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Optimization of the separation of a complex mixture of natural and synthetic anabolic steroids by micellar liquid chromatography

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Abstract

A systematic optimization of the HPLC separation of a complex mixture containing natural and synthetic anabolic steroids by micellar liquid chromatography using a Hypersil (150 mm × 3.0 mm i.d., 5 μm) C18 column and UV detection at 245 nm (exception is made for oxymetolone and danazol which were monitorized at 280 nm) 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 5% propanol and 40 mM surfactant allowed the separation of 12 steroids out of 14 tested in about 20 min. A bivariant optimization method for the micellar mobile phase propanol-surfactant corroborated the above results.

Keywords: Anabolic steroids

1. Introduction

Androgenic steroids are characterized by their biological effect on the primary and secondary sex characteristics of various male animals, most of them having an anabolic effect. For these reasons, they are named anabolic-androgenic steroids (AAS). Testosterone is the most representative natural AAS [1]. Numerous derivatives from testosterone (T) have been synthesized. Alkylation of T at the 17α position results in derivatives that are orally active (e.g. methyltestosterone), and removal of the 19-methyl group improves the anabolic to androgenic ratio (e.g. nandrolone) [2].

AAS are contained in different pharmaceuticals and used as therapeutic agents. However, their use is banned in the European Union for animals intended for consumption and by the International Olympic Committee (IOC) and National and International Sports Federations. It contributes to the black market demand for hormone and hormone cocktail [3,4]. For regulatory control of the illegal use of AAS, various complex matrices (edible tissues, kidney fat, urine and feces from animals [5], and urine, plasma and hair from humans) [6] have been described. Clean-up procedures have usually been employed to eliminate interferences [7,8]. These compounds have also been determined in pharmaceuticals [9].

Micellar liquid chromatography (MLC), which uses surfactants above the critical micellar concentration (cmc), provides hydrophobic interactions of the solute with both micelle and stationary phase. The addition of alcohols to the micellar phase can result in an additional interaction with the solute. The variety of possible interactions gives a large versatility to this technique as an alternative to conventional HPLC and makes it appropriate for a wide range of solute analysis. Another advantages are the low amount of organic solvent used (reducing the toxicity, flammability, environmental impact and cost of these phases) and the direct injection of physiological samples by solubilizing the protein components. However, the most important drawback of the MLC is the decrease of chromatographic efficiency (poor wetting of the stationary phase and restricted mass transfer) as compared to HPLC. To improve chromatographic efficiency in MLC, the use of columns with inner

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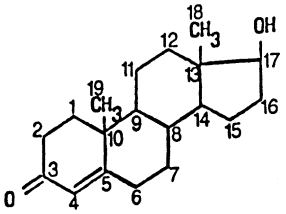
Table 1
Chemical structures for natural and synthetic anabolic steroids



	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	CH_3	
Nortestosterone (NT)											H
Boldenone (B)	=										
Metandrostenolone (DMT)	=									CH_3	
Norethindrone (NE)										C≡CH	H
Testosterone (T)										*	
Methyltestosterone (MT)										CH_3	
Androstendolone (AOO)	=				–						
Bolasterone (BLS)							CH_3			CH_3	
Dehydroepiandrosterone (DHEA)			OH		–	=					
Epitestosterone (ET)										*	
Oximetolone (OM)		=CHOH								CH_3	
Danazol (DZ)										C≡CH	

=: double bond; –: single bond; testosterone and epitestosterone are epimeric compounds.

diameter (i.d.) smaller than those employed in HPLC and the increase of the column temperature or the addition of small amounts of organic modifiers, such as short chain alcohols, are recommended [10].

GC-MS methods has been used to detect the abuse of anabolics in sport [8,11] and to identify residues of illegal growth promoters in urine and muscle tissue from animals [2,4]. However, a derivatization process is required and, in addition, the reproducibility obtained is not always sufficient [12].

HPLC has also been used to analyze single mixtures [13,14] of steroids and for optimizing complex mixtures of urinary steroids and natural and synthetic anabolics [15,16].

