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Optimization of the high-performance liquid chromatographic separation of a complex mixture containing urinary steroids, boldenone and bolasterone: application to urine samples

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Abstract

An HPLC separation of a complex mixture containing 13 urinary anabolics and corticoids, and boldenone and bolasterone (synthetic anabolics) has been carried out. The applied optimization method involved the use of binary, ternary and quaternary mobile phases containing acetonitrile, methanol or tetrahydrofuran as organic modifiers. The effect of different reversed-phase packings and temperature on the separation was studied. The optimum separation was achieved by using a water–acetonitrile (60:40, v/v) mobile phase in reversed-phase HPLC at 30°C, allowing the separation of all the analytes in about 24 min. Calibration graphs were obtained using bolasterone or methyltestosterone as internal standards. Detection limits were in the range 0.012–0.107 μg ml^{-1}. The optimized separation was applied to the analysis, after liquid–liquid extraction, of human urine samples spiked with steroids.

Keywords: Urinary steroids; Boldenone; Bolasterone

1. Introduction

Analysis of steroids in urine has been used to detect different hormonal actions in human beings such as testicular function in men, hyperandrogenic disorders in women and puberty problems in children, by the measurement of anabolic steroids, and some hormonal disorders such as adrenocortical adenoma and Cushing syndrome by the control of corticoids [1]. These steroids have been included in the International Olympic Committee (IOC) doping list due to their illegal use in some sports [2] and in the list of schedules drugs in several countries because of their use by young people [3]. In addition, the examination of endogenous steroids profile provided information about the health and the use of exogenous steroids. As an example, a small testosterone (T) level could indicate a dysfunction in the pituitary–testicular axis whereas a large amount of T could mean an administration of this hormone, e.g., by sportsmen. In doping control analysis, T abuse is detected by the ratio T/ET (epi-T)<6 [4,5], although there are some cases where an elevated ratio could be of natural origin. For this reason, the control of other ratios, such as T/CL, T/LH and T/HP (CL, cortisol; LH, luteinizing hormone; and HP, hydroxyprogesterone), have been proposed [6,7]. Therefore, the determination of urinary anabolic steroids along with corticoids have a great importance.

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The level of each steroid in biological fluids is usually very low, so that a previous sample pretreatment such as, solid-phase extraction (SPE) or liquid–liquid extraction (LLE) to preconcentrate and eliminate non-desirable species [8], is required. In some cases, it is necessary to perform a previous enzymatic hydrolysis of the glucuronic or sulphate conjugates to obtain the corresponding steroid [9]. The current tendency for the analysis of natural and synthetic anabolic agents and their metabolites in human urine includes GC–MS [10–12]; however a derivatisation process is required in this case. Nevertheless, HPLC methods have also been used for the analysis of urinary anabolics and corticoids related to testosterone doping. The majority of these HPLC methods have been applied to urine samples containing only few compounds and using UV absorbance [13] or fluorescence detection [14]. HPLC-UV absorbance detection has been used for the analysis of these compounds in plasma samples for the analysis of hypoandrogenic and hyperandrogenic populations [15] and HPLC-MS in tissues related to some tumours by controlling the cortisol-to-corticosterone ratio, and dehydroepiandrosterone [16]. In addition, a large number of anabolic and other related steroids have been separated using a gradient elution method [17]. However, little attention has been paid to HPLC isocratic methods for the separation of steroids complex mixtures with screening purposes [18], even though the optimization is easier than gradient methods [19].

In the present paper, an HPLC optimization method for the separation of a urinary steroids mixture, and boldenone and bolasterone (two synthetic anabolics) which are excreted as parent compounds in urine; see structures in Table 1 is described. The effect of several variables (mobile phase, column packing and temperature) that can affect the separation is discussed. This method was applied to human urine samples.

2. Experimental

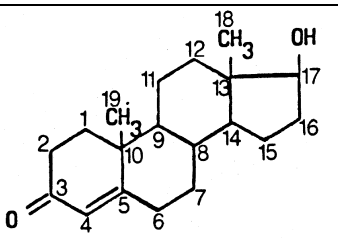
2.1. Chemicals

Cortisone (CS) (4-pregnene-17α,21-diol-3,11,20-trione), cortisol (CL) (11β,17α,21-trihydroxypregnene-4-en-3,20-dione), 11β-hydroxytestosterone (HT) (4-androstane-11β,17β-diol-3-one), 11-ketotestosterone (KT) (4-androstene-17β-ol-3,10-dione), corticosterone (CT) (4-pregnene-11β,21-diol-3,20-dione), boldenone (B) (1,4-androstadien-17β-ol-3-one), hydroxyprogesterone (HP) (4-pregnene-11α-ol-3,20-diene), testosterone (T) (17β-hydroxy-4-androsten-3-one), deoxycorticosterone (DOC) (4-pregnen-21-ol-3,20-dione), androstenolone (AOO) (17β-hydroxy-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), were purchased from Sigma (St. Louis, MO, USA). β-Glucuronidase from *Escherichia coli* was purchased from Boehringer Mannheim (Germany). Sodium acetate, sodium dihydrogenphosphate and disodium hydrogenphosphate were of analytical-reagent grade from Merck (Darmstadt, Germany). HPLC-grade methanol, acetonitrile and tetrahydrofuran were purchased from Promochem (Wesel, Germany) and dichloromethane 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) were also used. Other chemicals were of analytical reagent grade.

