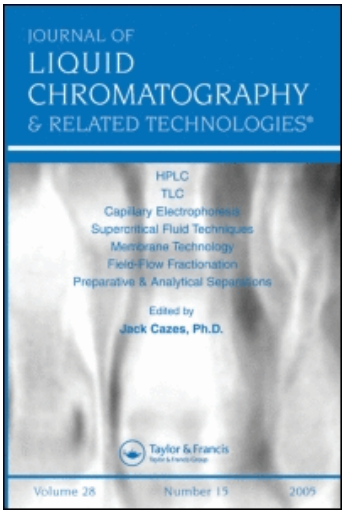

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Development of a Validated HPLC Method for the Simultaneous Determination of Anabolic Steroids in Biological Fluids

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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 (TES), epitestosterone (EPI), and nandrolone (NAN). The analytical column, Inertsil C_8, 5 μm, 250 × 4 mm, was operated at ambient temperature. Isocratic elution was performed using a mixture of 50% buffer solution CH_3COOH 0.11% –CH_3COONa 7.5 mmol/L, pH = 4, 45% CH_3CN and 5% CH_3OH, at a flow rate of 1.1 mL/min. UV detection was performed at 238 nm.

The detection limits of the method were 2.4 ng for NAN, 3.6 ng for TES, and 2.6 ng for EPI in blood plasma, and 2.7 ng for NAN, 1.1 ng for TES, and 3.8 ng for EPI in urine, per 20 μL injection volume. Alprazolam 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 × 6) and was found to be satisfactory, with high accuracy and precision results.

Sample preparation involved solid phase extraction on Nexus cartridges with high recoveries. The developed method was successfully applied to the analysis of urine samples of one female and 9 male volunteers.

Keywords: Anabolic steroids, Blood plasma, Epitestosterone, Nandrolone, Testosterone, Urine

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INTRODUCTION

Steroid hormones share cholesterol (Figure 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).[1]

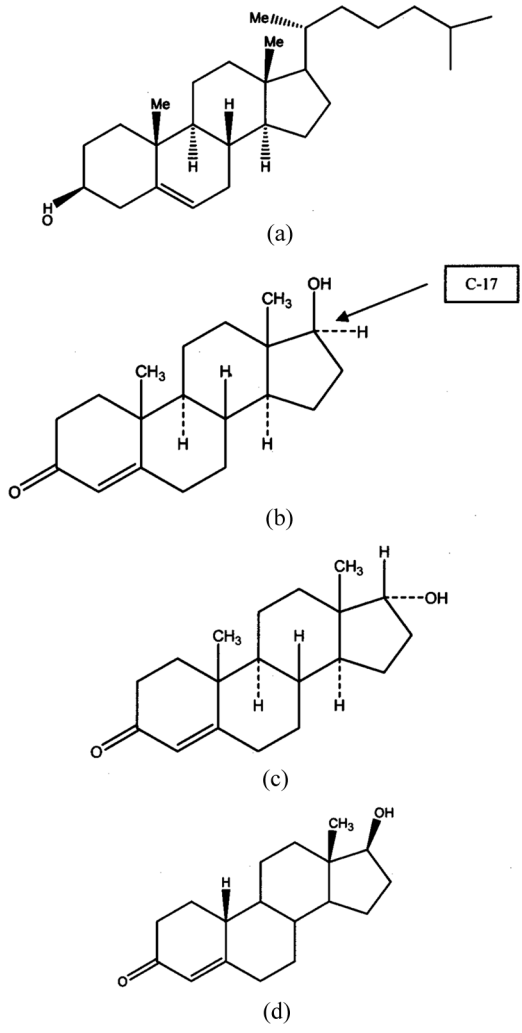

Figure 1. Chemical structures of (a) Cholesterol, (b) Testosterone, (c) Epitestosterone, and (d) Nandrolone.

Testosterone is the most important gonadal steroid, responsible for the secondary sex characteristics in the male, and supports spermatogenesis in synergy with follicle stimulating hormones (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). 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 anti-androgen flutamide are of particular importance.[2]

Testosterone (17β-hydroxyandrost-4-en-3-one) (Figure 1b) is a steroid hormone from the androgen group. Testosterone is primarily secreted by Leydig cells of the testes of males and the ovaries of females, although small amounts are also secreted by the adrenal glands. It is the principal male sex hormone and an anabolic steroid. In both males and females it plays a key role in health. Clinicians use serum testosterone to diagnose and monitor various disorders in both sexes such as hypogonadism, testicular dysfunction, hirsutism, virilization, alopecia, prostate disease, adrenal hyperplasia, ageing, breast cancer, protection against osteoporosis, and finally, menopause. On average, an adult human male body produces about eight to ten times more testosterone than an adult female body. The steroid hormone is also found in numerous animals. Testosterone's commercial names are: androlin, malestrone, perandren, synadrol F, testoviron T, testryl.[3–5]

