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High-performance liquid chromatographic optimization study for the separation of natural and synthetic anabolic steroids. Application to urine and pharmaceutical samples

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Abstract

An HPLC separation of a complex mixture containing 14 androgenic anabolic steroids (natural and synthetic) for screening purposes 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 using acetonitrile as organic modifier was studied. The optimum separation was achieved by using a water–acetonitrile (55:45, v:v) mobile phase and a Hypersil ODS (250 mm×4.6 mm) 5 μm column (30°C) in about 38 min, allowing the separation of 14 out of 14 compounds tested (when danazol is exluded, 13 out of 14 were separated in 23 min). Calibration graphs were obtained using bolasterone, methyltestosterone and canrenone as internal standards. Detection limits were in the range 0.012–0.11 μg ml^{-1}. The optimized separation was applied for monitoring the norethindrone acetate hydrolysis from tablets and to the analysis, after liquid–liquid extraction, of urine samples spiked with steroids.

Keywords: Anabolic steroids

1. Introduction

Androgenic anabolic steroids (AAS) are compounds mainly secreted by the testes and in a few amount by ovaries and adrenal cortex. The masculinizing (androgenic) effects are coupled with an anabolic effect (tissue building). Testosterone (T) is the most potent naturally occurring androgen [1]. Other AAS are synthesized from T in order to produce AAS with strong anabolic properties and low androgenicity. AAS can also be obtained by esterification with organic acids, e.g. testosterone propionate or norethisterone acetate. It is known that AAS esters are readily hydrolysed in tissues by esterases [2,3]. These synthetic AAS are contained in different pharmaceuticals (patches, creams, tablets and ampoules) and used for therapeutic (growth-disorder, breast cancer and osteoporosis) and, in sports, for doping purposes. Therefore, these AAS along with the natural ones are controlled in plasma or in urine for the diagnosis of some tumours [4,5] and for identifying the abuse of anabolic agents in sports [6,7]. All these compounds belong to one of

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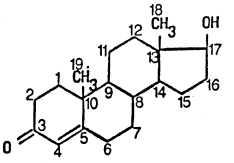
the doping classes banned by the International Olympic Committee (IOC) and National and International Sports Federations. In addition, they are also illegally used as growth-promoting agents individually or in ''cocktail'' form in cattle [8–10].

Most AAS are metabolized and excreted in urine as glucuronic derivatives. For this reason the analysis normally requires a previous hydrolysis and extraction procedure [11].

The majority of analytical methods for AAS and their metabolites use GC–MS which allows the identification of these compounds and metabolites with low detection limits, requiring, however, a previous derivatization [11]. These methods are referred to a single compound [9], mixtures including few compounds [10] and complex mixtures with screening purposes [12]. RP-HPLC is also used for the analysis of complex mixtures of AAS without derivatization process using UV absorbance [13–16], electrochemical [17], fluorescence [18], MS [19] and infrared spectroscopy detection [20].

In the present paper a RP-HPLC systematic optimization of a complex mixture of natural and synthetic AAS (structures in Table 1) is described. The influence on the separation of different mobile and stationary phases, and temperature is discussed. In this way 14 AAS out of 14 were separated in about 38 min using a water–acetonitrile (55:45, v:v) mobile phase and a Hypersil ODS (250 mm×4.6 mm I.D.) 5 μm column. This method was applied for

Table 1
Structures of androgenic anabolic steroids (AAS)



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

monitoring the norethindrone acetate hydrolysis and to the analysis, after liquid–liquid extraction, of human urine urine samples spiked with steroids.

