Development and Method Validation for Testosterone and Epitestosterone in Human Urine Samples by Liquid Chromatography Applications

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Abstract

An isocratic high-performance liquid chromatographic method for the determination of testosterone (T) and epitestosterone (ET) in human urine using liquid–liquid or solid-phase extraction (SPE) is developed and validated. The optimum separation is achieved using a Hypersil C_{18} column, water–acetonitrile (57:43, v/v) as the mobile phase and UV-absorbance detection at 245 nm. The recoveries obtained for T and ET in liquid–liquid and SPE demonstrate that these procedures are interchangeable. Quantitation limits for T and ET are 8.6 and 5.4 ng/mL using solvent extraction and 7.3 and 5.7 ng/mL using SPE, respectively. The proposed method is used to evaluate the urinary T, ET, and the T/ET ratio for a healthy male population using liquid–liquid extraction, and the T and ET excretion profile for nine healthy men using SPE.

Introduction

Testosterone (T) and epitestosterone (ET) are epimeric steroids belonging to the C19 steroids group (structures shown in Figure 1). T is the most representative natural steroid having anabolic-androgenic properties, and ET has little androgenic activity and no known function (1). However, similar compounds exist that are synthesized from T that show more effectiveness (e.g., nandrolone). The measurement of T and ET levels in body fluids is widely employed to evaluate the androgenic status in men, such as low levels of androgenic compounds in male (hipogonadism) or high levels in females (hyperandrogenic syndromes) (2). In addition, the examination of the endogenous steroids profile provides information about the health and the use of exogenous steroids. Excretion also shows how an endogenous compound's concentration varies during the day. Nowadays, measurement of urinary T has gained great relevance in the field of sports for the investigation steroid abuse by athletes or in animals engaged in races. Urine contains variable concentrations of T and ET with a ratio of T/ET that can vary in a wide range and it is not dependent, obviously, on the extent of urine dilution. For this reason, T/ET is evaluated in untimed urine collections (3,4) as a marker for T doping. According to the International Olympic Committee regulations, an athlete with a T/ET ratio greater than 6 is considered a potential user of T. Furthermore, a time profile is normally requested in these cases before penalizing the athlete because other factors can affect the T/E ratio value (5).

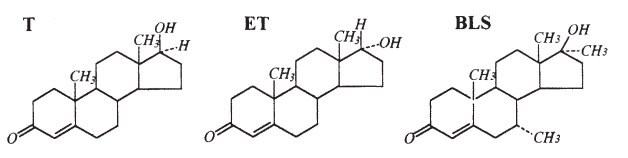

Figure 1. Chemical structures of T, ET, and bolasterone (BLS).

Steroids are mainly excreted in human urine as glucuronic or sulphate conjugates (conjugated fraction), and only 1% is excreted as free hormone (free fraction). For this reason, in most cases an enzymatic hydrolysis with β-glucuronidase is required to obtain the corresponding steroid. In addition, before chromatographic analysis, a sample pretreatment [including liquid–liquid extraction (LLE) or solid-phase extraction (SPE)] to preconcentrate and eliminate nondesirable species is normally required. LLE or SPE (or both) procedures have been used for T metabolites (6), for some androgenic anabolic steroids (including T and ET) (7), and to compare two LLE procedures for T and ET (3).

Radioimmunoassay is currently considered the method of choice for routine analysis of T and ET. However, there are restrictions caused by cross-reactivity of related compounds (8). Gas chromatography (GC) and GC–mass spectrometry (MS) have proven to be very suitable for urinary steroids in cases of endocrinological disorders and in doping control over the years (9–13). GC–MS methods for T determination in plasma (14) and hair (15) have also been developed. However, a derivatization process is required in this case and, in addition, the reproducibility obtained is not always sufficient (16,17). High-performance liquid chromatography (HPLC) methods have also been developed for the determination of T and some metabolites in rat liver (18) and human plasma (19), T and ET in doping control using gradient

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elution and UV detection (3), and recently in bovine serum and urine (20) and for T in plasma (21) with MS–MS detection.