2.2. Apparatus

The chromatographic system consisted of the following components all of them from TSP (Riviera Beach, FL, USA): a Constametric 4100 solvent delivery system, a spectromonitor 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, USA) and a Jones-Chromatography block heated series 7960 for thermostating columns in the range 30–60°C (Seagate Technology, Scotts Valley, CA, USA) were used. A Selecta (Barcelona, Spain) thermostated bath was used to control the column temperature below 30°C. The following reversed-phase columns were used: bonded-silica Hypersil ODS (250 mm×4.6 mm I.D., 5 μm), Hypersil BDS (250 mm×4.6 mm I.D., 5 μm), Spherisorb ODS (250 mm×4.6 mm I.D., 5 μm), Luna ODS (250 mm×4.6 mm I.D., 5 μm), Spherex ODS (250 mm×4.6 mm I.D., 5 μm), and a Hamilton PRP-1 (150 mm×4.6 mm I.D., 5

Table 1
Chemical structures of urinary anabolic steroids and corticoids, boldenone and bolasterone



	C1-2	C3	C4-5	C5-6	C7	C11	C17	C19
Hydroxytestosterone (HT)						-OH		
Ketotestosterone (KT)						=O		
Boldenone (B)								-H
Testosterone (T)								
Androstenolone (AOO)	=		—					
Bolasterone (BLS)					$-CH_3$		$-CH_3$	
Epitestosterone (ET)							...OH	
Cortisone (CS)						=O	$-COCH_2OH$ -OH	
Cortisol (CL)						-OH	$-COCH_2OH$ -OH	
Corticosterone (CT)						-OH	$-COCH_2OH$ -OH	
Hydroxyprogesterone (HP)							$-COCH_3$	
Deoxycorticosterone (DOC)							$-COCH_2OH$	
Dehydroepiandrosterone (DHEA)		-OH	—	=				
Methyltestosterone (MT)							$-CH_3$	

μm) from Phenomenex (Torrance, CA, USA). C_{18} extrasep cartridges (2.8 ml and 500 mg) (Phenomenex, Torrance, CA, USA). A vortex mixer Mixo-Tub-30 from Crison (Barcelona, Spain) was also used.

2.3. Mobile phase

The mobile phases were prepared daily by mixing Milli-Q water with methanol (MeOH), acetonitrile (ACN) or tetrahydrofuran (THF) at the required volume ratio by programming the pump. The flow-rate was 1.0 ml min^{-1}. All solvents and mobile phases were firstly filtered under vacuum through 0.45-μm Nylon filters and degassed using helium sparge.

2.4. Chromatographic analysis

Once the column had been conditioned with the mobile phase, chromatograms were obtained at the programmed temperature (range 10–40°C). For optimization purposes based on the use of different mobile phases, a methanolic solution containing a

single steroid or an appropriate mixture of them (5 μg ml^{-1}) was injected (20 μl). Peak identification and peak purity was performed by comparison of their retention time and UV spectra with those of compounds previously registered by injection of each one individually. With exception to DHEA which was monitored at 200 nm, steroids analysis was carried out at 245 nm.

2.5. Urine samples preparation

2.5.1. For steroid recoveries

In order to obtain recoveries of anabolic steroids from urine samples, steroid-free urine was initially prepared by percolating the urine through Extra-sep C_{18} cartridges. The eluates were collected and used as the matrix for steroid spikes. The same procedure as the one reported in Ref. [20] has been used to check endogenous steroids in the above eluates with negative result and to check steroid recoveries. Briefly, 4 ml of dichloromethane were added to 3 ml urine samples spiked with steroids (Table 1) in the range 0.13–0.66 μg ml^{-1}; 0.35 g of sodium chloride (to avoid the formation of emulsions) was added and pH was adjusted to 9 adding 0.5 g of disodium hydrogenphosphate. The mixture was shaken and centrifuged. The organic phase was removed and 3 ml of the resulting solution were dried over anhydrous Na_2SO_4 and then it was evaporated. The dry residue was dissolved using 200 μl of methanol, and 20 μl were injected into the HPLC system.