2. Experimental

2.1. Chemicals

11β-hydroxytestosterone (HT) (4-androstane-11β,17β-diol-3-one), 11-ketotestosterone (KT) (4-androstene-17β-ol-3,10-dione), 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), boldenone (B) (1,4-androstadien-17β-ol-3-one), testosterone (T) (17β-hydroxy-4-androsten-3-one, 17α-methyl-testosterone (MT) (17-hydroxy-17-methylandrost-4-en-3-one), androstenolone (AOO) (17β-hydroxy-5α-androst-1-en-3-one), bolasterone (BLS) (17-hydroxy-7,17-dimethylandrost-4-en-3-one), epitestosterone (ET) (17α-hydroxy-4-androsten-3-one), 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), canrenone (CAN) (17α-17-hydroxy-3-oxopregna-4,6-diene-21-carboxylic acid γ-lactone) were purchased from Sigma (St. Louis, MO, USA). 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 used 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 as recorder a 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 also 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 Hamilton PRP-1 (150×4.6 mm) 5 μm from 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 MeOH, acetonitrile (ACN), or tetrahydrofuran (THF) 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 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 AAS or an appropriate mixture of them (5 μg/ml) was injected (20 μl). The flow-rate was 1.0 ml min^{-1} (for the Hamilton PRP-1 column was 0.5 ml min^{-1}) and UV absorbance-DAD detection in the range 190–360 nm was used. Peaks identification and peak purity of AAS were performed by comparison of their retention time and UV spectra with those of compounds previously registered by injection of each one individually. Analysis was carried out at 245 with exception to OM and DZ which was monitorized at 280 nm.

2.5. Sample preparation

2.5.1. For AAS recoveries from human urine

In order to obtain recoveries of AAS under study from urine samples, the same procedure as the one reported [21] has been used. Briefly, 4 ml of dichloromethane were added to 3 ml urine samples. K_2HPO_4 was added to obtain pH 9 and NaCl to avoid the formation of emulsions. 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 MeOH, and 20 μl were injected into the HPLC system. In this way, the recoveries obtained for AAS in Table 1 were in the range 90–100% [22].

2.5.2. For human urine analysis

The above described solvent extraction procedure was applied to urine samples (3 ml) from a male and the dry residue was dissolved in 200 μl of a methanolic solution containing 5 μg/ml of each AAS and 20 μl were then injected into the HPLC system.

2.5.3. For hydrolysis of norethindrone acetate

Tablets (Primolut®-Nor from Schering AG, Germany) containing 5 mg norethindrone acetate were adequately pulverized. The fine powder was weighed and adequately dissolved in water. After this, samples were diluted with phosphoric acid solution (pH 3.0), phosphate buffer (pH 7.2) or sodium hydroxide (pH 12) to obtain three separate 5 μg/ml solutions and 20 μl were then injected into the HPLC system.

3. Results and discussion

3.1. Optimization of mobile phases

The method reported by Snyder et al. for complex mixtures optimization named ''Glajch's triangle'' [23] is based on the use of mobile phases containing aqueous solutions and three different organic modifiers with different selectivities. The organic solvents are ACN (which belong to group VIb of the Snyder's selectivity triangle), MeOH (group II) and THF (group III). In order to obtain the optimum results, resolution are mapped versus the 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). This method has shown to be applicable for the separation of complex mixtures of similar compounds such as natural and synthetic corticoids [24] using conventional (CLC) and micellar liquid chromatography (MLC) [25], and urinary steroids including boldenone and bolasterone [26] using CLC. In the present work, the method has been applied to the separation of a complex mixture of AAS.

For any mobile phase further assessed, DZ eluted for a retention time close to 40 min. For this reason in Table 2 are listed two run time analysis (RTA), one of these included DZ (RTA*) and the other included the remaining AAS.

The triangle vertices A, B and C (binary mobile phases) were assessed using MeOH, ACN and THF respectively and a Hypersil ODS (250 mm×4.6 mm I.D., 5 μm) column (Fig. 1). When retention factors ($\ln k$) for any compound are plotted vs. the organic modifier concentration, Φ, used (Fig. 2), linear plots are in agreement with the equation,

$$\ln k = -S\Phi + \ln k_w \quad (1)$$

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 [27,28]. The optimal separation for the A vertex was achieved with H_2O–MeOH (35:65, v/v). Under these conditions 9 AAS were separated in about 23 min coeluted: (FM, B), (NT, NE), (MT, AOO) and (ET, BLS). Similarly, the B vertex was achieved with H_2O–ACN (55:45, v/v). Under these conditions 13 AAS were separated in about 23 min without coelution. The C vertex was achieved with H_2O–THF (70:30, v/v) with which 10 AAS were separated in about 32 min and coeluted: (FM, B), (NT, DMT) and (NE, MT). DZ was eluted for a retention time higher than 40 min.