A naturally occurring inactive epimer of testosterone is epitestosterone (17α-hydroxy-4-androsten-3-one) (Figure 1c). 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*. Structurally it differs from testosterone only in the configuration at the OH- bearing carbon, C-17. It is believed to form in a similar way as the main hormone, although the exact pathway of its formation is still a subject of research. It has been shown to accumulate in mammary cyst fluid and in the prostate. 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. Epitestosterone levels are typically highest in young males. 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 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 the maximum around 20 years of age, followed by a 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 a continuous decrease later.[6–8]

The use of anabolic steroids to improve athletic performance in major sports has been rumoured for more than two decades. The International Olympic Committee (IOC) and other national and international sport governing agencies have developed programs to combat drug use. A critical component of any anti-drug program is effective testing frequently in urine samples. Since 1983, testosterone was forbidden in sports by the IOC. The detection of illicit use of testosterone is currently carried out measuring the ratio between the concentration of Testosterone (T) and its isomer Epitestosterone (E). Until 2005, if this ratio was greater than 6 in the urine of a competitor, it was mandatory that additional investigations should be conducted. On the other hand, because the T/E ratio can be artificially modified, a urinary concentration of epitestosterone above 200 ng/mL had been established as indicative of its misuse as a masking agent. In the 2005 Prohibited List published by the World Anti-Doping Agency (WADA) it is mentioned that the ratio T/E should no longer exceed 4. If the ratio is greater than 4, further investigation will be obligatory in order to determine whether the ratio is due to physiological or pathological conditions.[9–15]

19-Nortestosterone also named Nandrolone (17β-hydroxyestr-4-en-3-one) (Figure 1d) was first synthesized in 1950. The substitution of the C-19 methyl group by a hydrogen atom in testosterone changed appreciably the ratio between anabolic and androgenic activity. In humans, two main metabolites were isolated: 19-norandrosterone and 19-noretiocholanolone. It is used for the treatment of anaemia, osteoporosis, and breast carcinoma. Because of its anabolic properties, nandrolone is used among athletes as a doping agent to accelerate muscle growth, to increase lean body mass, strength, and aggressiveness. This occurs because it does not convert to estrogens and does not have androgenic side effects. Detection of nandrolone according to the protocols set down by the World Anti-Doping Agency (WADA) is based on the identification of 19-norandrosterone and 19-noretiocholanolone, which are, as mentioned before, the two principal urinary metabolites. Nandrolone has been identified in human ovarian follicular fluid as a possible intermediate in the multi-step enzymatic conversion of androgen to estrogen.

Exercise has been shown to affect the urinary excretion of nandrolone metabolites. Finally, nandrolone and the two main prohormones mentioned are used by athletes in the form of nutritional supplements such as Durabolin.[16–20]

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 either from man or animals, as well as pharmaceuticals and dietary supplements. Most of the reported methods involve RP-HPLC with UV detection and isocratic or gradient elution.[2,4,8,17,21–26]

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.[5,9,13,14,20,27,28]

Other chromatographic techniques used for nandrolone, testosterone, and epitestosterone determination include gas liquid chromatography, as well as GC/MS.[12,18,19,29,30] GC-MS methods have been reported for the quantitative determination of the anabolic steroids in nutritional supplements.[29]

Sample preparation techniques used for the steroids determination include SPE in plasma and urine,[8,9,17,27] LLE,[8] enzymatic hydrolysis,[8,18] extraction with various organic solvents in cell microsomes, followed by incubation of microsomes with testosterone.[21–24] Finally, for the extraction of steroid hormones in human urine aqueous two-phase systems of ionic liquid and salt have been used.[15,30]

The aim of the present study was to develop and validate an HPLC method for the simple, rapid, accurate, and sensitive simultaneous determination of three anabolic steroids, testosterone, epitestosterone, as well as nandrolone in biological fluids, blood plasma, and urine.

EXPERIMENTAL

Reagents and Materials

Anabolic steroids, as well as alprazolam (used as the internal standard) were supplied by 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. Three types of SPE cartridges were used for sample preparation: Nexus Abselut (30 mg/cm^3) by Varian (Harbor City,

CA, USA), C_{18} (500 mg/3 mL) Discovery by Supelco, (Bellefonte, PA, USA), and Lichrolut RP-18 cartridges 200 mg/3 mL, were supplied from Merck (Darmstadt, Germany).

