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Solvent and solid-phase extraction of natural and synthetic anabolic steroids in human urine

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Abstract

Liquid–liquid (using dichloromethane) and liquid–solid extraction processes (using disposable C_{18} cartridges) were applied to human urine samples spiked with 15 androgenic anabolic steroids (natural and synthetic). The extraction recoveries were assessed from different HPLC separations of anabolic steroids using water–acetonitrile mobile phase, and using calibration graphs obtained by injection into HPLC of standard samples of these compounds before and after extraction. The procedures, including sample preconcentration, showed extraction efficiencies over 90% which were independent on a wide range of concentrations tested. Solid phase extraction yielded poor results for oximetolone, danazol and dehydroepiandrosterone. For real urine samples, hydrolysis using β-glucuronidase and washing using sodium hydroxide before and after solvent extraction, respectively, is recommended.

1. Introduction

Anabolic androgenic steroids (AAS) have a natural and synthetic origin and are derived from testosterone. They are excreted in urine basically as conjugated metabolites and in few cases in non metabolized form and, therefore, the metabolism of AAS is well known [1].

A great interest exists in the development of rapid and reliable methods for the detection and/or determination of AAS in several matrices with different purposes, e.g. veterinary residue in foods, biomedical applications and doping control [2]. Usually, hydrolysis with β-glucuronidase is required, purification and then preconcentration of the samples before chromatographic analysis. Liquid–liquid extraction (LLE) has been used traditionally for steroids because it provides high recoveries and adequate selectivities, avoiding interfering matrix components, e.g. by using diethyl ether for unconjugated steroids [3,4]. The main drawbacks of LLE are emulsion formation and that the methods is time consuming. Solid-phase extraction (SPE) with cartridges packed with bonded silica C_{18} have also been used for cleaning-up of complex mixtures, providing highly purified extracts and reproducibility and recoveries at least as good as LLE [5] with high selectivities. Moreover, SPE allows the treatment of smaller sample volumes than LLE without emulsion formation [6] and, in addition, it is also quicker, offering the possibility of automation [7,8].

There are few data about steroid recoveries from urine samples [2,9]. A LLE procedure for the steroid extraction of some testosterone metabolites from urine samples was compared to a single SPE method

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performed on disposable extraction columns and it was shown that SPE improved the recoveries obtained in comparison with LLE [10]. This procedure has also been applied successfully to the quantitative extraction of steroids from urine. A routinely combined extraction procedure for AAS which involves SPE using Serdolit AD-2, enzymatic hydrolysis and LLE with diethyl-ether (DEE) was compared with a developed LLE method using *n*-pentane previous enzymatic hydrolysis [11]. The developed method provided high recoveries and cleaner chromatograms than those obtained using DEE. Nowadays, the most employed methods in athletic drug testing programs involve SPE for urine preconcentration before enzymatic hydrolysis and LLE, obtaining finally a concentrated dry extract (7). HPLC can be also considered as a clean-up procedure before GC–MS analysis [12]. This procedure has been demonstrated to be a key technique for the analysis of AAS after sample preparation [11,13].

In a previous paper, two extraction procedures (SPE and LLE) for natural and synthetic corticoids (CC) in human urine by an optimization of the main extraction variables were reported [14]. After optimization, a comparison between the recoveries obtained was also carried out. The LLE procedure has recently been applied to the determination of some corticoids in milk replacer powder with satisfactory results [15].

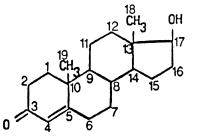
In the present paper the LLE and SPE procedures previously developed for CC [14] have been extended to sample preparation of human urine samples containing natural and synthetic AAS (see structures in Table 1). The AAS extraction efficiencies were assessed from HPLC separations using different mobile phases of water–acetonitrile and a Hypersil column (250 mm×4.6 mm, I.D, 5 μm).