The middle side points of the triangle D, E and F and the central G (ternary and quaternary mixtures, respectively) were also studied. The D point was obtained with H_2O–MeOH–ACN (40:32.5:22.5, v/v/v) and 10 AAS were separated in about 22 min

Table 2
Retention factors, *k*, for AAS using (A–G) mobile phases[a]. NR is the number of separated compounds (DZ and OM are included) and RTA the run time analysis. Conditions as in Fig. 1

AAS	A	B	C	D	E	F	G
HT	1.48	1.08	2.35	1.80	2.11	1.33	1.50
KT	0.99	1.17	1.97	1.43	1.43	1.25	1.15
FM	1.90	1.42	3.06	2.33	2.49	1.88	2.08
B	1.90	1.93	3.06	2.69	2.49	2.10	2.08
NT	2.16	2.35	3.78	3.01	3.01	2.43	2.40
DMT	2.40	2.60	3.78	3.01	3.15	2.61	2.62
T	2.85	3.08	5.01	4.03	3.97	3.18	3.20
NE	2.16	3.24	6.19	3.42	3.97	3.81	3.20
MT	3.72	4.03	6.19	5.31	5.28	4.07	4.18
AOO	3.72	4.33	7.91	5.31	5.84	4.78	4.70
ET	4.39	4.59	6.88	6.57	5.84	4.78	5.15
BLS	4.39	5.06	8.51	6.89	6.77	5.20	5.15
OM	7.55	8.64	11.36	>13	>13	7.88	9.36
DZ	>15	12.52	>15	>15	>15	>15	>15
NR	10	14	11	12	11	13	11
RTA	23	23	32	22	20	23	28
RTA*	>40	38	>40	>40	>40	>40	>40

[a] A–G mobile phases (v/ . . . /v). A: H_2O–MeOH (35:65); B: H_2O–ACN (55:45); C: H_2O–THF (70:30); D: H_2O–MeOH–ACN (40:32.5:22.5); E: H_2O–MeOH–THF (52.5:32.5:15); F: H_2O–ACN–THF (62.5:22.5:15) and G: H_2O–MeOH–ACN–THF (53.5:21.5:15:10).

and coeluted: (NT, DMT) and (MT, AOO). OM were also eluted for larger retention times (Table 2). The E point was obtained with H_2O–MeOH–THF (52.5:32.5:15, v/v/v) and 9 AAS were separated in about 20 min and coeluted: (FM, B), (NT, DMT), (T, NE) and (AOO, ET). OM were also eluted for larger retention times (Table 2). The F point was obtained with H_2O–ACN–THF (62.5:22.5:15, v/v/v). Under these conditions 12 AAS were separated in about 23 min and coeluted: (AOO, ET). DZ was eluted for a retention time higher than 40 min).

The G point was obtained with H_2O–MeOH–ACN–THF (53.5:21.5:15:10, v/v/v/v) and 10 AAS were separated in about 28 min and coeluted: (FM, B), (T, NE) and (ET, BLS). DZ was eluted for a retention time higher than 40 min). Other mobile phases (H and I) close to B vertex were tested. The H point was obtained with H_2O–ACN–THF (58.8:33.7;7.5, v/v/v) and 12 AAS were separated in about 23 min and coeluted: (HT, KT). Using H mobile phase resolution can be improved with regard to the above separations. The I point was obtained with H_2O–MeOH–ACN (50.1:33.7:16.2, v/v/v). Under these conditions 12 compounds were separated in about 32 min and coeluted: (ET, BLS). In Table 2 are summarized retention factors, *k*, obtained for AAS using (A–G) mobile phases. Taking into account the run time analysis and resolution, the optimum separation conditions selected for further experiments were those using B mobile phase (13 AAS were separated in about 23 min or 14 in 38 min). Other assays lead to good results, e.g. using H mobile phase resolution is improved.