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

Instrumentation

A Shimadzu (Kyoto, Japan) LC-10AD pump was used to deliver the mobile phase to the analytical column, Inertsil C_8 (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 238 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 (Alltech Associates, www.alltechweb.com) (Deerfield, IL, USA), was used for the filtration of buffer solutions through Whatman Cellulose Nitrate 0.2 μm WCN Type (47 mm DIA) membrane filters (Whatman Laboratory Division, Maidstone, England). 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 Supelco 6 port Mini-Vap concentrator/evaporaevaporator, Bellefonte, PA, USA). SPE was carried out on a 12 port vacuum manifold from Supelco.

The UV spectra of the three anabolic steroids 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).

Chromatographic Conditions

An Inertsil C_8 (5 μm, 250 × 4 mm) analytical column maintained at ambient temperature was used for the separation of the three anabolic steroids. UV detection was monitored at 238 nm. The elution was isocratic and the mobile phase consisted of a buffer solution 0.11% CH_3COOH –CH_3COONa 7.5 mmol/L, pH = 4/CH_3CN/Methanol

(50:45:5 v/v/v). Inlet pressure observed at a flow rate of 1.1 mL/min was 155 kg/cm^2. The injection volume was 20 μL. Alprazolam was used as internal standard at a concentration of 2 ng/μL.

Preparation of Standards

Stock solutions of 100 ng/μL were prepared in methanol by dissolving the appropriate amount of analyte. These solutions when kept at −18°C were found to be stable for 2 months. Working methanolic standards were prepared by the appropriate dilution at 0.2, 0.5, 1.0, 2.0, 5.0, 8.0, 10.0 ng/μL. A methanolic stock solution of alprazolam was prepared at 100 ng/μL. All working standards contained alprazolam at a concentration of 2 ng/μL as internal standard.

Sample Preparation

Solid Phase Extraction (SPE)–Sample Extraction Procedure

Three different sorbents were assayed for the extraction of the investigated steroids: C_{18} (500 mg) Discovery by Supelco, Nexus by Varian and Lichrolut RP-18 by Merck. 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 to avoid possible analytes' loss. Elution was performed by 2 mL of methanol. Optimal extraction protocol was then applied to standard solutions of NAN, TES, and EPI in biological fluids.

Blood Plasma Pretreatment

Aliquots of 50 μL human blood plasma were spiked with 200 μL of androgens solutions 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.

Calibration curves were constructed using spiked plasma at concentration levels in the range from 0.2 to 8.0 ng/μL for nandrolone and from 0.2 to 10.0 ng/μL for both testosterone and epitestosterone.

Urine Sample Preparation

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

Calibration curves were constructed using spiked urine samples at concentration levels in the range from 0.2 to 8.0 ng/μL for both NAN and EPI and from 0.2 to 10.0 ng/μL for TES.

Validation of Biological Assays

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

The linearity response was assessed in the range of 0.2–10.0 ng/μL using standard solutions and control plasma and urine samples. Calibration curves for each analyte with the respective correlation coefficient were calculated by least squares linear regression analysis of peak area ratio of each analyte to IS.

Method validation regarding accuracy and precision was achieved by replicate injections of spiked biological samples at three concentration levels: 0.5, 2.0, and 8 ng/mL for both urine and 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 six 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 calculations for the limits of detection (LODs) were based on the standard deviation of y-intercepts of regression analysis (σ) and the slope (S), using the following equation $LOD = 3.3\ \sigma/S$. Limits of quantitation (LOQs) were calculated by the equation $LOQ = 10\,\sigma/S$. For each analyte the calibration curve was compared between the examined tissues using the regression analysis, in order to check the uniformity of the methods.

Selectivity was assessed by the absence of interference in the same chromatographic windows as examined anabolic steroids in biological samples. It was demonstrated by the analysis of blank matrices.

For the stability study in biological fluids control steroid free samples were spiked with 2 ng/μL of a mixture of all three compounds and the internal standard. Long term stability was assessed after three weeks of storage in a freezer at −18°C. Stability was also investigated after several freeze-thaw cycles. Aliquots of frozen spiked biological matrices were left to thaw at room temperature and analyzed.