2.5.2. For urine analysis

The above-described solvent extraction procedure was applied to two samples of urine (3 ml) from a male and the dry residues were dissolved in 200 μl MeOH and 200 μl of a methanolic solution containing 5 μg ml^{-1} of each steroid, respectively. A volume of 20 μl of each solution was then injected into the HPLC system. The absolute preconcentration factor was close to 11.

Another solution was prepared with 3 ml of urine sample from a male, 1 ml 20 m*M* buffer phosphate (pH 7) and 50 μl of β-glucuronidase. The mixture was heated at 55°C for 1 h and then was extracted by applying the solvent extraction procedure described above. In this case the dry residue was dissolved using 50 μl of MeOH and 20 μl injected into the HPLC system. The absolute preconcentration factor was close to 45.

3. Results and discussion

3.1. Optimization of mobile phases

The method reported by Snyder et al. for complex mixtures optimization [19] named 'Glajch's triangle' has shown to be adequate for the separation of complex mixtures of corticoids [18,21]. This method is based on the use of three different organic modifiers with different selectivities, e.g., acetonitrile, methanol and tetrahydrofuran, and pure water as mobile phases. In order to obtain the optimum results, resolution is mapped versus composition of the mobile phases (ranged from binary to quaternary) and the retention factors, *k*, obtained with them have to be adequate depending on the sample complexity (normally in the range 1–15). In the present work, the method has been applied to a complex mixture of urinary anabolic steroids and corticoids, boldenone and bolasterone.

The triangle vertices A–C (binary mobile phases) were assessed using MeOH, ACN and THF, respectively (Fig. 1). When retention factors ($\ln k$) for any compound are plotted versus the organic modifier concentration, Φ, used, linear plots are in agreement with the retention equation, $\ln k = -S\Phi + \ln k_w$, where S and $\ln k_w$ (slope and intercept) are the solvent strength parameter and the retention factors in a purely aqueous mobile phase, respectively. The retention factors, *k*, obtained for each compound using the A–G mobile phases are summarized in Table 2. The optimal separation for the A vertex was achieved with H_2O–MeOH (40:60, v/v). Under these conditions 11 steroids were separated in about 22 min and coeluted: (B, HP) and (ET, BLS). Similarly, the B vertex was achieved with the mixture H_2O–ACN (60:40, v/v). Under these conditions 13 compounds were separated in about 24 min. The C vertex was achieved with H_2O–THF (70:30, v/v). Under such conditions 13 compounds were also separated in about 24 min.

The middle side points of the triangle D–F (ternary mixtures) and the centroid G (quaternary mixture) were also studied. The D point was ob-