Selectivity between solutes was examined qualitatively for the binary mobile phases by analysis of the ln *k* vs. Φ plots (Fig. 2). The selectivity between those solutes whose slopes and intercepts (Eq. 1) are directly related to one another would decrease with an increase in Φ. In contrast, for cases where there is no direct relationship between the slope and intercept, the selectivity would increase with Φ. In this way, no correlation (different selectivity behavior) was observed between *S* and ln k_w values for MeOH, ACN and THF. Selectivity, α, was also studied for the different solvents by taking into account consecutive peaks. The pairs of compounds under study exhibited different behaviors. Examples of them are in Fig. 2 (plots of retention factors vs. percent of

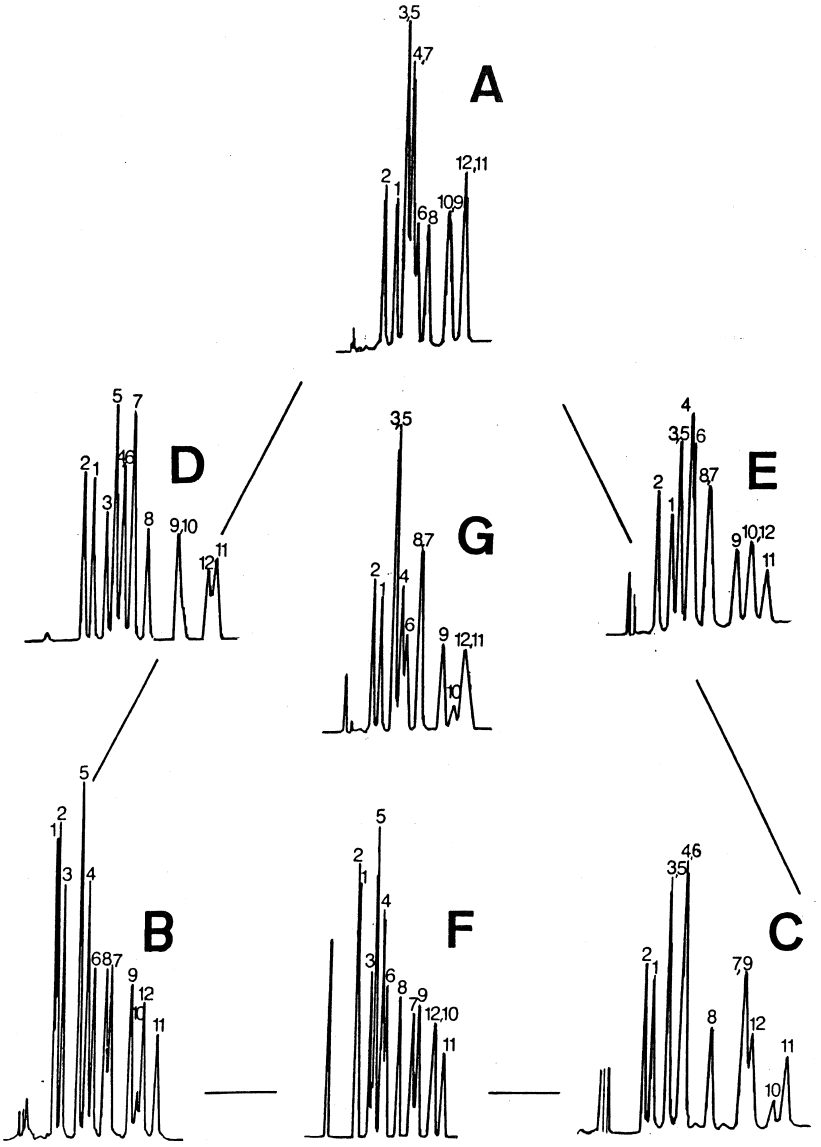


Fig. 1. Glajch's triangle applied to CLC using a standard mixture of AAS. Peak numbers: 1 (HT), 2 (KT), 3 (FM), 4 (NT), 5 (B), 6 (DMT), 7 (NE), 8 (T), 9 (MT), 10 (AOO), 11 (BLS), 12 (ET), 13 (OM), 14 (DZ). Conditions: flow-rate 1 ml min^{-1}. Hypersil ODS (250 mm×4.6 mm I.D. I.D.) 5 μm column (30°C).

organic modifier). As can be seen, selectivity decreases sligthly (some lines tend to converge) e.g. ET/AOO, HT/KT, KT/FM, NE/DMT for ACN; AOO/BLS and HT/KT using THF or is not modified (parallel lines) in a significant way as Φ increases for the majority of pairs. However, for the pairs, e.g. AOO or MT/ET or BLS using MeOH and MT/AOO using ACN, the behavior is the opposite (some lines tend to diverge). Likewise, reversals in the elution order for solvents and several coelutions for a solvent given, can also be observed (Table 2 and Fig. 2).

