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Journal of Chromatography B, 794 (2003) 215–225
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Method development for corticosteroids and anabolic steroids by micellar liquid chromatography

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Received 20 September 2002; received in revised form 21 May 2003; accepted 22 May 2003

Abstract

A systematic optimization of the HPLC separation of a complex mixture containing urinary steroids (anabolics and corticoids), boldenone and bolasterone (synthetic anabolics) by micellar liquid chromatography has been carried out. The isocratic micellar mobile phases (from binary to quaternary) consisted of sodium dodecyl sulphate and organic modifiers such as acetonitrile, tetrahydrofuran, propanol, butanol or pentanol. The effect of the organic modifiers, surfactant concentration, temperature, ionic strength and flow-rate on the separation has been studied. A micellar mobile phase made of 5% propanol and 40 m*M* surfactant allowed the separation of 13 steroids in about 23 min. A bivariant optimization method for the micellar mobile phase surfactant-propanol corroborated the above results. The separations obtained show good perspectives for future developments.

Keywords: Corticosteroids; Anabolic steroids

1. Introduction

Steroid hormones are compounds of endogenous origin chemically derived from cholesterol [1]. A group of these hormones named androgenic anabolic steroids (AAS) (testosterone (T) and synthetic compounds derived from T) have an anabolic effect (tissue building) coupled with an androgenic effect (masculinizing). AAS are used as therapeutic agents for the restoration of muscle size and strength, to increase muscle bulk in sports and as growth-promotion agents in meat producing animals to develop muscle and fatty tissue [2].

Several matrices such as meat, injection sites, urine, serum and faeces in animals [3–5] or human urine and hair [6–8], have been used to control the illegal use of steroids. These compounds have also been determined in pharmaceuticals [9].

Ion-pair chromatography (IP-HPLC) allows the separation of hydrophobic and ionic analytes using ionic surfactants added generally to the polar hydro-organic mobile phases and adsorbed on the alkyl-bonded stationary phases. The concentration of surfactant in the mobile phase should not pass the critical micelle concentration value (cmc) since solute retention is different when micelles are present in the mobile phase [10]. Micellar liquid chromatog-

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1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00426-4

raphy (MLC) using mobile phases containing surfactant concentration above its cmc is an alternative method to HPLC because of the large number of interactions of solutes with the mobile and stationary phases (enhanced selectivity). In addition, micellar mobile phases are less flammable and expensive, non-toxic and biodegradable. Moreover, the solubilizing ability of micelles is one of their most important properties and provides direct injection of untreated samples. The most important drawback of the MLC is the decrease in chromatographic efficiency (poor wetting of the stationary phase and restricted mass transfer) compared to that obtained in HPLC. To improve chromatographic efficiency in MLC, the use of columns with inner diameter (I.D.) smaller than those employed in HPLC has been proposed. In addition, the increase in column temperature and addition of small amounts of organic modifiers, such as short chain alcohols, are recommended. The range of concentration of organic modifiers must not be very high, because it might reduce the role of micelles and bring the system closer to a hydroorganic system. Alcohols reduce the loading of the surfactant in the stationary phase (improving the mass transfer and wetting) [10–12].

GC and GC–MS have been shown to be very suitable for urinary steroids in the case of endocrinological disorders and in doping control [6]. However, a derivatisation process is required and, in addition, the reproducibility obtained is not always sufficient [13]. HPLC has been described for complex mixtures of corticoids [14], urinary steroids (anabolics and corticoids) [15] and anabolics (natural and synthetic) [16]. MLC has been used for corticoids [11] and anabolics [17] employing binary mobile phases containing sodium dodecyl sulphate (SDS).

This paper describes the systematic optimization of the separation of a complex mixture of urinary steroids (anabolics and corticoids), boldenone and bolasterone (two synthetic anabolics analyzed as parent compounds in urine) [8,18,19] (see structures in Table 1) by MLC using SDS. The complex mixture was named URST. The HPLC optimization method based on the ''Glajch triangle'' has been extended to MLC and applied to URST using a pentagonal experimental design and PrOH, BuOH, PeOH, AcN and THF as organic modifiers, allowing the use of a plethora of mobile phases ranging from binary to quaternary. The effect of several variables affecting MLC, such as the nature and concentration of the organic modifiers, SDS concentration, flow-rate, salts added to the mobile phase and temperature is also discussed.