2. Experimental

2.1. Chemicals

11β-hydroxytestosterone (HT) (4-androstene-11β, 17β-diol-3-one), 11-ketotestosterone (KT) (4-androstene-17β-ol-3,10-dione), boldenone (B) (1,4-androstadien-17β-ol-3-one), testosterone (T) (17β-hydroxy-4-androsten-3-one), androstenolone (AOO) (17β-hy-

Table 1
Structures of natural and synthetic androgenic anabolic steroids (AAS)



	C1–2	C2	C3	C2–3	C4–5	C5–6	C7	C9	C11	C17	C19
Hydroxytestosterone (HT)									–OH		
Ketotestosterone (KT)									=O		
Fluoxymesterone (FM)								–F	–OH	$-CR_3$	
Nortestosterone (NT)											–H
Boldenone (B)	=										
Metandrostenolone (DMT)	=									$-CH_3$	
Norethindrone (NE)										–C≡CH	–H
Testosterone (T)										β	
Methyltestosterone (MT)										$-CR_3$	
Androstenolone (AOO)	=				–						
Bolasterone (BLS)							$-CH_3$			$-CH_3$	
Dehydroepiandrosterone (DHEA)			–OH		–	=					
Epitestosterone (ET)										α	
Oximetolone (OM)		=CHOH								$-CH_3$	
Danazol (DZ)										–C≡CH	

droxy-5α-androst-1-en-3-one), bolasterone (BLS) (17-hydroxy-7, 17-dimethylandrost-4-en-3-one), dehydroepiandrosterone (DHEA) (5-androsten-3β-ol-17-one), epitestosterone (ET) (17α-hydroxy-4-androsten-3-one), fluoxymesterone (FM) (9α-fluoro-11β, 17β-dihydroxy-17-methyl-4-androsten-3-one), norethindrone (NE) (19-nor-17α-ethynyl-4-androsten-17β-ol-3-one), 19-nortestosterone (NT) (17β-hydroxy-19-norandrost-4-en-3-one), methandrostenolone (DMT) (17β-hydroxy-17α-methyl-1, 4-androstadien-3-one), 17α-methyl-testosterone (MT) (17-hydroxy-17-methylandrost-4-en-3-one), androstenolone, oximetolone (OM) (17β-hydroxy-2-hydroxymethylene-17-methyl-5α-androst-3-one), danazol (DZ) (pregna-2,4-dien-20- inol(2,3-d)isoxazol-17-ol) (see structures in Table 1), α-Zearalanol (Z) (2, 4-dihydroxy-6-[6α, 10-dihydroxy-undecyl] benzoic acid μ-lactone and canrenone (CAN) (17-hydroxy-3-oxopregna-4, 6-diene-21-carboxylic acid) were purchased from Sigma (St. Louis, MO, USA). β-glucuronidase from Escherichia coli was purchased from Boehringer Mannheim (Germany). HPLC-grade acetonitrile (ACN) and diethylether (DEE), reagent-grade were purchased from Promochem (Wesel, Germany) and dichloromethane (DCM) was purchased from Carlo Erba (Milan). Water was purified with a Milli-Q system (Millipore, Molsheim, France). Millipore 0.45 μm Nylon filters (Bedford, MA, USA) and C_{18} Extrasep cartridges (2.8 ml and 500 mg) (Phenomenex, Torrance, CA, USA) were also used. Other chemicals were of analytical reagent grade.

2.2. Apparatus

The chromatographic system consisted of the following components, all from TSP (Riviera Beach, FL, USA): a ConstaMetric 4100 solvent delivery system; spectra Monitor 5000 photodiode-array detector (DAD), covering the range 190–360 nm and interfaced to a computer for data acquisition; recorder Model CI 4100 data module. A Rheodyne 20 μl loop injector (Cotati, CA, USA), a Jones–Chromatography block heated series 7960 for thermostatting columns (Seagate Technology, Scotts Valley, CA, USA), a 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, USA) were used. A vortex mixer Mixo-Tub-30 from Crison (Barcelona, Spain) and a Visiprep vacuum manifold system from Supelco (Bellefonte, PA, USA) were also used.