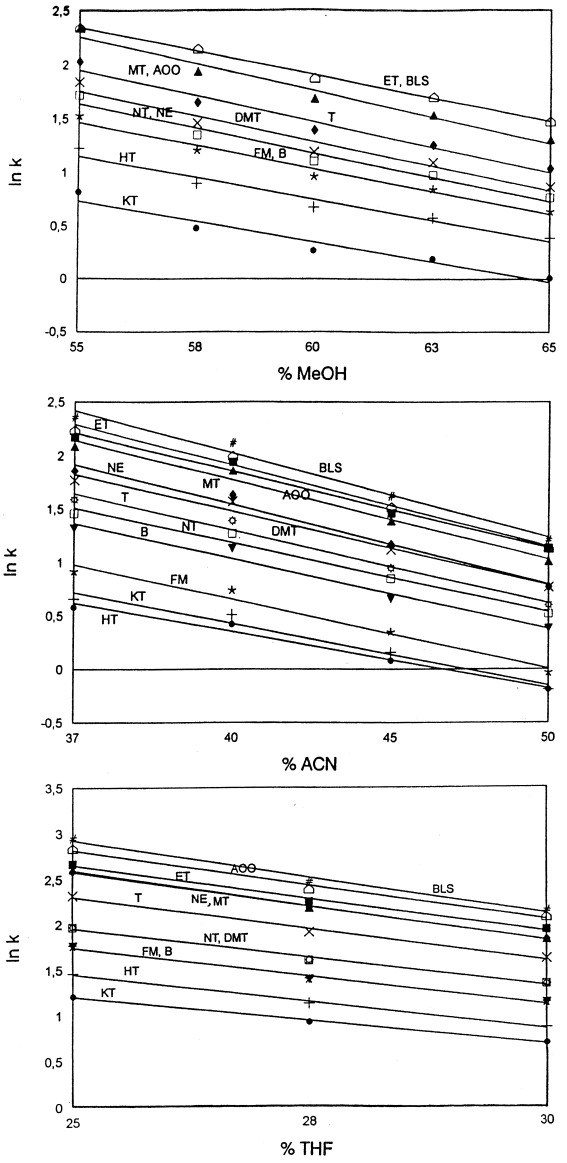


Fig. 2. Effect of different organic modifiers on AAS retention.

3.2. pH and salting out effects

The pH and salting out effects on AAS retention was studied in the range 3.2–7.2 using 20 mM phosphate, 50 mM phosphate (pHs 3.2 and 7.2 in both cases) or 20 mM, 50 mM acetate (pH 4.6), using B mobile phase. As expected, no significant changes in the retention of AAS were observed.

Table 3
Retention factors, k, for AAS obtained at different temperatures using water–acetonitrile (55:45, v/v) as mobile phase and enthalpy, ΔH, values obtained from the slope of van't Hoff plots. Other conditions as in Fig. 1

AAS	10°C	23°C	30°C	40°C	ΔH, Kcal mol^{-1}
HT	1.14	1.10	1.08	1.06	−0.933
KT	1.39	1.27	1.17	1.06	−1.064
FM	1.55	1.49	1.42	1.40	−0.533
B	2.50	2.27	1.93	1.91	−1.308
NT	3.08	2.71	2.35	2.32	−1.797
DMT	3.08	2.86	2.60	2.58	−0.878
T	3.96	3.57	3.08	3.05	−1.704
NE	4.98	3.92	3.24	3.05	−2.016
MT	5.06	4.62	4.03	3.96	−1.215
AOO	5.66	5.21	4.33	4.13	−2.396
ET	5.66	5.21	4.59	4.39	−1.274
BLS	6.28	5.81	5.06	4.89	−1.156
OM	11.71	10.55	8.64	8.56	−1.596
DZ	>15	>15	12.52	12.43	–

3.3. Temperature effect

The temperature effect on AAS retention was studied in the range 10–40°C using B mobile phase. In Table 3 are listed the k values for AAS at different temperatures and in Fig. 3 the Van't Hoff plots ($\ln k$ vs. $1/T$) constructed from the data of Table 3, showing a linear behavior for these compounds ($r>0.99$). As the temperature increases, k values decrease and the chromatographic resolution improves in the range 10–30°C. In addition, selectivity is also modified. Thus, at 10°C, 12 AAS were