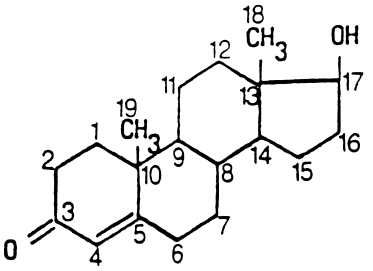
2. Experimental

2.1. Chemicals

Cortisone (CS) (M_r=360.4 g/mol) (4-pregnene-17α,21-diol-3,11,20-trione), cortisol (CL) (M_r=362.5 g/mol) (11β,17α,21-trihydroxypregnene-4-en-3,20-dione), 11β-hydroxytestosterone (HT) (M_r=304.4 g/mol) (4-androstane-11β,17β-diol-3-one), 11-ketotestosterone (KT) (M_r=302.4 g/mol) (4-androstene-17β-ol-3,10-dione), corticosterone (CT) (M_r=346.5 g/mol) (4-pregnene-11β,21-diol-3,20-dione), boldenone (B) (M_r=286.5 g/mol) (1,4-androstadien-17β-ol-3-one), hydroxyprogesterone (HP) (M_r=330.5 g/mol) (4-pregnene-11α-ol-3,20-diene), testosterone (T) (M_r=288.4 g/mol) (17β-hydroxy-4-androsten-3-one), deoxycorticosterone (DOC) (M_r=330.5 g/mol) (4-pregnen-21-ol-3,20-dione), androstenolone (AOO) (M_r=288.4 g/mol) (17β-hydroxy-5α-androst-1-en-3-one), bolasterone (BLS) (M_r=316.5 g/mol) (17-hydroxy-7,17-dimethylandrost-4-en-3-one), dehydroepiandrosterone (DHEA) (M_r=288.4 g/mol) (5-androsten-3β-ol-17-one) and epitestosterone (ET) (M_r=288.4 g/mol) (17α-hydroxy-4-androsten-3-one), were purchased from Sigma (St Louis, MO, USA). Stock solutions of these analytes (1000 μg ml^{-1}) were prepared in methanol. Working solutions (2–10 μg ml^{-1}) of a single steroid or an appropriate mixture of them were also prepared in methanol from stock solutions. Sodium dodecyl sulphate (SDS), sodium acetate, diammonium hydrogen phosphate and disodium hydrogenphosphate were of analytical reagent-grade from Merck (Darmstadt, Germany).

HPLC-grade methanol (MeOH), 1-propanol (PrOH), 1-butanol (BuOH), 1-pentanol (PeOH), acetonitrile (ACN) and tetrahydrofuran (THF) were purchased from Promochem (Wesel, Germany). Water was purified with a Milli-Q system (Millipore, Molsheim, France). Millipore 0.45-μm nylon filters

Table 1
Chemical structures for some steroids derived from testosterone



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

–, single bond; =, double bond.
[a] T and ET are epimeric compounds.

(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 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 six-port Rheodyne valve with a 20-μl sample loop injector (Cotati, CA, USA), a Jones-Chromatography block heated series 7960 for thermostating columns in the range 30–70 °C (Seagate Technology, Scotts Valley, CA, USA), a vacuum membrane degasser Model Gastor (SAS Corporation, Tokyo, Japan) and a bonded-silica Hypersil ODS (150 mm×3.0 mm I.D., 5 μm) column were used. A vortex mixer Mixo-Tub-30 from Crison (Barcelona, Spain) was also used.

2.3. Mobile phase

Isocratic micellar mobile phases were prepared daily mixing well known volumes of THF, ACN, MeOH, PrOH or BuOH with aqueous solutions of SDS (prepared with Milli-Q water) by programming the pump (e.g. 2% BuOH and 40 m*M* SDS). Binary mobile phases consisted of PrOH (1–10%) and 40 m*M* SDS; BuOH (1.5–5%) and 40 m*M* SDS; PeOH (0.15–1.5%) and 40 m*M* SDS; ACN (10–18%) and 40 m*M* SDS or THF (4–12%) and 40 m*M* SDS. Optimal A–E binary mobile phases were 5% PrOH (A); 2% BuOH (B); 1.5% PeOH (C); 15% AcN (D) or 4% THF (E), and 40 m*M* SDS. Ternary

and quaternary mobile phases were obtained from 1/2 or 1/3 of corresponding A–E binary mobile phases, respectively.