2.3. Mobile phase

The mobile phases were prepared daily by mixing Milli-Q water with acetonitrile (ACN) at the required volume ratio by programming the pump. All solvents and mobile phases were firstly filtered under vacuum through 0.45 μm nylon filters and degassed using a vacuum membrane degasser.

2.4. Sample preparation

Steroids-free urine samples (SFUS) were prepared by percolating urine samples through Extra-Sep C_{18} cartridges. In this way, urinary steroids and other potential interfering compounds are retained in the cartridges. After this, the eluates were collected and checked for endogenous steroids (with negative result following the solvent extraction procedure described in the following section), and used as matrix for steroid spikes.

2.4.1. Liquid–liquid extraction (LLE)

(i) To obtain calibration graph 1

SFUS (3 ml) was placed in a stoppered centrifuge tube and then spiked with anabolic steroids (see chemicals and Table 1) in the range 0.13–0.66 μg/ml. In order to obtain recoveries of the anabolics, a similar extraction procedure as the one reported in [14] was applied. Briefly, 0.35 g NaCl was added to avoid emulsions and a pH of 9 was obtained using 0.5 g K_2HPO_4 and 4 ml of DCM. 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 and dried over anhydrous Na_2SO_4 and 3 ml of the solution was evaporated to dryness under vacuum. The dried residue was dissolved using 200 ml of 5 mg/ml I.S. (BLS, MT, Z or CAN) and 20 μl was injected into the HPLC system.

The absolute preconcentration factor was close to 11.

(ii) To obtain calibration graph 2

A modified extraction process with respect to that described above was followed. In this case SFUS samples were not spiked with steroids and the dry residue was dissolved using 200 μl of steroids in the range 2–10 μg/ml using 5 μg/ml I.S.

2.4.2. Extraction with C_{18} cartridges (SPE)

(i) To obtain calibration graph 1

SFUS (3 ml) was placed in a stoppered centrifuge tube and then spiked with anabolic steroids (see chemicals and Table 1) in the range 0.13–0.66 μg/ml. In order to obtain recoveries of the anabolics, the same procedure as the one reported in [14] was applied. NaOH was added to the urine samples to adjust the pH to 7.5 and were processed with a vacuum manifold system (see apparatus) through Extra-Sep C_{18} cartridges which previously had been conditioned with 5 ml volumes of MeOH followed by 2×5 ml 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 anabolics was performed with 2×2 ml volumes of diethyl ether. The eluate was evaporated to dryness and dissolved with 200 μl of 5 μg/ml I.S., and 20 μl was injected into the HPLC system.

The absolute preconcentration factor was close to 15.

(ii) To obtain calibration graph 2

A modified extraction process with respect to that described above was followed. In this case SFUS samples were not spiked with steroids and the dry residue was dissolved using 200 μl of steroids in the range 2–10 μg/ml using 5 μg/ml I.S.

2.4.3. LLE and SPE for real urine samples

The above procedures (LLE and SPE) were directly applied or modified as follows. Urine samples (3 ml) from males were hydrolysed using 1 ml 20 m*M* buffer phosphate (pH 7) and β-glucuronidase (25 μl). The mixture was heated at 55°C for 1 h. In SPE, the procedure described in Section 2.4.2(i) was applied. However, in LLE, after solvent extraction (Section 2.4.1(i)), the organic layer was washed using 1 ml 2 *M* NaOH. In all cases, the dry residue was dissolved using 100 μl MeOH.

The absolute preconcentration factor was close to 22 (LLE) and 30 (SPE).