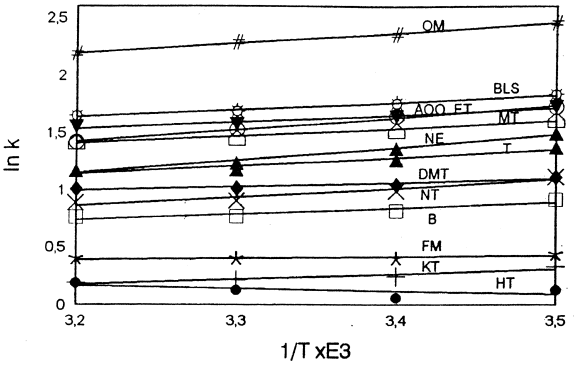


Fig. 3. Effect of temperature on AAS retention.

separated in about 29 min and coeluted (NT/DMT) and (ET/AOO); at 23°C, 13 AAS in about 27 min and coeluted (AOO/ET); at 30°C, 14 AAS in about 23 min and no coelutions were observed; at 40°C, 11 AAS in about 21 min and coeluted (KT/HT), (T/NE) and (MT/AOO). From these results, a temperature of 30°C was finally chosen as a compromise between run time, resolution and number of compounds separated.

Enthalpy (ΔH) and entropy (ΔS) can be derived from the slope and intercept (Fig. 3), 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.

3.4. Stationary phase effect

A comparison between different reverse phase columns at 30°C (I–VI in Table 4), using B mobile phase conditions obtained with the I column (Hypersil ODS), was carried out. In Table 4 are summarized the k values for the different compounds and packings. Poor results were obtained using the VI packing (Hamilton PRP-1): 9 compounds were separated and coeluted (KT, FM), (NT, DMT) and (NE, MT, ET, AOO). The V packing (Spherex) provided the separation of 11 compounds and coeluted (HT, KT), (NT, DMT) and (ET, AOO); III (Spherisorb) and II (Hypersil BDS) packings provided the separation of 13 compounds and coeluted (ET, AOO). Finally, IV (Luna) and I (Hypersil ODS) provided the separation of 14 compounds. Hypersil ODS packing was selected based on the number of separated compounds (14 out of 14) and run time analysis (lower than IV column). No changes in the elution order for these compounds using these columns were observed. However, different selectivities were obtained.

Table 4
Retention factors, (k), obtained for AAS using different reverse phase columns (30°C) I: Hypersil ODS (250 mm×4.6 mm I.D.) 5 μm; II: Hypersil BDS (250 mm×4.6 mm I.D.) 5 μm; III: Spherisorb ODS (250 mm×4.6 mm I.D.) 5 μm; IV: Luna ODS (250 mm×4.6 mm I.D.) 5 μm; V: Spherex ODS (150 mm×4.6 mm I.D.) 5 μm; VI: Hamilton PRP-1 (150 mm×4.1 mm I.D.) 5 μm. Mobile phase (ACN/H_2O, 45:55, v:v)

AAS	I	II	III	IV	V	VI
HT	1.08	0.98	1.58	1.64	2.11	1.07
KT	1.17	0.98	1.68	4.80	2.11	1.43
FM	1.42	1.22	2.00	2.22	2.50	1.43
B	1.93	1.73	3.61	3.14	4.81	2.75
NT	2.35	1.99	4.48	3.64	5.94	3.55
DMT	2.60	2.19	4.71	4.02	5.94	3.55
T	3.08	2.61	5.16	4.80	6.40	4.34
NE	3.24	2.83	5.76	5.40	7.63	5.64
MT	4.03	3.45	6.98	6.36	8.86	5.64
AOO	4.33	3.74	7.77	6.98	10.40	5.64
ET	4.59	4.00	7.77	7.44	10.40	5.64
BLS	5.06	4.11	9.30	8.18	12.24	6.73
OM	8.64	7.16	20.11	13.16	21.02	12.74
DZ	12.52	11.92	>22	>20	23.40	>15

3.5. Calibration graphs

Several compounds were tested for their suitability as an internal standard (IS) under optimal conditions. For OM and DZ, canrenone (CAN) was the judged best for quantitative purposes. However, for BLS, MT and for the remainder AAS, BLS.