RESULTS AND DISCUSSION

Chromatography

The mobile phase used, 0.11% CH_3COOH–CH_3COONa 7.5 mmol/L, –CH_3CN–MeOH (50:45:5 v/v/v) was delivered isocratically. The separation of the three anabolic steroids and internal standard was achieved within 8 min. Resolution factors were in the range 1.6–3.2 indicating an excellent separation. Retention times of the examined anabolic steroids are 5.1 min for nandrolone, 5.9 min for testosterone, 7.4 min for epitestosterone, and finally 4.4 min for the internal standard.

Linearity of the Method in Standard Solutions

Linearity for standard solutions was evaluated using calibration curves by a plot of peak area ratio of analyte to internal standard as a function of analyte amount in the examined range. The assay showed linearity up to 160 ng for NAN and TES, and up to 200 ng for EPI. The responses versus nominal concentrations fitted well to a straight line. Regression equations revealed good correlation coefficients ranging between 0.9906 and 0.9982 across the range examined. Linearity and sensitivity results are shown in Table 1.

Solid Phase Extraction (SPE)

SPE protocol development was executed prior to the application of the method to biological fluids. After investigating three SPE sorbents and various elution systems the optimum conditions were found involving the use of Nexus by Varian 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 greater than 76% for all analytes in both biofluids.

Validation of Biological Assays

Optimum SPE protocol was subsequently applied to biological fluids. Blood samples were first deproteinized by CH_3CN and after SPE protocol was applied.

Table 1. Calibration and sensitivity data of the three examined anabolic steroids in standard solutions, blood plasma and urine samples. x = ng of analyte and y = peak area ratio of analyte versus peak area of alprazolam (IS) (for standard solutions and biological samples)

Analyte	Regression data	r	LOD (ng)	LOQ (ng)	Upper limit (ng)
Standards					
NAN	$y = (0.1991 \pm 0.0042)\ x + (0.0799 \pm 0.0166)$	0.9982	1.4	4.2	160
TES	$y = (0.1922 \pm 0.0094)\ x + (0.0681 \pm 0.0371)$	0.9906	3.3	10.0	160
EPI	$y = (0.1627 \pm 0.0058)\ x + (0.0745 \pm 0.0304)$	0.9937	3.8	11.5	200
Blood Plasma					
NAN	$y = (0.2325 \pm 0.0102)\ x + (0.0396 \pm 0.040)$	0.9924	2.4	7.4	160
TES	$y = (0.2097 \pm 0.0091)\ x + (0.0628 \pm 0.0480)$	0.9907	3.6	10.9	200
EPI	$y = (0.1733 \pm 0.0044)\ x + (0.0969 \pm 0.0232)$	0.9968	2.6	7.7	200
Urine					
NAN	$y = (0.2259 \pm 0.0106)\ x + (0.0481 \pm 0.0419)$	0.9913	2.7	8.2	160
TES	$y = (0.2213 \pm 0.0032)\ x + (0.1257 \pm 0.0170)$	0.9992	1.1	3.5	160
EPI	$y = (0.1934 \pm 0.010)\ x + (0.0864 \pm 0.0432)$	0.9918	3.8	11.6	200

Linearity and Sensitivity

All calibration curves were linear in the range of 0.2–8 μg/mL for NAN and 0.2–10 μg/mL for TES and EPI with r values greater than 0.9907. The detection limit of the method was calculated as 2.4 ng for NAN, 3.6 ng for TES, and 2.6 ng for EPI in blood plasma, and in urine the LOD was 2.7 ng for NAN, 1.1 ng for TES, and finally, 3.8 ng for EPI, per 20 μL injection volume. The validation data for the anabolic steroids are included in Table 1.

Accuracy

Accuracy was assessed by a recovery percentage of steroid hormones in six determinations at samples spiked at three levels. The results for blood plasma lay in the range between 76.0% and 118.0%. Meanwhile, the results for urine lay in the range between 92.0% and 109.5%.

Repeatability

Repeatability was evaluated by a relative standard deviation of 3 determinations at samples spiked with the analytes at three levels. Relative standard deviations (RSD) for the three anabolic steroids were lower than 13.3% for plasma samples and lower than 7.9% for urine samples.

Intermediate Precision

Intermediate precision was assessed in the focus of between-day variation. The relative standard deviations obtained in total were lower than 9.6%. Accuracy and precision results in biological samples are summarized in Tables 2 and 3 for plasma and urine, respectively.

Recovery Experiment

The absolute recovery of anabolic steroids from biological matrices was measured by comparison of peak area ratios of extracted samples of spiked matrices versus non-extracted standard solutions at three concentration levels. Mean recovery rates were within the range 106.5–118.9% in both matrices.