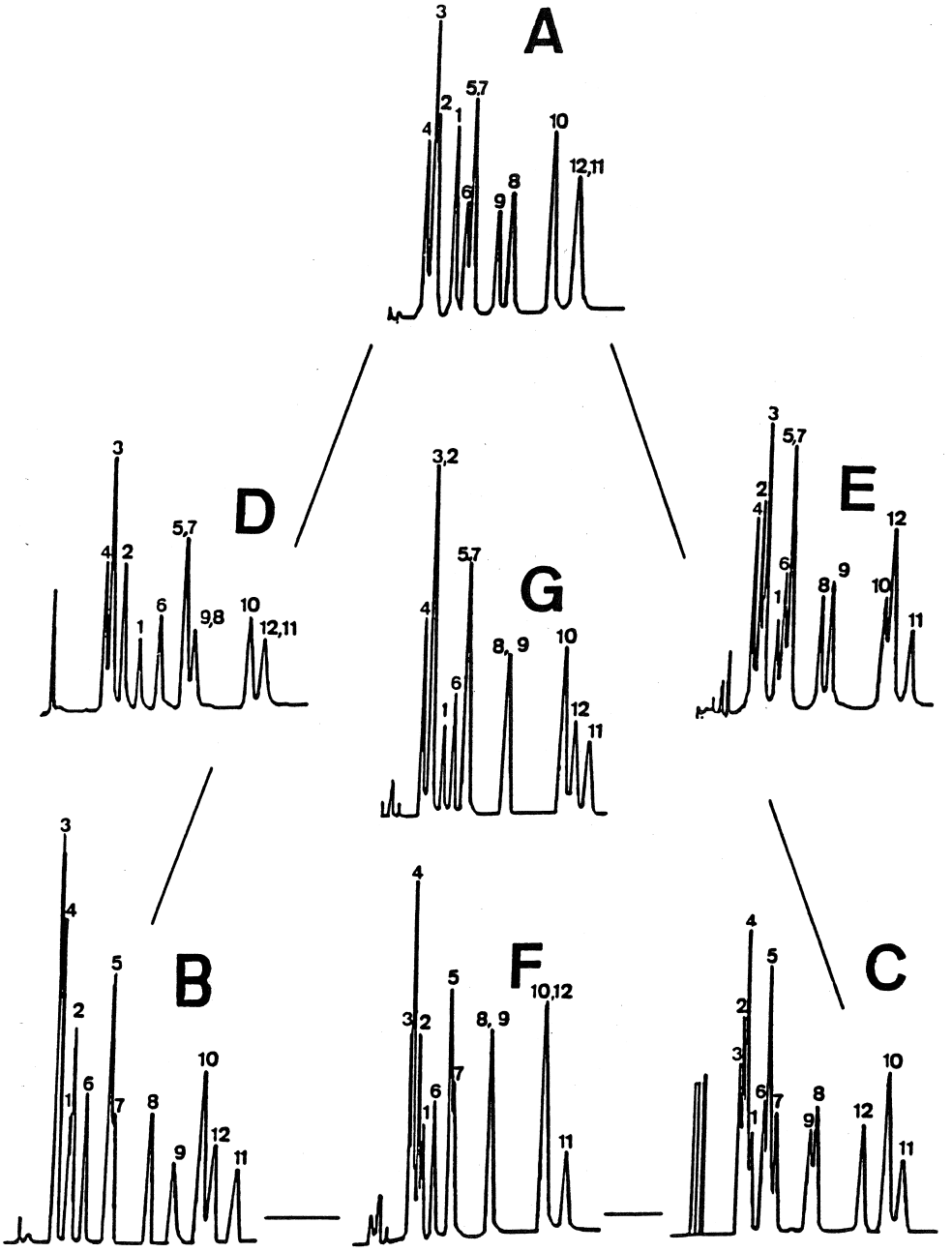


Fig. 1. Glajch's triangle for the optimization of a mixture of steroids (10 μg ml^{-1}) using different mobile phases: binary (A–C), ternary (D–F) and quaternary (G). A–C were obtained using MeOH, ACN and THF as organic modifiers and pure water, and D–F by combining appropriately the binary mobile phases. Peak numbers as in Table 2. Conditions: UV detection at 245 nm for peaks numbers 1–12; flow-rate, 1 ml min^{-1}; and Hypersil ODS (250 mm×4.6 mm, 5 μm) column (30°C).

Table 2
Retention factors, *k*, for binary (A–C), ternary (D–F) and quaternary (G) mobile phases (for identification see text)[a]

Compounds	A	B	C	D	E	F	G
3.CL	1.17	1.13	2.15	2.06	1.75	1.39	1.69
4.CS	0.89	1.20	1.84	1.81	1.29	1.54	1.29
1.HT	1.99	1.64	2.36	3.17	2.32	1.79	2.17
2.KT	1.28	1.79	1.99	2.54	1.57	1.96	1.69
6.CT	2.45	2.34	2.81	3.96	2.59	2.47	2.59
5.B	2.62	3.31	3.09	4.97	2.81	3.18	3.09
7.HP	2.62	3.49	3.39	4.97	2.81	3.30	3.09
8.T	4.04	5.13	5.03	5.35	4.16	4.96	4.80
9.DOC	3.55	6.28	4.85	5.35	4.58	4.96	4.80
13.DHEA	4.59	7.34	8.12	4.98	5.59	7.37	6.86
10.AOO	5.61	7.39	8.05	7.58	6.78	7.34	7.17
12.ET	6.60	7.90	7.02	8.18	7.08	7.34	7.65
11.BLS	6.60	8.88	8.67	8.18	7.91	8.33	8.23

[a] Conditions as in Fig. 1

tained with H_2O–MeOH–ACN (50:30:20, v/v/v). Under these conditions 10 compounds were separated in about 24 min and coeluted: (B, HP, DHEA), (DOC, T) and (ET, BLS). The E point was obtained with H_2O–MeOH–THF (55:30:15, v/v/v). Under these conditions 12 steroids were separated in about 23 min and coeluted: (B, HP). The F point was obtained with the mixture H_2O–ACN–THF (65:20:15, v/v/v) and 9 compounds were separated in about 23 min and coeluted: (T, DOC) and (DHEA, AOO, ET). The G point was obtained with H_2O–MeOH–ACN–THF (56.7:20:13.3:10, v/v/v/v). Under these conditions 10 compounds were separated in about 20 min and coeluted: (CL, KT), (B, HP) and (T, DOC). Taking into account the run time analysis and resolution, the optimum separation conditions for further experiments were those using B mobile phase (13 steroids were separated in about 24 min). Selectivity, α, was examined for the binary mobile phases in different ways. No correlation was found between S and $\ln K_w$ values obtained from Hooke's equation for the steroids and the three solvents tested [22]. Selectivity, α, was also studied for the different solvents tested by taking into account consecutive peaks. Fig. 2, in which plots of α values of pairs of steroids versus percent of the organic modifier are shown, indicates different behaviors depending on the organic modifier used. α decreases as the solvent concentration increases, e.g., for CL/KT and HP/DOC pairs using MeOH, for