Calibration graphs were obtained by adding to steroid free-urine samples (Table 1), standards of AAS at five different concentrations in the range (2–8 μg/ml) using MT, BLS or CAN (5 μg/ml), as IS, under solvent extraction conditions (Section 2.5). These mixtures were separated using H_2O:ACN (55:45, v/v), a flow rate 1 ml min^{-1} and UV absorbance-DAD detection at 245 and 280 nm (OM and DZ). Plotting each AAS peak area to IS ratio (PAR) vs. the concentration (x) of each AAS, the calibration equation $PAR = A + Bx$ (μg/ml) was obtained. In Table 5 the parameters A (intercept), B (slope) and r (regression coefficient) are summarized. In all cases the intercepts were not significantly different from zero.

3.6. Precision, accuracy and detection limits

The precision was examined by analyzing 10 different samples of each AAS containing 5 μg/ml using the calibration graphs. In Table 5 are shown the CV values obtained. The accuracy was examined in the 2–20 μg/ml range for each AAS. The Barlett and Harley test [29] was applied to corroborate the randomness of the variances. This test confirmed that the above mentioned method does not present sys-

Table 5
Linear regression equations (PAR = $A + Bx$), detection limits (LODs) and CV for AAS. PAR is the steroids peak area ratio to IS. Conditions: mobile phase (ACN/H_2O, 45:55, v:v), flow-rate 1 ml min^{-1} and Hypersil ODS (250 mm × 4.6 mm I.D.) 5 μm column

AAS	*A*	*B*	*r*	LODs (μg ml^{-1})	CV (%)
KT	0.056	0.243	0.993	0.026	1.44
HT	0.178	0.137	0.999	0.038	1.06
FM	0.158	0.136	0.994	0.033	3.46
B	0.267	0.280	0.998	0.014	5.52
NT	0.270	0.163	0.995	0.038	2.91
DMT	0.188	0.156	0.995	0.029	5.49
T	0.095	0.224	0.999	0.026	5.12
NE	0.324	0.143	0.998	0.033	3.34
MT	0.118	0.149	0.995	0.031	2.41
AOO	−0.012	0.068	0.999	0.076	3.66
ET	0.055	0.230	0.999	0.026	5.12
BLS	0.165	0.112	0.996	0.038	5.35
OM	0.073	0.062	0.999	0.126	3.04
DZ	−0.320	0.288	0.992	0.027	3.36

tematic error and does not require blank correction. Detection limits (LODs) were also calculated from the calibration graphs for a signal-to-noise ratio (S/N) of 3 ($n = 10$) (Table 5).

3.7. Applications

3.7.1. Urine samples

In Fig. 4(A, B) are shown the chromatograms obtained from a standard mixture of AAS with UV absorbance detection at 280 and 245 nm respectively, and in Fig. 4C (280 nm) and D (245 nm) typical chromatograms obtained from a human urine sample under solvent extraction (see Section 2.5) and further spiked with these compounds (5 μg/ml). The comparison between Fig. 4(A and C) and (B and D) 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 Fig. 4(A, C) and (B, D) conditions was lower than 1% for each one]. Impurities were investigated further using a diode array detector (DAD) by displaying the spectra obtained at different points across the peak and compared with those obtained in Section 2.4. The possible impurities in each peak detected were negligible. This separation showed the possibility of solving different problems related with the separation and determination of these compounds in human urine samples.

3.8. Hydrolysis of norethindrone acetate

Norethisterone acetate (NEA) is the precursor of NE (norethisterone or norethindrone), the active hormone at the reception site. As an example, after transdermal application through patches, NEA is hydrolysed enzymatically in skin and blood to NE [30]. Hydrolysis of glucocorticoids esters including applications to pharmaceuticals has also been reported [31].

As an application of the proposed method and in order to obtain preliminary information about the hydrolysis of NEA, standards samples were maintained for 24 h under several conditions of pH, range (3–12), and temperature (25°C and 37°C). In such conditions, retention times and UV absorption spectra obtained from chromatographic peaks of reaction products were compared with those obtained for NE. In this way, NE was only identified as reaction product at pH 12. As expected, chemical hydrolysis in the stomach or duodenum for tablets practically did not take place. Based on the behavior of NEA at pH 12, quantitative assessment of NEA percent hydrolysis (25°C) vs. reaction time was carried out from calibration graphs. Hydrolysis was stopped by adding concentrated phosphoric acid to samples. In Fig. 5 the results obtained, are shown. As can be observed, the hydrolysis reaction was completed in about 90 min.

