. Recovery after SPE was measured by comparison of peak area ratios of extracted standard solutions versus non-extracted solutions. Optimum protocol yielded high absolute recovery rates of 95%.

Chromatograms of blank and spiked samples of blood plasma are presented in Fig. 2B and Fig. 2C. Figures 3A and B present chromatograms of urine samples. No endogenous interference was noticed in biological matrices. High recovery rates are obtained and the assay procedures are simple with satisfactory precision and accuracy.

Validation of Biological Assays

Optimum SPE protocol was subsequently applied to biological fluids. Blood samples were deproteinised by acetonitrile, while urine samples were simply filtered.

Recovery Experiment

The absolute recovery of steroid hormones from biological matrices was measured by comparison of peak area ratios of extracted samples of spiked

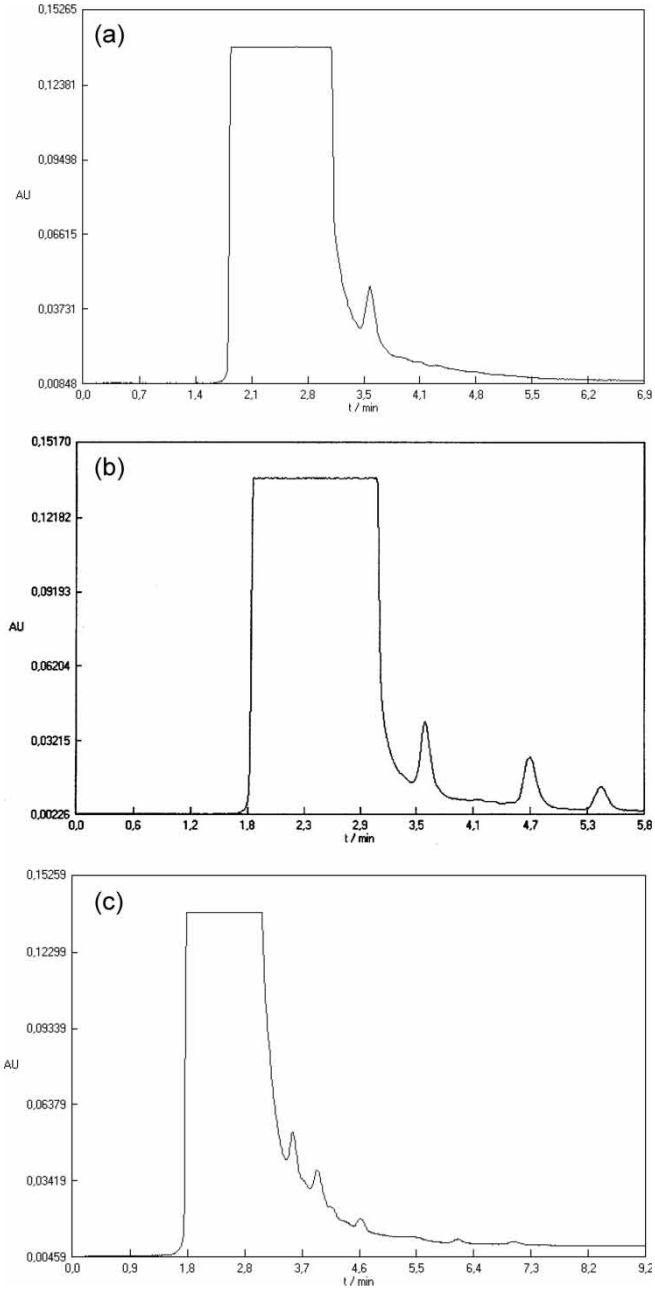

Figure 3. A. Blank chromatogram of blank urine sample (female volunteer). B. Chromatogram of spiked urine sample testosterone (4.6 min) and epitestosterone (5.3 min) in the presence of p-Cresol (3.6 min). C. Chromatogram of healthy 21 years old male volunteer.

matrices versus non-extracted standard solutions at five concentration levels. Recovery rates are in the range from 93.0–106.7% in plasma samples and 89.8–98.4% in urine.