In a previous work, an HPLC optimization of the chromatographic separation of a complex mixture containing urinary steroids (including T and ET), boldenone, and bolasterone was reported (22). The optimal separation method was applied to human urine samples under LLE conditions (7).

In this paper, an isocratic HPLC method, which employed a Hypersil octadecylsilane (ODS) column and water–acetonitrile as mobile phase, was developed and validated for T and ET determination in human urine. Two separate sample preparation procedures (LLE and SPE) were used. These methods, which were found to be interchangeable, were applied to urine samples.

Experimental

Chemicals

The T (17β-hydroxy-4-androsten-3-one), ET (17α-hydroxy-4-androsten-3-one), and BLS (17-hydroxy-7,17-dimethylandrost-4-en-3-one) (shown in Figure 1), were purchased from Sigma (St. Louis, MO). β-Glucuronidase from Escherichia coli K12 was purchased from Boehringer (Mannheim, Germany). HPLC-grade acetonitrile (ACN), methanol (MeOH), tetrahydrofuran (THF), and diethylether (DEE) were purchased from Promochem (Wesel, Germany). Dichloromethane (DCM) was from Carlo Erba (Milan, Italy). Water was purified with a Milli-Q system (Millipore, Molsheim, France). Millipore 0.45-µm Nylon filters (Bedford, MA) and C_{18} Bond elut cartridges (3.0 mL and 500 mg) (Varian, Harbor City, CA) were also used. Other chemicals were of analytical reagent grade.

Apparatus

The chromatographic system consisted of the following components, all of which were from TSP (Riviera Beach, FL): a ConstaMetric 4100 solvent delivery system, a spectra Monitor 5000 photodiode-array detector (DAD) covering the range 190–360 nm and interfaced to a computer for data acquisition and a recorder Model CI 4100 data module. A Rheodyne 20-µL loop injector (Cotati, CA), Jones-Chromatography block heated series 7960 for thermostating columns (Seagate Technology, Scotts Valley, CA), vacuum membrane degasser Model Gastor (SAS corporation, Tokyo, Japan), and a bonded-silica Hypersil ODS (250-mm × 4.6-mm i.d., 5 µm) column from Phenomenex (Torrance, CA) were used. A vortex mixer Mixo-Tub-30 from Crison (Barcelona, Spain) and a Visiprep vacuum manifold system from Supelco (Bellefonte, PA) were also used.

Mobile phase and chromatographic analysis

The mobile phases were prepared daily by mixing Milli-Q water with ACN, THF, or MeOH at the required volume ratio by programming the pump. All solvents and mobile phases were first filtered under vacuum through 0.45-µm Nylon filters and degassed using a vacuum membrane degasser.

Once the column had been conditioned with the mobile phase at 25°C, chromatograms were obtained. In order to optimize the mobile phase, a methanolic solution containing a single steroid or an appropriate mixture of them (5 mg/mL) was injected (20 mL). The flow rate was 1.0 mL/min and UV absorbance–DAD detection in the range 190–360 nm was used. Peak identification and purity was performed by comparison of their retention time (t_R) and UV spectra with those of compounds previously registered by injection of each one individually. Steroid analysis was monitored at 245 nm, which was the wavelength where T, ET, and BLS exhibited maximum absorption.

Urine sample preparation

Urine collection

Urine samples were collected from nine healthy males (25–35 years old) several times during a period of 24 h to determine the T and ET excretion profiles. In addition, samples from twelve non-doped males (25–30 years old) were collected early in the morning to evaluate T, ET, and the T/ET ratio. After collection, samples were added with some toluene drops and stored at 4°C for further analysis.

Blank samples

Steroids-free urine samples (SFUS) were prepared by percolating urine samples through Bond elut C_{18} cartridges. By doing this, urinary steroids and other potential interfering compounds were retained in the cartridges. Then, the unretained fraction was collected and checked for endogenous steroids (with negative results following the solvent extraction procedure described in the following section) and used as matrix for steroid spikes.