Other mobile phases consisted of 5% PrOH and SDS in the range 20–52 m*M*; 5% PrOH and 40 m*M* SDS in (50 m*M*, pH 6) disodium hydrogen phosphate or 5% PrOH and 40 m*M* SDS in (50 m*M*, pH 6) diammonium hydrogen phosphate were also used. 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. Chromatographic analysis

Once the column had been conditioned with the micellar mobile phase (30 min), chromatograms were obtained at the programmed temperature. For optimization purposes based on the use of different isocratic micellar mobile phases, a methanolic solution containing a single steroid or an appropriate mixture of them (5 $\mu g\,ml^{-1}$) was injected. The flow-rate was 0.5 $ml\,min^{-1}$ and UV detection was used. Peak identification and peak purity was carried out by comparing the retention time and UV spectra of the chromatographic peaks with those of reference compounds previously registered by injection of each one individually. In addition, single steroid standards (3 $\mu g\,ml^{-1}$) were spiked to the steroid mixture, and the increase in the corresponding peak area in the chromatogram was checked. Steroid analysis was carried out at 245 nm, with the only exception of DHEA, which was monitored at 200 nm.

3. Results and discussion

3.1. Preliminary experiments

To improve column efficiency in MLC, lower flow-rates and higher temperatures than those employed in HPLC were used [11]. However, retention times were increased. To keep them constant, smaller I.D.s of the column can be used to operate at similar linear velocities. For that purpose, MLC for URST was initiated using a 3.0 mm I.D. Hypersil column (60 °C).

A micellar mobile phase ACN and 40 m*M* SDS (larger than the critical micelle concentration, cmc = 8.1 m*M*) [20], was initially selected. In order to obtain preliminary MLC data, testosterone and bolasterone were selected and the concentration of ACN was varied in a wide range. Satisfactory results were obtained using 18% ACN (retention factors lower than 15). In addition, the measured UV absorption spectra using these micellar mobile phases did not show significant differences with respect to those obtained in HPLC using different mobile phases [15].

3.2. Retention characteristics of URST using organic modifiers

The influence of various organic modifiers and 40 m*M* SDS on URST retention has been studied. The solvents and concentration range (SCR) were: PrOH (1–10%); BuOH (1.5–5%); PeOH (0.15–1.5%); ACN (10–18%) and THF (4–12%).

The retention factors, *k*, were obtained from the retention times of URST and from the retention time of a solution of KNO_3. When ln *k* was plotted versus the organic modifier concentration, *Φ*, linear plots were obtained, which are in agreement with the simplified retention equation:

$$\ln k = -S_{hyb}\Phi + \ln k_w \quad (1)$$

In this equation, the slope, S_{hyb}, and the intercept, ln k_w, represent the solvent strength parameter and the retention factor, respectively, at a given micelle concentration in the absence of modifier. S_{hyb} values for all solvents have been obtained from the slopes of the straight lines corresponding to the representation of ln *k* versus *Φ*. S_{hyb} values for any URST generally follow the sequence: PeOH > BuOH > PrOH > THF > ACN. This indicates that the solvent strength in MLC depends on the organic modifier nature. In particular for the alcohols, the longer the alkyl chain of the alcohol, the larger the solvent strength. In other words, as the length of the alkyl chain of the alcohol increases, the interaction with the solutes is stronger and the alcohol can compete efficiently with micelles [12].

Table 2 summarizes the retention factors, *k*, number of separated compounds (*n*) and analysis

Table 2
Chromatographic characteristics for URST: retention factors, k (RSDs<2%); analysis time (t_{tot}) and number of separated compounds (n) using concrete binary mobile phases in 40 mM SDS