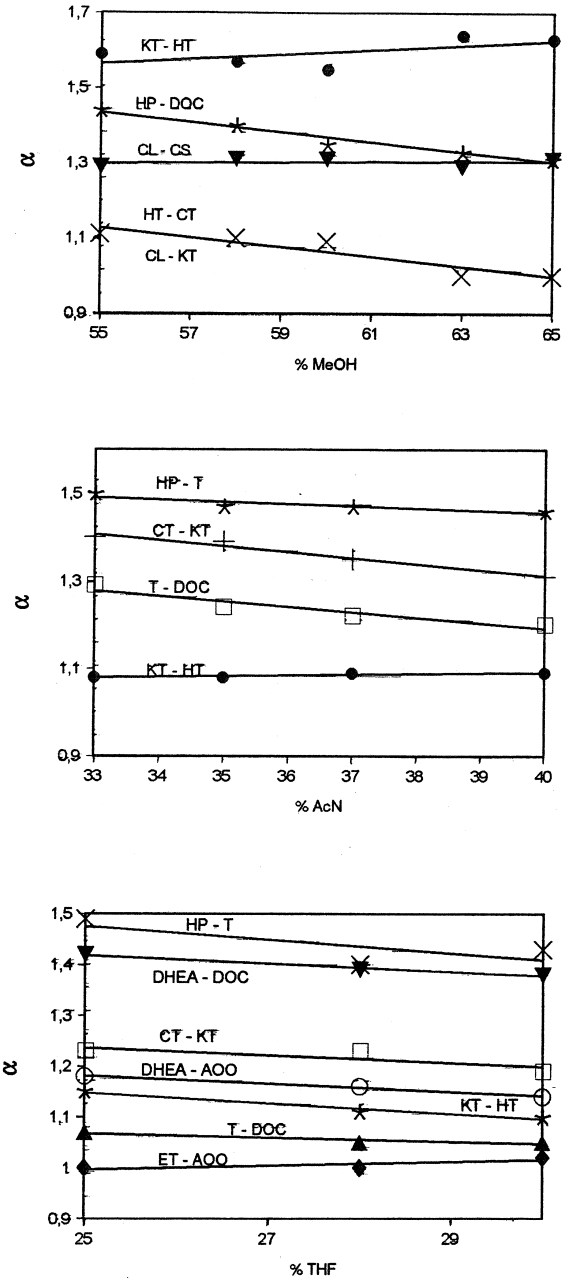


Fig. 2. Influence of different organic modifiers on the steroid selectivity, α.

CT/KT and T/DOC pairs using ACN, and for HP/T, CT/KT, DHEA/AOO or DOC, KT/HT pairs using THF. However, for other pairs, e.g., KT/HT using MeOH and ET/AOO using THF the behavior is the

opposite and for the remaining pairs selectivity is not modified in a significant way as the solvent concentration increases, e.g., CL/CS using MeOH, KT/HT using ACN and T/DOC using THF. Moreover, changes in the elution order for compounds were not observed for any organic modifier in the concentration range tested. However, changes in the elution order take place when comparing the A–G mobile phases (see Fig. 2 and Table 2 data).

3.2. Effect of salts added to the mobile phase

The influence of salts added to the mobile phase on the steroid retention was studied using mobile phases consisting of 20 m*M* phosphate, 50 m*M* phosphate (pH values 3.2 and 7.2 in both cases) or 20 m*M* or 50 m*M* acetate (pH 4.6) buffer and ACN (60:40, v/v). Under these conditions no significant changes in the retention of steroids were observed.

3.3. Temperature effect

The temperature effect on steroid retention was studied in the range 10–40°C under the optimum separation conditions (B triangle vertex). The retention factors, *k*, obtained at different temperatures are listed in Table 3. As the temperature increases, *k* values decrease and the chromatographic resolution improves. In addition, selectivity is also modified. Thus, at 10°C, 12 steroids were separated in about 26 min and coeluted (B/HP); at 23°C, 11 steroids in about 25 min and coeluted (B/HP) and (AOO/ET); at 30°C, 13 steroids in about 24 min and no coelutions were observed; at 40°C, 11 steroids in about 22 min and coeluted (CL/CS) and (HT/KT). From these results, a temperature of 30°C was finally chosen as a compromise between run time, resolution and number of compounds separated. Van't Hoff plots ($\ln k$ vs. $1/T$) constructed from the data of Table 3 show a linear behavior for these compounds ($r > 0.99$). Enthalpy (ΔH) and entropy (ΔS) can be derived from the slope and intercept, respectively. The intercept negative sign indicates that the entropy decreases while the compounds are transferred from the mobile to the stationary phases and the negative enthalpy values (Table 3) indicate that the mass transfer process is exothermic.