Selectivity

Selectivity was assessed by the absence of interference in the same chromatographic windows as examined anabolic steroids in biological samples and it was demonstrated by the analysis of blank matrices.

Table 2. Within-day repeatability, between-day precision and accuracy for the determination of the examined steroids in blood plasma, at 0.5, 2, and 8 ng/μL

		Within-day (n = 6)			Between-day (n = 6)		
Analyte	Added (ng)	Found ± SD (ng)	RSD	Recovery (%)	Found ± SD (ng)	RSD	Recovery (%)
NAN	10	9.8 ± 0.9	9.4	98.0	11.8 ± 0.7	5.7	118.0
	40	30.4 ± 3.0	9.9	76.0	31.9 ± 1.7	5.4	80.0
	160	154.0 ± 5.7	3.7	96.3	164.8 ± 7.2	4.4	100.3
TES	10	9.6 ± 1.3	13.3	96.4	10.7 ± 1.0	9.6	107.2
	40	36.1 ± 3.0	8.4	90.3	31.7 ± 1.0	3.3	79.3
	160	143 ± 5.0	3.5	89.4	161.8 ± 6.6	4.1	101.2
EPI	10	9.6 ± 0.9	9.6	95.7	11.8 ± 0.7	5.8	117.6
	40	37.8 ± 3.3	8.6	94.6	33.3 ± 2.9	8.6	83.3
	160	162.7 ± 13.9	8.6	101.7	164.2 ± 8.0	4.9	102.6

Table 3. Within-day repeatability, between-day precision and accuracy for the determination of the examined steroids in urine, at 0.5, 2 and 8 ng/μL

		Within-day (n = 6)			Between-day (n = 6)		
Analyte	Added (ng)	Found ± SD (ng)	RSD	Recovery (%)	Found ± SD (ng)	RSD	Recovery (%)
NAN	10	10.0 ± 0.5	5.0	100.0	9.8 ± 0.4	4.4	98.0
	40	40.7 ± 2.0	4.8	101.8	36.8 ± 1.6	4.4	92.0
	160	158.3 ± 3.2	2.0	98.9	155.1 ± 2.5	4.4	96.9
TES	10	9.9 ± 0.8	7.9	98.7	9.9 ± 0.3	3.1	99.8
	40	38.4 ± 1.8	4.6	96.0	38.3 ± 1.9	4.8	95.8
	160	158 ± 2.7	1.7	98.8	156.2 ± 5.3	3.4	97.6
EPI	10	10.9 ± 0.7	6.2	109.5	10.1 ± 0.5	4.9	101.3
	40	39.9 ± 1.8	4.5	99.9	37.9 ± 1.2	3.3	94.8
	160	158.5 ± 6.7	4.2	99.1	155.7 ± 2.7	1.7	97.3

Typical blank and spiked chromatograms of each examined matrix are given in Figures 2–3.

Stability

For the stability study in plasma and urine, control drug free samples were spiked with 2 ng/μL of a mixture with all three compounds and the internal standard. Long term stability was assessed during storage at −18°C of spiked plasma and urine samples at 2 ng/μL for three weeks. All examined compounds were found to be stable for two weeks. The stability of anabolic steroids in biological fluids samples was investigated after five freeze–thaw cycles (from −18°C to room temperature). Degradation is decided using the −10% criterion. All examined compounds were found to be stable for four freeze-thaw cycles, apart from TES in urine, which found to be stable for three cycles. Figure 4 illustrates stability's assay results.

Application to Real Urine Samples of Healthy Volunteers

The last step of method development was the application of the method to real urine samples from 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.

Ten urine samples from male and female volunteers were analyzed in total, in order to measure the amount of endogenous hormones. The