LLE

Urine samples (3 mL) were placed in a stoppered centrifuge tube and hydrolyzed using 1 mL 20mM buffer phosphate (pH 7) and β-glucuronidase (25 µL), which was previously checked using testosterone-glucuronide. The mixture was heated at 55ºC for 1 h. After hydrolysis, 230 ng/mL internal standard (IS) (BLS) was added and a similar extraction procedure as the one reported in (7) was applied. Added was 0.35 g NaCl to avoid emulsions and pH = 9 was obtained using 0.5 g K_2HPO_4 and 4 mL of DCM as extractant solvent. The mixture was shaken for 1 min and centrifuged for 3 min at 3700 g. The organic phase (the lower layer in the extraction flask) was removed, washed using 1 mL 2M NaOH, dried over anhydrous Na_2SO_4, and 3 mL of the solution were evaporated to dryness under vacuum. The dried residue was dissolved using 100 µL MeOH and 20 µL were injected into the HPLC system. The absolute preconcentration factor was close to 22.

Extraction with C_{18} cartridges (SPE)

Urine samples (3 mL) were placed in a stoppered centrifuge tube and hydrolyzed (just as in the previous section). After hydrolysis, 170 ng/mL of IS was added, and the same procedure as the one reported by Gonzalo-Lumbreras et al. (7) was applied. The above hydrolyzed samples were processed with a vacuum manifold system (see the Apparatus section) through Bond elut C_{18} cartridges, which had been previously conditioned with 5-mL volumes of MeOH followed by 2- × 5-mL volumes of water. The cartridges were washed with 5 mL of a mixture water–acetone (4:1, v/v) close to dryness and 1 mL of *n*-hexane. The elution of T and ET was performed with 2- × 2-mL volumes of DEE. The eluate was evaporated to dryness and dissolved with 100 µL MeOH, and

20 µL were injected into the HPLC system. The absolute preconcentration factor was close to 30.

Results and Discussion

Optimization of the chromatographic separation of T and ET

Mobile phases containing water and organic modifiers (ACN, THF, and MeOH) were examined and the separation characteristics for T and ET further evaluated using a Hypersil ODS column as the stationary phase and BLS as the IS (if the presence of BLS is detected in a preliminary urine assay, a change in the selection of IS is required). The solvent concentration range studied was 65–74% for MeOH, 40–51% for ACN, and 35–39% for THF. Table I summarizes the optimum composition achieved for T, ET, and BLS according to the resolution between peaks (R_s), run time analysis (RTA), and the retention factors (k) obtained. As can be observed (Figure 2), good results were obtained using THF and ACN (ET and BLS overlapped using MeOH). When urine samples were tested, ACN allowed the detection of T and ET without interferences. However, interferences of T with endogenous compounds present in urine were detected when using THF. These interferences constitute the major impediment to reduce the run time. Ternary mobile phases involving the above solvents were also tested with unsatisfactory results. In this way, a mobile phase water–ACN (57:43, v/v) (Figure 2) was finally selected for further application to urine samples. Based on this separation, the repeatability as coefficient of variation (CV) for five different standard samples containing 2 µg/mL each was estimated using peak areas (CV_a) and t_Rs (CV_t).

Table I. Performances of the HPLC Separation for T, ET, and BLS (IS) Using Organic Modifiers (OM)*

OM	SCR† (%)	OPT‡ (%)	k			Rs		RTA
			T	ET	BLS	T-ET	ET-BLS	
ACN	40-51	43	4.14	6.43	7.06	5.00	1.11	20 min
THF	35-39	35	4.34	5.86	7.21	3.50	3.20	21 min
MeOH	65-74	70	3.09	4.73	4.73	3.20	0	16 min

* k is the retention factor, R_s is the resolution between the peaks, and RTA is the run time analysis.
† SCR, solvent concentration range.
‡ OPT, optimum composition achieved.

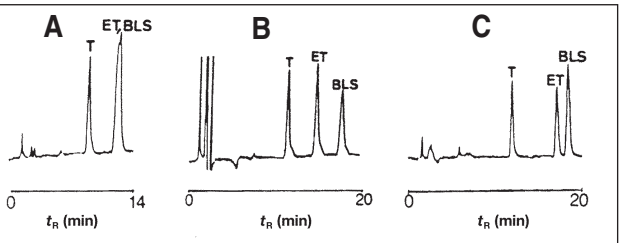

Figure 2. Chromatograms obtained from a standard mixture containing T (5 µg/mL), ET (5 µg/mL), and BLS (5 µg/mL) using 70% MeOH (A), 35% THF (B), and 43% ACN(C) as organic modifiers. Conditions: UV-absorbance detection at 245 nm, flow rate 1 mL/min and Hypersil ODS (250 × 4.6 mm, 5 µm) column (25°C).