URST	HPLC ACN 40%	Retention factors (k)				
		5% PrOH A	2% BuOH B	1.5% PeOH C	15% ACN D	4% THF E
3. CS	1.17	4.30	2.54	1.60	4.45	4.35
4. CL	1.08	4.66	2.70	1.81	5.12	4.98
1. HT	1.59	5.15	2.99	2.19	5.40	4.98
2. KT	1.73	5.42	3.19	2.37	6.10	5.55
6. CT	2.26	6.86	3.84	2.62	7.55	6.28
5. B	3.22	7.45	4.31	3.14	8.88	7.42
7. HP	3.40	8.34	5.31	3.60	10.84	8.13
8. T	4.98	9.00	5.16	3.78	12.39	9.13
9. DOC	5.96	9.92	6.30	4.71	13.34	10.13
10. AOO	7.19	11.01	6.30	4.90	13.34	12.00
11. BLS	8.67	12.08	6.74	4.90	14.56	12.00
13. DHEA	7.11	14.37	8.89	6.84	15.04	14.21
12. ET	7.68	15.42	7.80	4.90	22.57	13.68
n	13	13	12	11	12	11
t_{tot} (min)	24	23	15	15	30	28

time (t_{tot}) obtained for URST using concrete binary mobile phases, which were selected as a compromise between t_{tot} and n from the binary mobile phases tested. The range of RSDs ($n=3$) of the retention factors for these compounds was lower than 2%. As can be observed, satisfactory separations were achieved using PrOH and BuOH. However, higher t_{tot} for ACN and THF, and lower n for PeOH and THF, were found. For comparison purposes, Table 2 also summarizes the k-values obtained in HPLC for URST using 40% ACN.

3.3. Systematic optimization based on Glajch's method

In previous studies, Glajch's method [21] based on the use of mobile phases containing aqueous solutions and three organic modifiers with different selectivities has been applied to optimize the separation of solutes of complex mixtures of steroids in HPLC [14–16], and extended to MLC for complex mixtures of corticoids [11] using SDS and different organic modifiers. Based on ''Glajch's triangle'', a pentagonal experimental design has been applied in the present work to URST in MLC, using PrOH, BuOH, PeOH, ACN and THF as organic modifiers (the most commonly used solvents in MLC) [10]. The selected separations achieved in Section 3.2 using binary mobile phases (Table 2), describe the A–E vertices of a pentagon consisting, in turn, of several triangles. A–E binary mobile phases were mixed appropriately (see captions of Tables 3 and 4) to obtain ternary (middle side points of the triangles) and quaternary ones (centroid of each of 10 triangles). Tables 3 and 4 summarize retention factors, k, for URST using ternary and quaternary mobile phases, respectively. In summary, 13 out of 13 URST were separated using A (Table 2), AB (Table 3), and BCD and CDA (Table 4) mobile phases. From these results, the A binary mobile phase (5% PrOH and 40 mM SDS) was finally selected as a compromise between t_{tot}, n and resolution between peaks. Nevertheless, the information shown in Tables 2–4 is very useful to solve specific analytical problems or, for different purposes, since several separations with different performances are obtained. In other words, other separations of interest can be carefully selected (e.g. only for CC, only for anabolics, or for both). In addition, these separations can be considered as the starting point for the

Table 3
Chromatographic characteristics for URST: retention factors, k (RSDs<2%); analysis time (t_{tot}) and number of separated compounds (n) using ternary mobile phases[a] containing 40 m*M* SDS and two organic modifiers

URST	Retention factors (k)									
	AB	AC	AD	AE	BC	BD	BE	CD	CE	DE
CS	2.63	1.98	4.15	4.47	1.88	4.23	4.79	2.60	3.45	4.35
CL	2.90	2.20	4.74	5.18	2.11	4.79	5.34	2.60	3.88	5.16
HT	3.20	2.39	4.74	5.18	2.46	5.18	5.34	2.82	4.35	5.16
KT	3.41	2.92	5.62	5.68	2.46	5.62	5.34	3.16	4.35	5.84
CT	4.30	3.16	7.62	7.37	3.03	7.65	7.50	3.93	5.28	7.73
B	4.65	3.73	7.62	7.37	3.48	7.65	7.50	4.29	5.73	7.73
HP	5.02	3.73	9.60	7.37	3.48	8.24	7.50	4.80	5.73	9.71
T	5.55	4.54	9.60	8.13	4.19	9.54	8.70	5.24	6.82	9.71
DOC	5.95	5.59	10.79	8.92	5.20	13.00	10.56	6.60	8.35	12.01
AOO	6.88	5.59	11.56	9.90	5.20	11.45	10.84	6.60	8.35	11.11
BLS	7.28	6.03	12.41	11.15	5.55	12.34	10.84	7.07	8.35	12.01
ET	8.94	6.62	17.58	14.09	5.90	17.27	14.16	8.67	9.87	17.15
DHEA	9.28	7.86	13.82	14.27	7.48	13.78	14.31	8.32	11.59	14.06
n	13	11	10	10	10	12	8	11	9	9
t_{tot} (min)	16	15	23	19	14	23	19	16	16	23