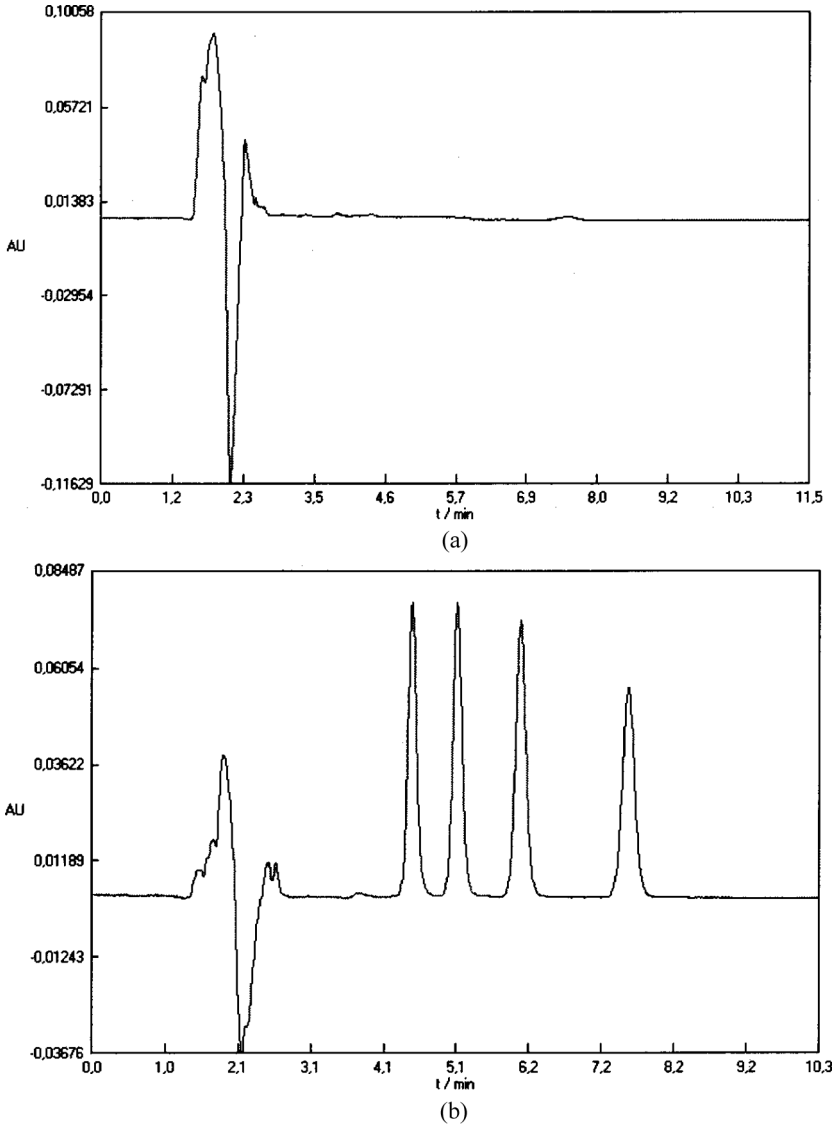

Figure 2. (a) High performance liquid chromatogram of anabolic steroids in blank human plasma, (b) spiked human plasma (8 ng/μL) in the presence of alprazolam as internal standard.

male volunteers were 8, 12, 25, 26, 27, 29, 38, 45, and 52 years old, and one female sample was analyzed (the woman was 24 years old) in which no steroid hormones were detected. The pre-treatment was the same as