The CV_a and CV_t obtained were lower than 1%. In summary, the data obtained from these compounds were adequate enough to develop an analytical method. These T and ET results improved upon those reported previously (22) because the mobile phase herein optimized employs a higher ACN content (RTA decreased) and better conditions for quantitation purposes.

Analytical characteristics

Calibration graphs and detection and quantitation limits

Standards containing methanolic mixtures of T and ET were prepared in the range 200–8000 ng/mL using 5000 ng/mL BLS as the IS. These mixtures were analyzed using the optimal mobile phase: H_2O–ACN (57:43, v/v), a flow rate of 1.0 mL/min, and UV absorbance–DAD at 245 nm. The results were analyzed by linear regression. By plotting each steroid peak area to IS ratio (PAR) versus the concentration (x) of each one, the calibration equation PAR = A + Bx (ng/mL) was obtained. The parameters were: A (intercept), –0.003 and 0.002 B · 10^4 (slope), 1.54 and 1.69; and r (regression coefficient), 0.998 and 0.999; for T and ET, respectively. In all cases the intercepts were not significantly different from zero. The limits of detection (LOD) and quantitation (LOQ) obtained for a signal-to-noise (S/N) ratio of 3 and 10, respectively, using calibration graphs were 40 and 150 ng/mL, and 40 and 140 ng/mL for T and ET, respectively.

Analysis of T and ET in urine using LLE or SPE and method validation

Calibration graphs, LODs, and LOQs

Calibration graphs were obtained by adding to SFUS standards of T and ET at nine concentrations in the range 9.0–360 ng/mL, using 230 ng/mL BLS as IS under LLE. In the same way, 8–270 ng/mL were added to SFUS using 170 ng/mL BLS as the IS under SPE conditions. These mixtures were analyzed as described in the "Optimization of the chromatographic separation of T and ET" section. Table II summarizes the data obtained by linear regression for T and ET (the errors in the slopes and intercepts do not reveal significant differences) and LOD and LOQ. The slope, LOD, and LOQ values in Table II include the preconcentration factors assessed in previous sections for LLE and SPE. The calibration equations (Table II) allow the calculation of T and ET concentration levels in urine samples.

Precision (repeatability and reproducibility)

Repeatability (Rep) (within-run precision) was examined for

Table II. Linear Regression Equations (PAR = A + Bx) Using Spiked SFUS for T and ET; LOD and LOQ and Within- and Between-Run Precision*

SP	Steroid	A	B × 103	r	LOD†	LOQ†	CV_{Rep}	CV_{Repr}
LLE	T	–0.004	3.45	0.997	2.7	8.6	7.2	10.5
	ET	0.004	3.81	0.999	1.4	5.4	8.5	13.5
SPE	T	–0.002	3.41	0.998	2.7	7.3	7.2	9.1
	ET	0.005	3.92	0.996	1.7	5.7	3.5	6.0

* PAR is the peak area ratio of T or ET to BLS (IS), x = ng/mL of T and ET, r = correlation coefficient, and SP = sample preparation.
† ng/mL.

T and ET by analyzing 6 different urine samples by only one operator within a day. Individual concentrations of 230 ng/mL (T or ET) in LLE and 170 ng/mL (T or ET) in SPE were used, and each sample was run once (n = 6). Reproducibility (Repr) (between-run precision) was evaluated for three different days (n = 18) using calibration graphs. The CV values (CV_{Rep} and CV_{Repr}) obtained are shown in Table II.

Accuracy

Accuracy was assessed at three different concentration levels of T and ET by replicate measurements (n = 6). Standards of T and ET were added to SFUS, processed under LLE and SPE conditions, and analyzed using the proposed HPLC method. Table III shows the amounts spiked and recoveries, R (%) and CV, found for T and ET in LLE and SPE. These values are similar to those previously found (7).