2.5. Chromatographic analysis

Once the column had been conditioned with the mobile phase at 30°C (for reproducible measurements), chromatograms were obtained. For optimization purposes based on the use of different mobile phases of water–ACN, a standard solution containing one of the compounds or a mixture of them were injected. Peak identification and peak purity were performed by comparison of their retention time and UV spectra with those of compounds previously registered by injection of each one individually. The analysis of the anabolic was carried out at 245 nm with the exception of DHEA and Z (I.S.) which were monitorized at 200 nm, and OM, DZ and CAN (I.S.) monitorized at 280 nm (see Table 2).

3. Results and discussion

3.1. Preliminary experiments

In a previous paper, a HPLC optimization of complex mixtures containing natural and synthetic AAS using conventional mobile phases of water–organic modifiers (MeOH, ACN or THF) was de-

Table 2
Detection conditions and water–acetonitrile (ACN) mobile phases for AAS mixtures. Flow-rate, 1 ml min^{-1}

AAS mixture	Composition	ACN (%)	λ (nm)
A	HT, NE, MT, BLS (IS)	43	245
B	FM, DMT, BLS (IS)	45	245
C	T, E, BLS (IS)	45	245
D	KT, B, NT, MT (IS), AOO, BLS	37	245
E	Z (IS), DHEA,	55	200
F	CAN (IS), OM, DZ	50	280

veloped [16]. In order to obtain LLE and SPE recoveries for AAS added to urine samples, the above separations (using ACN as organic modifier), were used in the present work. In Table 2 the name and the composition of the AAS mixture, ACN concentration in the mobile phase for their separation and detection wavelength are summarized. Fig. 1 shows the chromatograms obtained for a standard methanolic solution containing 5 μg ml^{-1} of AAS under such conditions.

3.2. LLE and SPE recoveries using calibration graphs

LLE and SPE recoveries (%*R*), for AAS were assessed using calibration graphs (see Sections 2.4.1 and 2.4.2). A calibration graph was prepared using five different concentrations of AAS in the range 2–10 μg/ml added over the dry residues of SFUS obtained after applying LLE and SPE (calibration graph of 2.4.1(ii) and 2.4.2(ii) sections). The chromatograms obtained do not differ significantly with respect to those obtained using methanolic solutions of standards (Fig. 1) [matrix interferences were not detected and the relative standard deviation, RSD ($n=6$), of the retention factors for the compounds under study was lower than 1% for each one]. The results were analyzed by linear regression. Plotting the ratio of AAS peak area to I.S. (PAR) against the concentration (x) of each AAS, the calibration equations, $PAR=A+S_2\times(\mu g\ m1^{-1})$, were obtained. Table 3 shows the parameters A (intercept), S_2 (slope) and r (regression coefficient). In all cases the intercepts were not significantly different than zero.

Similarly, another calibration graph was obtained from SFUS spiked with AAS in the range 0.13–0.66 μg $m1^{-1}$ before applying the LLE and SPE procedures described in Sections 2.4.1(i) and 2.4.2(i), and the slope, S_1, was also calculated. The %*R* values were calculated using the equation $R(\%)=(S_1/S_2)\times 100$.

The results are shown in Table 4. As can be observed, similar results were obtained using SPE or LLE. However, SPE yielded poor results for DZ, OM and DHEA SPE. This behavior is probably due to the polarity of substituents on C2 (OM), C3 (DHEA) and C2–3 (DZ) (see Table 1). In addition,