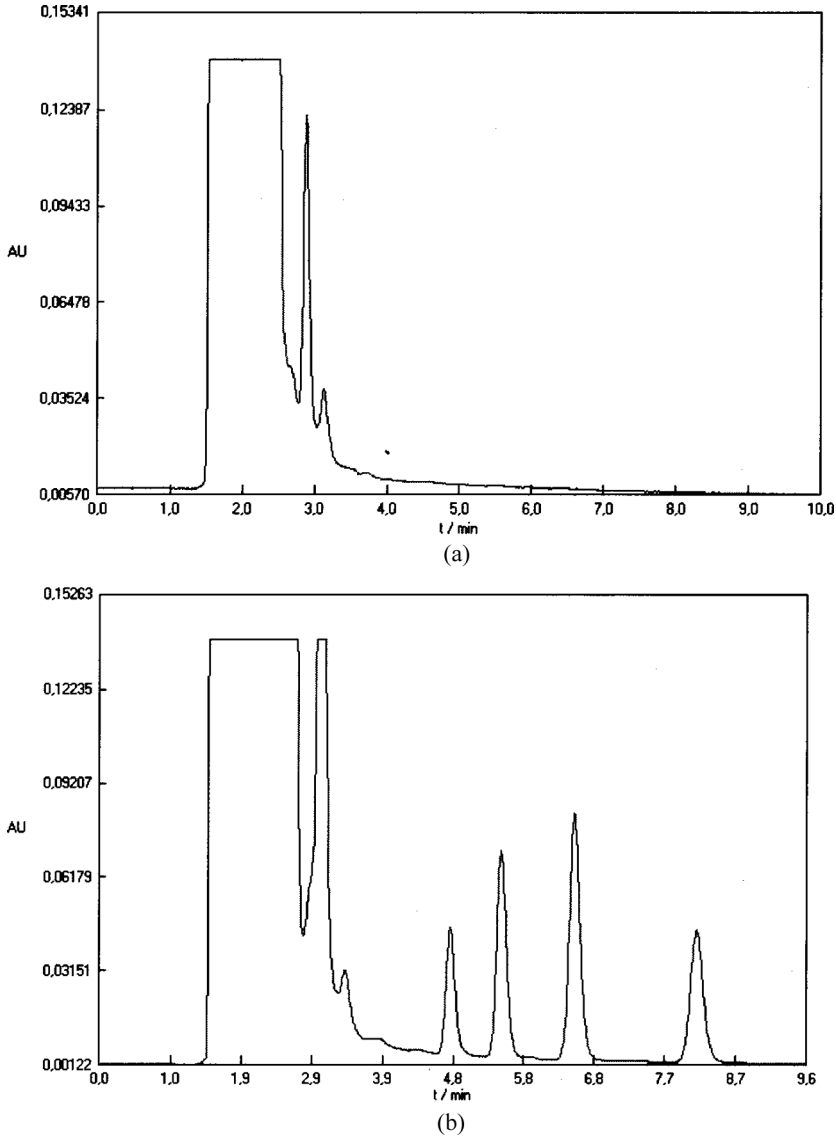

Figure 3. (a) High performance liquid chromatogram of anabolic steroids in blank human urine. (b) spiked human urine (10 ng/μL) in the presence of alprazolam as internal standard.

described under the sample preparation paragraph. Chromatograms of real samples of urine in which testosterone were detected are presented in Figure 5. The method of standard addition was applied, and finally,

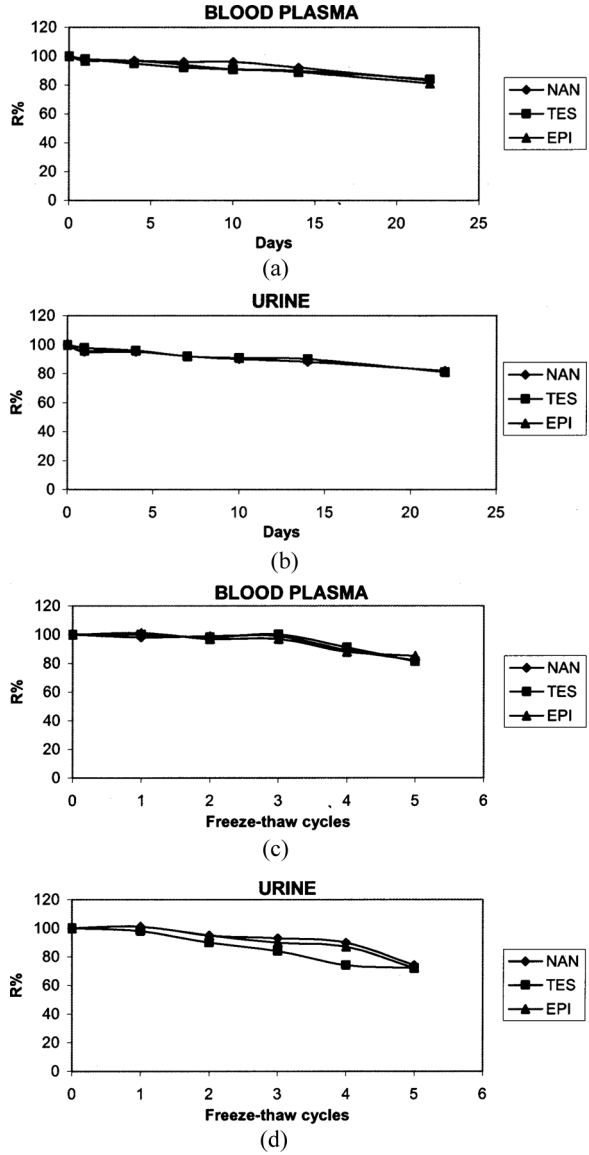

Figure 4. (a) Long term stability assay of steroids in blood plasma. (b) Long term stability assay of steroids in urine. (c) Stability of steroids after five freeze-thaw cycles in blood plasma. (d) Stability of steroids after five freeze-thaw cycles in urine.