[a] These mobile phases were obtained from 50% of A–E compositions (Table 2).

development of different analytical methods for simple mixtures or for only one URST [9,22]. In such cases, in order to obtain an adequate separation, the selection and/or modification of the mobile phase will require little chromatographic work.

3.4. Effect of the organic modifier on selectivity

Selectivity has been examined qualitatively for the binary mobile phases by analysis of the retention factors (ln k) versus percentage of organic modifier,

Table 4
Chromatographic characteristics for URST: retention factors, k (RSDs<2%); analysis time (t_{tot}) and number of separated compounds (n) using quaternary mobile phases[a] containing 40 m*M* SDS and three organic modifiers

URST	Retention factors (k)									
	ABC	ABD	ABE	BCD	BCE	CDE	CDA	DEA	DEB	EAC
CS	2.11	3.89	3.52	2.40	3.17	3.96	3.48	4.90	4.58	3.61
CL	2.39	4.42	3.91	2.70	3.45	4.36	3.77	4.90	5.10	4.03
HT	2.62	4.74	4.15	2.89	3.45	4.36	4.06	5.62	5.88	4.23
KT	3.00	5.16	4.40	3.16	3.45	4.95	4.45	5.62	6.38	4.23
CT	3.43	6.92	5.81	3.88	5.02	6.45	5.51	8.19	8.54	5.84
B	3.91	6.92	5.81	4.27	5.02	6.45	5.95	8.19	8.54	6.09
HP	3.91	8.30	6.36	4.64	5.98	7.27	6.56	9.64	10.04	6.09
T	4.76	8.30	6.83	5.15	5.98	7.70	7.15	11.34	10.04	7.23
DOC	5.85	9.47	7.31	5.37	6.84	7.70	7.39	11.81	11.69	7.23
AOO	5.85	10.22	8.39	6.51	6.84	8.35	8.95	11.81	11.69	8.81
BLS	6.27	10.91	8.39	6.85	6.84	9.56	9.48	16.96	12.29	10.38
ET	7.05	14.55	11.25	8.27	8.54	12.43	11.62	16.96	17.70	10.79
DHEA	8.24	12.83	11.25	8.37	9.74	12.31	11.36	14.61	15.38	11.87
n	11	11	10	13	7	10	13	8	10	10
t_{tot} (min)	16	20	18	16	15	17	16	22	23	16

[a] These mobile phases were obtained from 1/3 of A–E compositions (Table 2).

Φ, plots (Eq. (1)). Likewise, to study selectivity between binary, ternary and quaternary mobile phases, adequate plots of retention factors (ln k) versus given mobile phase compositions can also be used. In this way, useful information can be obtained by comparing selected mobile phases (e.g. only binary or ternary or even binary versus ternary or quaternary). As an example, for PrOH mixtures, selectivity decreases slightly as Φ increases (lines tend to converge for DHEA/ET, ET/BLS, CL/CS) or it is not modified in a significant way (parallel lines for DOC/T, HP/B, HP/T, B/CT, CT/KT). However, for some pairs (BLS/AOO, AOO/DOC, KT/HT and CL/HT), the behavior is just the opposite (lines tend to diverge). Likewise, several coelutions (e.g. BLS/AOO) and reversals in the elution order (crossing between lines), e.g. ET and DHEA, have been observed. The rest of the solvents exhibit a similar behavior versus selectivity.

Changes in selectivity for compounds also take place when comparing binary mobile phases between them. As an example for A, B, C, D and E mobile phases described in Table 2. An exception is made for ET/DHEA pair which shows reversals in the elution order when A is compared versus B or D versus E. Likewise, additional information can also be drawn using ternary (e.g. AB, AC, AD, AE) or quaternary (e.g. ABC, ABD, ABE, ACD, ACE, ADE) mobile phases from the data presented in Tables 3 and 4.