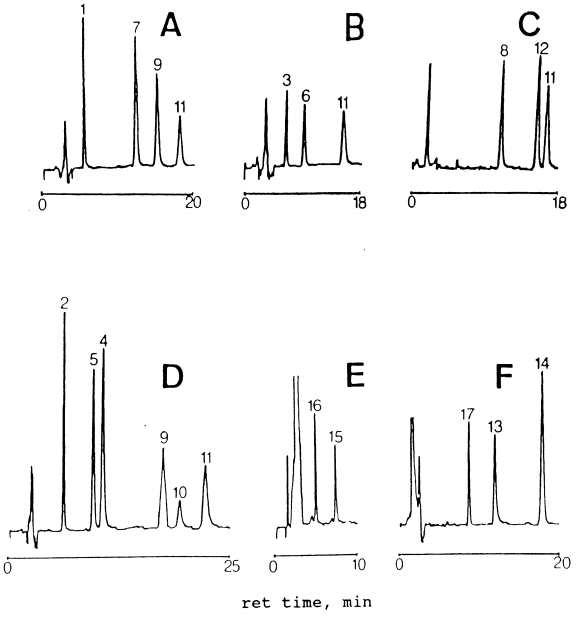


Fig. 1. HPLC chromatograms of a standard mixture of AAS (5 μg/ml) prepared in methanol. The A–F chromatograms were achieved using A–F water–acetonitrile mixtures as mobile phases (see Table 2), a Hypersil (250 mm×4.6 mm I.D., 5 μm) column (30°C) and flow-rate of 1.0 ml min^{-1}. Peak numbers of AAS as in Table 2; 16 (Z) and 17 (CAN) are I.S.

Table 3
Linear regression equations ($PAR=A+S_2x$) and detection limits (LODs) for steroids. PAR is the steroid peak area ratio to I.S. Conditions as in Fig. 1

AAS	A	S_2	r	LODs (ng $m1^{-1}$)
1. HT	−0.090	0.234	0.999	0.059
2. KT	0.066	0.155	0.999	0.059
3. FM	0.071	0.175	0.997	0.067
5. B	−0.013	0.129	0.999	0.071
4. NT	0.023	0.211	0.997	0.044
6. DMT	0.071	0.182	0.997	0.065
8. T	0.034	0.223	0.997	0.070
7. NE	−0.050	0.254	0.999	0.054
9. MT	−0.070	0.215	0.999	0.064
10. AOO	0.066	0.055	0.998	0.129
12. ET	0.088	0.244	0.999	0.065
11. BLS	0.065	0.152	0.998	0.061
13. OM	−0.030	0.308	0.998	0.066
14. DZ	−0.054	0.235	0.999	0.086
15. DHEA	0.013	0.114	0.999	0.148

Table 4
LLE and SPE recoveries, (%E)±RSD, for AAS added to urine samples (3 ml). Amount added, 0.13–0.66 μg ml^{-1}

AAS	LLE	SPE
HT	92.7±6.0	102±2.1
KT	93.8±6.3	93.3±7.1
FM	101±1.3	97.8±1.8
B	92.0±7.1	97.2±3.0
NT	95.5±3.9	104±4.6
DMT	101±0.6	93.5±7.3
T	92.2±6.9	101± 6.0
NE	91.3±8.0	106±7.3
MT	91.2±5.9	99.2±2.7
AOO	94.4±5.2	91.5±5.8
ET	95.1±5.6	106±6.8
BLS	103±4.5	91.4±6.4
OM	96.0±3.6	–
DZ	97.3±2.9	–
DHEA	102±2.0	–

C_{18} cartridges provided the lowest time analysis and the cleanest chromatograms.

3.3. Precision, accuracy and detection limits

The precision was evaluated by analyzing five different samples of AAS containing 5 μg/ml each, using the calibration graphs under LLE or SPE conditions. The relative standard deviation (RSD) for each steroid is shown in Table 3.

The accuracy was assessed in the 2–10 μg/ml range for each steroid by applying the Barlett and Harley test [17]. The results confirmed that the developed methods do not present a systematic error and do not require a blank correction.

The limits of detection (LODs) obtained for a signal-to-noise ratio=3 (S/N=3) (n=5) are also listed in Table 3.