MLC has been employed for separation studies of complex mixtures of corticoids, urinary steroids (corticoids and anabolics) [17,18] and for the determination of single anabolic compounds in pharmaceuticals [9].

In this paper, the systematic optimization of the separation of a complex mixture of natural and synthetic anabolic steroids (structures in Table 1) by MLC using sodium dodecyl sulphate (SDS) is described. The HPLC optimization method based on a partial version of "Glajch triangle" has been applied to AAS using a pentagonal experimental design and PrOH, BuOH, PeOH, ACN and THF as organic modifiers, allowing the use of a plethora of mobile phases ranged 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

11β-hydroxytestosterone(HT) (MW = 304.4 g/mol) (4-androstane-11β,17β-diol-3-one), 11-ketotestosterone(KT) (MW = 302.4 g/mol) (4-androstene-17β-ol-3,10-dione), fluoxymesterone(FM) (MW = 336.4 g/mol) (9α-fluoro-11β,17β-dihydroxy-17-methyl-4-androsten-3-one), norethindrone (NE) (MW = 298.4 g/mol) (19-nor-17α-ethynyl-4-androsten-17β-ol-3-one), nandrolone or 19-nortestosterone-(NT) (MW = 274.4 g/mol) (17β-hydroxy-19-norandrost-4-en-3-one), 1-dehydro-17α-methyltestosterone (MW = 300.4 g/mol) (DMT) (17β-hydroxy-17α-methyl-1,4-androstadien-3-one), boldenone(B) (MW = 286.5 g/mol) (1,4-androstadien-17β-ol-3-one), testosterone(T) (MW=288.4 g/mol) (17β-hydroxy-4-androsten-3-one, 17α-methyl-testosterone (MT) (MW = 302.5 g/mol) (17-hydroxy-17-methylandrost-4-en-3-one), androstenolone (AOO) (MW = 288.4 g/mol) (17β-hydroxy-5α-androst-1-en-3-one), bolasterone (BLS) (MW = 316.5 g/mol) (17-hydroxy-7,17-dimethylandrost-4-en-3-one), epitestosterone (ET) (MW = 288.4 g/mol) (17α-hydroxy-4-androsten-3-one), oxymetolone (OM) (MW = 332.5 g/mol) (17β-hydroxy-2-hydroxymethylene-17-methyl-5α-androst-3-one), danazol(DZ) (MW = 337.5 g/mol) (pregna-2,4-dien-20-inol (2,3-d)isoxazol-17-ol), were purchased from Sigma (St. Louis, MO, USA). Stock solutions of these analytes (1000 $\mu g\,ml^{-1}$) were prepared in methanol. Working solu-

tions (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 (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 a recorder model CI 4100 data module. A 6-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 mM SDS). Binary mobile phases consisted of PrOH (1–10%) and 40 mM SDS; BuOH (1.5–5%) and 40 mM SDS; PeOH (0.15–1.5%) and 40 mM SDS; ACN (10–18%) and 40 mM SDS or THF (4–12%) and 40 mM SDS. Optimal A–E binary mobile phases were 5% PrOH (A); 2.5% BuOH (B); 0.3% PeOH (C); 18% ACN (D) or 4% THF (E), and 40 mM 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 mM; 5% PrOH and 40 mM SDS in (50 mM, pH 6) disodium hydrogen phosphate or 5% PrOH and 40 mM SDS in (50 mM, 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 micellar mobile phases, a methanolic solution containing a single steroid or an appropriate mixture of them (5 μg ml^{-1}) was injected. The flow-rate was 0.5 ml min^{-1} and UV detection was used. Peaks identification and peak purity was performed 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 μg ml^{-1}) were spiked to the steroids mixture, and the increase of the corresponding peak area in the chromatogram was checked. With exception to OM and DZ which was monitorized at 280 nm, steroids analysis was carried out at 245 nm.