Table 3
Retention factors, *k*, obtained at different temperatures using water–acetonitrile (60:40, v/v) as mobile phase and enthalpy, ΔH, values obtained from the slope of van't Hoff plots[a]

Compounds	10°C	23°C	30°C	40°C	ΔH (KJ mol^{-1})
CL	1.30	1.20	1.13	1.13	−1.735
CS	1.51	1.28	1.20	1.13	−7.231
HT	1.67	1.59	1.64	1.76	−2.784
KT	2.09	1.84	1.79	1.76	−5.998
CT	2.68	2.40	2.34	2.30	−1.747
B	4.00	3.46	3.31	3.26	−6.805
HP	4.00	3.60	3.49	3.45	−5.221
T	6.50	5.65	5.13	5.02	−8.602
DOC	8.45	7.00	6.28	5.93	−10.542
DHEA	10.40	8.54	7.34	7.03	−7.382
AOO	9.66	8.49	7.39	7.17	−7.265
ET	10.06	8.49	7.90	7.76	−7.300
BLS	10.98	9.68	8.88	8.76	−8.360

[a] Other conditions as in Fig. 1.

3.4. Stationary phase effect

A comparative study on the steroids separation using different reversed-phase columns at 30°C (I–VI in Table 4) and B mobile phase conditions, was carried out. The retention factors, *k*, obtained are

Table 4
Retention factors (*k*), number of compounds separated (NS) and run time analysis (RTA) obtained using different reversed-phase columns (30°C): (I) Hypersil ODS, (II) Hypersil BDS, (III) Spherisorb ODS, (IV) Luna ODS, (V) Spherex ODS and (VI) PRP-1[a]

Compounds	I	II	III	IV	V	VI
CL	1.08	0.90	1.39	1.57	1.47	0.93
CS	1.17	0.90	1.60	1.72	1.64	1.15
HT	1.59	1.31	2.34	2.32	3.01	1.41
KT	1.73	1.31	2.57	2.56	3.68	1.95
CT	2.26	1.84	3.35	3.37	6.18	2.32
B	3.22	2.55	5.04	4.68	7.35	3.52
HP	3.40	2.79	5.76	5.20	7.35	3.82
T	4.98	3.99	9.70	7.38	11.97	6.20
DOC	5.96	4.77	11.14	8.92	14.17	8.33
DHEA	7.11	5.73	11.56	10.71	15.29	8.33
AOO	7.19	5.89	11.95	11.08	16.52	8.33
ET	7.68	6.34	14.07	11.84	16.52	9.73
BLS	8.67	6.99	16.62	13.28	20.19	10.68
NS	13	11	13	13	11	11
RTA	24	21	27	31	35	24

[a] Mobile phase: water–acetonitrile (60:40, v/v) and flow-rate 1 ml min^{-1}.

summarized in Table 4. Poor results were obtained using the polymeric phase PRP-1: 11 compounds were separated with poor resolution and coeluted (DOC/DHEA/AOO). However, using the Spherex and Hypersil BDS columns, resolution was improved in comparison with the PRP-1 column, allowing also the separation of 11 steroids (the pairs B/HP; AOO/ET were coeluted using the Spherex column, and CL/CS; HT/KT using the Hypersil BDS packing). Similar results, comparing run time analysis (RTA) and number of compounds separated (NS), were observed using Hypersil ODS, Spherisorb and Luna packings (Table 4). However, different selectivities were obtained with these columns. Taking into account the results obtained (RTA and NS), a Hypersil ODS column was finally chosen for further experiments.

3.5. Calibration graphs

Calibration graphs were obtained by adding to steroid-free urine samples (Table 1), standards of steroids at five different concentrations in the range 2–10 μg ml^{-1}, using 5 μg ml^{-1} BLS as internal standard (I.S.) (or MT for BLS) under solvent extraction conditions (Section 2.5). These mixtures were separated using the optimal mobile phase: H_2O–ACN (60:40, v/v), a flow-rate of 1.0 ml min^{-1} and UV absorbance-DAD detection at 245 and 200 nm (DHEA). The results were analyzed by linear regression. Plotting each steroid peak area to I.S. ratio (PAR) versus the concentration (x) of each one, the calibration equation $PAR = A + Bx$ (μg ml^{-1}), was obtained. The parameters A (intercept), B (slope) and r (regression coefficient) are summarized in Table 5. In all cases the intercepts were not significantly different from zero.