4. Conclusions

The optimization method reported by Snyder was applied to the separation of a mixture of 14 AAS (natural and synthetic). Different separations and consequently different selectivities were found using binary, ternary and quaternary mobile phases tested. 14 out of 14 of them were separated in 38 min (13 out of 14 in 23 min when excludes DZ) using a isocratic binary mobile phase H_2O–ACN (55:45, v/v) and a Hypersil ODS column (30°C). Using this mobile phase, different selectivities were also found for other stationary phases and temperatures. The

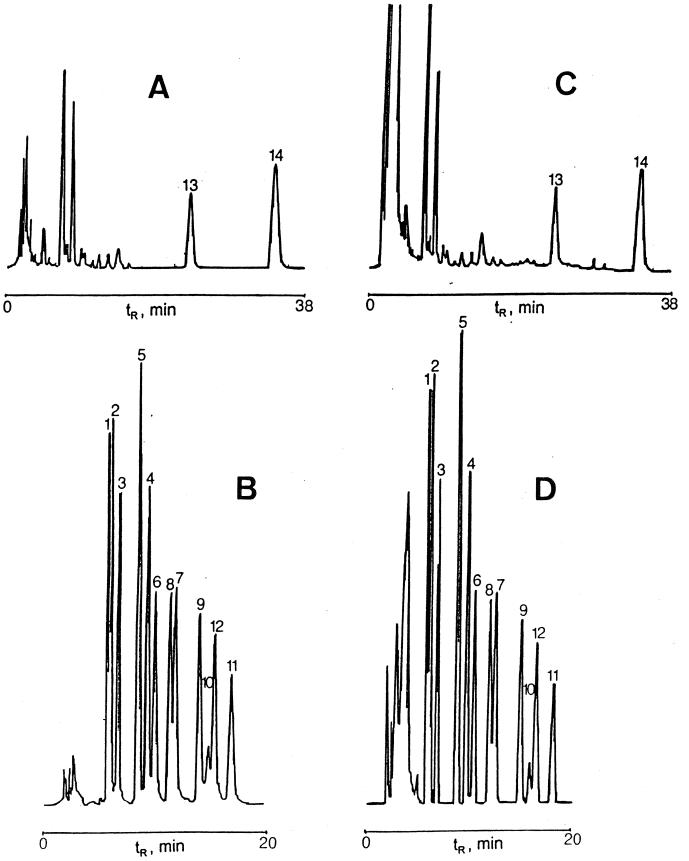


Fig. 4. Chromatograms obtained at 280 nm (A and C) and 245 nm (B and D) under optimal conditions for a standard mixture of AAS (A and B) and for a human urine sample spiked with these compounds (C and D).

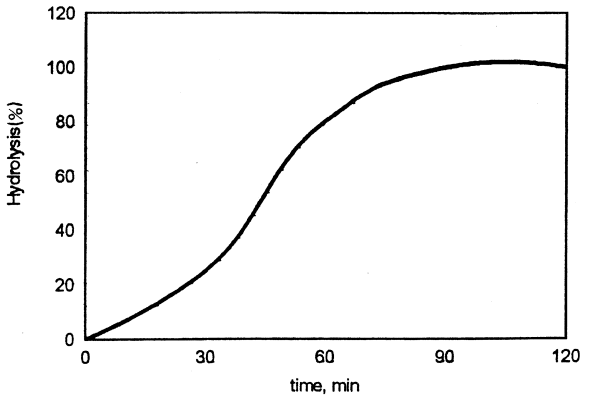


Fig. 5. Hydrolysis percentage for norethisterone acetate (NEA) vs. time at pH 12 and 25°C.

above separation was applied to a AAS screening in spiked urine samples without detecting impurities and the monitorization of NEA hydrolysis. The present method showed the possibilities for future applications related to the separation, detection and/or determination of these compounds for screening purposes, e.g. doping, animal feed (''drug cocktail''), growth-promoting agents (multiresidue analysis in urine or tissues of animals) controls. In addition, other simpler methods (obtained from the separations studied) for samples containing a few of these compounds can be developed and used for concrete applications, e.g. T/ET ratio in doping control, method validation or hydrolysis studies for pharmaceuticals.

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