3. Results and discussion

3.1. Preliminary considerations

In previous papers, the optimization of the separation of a complex mixture of natural and synthetic corticoids [17] and urinary steroids (corticoids and anabolics) [18] in MLC using SDS and different organic modifiers has been reported. To improve column efficiency, higher temperatures and smaller flow-rates than those used in HPLC were proposed. However, retention times were increased. To keep them constant, smaller IDs of the column can be used to operate at similar linear velocities. From these data a 3.0 mm i.d. Hypersil C18 column (60 °C), a flow-rate of 0.5 ml min^{-1} and 40 mM SDS (larger than the critical micellar concentration, cmc = 8.1 mM) [19] were initially selected.

3.2. Retention characteristics of AAS using organic modifiers

The influence of various organic modifiers and 40 mM SDS on AAS retention has been studied. The solvents and concentration range studied (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 AAS and from the retention time of a solution of KNO_3. When $\ln k$ were 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

Table 2
Chromatographic characteristics for AAS

AAS	k (HPLC)	Retention factors, k				
	ACN 45%	5% PrOH (A)	2.5% BuOH (B)	0.3% PeOH (C)	18% ACN (D)	8% THF (E)
1. HT	1.08	5.19	3.11	5.20	4.52	5.13
2. KT	1.17	5.40	3.28	5.20	5.18	5.81
3. FM	1.42	6.11	3.66	5.70	6.25	6.69
4. NT	2.35	7.47	4.47	7.12	6.57	7.40
5. B	1.93	7.47	4.47	7.12	7.38	7.40
6.DMT	2.60	7.79	4.76	8.16	7.38	7.40
7. NE	3.24	7.79	4.76	8.16	8.50	8.43
8. T	3.08	9.04	5.36	8.63	9.81	9.23
9. MT	4.03	10.15	6.01	10.14	11.27	10.30
10. AOO	4.33	10.99	6.62	13.18	12.40	12.07
11. BLS	5.06	12.08	7.18	13.18	13.63	12.07
12. ET	4.59	15.47	8.03	14.65	20.92	14.29
13. OM	8.64	15.63	9.18	13.10	16.96	14.64
14. DZ	12.52	17.48	9.77	15.48	23.16	20.06
n	14	12	12	10	13	11
t_{tot} (min)	38	25	16	26	30	34

Retention factors, k (R.S.D.s < 2%); analysis time (t_{tot}) and number of separated compounds (n) using optimized mobile phases in 40 mM SDS.

obtained from the slopes of the straight lines corresponding to the representation of $\ln k$ versus Φ. S_{hyb} values for any AAS 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 [20].

Table 2 summarizes the retention factors, k, number of separated compounds (n) and analysis time (t_{tot}) obtained for AAS using optimized binary mobile phases, which were selected as a compromise between t_{tot} and n from the binary mobile phases tested. The range of R.S.D.s ($n = 3$) of the retention factors for these compounds were lower than 2%. As can be observed, satisfactory separations (defined by the visible presence of two peaks in the chromatograms) were achieved using ACN, 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 AAS using 45% ACN.

3.3. Systematic optimization based on the Glajch's method

Glajch's method [21] based on the use of mobile phases containing aqueous solutions and three organic modifiers (belonging to different families in the Snyder selectivity triangle) has been applied to optimize the separation of solutes of complex mixtures of steroids in HPLC [15,16] by mapping resolution versus the mobile phase composition. It has been extended to MLC for complex mixtures of corticoids and urinary steroids (corticoids and anabolics) [17,18] using SDS and different organic modifiers. A pentagonal experimental design has been applied to AAS in MLC, using PrOH, BuOH, PeOH, ACN and THF as organic modifiers (the most commonly solvents used in MLC) [10] using a partial version of Glajch's method (note that the logical method only can be constructed using THF, ACN and one of the proposed alcohols). The selected separations previously achieved 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, n and t_{tot} obtained for AAS using ternary and quaternary mobile phases, respectively. In summary, 13 steroids were separated using D mobile phase (18% ACN) (the resolution was poor) and 12 using A (5% PrOH), B (2.5% BuOH) (Table 2), AB and CD (Table 3). 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 natural AAS, only for synthetic AAS, or for both). In addition, these separations can be considered as the starting point for the development of different analytical methods for simple mixtures or for only one AAS [9]. In such cases, to obtain an adequate separation the selection and/or modification of the mobile phase it will be required few chromatographic work.