3.6. Precision, accuracy and detection limits.

The repeatability (within run precision) was examined by analyzing 10 different mixtures of the steroids within a day using an individual concentration of 5 μg ml^{-1} and by running each mixture once ($n = 10$), whereas reproducibility (between run precision) was evaluated for three different days ($n = 30$) using the calibration graphs. The C.V. values obtained are shown in Table 5. The accuracy was examined in the range 2–10 μg ml^{-1} for each steroid. The Barlett and Harley test [23] was applied to corroborate the randomness of the variances. This test confirmed that this chromatographic method does not present systematic error and does not require blank correction. Detection limits (LODs) were also calculated from the calibration graphs for a signal-to-noise (S/N) ratio of 3 ($n = 10$) [24] (Table 5).

Table 5
Linear regression equations ($PAR = A + Bx$), detection limits (LODs) and C.V. for repeatability (RPT) and reproducibility (RPC) of steroids. PAR is the steroids peak area ratio to BLS(I.S.)[a]

	A	B	r	LODs (μg ml^{-1})	RPT C.V. (%)	RPC C.V. (%)
CS	0.043	0.138	0.999	0.041	2.23	4.02
CL	0.070	0.153	0.998	0.026	5.68	7.58
KT	0.056	0.243	0.999	0.041	2.47	5.70
HT	0.035	0.195	0.996	0.038	5.74	7.41
CT	0.021	0.148	0.999	0.037	8.05	11.08
B	0.060	0.340	0.999	0.012	4.74	6.94
HP	0.040	0.150	0.998	0.040	2.57	4.67
DOC	0.032	0.145	0.999	0.025	1.26	3.62
T	0.053	0.176	0.998	0.040	1.54	3.48
DHEA	0.036	0.092	0.999	0.058	6.20	8.36
AOO	0.058	0.069	0.996	0.107	5.37	7.53
ET	0.055	0.230	0.998	0.039	2.64	4.22
BLS	0.046	0.117	0.999	0.034	5.63	8.26

[a] A retention factor, $k = 6.44$, was obtained for MT used as I.S. for BLS. Conditions: mobile phase (ACN/H_2O, 40:60, v/v), flow-rate 1 ml min^{-1} and Hypersil ODS column.

3.7. Analysis of urine samples

A solvent extraction procedure (Section 2.5) for some of the corticoids under study (CL, CS, CT, HP and DOC) has been previously developed. Recoveries (%E) were larger than 91.0% [20]. This procedure was also applied to the remaining steroids under study. The individual values of the recoveries (%E)±C.V. obtained were: (93.8±6.3) for KT, (92.7±6.0) for HT, (92.0±7.1) for B, (92.2±6.9) for T, (102±2.0) for DHEA, (94.4±5.2) for AOO, (95.1±5.6) for ET, (103±4.5) for BLS (R. Gonzalo-Lumbreras, R. Izquierdo-Hornillos, unpublished work).

The chromatogram obtained under the optimal separation conditions for a standard mixture of

steroids prepared in methanol is shown in Fig. 3A. Under these conditions, the method was applied to human urine samples using LLE. Fig. 3B–D show the chromatograms obtained. Fig. 3B corresponds to a real sample spiked with steroids and Fig. 3D and Fig. 3C were obtained with and without hydrolysis with β-glucuronidase (see Section 2.5). Comparing Fig. 3A with 3B indicates that this method is adequate for analysis of urine samples containing these steroids without matrix interferences (the relative standard deviation, RSD ($n=6$) of the retention factors for the compounds under the conditions in Fig. 3A,B was lower than 1% for each one). In Fig. 3C, free CL, CS and CT appeared (the preconcentration factor used is close to 11). However, under hydrolysis conditions, peaks corresponding to free and conjugated CL, CS, KT, CT, HP, T and ET are observed (the preconcentration factor used is close to 45). UK in Fig. 3B–D denotes an unknown peak. In addition, the area of the steroid peaks were ex-