4. Application of LLE and SPE to urine samples

Based on LLE and SPE procedures herein described, the quantitation of AAS in human urine is possible (e.g. for SFUS spiked with steroids when the dry residue is reconstituted in 200 μl). However, most of these compounds (natural and synthetic) are excreted in urine at very low concentrations, free or conjugated as glucuronides. In order to increase the concentration of free AAS, a higher preconcentration level (e.g. using 100 μl to reconstitute the dry residue) or hydrolysis with β-glucuronidase before LLE, is required. In this way, the concentration of endogenous compounds (e.g. metabolites) can also be increased [1,18]. For these reasons, assays for AAS in human urine require further assessment on sensibility and specificity.

In order to increase the assay specificity for real samples in LLE, 2 ml of 1 *M* NaOH was used to wash the DCM phase without significant changes on recoveries. As an example, Fig. 2 shows the chromatograms obtained (after LLE) from a urine sample with (Fig. 2B) and without hydrolysis (Fig. 2A) and washing the DCM phase using l ml NaOH (Fig. 2C) (Section 2.4.3). As can be observed in Fig. 2A, T and ET are not detected, however, using hydrolysis,

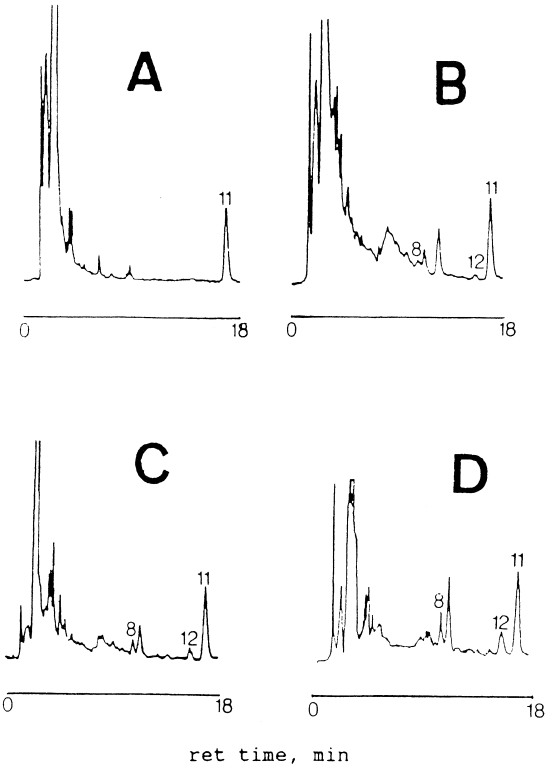


Fig. 2. Chromatograms obtained from urine samples under LLE and SPE conditions. In LLE: without hydrolysis, (A); hydrolysed with β-glucuronidase, (B); by washing the DCM phase with NaOH, (C). In SPE: hydrolysed with β-glucuronidase, (D). Peak numbers: 8 (T), 12 (ET) and 11 (BLS).

T and ET detection is possible (Fig. 2A and B), obtaining a cleaner chromatogram by washing the organic layer with NaOH (Fig. 2C). In addition, the peak area of T and ET were examined, showing each compound concentration over its LOD.

In Fig. 2D is also shown a chromatogram obtained from a real urine sample treated with β-glucuronidase before SPE. As in LLE (Fig. 2B and C) T and ET can be detected, showing each compound concentration over its LOD. A detection and identification process of these steroids based on retention times and a diode array detector (DAD) was carried out [19]. The instrument 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 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, for investigating peak purity the second derivatives of the spectra and absorbance ratios (A_{275}/A_{245}) across the peak were also obtained.

5. Conclusions

In this paper two different extraction procedures have been applied to urine samples containing 15 AAS. With the exception of OM, DZ and DHEA using SPE, similar recoveries using LLE or SPE have been obtained. Recoveries showed to be independent of concentration. In addition, new extractions and improvements on literature data are reported. Similar clean-up of urine samples was obtained in LLE (using NaOH as washing reagent) and in the case of SPE using disposable C_{18} cartridges. For real urine samples, hydrolysis using β-glucuronidase and washing using sodium hydroxide before and after solvent extraction, respectively, is recommended.

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