3.4. Effect of the organic modifier on the selectivity

Selectivity has been examined qualitatively for the binary mobile phases by analysis of the retention factors ($\ln k$)

Table 3
Chromatographic characteristics for AAS

AAS	Retention factors, k									
	AB	AC	AD	AE	BC	BD	BE	CD	CE	DE
HT	3.53	4.36	4.86	5.26	3.83	4.97	5.82	6.38	5.21	5.24
KT	3.72	4.61	5.40	5.71	3.99	5.45	5.82	6.85	5.43	5.89
FM	4.22	5.02	6.06	6.40	4.30	6.08	6.30	7.50	5.80	6.73
NT	5.06	5.91	7.42	7.36	5.27	7.74	7.54	8.73	7.07	7.74
B	5.06	6.20	7.42	7.36	5.27	7.74	7.54	9.21	7.07	7.74
DMT	5.40	6.20	7.42	7.36	5.27	7.74	7.54	9.21	7.07	7.74
NE	5.40	6.20	7.42	7.36	5.27	7.74	7.54	9.21	7.07	9.00
T	6.04	7.13	9.32	8.03	6.15	9.18	8.75	10.99	8.04	9.81
MT	6.73	7.72	10.31	8.89	6.74	10.14	9.42	11.99	8.50	10.91
AOO	7.54	9.11	11.24	9.94	7.22	10.75	11.10	13.11	9.78	12.88
BLS	8.05	9.11	12.09	11.47	7.77	11.80	11.10	13.90	9.78	12.88
ET	9.65	12.13	17.12	14.08	9.82	16.30	14.21	10.49	14.05	17.27
OM	10.01	11.17	14.52	13.85	9.99	14.20	13.65	16.74	11.90	15.33
DZ	11.26	13.08	18.46	17.45	11.11	17.47	16.21	20.70	14.74	20.63
n	12	11	11	11	11	11	9	12	10	11
t_{tot} (min)	18	20	25	23	18	23	21	26	23	27

Retention factors, k (R.S.D.s < 2%); analysis time (t_{tot}) and number of separated compounds (n) using ternary mobile phases containing 40 mM SDS and two organic modifiers. These mobile phases were obtained from 50% of A–E compositions (see Table 2).

versus percentage of organic modifier, 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 ACN mixtures, selectivity decreases slightly as Φ increases (lines tend to converge for FM/NT, OM/DZ, NT/B) or it is not modified in a significant way (parallel lines for MT/AOO, AOO/BLS, BLS/OM, NE/T). However, for some pairs (HT/KT, KT/FM, DMT,B/NE) the behavior is just the opposite (lines tend to diverge). Likewise, several coelutions (e.g. DMT and B) can also be observed. The rest of solvents exhibits 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–E mobile phases described in Table 2. A exception is made for ET/OM pair which shows reversals in the elution order when compare A or B versus C, and C or D versus E. Additional information can also be drawn using ternary or quaternary mobile phases from the data presented