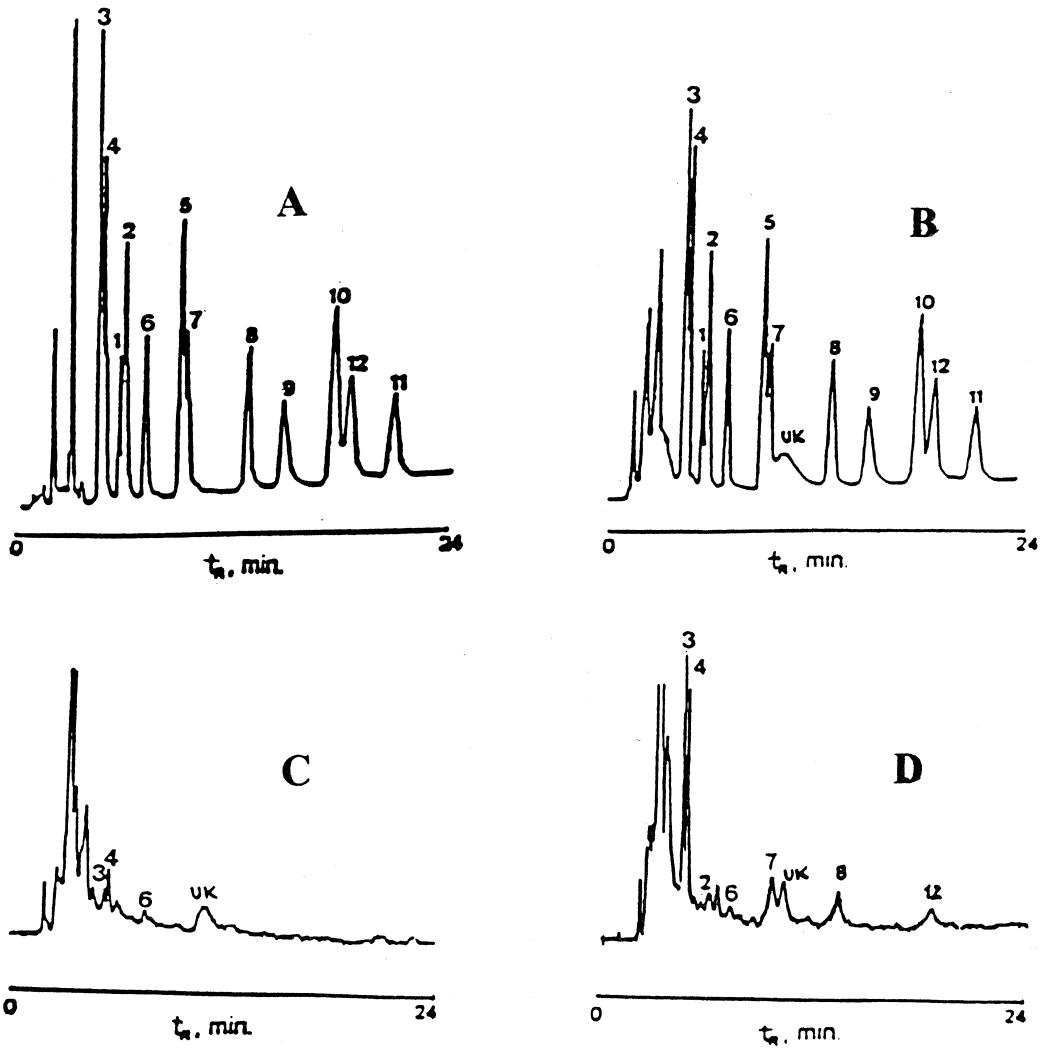


Fig. 3. Chromatograms obtained from a standard mixture of steroids prepared in methanol (A) (10 μg ml^{-1}), and from urine samples under LLE: (B) spiked with steroids (10 μg ml^{-1}), and (D,C) with and without a previous treatment with β-glucuronidase. Peak numbers as in Table 2 and UV detection at 245 nm. UK denotes an unknown peak.

amined, showing each compound a concentration over its LOD.

A detection and identification process of steroids based on retention times and a diode array detector (DAD) was carried out [25]. 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. The possible impurities in each peak detected were negligible. When interferences occur, a change in the mobile phase composition is recommended. The present method shows the ability to analyze steroids in urine samples with good analytical performances (e.g., in doping control by means of several ratios: CL/T, T/ET or T/HP from Fig. 3D) or for clinical purposes (e.g., Cushing syndrome by the CL/CS ratio from Fig. 3C) [26].

4. Conclusions

A mixture of 13 steroids (urinary and synthetic) was separated in about 24 min using H_2O–ACN (60:40, v/v), a Hypersil ODS 5 μm column (30°C) and UV absorbance-DAD detection at 245 and 200 nm (DHEA) by applying the Glajch method. Different separations and consequently different selectivities were obtained depending on the mobile and stationary phases and temperatures studied. The optimal separation was applied to human urine samples allowing qualitative detection of free urinary CL, CS and CT. However, when hydrolysis is performed, qualitative detection in urine samples of free and conjugated CL, CS, CT, KT, HP, T and ET is achieved. In both cases the concentration factor required was different.The present method seems to be potentially applicable in urine analysis for different purposes (clinical purposes or in doping control). Obviously, these applications must be studied (and validated) separately because differ from each other and the performances required are not identical.

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