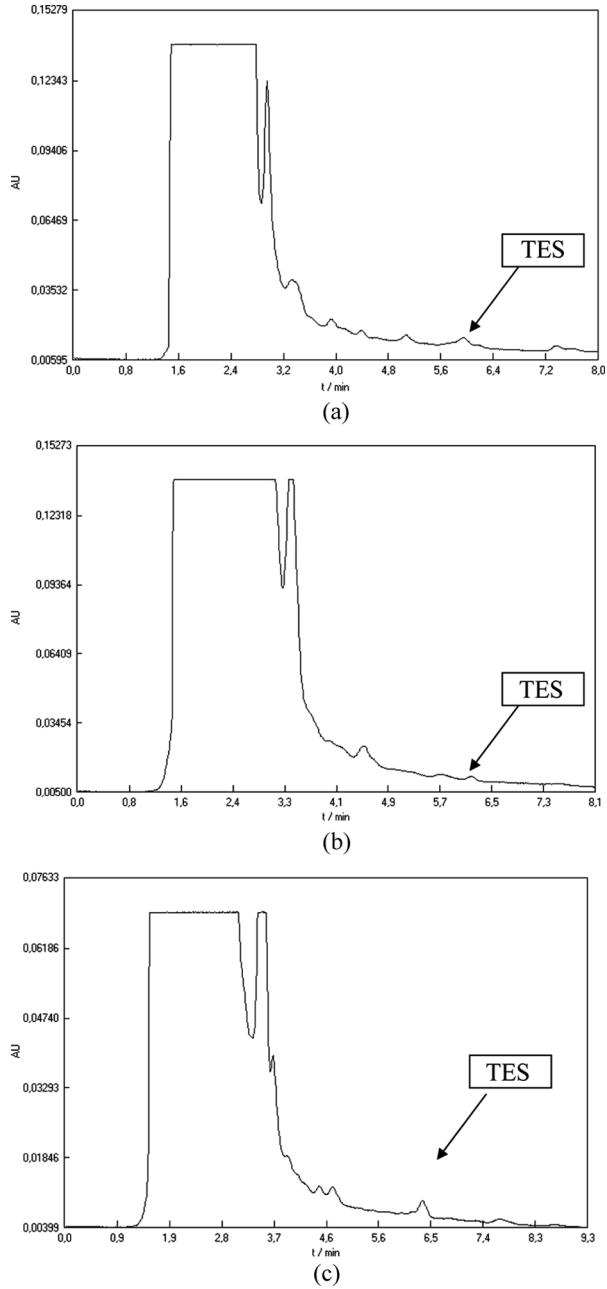

Figure 5. HPLC chromatogram of urine samples (a) from a healthy 25 years old male volunteer. (b) from a healthy 26 years old male volunteer. (c) from a healthy 27 years old male volunteer.

testosterone was found to be: 0.6 ng/μL for the sample of 25 years old male volunteer, 0.9 ng/μL for the sample of 26 years old male volunteer, and 0.7 ng/μL for the sample of 27 years old male volunteer.

CONCLUSIONS

The method described herein is a simple validated assay that can be readily used for the quantitative determination of steroid hormones: nandrolone, testosterone, and epitestosterone in biological matrices. Selective extraction of analytes from the biological matrices was achieved by means of SPE. Chromatographic analysis was performed by isocratic elution on an Inertsil C_8 analytical column (250×4 mm, 5 μm), with A: CH_3COOH 0.11% –CH_3COONa 7.5 mmol/L, pH = 4, B: CH_3CN and C: MeOH (50:45:5 v/v) as mobile phase within 8 min.

Validation was conducted on the developed method in terms of selectivity, linearity, accuracy, precision, stability, and sensitivity. Alprazolam was used as IS.

The method was proven to be accurate and precise (RSD values lower than 13.3% in plasma and lower than 7.9% in urine). Recoveries ranged from 76.0% and 118.0% for blood plasma and between 92.0% and 109.5% for urine samples.

The detection limit of the method was calculated as 2.4 ng for NAN, 3.6 ng for TES, and 2.6 ng for EPI in blood plasma, and in urine the LOD was 2.7 ng for NAN, 1.1 ng for TES, and finally 3.8 ng for EPI, per 20 μL injection volume.

Selectivity was demonstrated by the analysis of blank matrices. All examined anabolic steroids were found to be stable for two weeks at −18°C and for four freeze-thaw cycles apart from TES in urine, which was found to be stable for three cycles, using the −10% criterion and proved that they were stable for four cycles.

The method can be finally applied to real urine samples in order to find the amount of the endogenous hormone testosterone successfully. This fact can help clinicians in their research, because serum testosterone is used to diagnose and monitor various disorders in both sexes such as hypogonadism, prostate disease, ageing, breast cancer, protection against osteoporosis, menopause. Finally, all the three examined anabolic steroids in urine have to be determined in anti-doping control.

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