Table 4
Chromatographic characteristics for AAS

AAS	Retention factors, k									
	ABC	ABD	ABE	ACD	ACE	ADE	BCD	BCE	BDE	CDE
HT	3.63	4.80	4.16	5.57	4.68	6.01	5.56	4.46	5.98	5.61
KT	3.63	5.21	4.40	5.87	4.89	6.01	5.95	4.61	6.39	6.09
FM	4.08	5.74	4.79	6.41	5.28	6.74	6.46	4.96	7.01	6.75
NT	5.00	7.19	5.83	7.97	6.43	8.04	7.95	6.07	8.52	7.96
B	5.00	7.19	5.83	7.97	6.43	8.04	7.95	6.07	8.52	7.96
DMT	5.00	7.19	5.83	7.97	6.43	8.04	7.95	6.07	8.52	7.96
NE	5.00	7.19	5.83	7.97	6.43	8.04	7.95	6.07	8.52	7.96
T	5.88	8.38	6.82	8.96	7.42	9.69	9.23	6.99	9.98	9.47
MT	6.47	9.51	7.45	10.18	7.98	10.44	10.14	7.53	11.04	10.43
AOO	7.13	10.21	8.70	11.20	9.28	11.96	11.28	8.65	12.66	12.15
BLS	7.60	10.91	8.70	11.84	9.28	17.04	11.81	8.65	12.66	12.15
ET	9.55	14.61	11.19	16.48	12.68	17.04	16.18	10.84	17.49	16.19
OM	9.63	13.11	10.79	14.44	11.36	14.59	14.26	11.65	15.32	14.58
DZ	10.82	15.97	12.66	17.21	13.75	18.24	17.00	12.80	18.81	18.46
n	10	11	10	11	10	9	11	10	10	10
t_{tot} (min)	18	21	20	23	21	23	22	20	24	24

These mobile phases were obtained from 1/3 of A–E compositions (see Table 2). Retention factors, k (R.S.D.s < 2%); analysis time (t_{tot}) and number of separated compounds (n) using quaternary mobile phases containing 40 mM SDS and three organic modifiers.

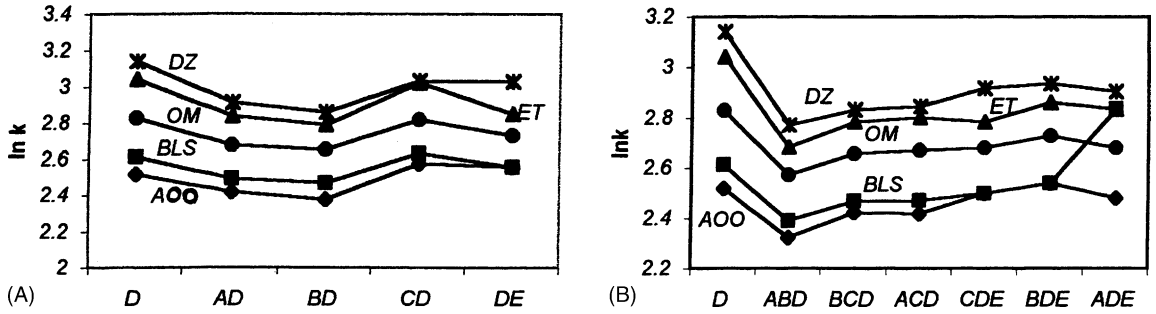


Fig. 1. Changes in selectivity for some AAS using: binary and ternary mobile phases (A) and binary and quaternary mobile phases (B).

in Tables 3 and 4. Fig. 1 illustrates changes in selectivity for AAS as compared the binary mobile phase D and ternary (Fig. 1A) or quaternary ones (Fig. 1B) obtained from D.

3.5. Effect of SDS concentration

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

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

where k is the retention factor, [M] the micelle concentration and the constants K_{AS} and K_{AM} 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 of SDS concentration produced shorter retention factors for all AAS. In addition, the curves obtained tend to converge (e.g. HT/KT, DMT, NE/NT, B), to diverge (e.g. KT/FM) or are closely parallels (e.g. DZ/OM). Thus, not only the retention factors, k, but also the selectivity depend on the SDS concentration. 40 mM SDS was finally selected as compromise between resolution and t_{tot}.

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 mM and 4–10%, respectively. Owing to the poor chromatographic performances detected in this study, a new column with identical characteristics was examined using 5% PrOH and 40 mM SDS. Retention varied from column to column without significant changes in the elution order, t_{tot} and selectivity. Table 6 summarizes the retention factors for AAS using the bivariant optimization method described above. Using the new column and a mobile phase 4% PrOH and 44 mM SDS allowed the separation of nine compounds. However, for four mobile phases tested (5–10% PrOH and 20–40 mM SDS), n was 12 (out of 14).