3.5. Effect of SDS concentration

The effect of SDS concentration on the separation of URST has been studied using mobile phases consisting of 5% PrOH and 20–52 mM SDS. Retention factors, k, for URST have been obtained at 60 °C (Table 5). The results obtained using $1/k$ versus [SDS] plots are in agreement with the simplified retention equation [10]

$$\frac{1}{k} = \frac{K_{AM}}{K_{AS}} \cdot [M] + \frac{1}{K_{AS}} \quad (2)$$

where k is the retention factor, [M] the micelle concentration and the constants K_{AM} and K_{AS} describe the partition of the solute between bulk water and stationary phase or micelle, respectively. K_{AM} and K_{AS} values (Table 5) have been calculated from the slopes (K_{AM}/K_{AS}) and intercepts ($1/K_{AS}$) of the linear plots $1/k$ versus SDS concentration. These plots also show that an increase in SDS concentration produces shorter retention factors for all URST. In addition, the curves obtained tend to converge (e.g. CS and CL), to diverge (e.g. BLS and DHEA) or are closely parallel (e.g. AOO and BLS). Thus, not only the retention factors, k, but also the selectivity depends on the SDS concentration; 40 mM SDS was finally selected as a compromise between resolution and run time analysis.

Table 5
Retention factors of URST (k) for different SDS concentrations and PrOH 5%; RSD<2%

URST	Retention factors (k)				K_{AS}	K_{AM}
	20 mM	28 mM	40 mM	52 mM		
CS	6.05	4.50	3.70	2.93	15.9	0.086
CL	6.57	4.82	3.85	2.93	25.7	0.132
HT	7.22	5.65	4.26	3.41	24.2	0.117
KT	8.02	6.13	4.46	3.41	57.5	0.304
CT	10.35	7.82	5.65	4.43	64.5	0.832
B	11.30	8.42	5.97	4.70	103.0	0.403
HP	12.48	9.25	7.44	5.09	146.0	0.530
T	13.87	10.01	8.20	5.59	168.3	0.561
DOC	15.99	11.50	8.83	6.26	926.0	2.83
AOO	17.62	12.77	9.60	6.91	740.7	2.04
BLS	18.76	13.59	10.70	7.37	1587.3	4.13
ET	23.67	17.05	13.60	9.09	2000	4.26
DHEA	22.41	16.03	12.73	9.21	155.3	0.304

3.6. Bivariant optimization method for the SDS–PrOH system

A bivariant method for the optimization of an adequate composition of the micellar mobile phase SDS–PrOH (SDS was decreased when PrOH increased), has been carried out. The ranges of SDS and PrOH were 20–44 m*M* and 4–10%, respectively. Owing to the poor chromatographic performances detected in this study, a new Hypersil column with identical characteristics was examined using 5% PrOH and 40 m*M* SDS. Retention varied from column to column without significant changes in the elution order, t_{tot} and selectivity. Using the new column and mobile phases 10% PrOH and 20 m*M* SDS, and 4% PrOH and 44 m*M* SDS, the separation of 12 and 11 compounds (out of 13), respectively, was achieved. However, for 5–8% PrOH and 28–40 m*M* SDS, the number of separated compounds was 13.

These results are not only consistent with those presented above, but also indicate that by controlling adequate SDS/PrOH concentration ratios in the range 3.5–8, a complete separation can be achieved. In other words, the method presents a certain robustness since a slight variation of the SDS and PrOH concentrations does not change the separation characteristics in a significant way.

3.7. Temperature effect

The effect of temperature on URST retention has been studied in the range 40–70 °C using the above optimized mobile phase. Fig. 1 presents the chromatograms obtained at different temperatures and the corresponding *k*-values are shown in Table 6. Selectivity (and resolution) are modified by temperature: at 40 °C, HT and KT (HT/KT), B/CT and AOO/BLS coeluted. However, in the range 50–70 °C, a complete separation is achieved, although at 70 °C peaks T/DOC tend to coelute (Fig. 1). This indicates that temperature is not critical for the complete separation of these compounds. Taking into account t_{tot}, resolution and *n*, a temperature of 60 °C was finally chosen. In addition, two different behaviors versus retention can be observed in the data presented in Table 6: as temperature increases, retention decreases for CS, CL, HT and CT and increases for the rest. Nevertheless, for many compounds, retention does not change in a significant way with temperature.