LLE versus SPE

An analysis of variance test was applied to the T and ET results (Table III). Because for T and ET the P values of the F-test were 0.13 and 0.71 (greater than 0.05), respectively, there were not statistically significant differences between LLE and SPE at the significance level of 5%. In other words, LLE and SPE procedures can be considered as interchangeables.

Table III. Accuracy Using LLE and SPE for Three Different Concentrations of T and ET (n = 6)

SP*	Compounds	Spiked (ng/mL)	Found (ng/mL)	R ± CV (%)
LLE	T	9.0	8.8	98 ± 7.8
		227	218	96 ± 7.2
		350	338	97 ± 5.3
	ET	9.0	8.6	95 ± 9.1
		227	225	99 ± 8.5
		350	337	96 ± 4.2
SPE	T	8.0	8.1	101 ± 7.5
		167	165	99 ± 7.2
		250	248	99 ± 6.1
	ET	8.0	8.2	102 ± 6.2
		167	167	100 ± 3.5
		250	253	101 ± 3.4

* SP, sample preparation.

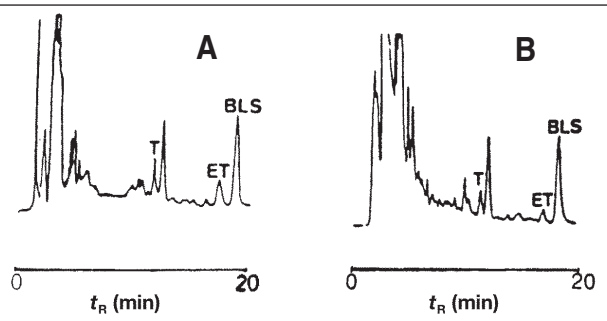

Figure 3. Chromatograms obtained from urine samples under LLE (A) and SPE (B) using a mobile phase of water–ACN (57:43, v/v). All other conditions are the same as in Figure 2.

Selectivity

Urine samples were analyzed under LLE or SPE conditions. A detection and identification process of T and ET based on t_R and a DAD was carried out (23). The CV (n = 6) of the retention factors for T and ET was lower than 1% for each one. DAD can provide a contour plot, showing the relationship between absorbance, wavelength, and time. The UV spectrum of each peak in the chromatogram was stored and subsequently compared with the standards. The spectra were normalized and overlaid. Impurities were investigated further by displaying the spectra obtained at different points across the peak. As urinary endogenous steroids can present similar spectra, the second derivatives of the spectra and absorbance ratios (A_{275}/A_{245}) across the peak were also obtained to check for peak purity. The possible impurities in each peak were not detected. The present method shows the ability to analyze T and ET in urine samples with good analytical performances.

Applications

T and ET excretion profile in urine samples using SPE

The proposed method was used to determine the excretion profile in urine of T and ET of nine healthy male volunteers (25–35 years old) who were selected because they didn't smoke, drink alcohol, or take drugs. Samples were collected as indicated and processed under the mentioned SPE conditions. A representative chromatogram is shown in Figure 3.

The excretion profiles (circadian rhythm) for T and ET were

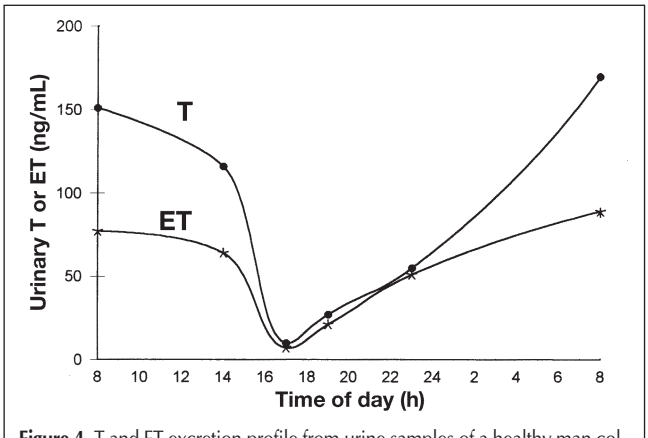

Figure 4. T and ET excretion profile from urine samples of a healthy man collected during 24 h (circadian rhythm).