Table 5
Retention factors of AAS, k, for different SDS concentrations and PrOH 5%

AAS	Retention factors, k				K_{AS}	K_{AM}
	20 mM	28 mM	40 mM	52 mM		
HT	7.19	5.33	4.26	3.39	20.8	98.6
KT	7.98	5.78	4.46	3.39	43.3	224
FM	9.10	6.53	4.70	3.86	49.5	231
NT	11.10	7.97	5.97	4.64	69.4	268
B	11.10	7.97	5.97	4.64	69.4	268
DMT	11.90	8.43	6.20	4.64	313	1264
NE	11.90	8.43	6.20	4.64	313	1264
T	13.83	9.75	7.35	5.55	137	452
MT	16.36	11.40	8.93	6.34	357	1039
AOO	17.53	12.37	9.62	6.85	345	926
BLS	18.71	13.19	10.71	7.30	385	972
ET	23.62	16.62	13.60	8.99	5000	10224
OM	23.70	16.73	13.78	9.34	345	673
DZ	28.89	19.76	14.23	10.29	244	469

R.S.D.s < 2%. K_{AM} and K_{AS} data were obtained from Eq. (2).

Table 6
Retention factors, k, for AAS obtained with bivariant optimization

SDS (mM)	20	28	36	42	44
% PrOH	10	8	6	5.25	4
HT	3.23	3.44	3.46	3.51	3.71
KT	3.85	3.90	3.73	3.61	3.71
FM	4.76	4.57	4.21	4.13	4.21
NT	5.99	5.64	5.15	5.03	5.31
B	5.99	5.64	5.15	5.03	5.31
DMT	6.67	6.16	5.50	5.31	5.31
NE	6.67	6.16	5.50	5.31	5.31
T	8.05	7.17	6.33	6.82	6.17
MT	9.58	8.44	7.36	6.98	6.84
AOO	10.35	9.12	7.95	7.59	7.59
BLS	11.67	10.01	8.58	8.13	8.04
ET	13.33	11.70	10.40	10.15	10.41
OM	14.78	12.63	10.94	10.47	10.40
DZ	18.27	14.80	12.32	11.59	11.44
n	12	12	12	12	9

R.S.D.s < 2%.

Table 7
Retention factors, k for AAS obtained at different temperatures, using PrOH 5% and 40 mM SDS

AAS	Retention factors, k				$\Delta H \pm$ R.S.D. (kJ mol^{-1})	$(\Delta S + R\ln\phi) \pm$ R.S.D. (J mol^{-1})
	40 °C	50 °C	60 °C	70 °C		
HT	3.80	3.80	3.82	3.84	0.89 ± 0.48	8.31 ± 1.7
KT	4.01	4.08	4.12	4.08	1.32 ± 0.33	15.8 ± 0.52
FM	4.40	4.49	4.58	4.60	1.76 ± 0.26	17.9 ± 0.70
NT	5.37	5.44	5.52	5.58	1.65 ± 0.31	17.9 ± 0.10
B	5.37	5.44	5.52	5.58	1.65 ± 0.31	17.9 ± 0.10
DMT	5.37	5.65	5.83	6.03	3.50 ± 0.36	26.5 ± 0.80
NE	5.37	5.65	5.83	6.03	3.50 ± 0.36	26.5 ± 0.80
T	6.32	6.49	6.70	6.91	2.69 ± 0.22	23.8 ± 0.17
MT	6.91	7.26	7.73	7.52	4.81 ± 0.41	31.3 ± 1.0
AOO	8.17	8.10	8.32	8.42	1.89 ± 0.28	20.3 ± 1.1
BLS	8.17	8.52	9.01	9.46	4.56 ± 0.37	31.9 ± 0.46
ET	10.06	10.87	11.18	11.40	1.77 ± 0.24	25.6 ± 2.0
OM	10.10	10.60	11.23	11.80	4.84 ± 0.34	34.6 ± 0.27
DZ	11.96	12.35	12.77	13.10	2.78 ± 0.28	29.4 ± 0.31

R.S.D.s < 2%.