Stability

For the stability study in plasma, control plasma samples were spiked with 1 ng/μL of testosterone and epitestosterone. Long term stability was assessed during storage at −18°C. Spiked plasma samples at 1 ng/μL stored at −18°C were analysed and stability was demonstrated for at least 1 month.

Precision and Accuracy

The accuracy and precision of the method based on within-day repeatability was performed by replicate injections (n = 6) directly in biological fluids (plasma and urine) of three spiked solutions, covering different concentration levels: low, medium, and high. The reproducibility (between day variation) of the method was established again in biological fluids using the same concentration range as above. A triplicate determination of each concentration was conducted during routine operation of the system over a period of five consecutive days. Statistical evaluation revealed relative standard deviations, at different values. Satisfactory repeatability and precision was achieved with RSD values lower than 11%. Tables 2 and 3 summarize the results of the method validation regarding accuracy, within-day, and between-day precision assays for epitestosterone and testosterone, respectively.

Application to Real Urine Samples of Healthy Volunteers

The next step of method development was its application to real urine samples of healthy male and female volunteers.

In men, the highest amount of testosterone is excreted from 20 to 35 years, while in women the amount excreted can not be easily estimated, since estrogens are the main female hormones.

Four urine samples from male volunteers were analyzed and the donators were 10, 21, 33, 43 years old and one female sample (the woman was 22 years old) and no hormones were detected. The pretreatment was exactly the same with the standard samples. A chromatogram of real samples of urine is presented in Fig. 3C. The method of standard addition was applied and finally testosterone was found to be 0.4 ng/μL.

Table 2. Within-day and between-day precision and accuracy study for epitestosterone in blood plasma and urine samples

	Within-day n = 6			Between-day n = 5		
Added (ng)	Found (ng) ± SD	RSD	Recovery (%)	Found (ng) ± SD	RSD	Recovery (%)
			Blood plasma			
10	8.5 ± 0.6	7.1	85.0	9.5 ± 0.7	7.4	95.0
20	16.8 ± 1.1	6.5	84.0	16.5 ± 1.2	7.3	82.5
40	38.7 ± 1.7	4.4	96.8	39.5 ± 3.3	8.3	98.8
			Urine			
Added (ng)	Found (ng) ± SD	RSD	Recovery (%)	Found (ng) ± SD	RSD	Recovery (%)
10	8.6 ± 0.9	10.5	86.0	10.3 ± 0.6	5.8	103.0
20	17.1 ± 1.4	8.2	85.5	19.9 ± 0.8	4.0	99.5
30	29.9 ± 0.3	1.0	99.7	29.9 ± 1.3	4.3	99.7

Table 3. Within-day and between-day precision and accuracy study for testosterone in blood plasma and urine samples

	Within-day n = 6			Between-day n = 5		
Added (ng)	Found (ng) ± SD	RSD	Recovery (%)	Found (ng) ± SD	RSD	Recovery (%)
			Blood plasma			
10	9.2 ± 0.7	7.6	92.0	9.6 ± 1.0	10.4	96.0
20	18.8 ± 1.4	7.4	94.0	21.5 ± 2.3	10.7	107.5
40	38.5 ± 1.5	3.9	96.3	37.7 ± 3.2	8.5	94.3
			Urine			
Added (ng)	Found (ng) ± SD	RSD	Recovery (%)	Found (ng) ± SD	RSD	Recovery (%)
10	8.5 ± 0.7	8.2	85.0	10.8 ± 1.0	9.2	108.0
20	19.1 ± 1.4	7.3	95.6	20.5 ± 2.1	10.2	102.5
30	25.8 ± 0.6	2.3	86.0	30.8 ± 0.8	2.6	102.7

CONCLUSIONS

The method described herein is a simple validated assay that can be readily used for the determination of steroid hormones: testosterone and epitestosterone. The assay procedures are simple with satisfactory precision and accuracy. High percentage recoveries of testosterone and epitestosterone from biological fluids were noticed without endogenous interference. The method can be finally applied to real urine samples in order to find the amount of the endogenous hormone testosterone successfully.

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