Van't Hoff plots (ln *k* vs. $1/T$) for URST were constructed with the data of Table 6, showing good linearity ($r>0.99$). This behavior shows that the integrity of the micelle structure is maintained over the temperature range studied [23,24]. The negative or positive enthalpy values (ΔH) (Table 6), obtained

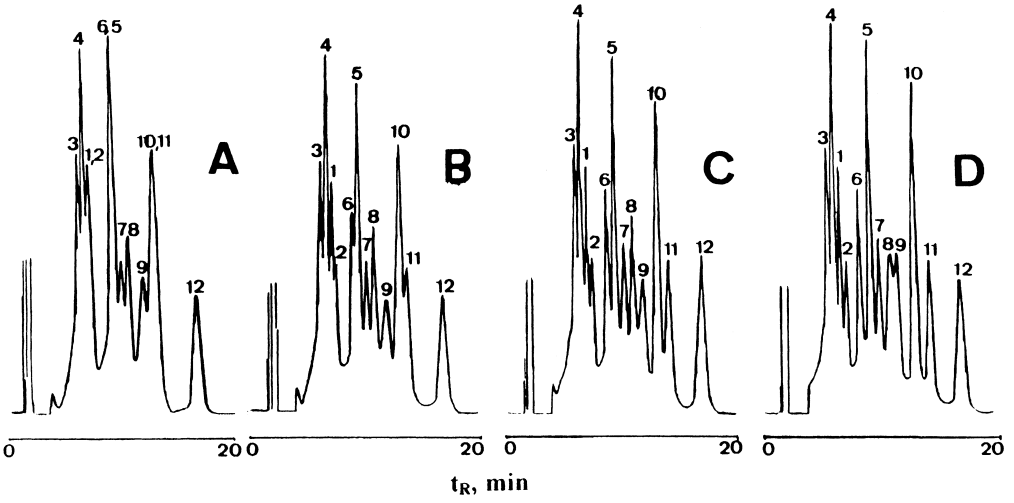


Fig. 1. Chromatograms for 10 μg ml^{-1} URST (AOO = 15 μg ml^{-1}) obtained at different temperatures using 5% PrOH and 40 m*M* SDS mobile phase. (A) 40 °C; (B) 50 °C; (C) 60 °C; (D) 70 °C. Other conditions: Peak numbers as in Table 2, UV detection at 245 nm. Limits of detection for a signal-to-noise ratio (*S*/*N*) of 3 ($n=10$) were in the range 13 (CL)–98 (AOO) ng ml^{-1}.

Table 6
Retention factors, *k*, for URST obtained at different temperatures, using PrOH 5% and 40 m*M* SDS; RSDs<2%

URST	Retention factors (*k*)				$\Delta H \pm$RSD (kJ mol^{-1})
	40 °C	50 °C	60 °C	70 °C	
CS	3.23	3.15	3.04	2.91	−3.033±0.36
CL	3.55	3.49	3.41	3.28	−2.321±0.34
HT	3.83	3.83	3.79	3.70	−0.892±0.30
KT	3.83	4.07	4.08	4.08	1.321±0.31
CT	5.36	5.17	5.09	4.94	−2.250±0.31
B	5.36	5.46	5.57	5.58	1.754±0.05
HP	5.93	6.04	6.18	6.20	1.754±0.07
T	6.34	6.51	6.71	6.96	2.950±0.19
DOC	7.27	7.28	7.40	7.38	1.850±0.26
AOO	7.97	8.12	8.33	8.46	1.883±0.23
BLS	7.97	8.58	9.06	9.44	5.042±0.49
ET	10.74	10.90	11.16	11.38	1.792±0.03
DHEA	10.71	10.68	10.68	10.54	−0.833±0.45

from the slopes, indicate that the mass transfer process is exothermic or endothermic, respectively. These enthalpic differences can be due to the entropic factor ($T\Delta S$ values) in the Gibb's equation, which probably overcomes the enthalpy factor, changing in this way the sign of the slope. This dual mechanism has also been observed for some corticoids in MLC using SDS [11].