Table IV. Urinary T, ET, and T/ET Levels in Male Subjects Using LLE*

Statistic (n = 12)	T†	ET†	T/ET
Mean	125	75	1.7
SD	43	32	0.5
CV (%)	35	43	27
Minimum value	54	34	1.1
Maximum value	191	159	2.9
Median	122	66	1.8

* (n = sample size)
† ng/mL.

obtained by plotting the urinary T and ET levels (ng/mL) versus the time of the day considered for each subject. The plots obtained show a similar qualitative pattern because T and ET exhibit a maximum and a minimum of excretion. Figure 4 shows a typical excretion profile for one of the tested volunteers. However, there were quantitative differences because the maxima (or minima) in the plots were reached at different times during the day. The maximum of the excretion curves was obtained in the morning (7–10 h), ranging from 194 to 72 ng/mL for T and 170 to 65 ng/mL for ET. The minimum was obtained later (17–20 h), ranging from 44 to 10 ng/mL for T and 38 to 7 ng/mL for ET. A further increase was observed later, reaching again high levels the morning of the following day. Therefore, it was extremely important to take into account the collection time of the sample because the data and the conclusions can change appreciably (e.g., in Figure 4, at a time close to 17–19 h, a minimum for both T and ET was observed). This behavior was in accordance with Kaplan and Pesce (2), who reported that the excretion of T is episodic, with a maximum in the early morning (~ 7 h) and a minimum approximately 13 h later. Nevertheless, excretion profiles are dependent on numerous environmental/physiological factors that vary from subject to subject and from population to population (25,26).

The values for the T/ET ratio (useful in doping control) obtained from T and ET data were in the range of 1.08–3.08 (typical values for a healthy male population). As expected, the CV values found for each subject were less than 30%.

Evaluation of urinary T, ET, and T/ET ratio for a male population using LLE

The proposed method was also used to determine the urinary T, ET, and T/ET ratio under LLE conditions. For this purpose a male population of twelve nondoped subjects was selected. Urine samples were collected early in the morning. A typical chromatogram obtained from these urine samples is shown in Figure 3. The urinary T, ET, and T/ET data were assessed using a Kolmogorov-Smirnov (KS) test. This test shows that the T, ET, and T/ET ratio data may not be discarded as normal distributions at any usual significance level. In spite of that, Table IV summarizes further statistical information for the T, ET, and T/ET variables. The mean and the CV values of T, ET, and T/ET can be considered as typical values (24). Nevertheless, the amounts excreted (and even the T/ET ratio) could be influenced by endogenous and exogenous factors such as age and development, endocrinological diseases, ethnic origin, circadian rhythm (the variation in T/ET ratio is expected to be less than 30% in males) (25), exercise, and the consumption of alcohol or anabolic androgenic steroids (26).

Conclusion

Different conventional mobile phases of water and organic modifiers (MeOH, ACN, or THF) were examined using a Hypersil ODS column with the aim to develop an HPLC method for T and ET in human urine samples using BLS as IS. The characteristics of the selected separation were shown to be adequate for the development of an analytical method. The HPLC method was validated for urine samples using two different sample preparation procedures (LLE and SPE). The validation parameters (linearity, LOD, LOQ, reproducibility, repeatability, accuracy, and selectivity) obtained using LLE or SPE have shown to be adequate for the determination of these hormones in human urine. The SPE and LLE procedures are interchangeable; however, SPE uses a higher concentration factor and is in general quicker, and automation is possible. The method has been applied to the determination of T, ET, and the T/ET ratio in a normal male population under LLE conditions and also to the evaluation of the excretion profile in urine of T and ET for nine normal male subjects under SPE conditions.

The wide variability on the urinary levels of T and ET could be explained by endogenous and exogenous factors of each subject. Urinary T and ET levels (and the T/ET ratio) are useful data for early detection of some hormonal diseases or doping control. Recent methods for the determination of T and ET utilize highly specific HPLC–MS or HPLC–MS–MS techniques (27).

Acknowledgments

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