3.8. Effect of the flow-rate

The flow-rate has a relevant influence on the chromatographic efficiency in MLC [25]. The effect of flow-rate has been studied in the range 0.4–0.6 ml min^{-1} under optimum working conditions for URST. t_{tot} decreases as flow-rate increases, showing a tendency to coelute at higher flow-rates. A slight increase in resolution at lower flow-rates was also observed. In addition, the achieved separations always yielded $n=13$. A flow-rate of 0.5 ml min^{-1}, was finally selected as a compromise between t_{tot} and resolution.

3.9. Effects of salts added to the mobile phase

The addition of salts to a mobile phase containing micelles can influence the retention and separation characteristics of solutes, since the ionic surfactant cmc is greatly decreased (the degree of counterion binding is affected and the micelle size is increased) [26]; 50 m*M* Na_2HPO_4 (pH 6) or 50 m*M* $(NH_4)_2HPO_4$ (pH 6), 5% PrOH and 40 m*M* SDS mobile phases were used to study this effect. No significant changes in retention and resolution were observed when using sodium salt. However, when ammonium salt was added, retention and resolution were affected. For those compounds which are retained more (B, T, DOC, AOO, BLS), retention increases and resolution decreases (e.g. BLS/ET), showing a clear tendency to coelute for some of them (e.g. DOC/AOO). However, for the compounds which are retained less (CS, CL, HT, KT), retention is modified slightly and an overall better resolution is obtained than in the absence of salt. In HPLC, however, the addition of salts to the mobile phase did not show a significant effect on steroids [15]. Consequently, the presence of salts in MLC for URST is not recommended to improve the separation previously obtained.

3.10. MLC versus HPLC

The results obtained from HPLC [15] and those herein obtained in the MLC optimization of the separation of steroids have been compared. Some similarities have been found when t_{tot} and n are compared. In both cases, the complex mixture of steroids was separated in about 25 min. However, MLC is cheaper, less toxic and less contaminating than HPLC, as a consequence of the flow-rate

(0.5 ml/min in MLC, and 1 ml/min in HPLC) and mobile phase composition (40% ACN in HPLC and 5% propanol and 40 m*M* SDS in MLC). Moreover, the optimal separation achieved in MLC (see Sections 3.6 and 3.7) has a certain robustness when compared with that obtained in HPLC (especially with regard to temperature). Differences in selectivity are also found.

One problem of MLC in comparison with HPLC is the lower efficiency (poor wetting of the stationary phase and restricted mass transfer). However, this inconvenience can be overcome by increasing temperature. For instance, the resolution, R_s, between CL and CT at 40 °C is greater in HPLC (2.0) than in MLC (1.6). However, a wider temperature range can be used in MLC in comparison with HPLC (the boiling points of micellar mobile phases are higher than those of the HPLC mobile phases). Fig. 1A–D shows the influence of temperature on chromatographic efficiency in MLC. As can be seen, efficiency increases with temperature for compounds 10–11 (AOO/BLS), 1–2 (HT/KT) and 5–6 (B/CT). However, efficiency for compound 12 (ET) is apparently not modified. In summary, MLC can be considered as an alternative method and in many aspects can compete with HPLC.

4. Conclusions

Several micellar mobile phases were prepared with different organic modifiers and SDS and used for the separation of a complex sample containing urinary steroids, boldenone and bolasterone by applying an experimental design based on Glajch's method. Different separations and, consequently, different selectivities were obtained depending on the micellar mobile phase composition (defined by SDS concentration and several organic modifiers of different nature) and temperatures studied. As an example, using the final chromatographic conditions (5% PrOH and 40 m*M* SDS mobile phase, a 3.0 mm I.D. Hypersil C_{18} column (60 °C) and a flow-rate of 0.5 ml min^{-1}), 13 out of 13 urinary steroids were separated in about 23 min. In addition, this separation presents a certain robustness since a slight variation of the SDS (28–40 m*M*) and PrOH (5–8%) concentrations in the mobile phase, and column temperature (50–70 °C) does not change the separation characteristics in a significant way. This also indicates that the final separation method is potentially applicable in future developments to urine samples.

Acknowledgements

This work was supported by the Spanish Dirección General de Investigación Científica y Técnica (DGICYT), grant SAF95-296/94.

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