These results were not only consistent with those presented above, but also indicates that handling adequate SDS/PrOH ratios in the range 2–8 similar separations can be achieved. In other words, the method presents a certain robustness since a slight variation of the SDS and PrOH concentrations does not changes in a significant way the separation performances.

3.7. Temperature effect

The effect of the temperature on AAS retention was studied in the range 40–70 °C using a mobile phase 5% PrOH and 40 mM. In Table 7 are listed the k-values obtained at different temperatures from Fig. 2 chromatograms. As can be observed, as temperature increases retention and resolution increases slightly. The increased retention with increased temperature can be due to changes in the aggregation number or concentration of the micelles, which decreases generally as temperature increases [26]. In addition, selectivity is also modified by temperature: peaks corresponding to pairs NT/B and DMT/NE coelute in the range 40–70 °C, and AOO/BLS and DMT/NE/NT/B do it only at 40 °C. This indicates that a control of temperature for an adequate separation of AAS is required. Taking into account t_{tot}, resolution and n, a temperature of 60 °C was finally chosen.

Van't Hoff plots ($\ln k$ versus $1/T$) were constructed with the data of Table 7, showing good linearity ($r > 0.99$). This behavior evidences that the integrity of the micelle structure is maintained over the temperature range studied [22,23]. The positive enthalpy values (ΔH) (Table 7), obtained from the slopes, indicate that the mass transfer process is endothermic. Table 7 lists the positive values for the term $(\Delta S + R\ln\phi)$, where ΔS are the entropy changes and ϕ the phase ratio (unknown value). This term is related to

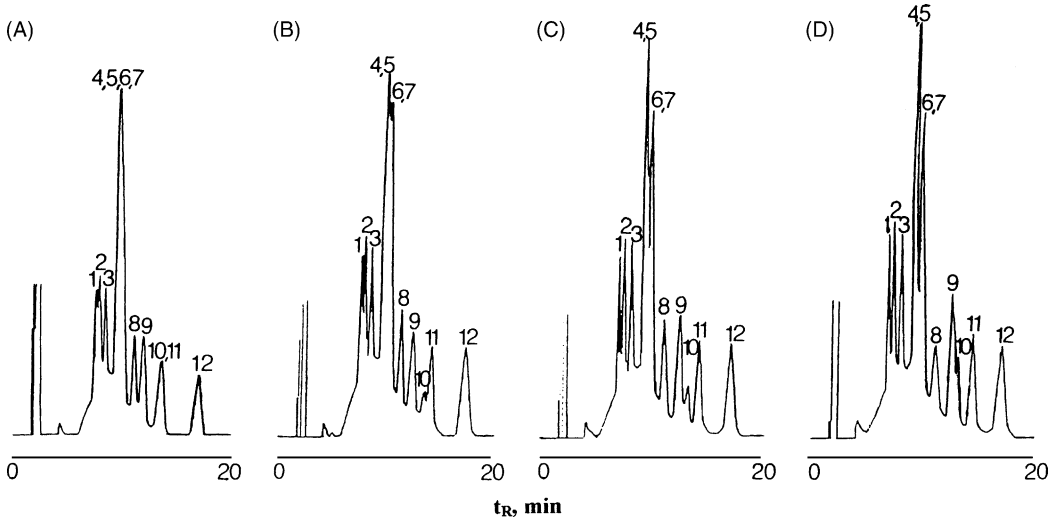


Fig. 2. Chromatograms for 10 μg ml^{-1} AAS (AOO = 15 μg ml^{-1}) obtained at different temperatures using 5% PrOH and 40 mM SDS mobile phase. (A) 40 °C, (B) 50 °C, (C) 60 °C, (D) 70 °C. Other conditions: peak numbers as in Table 2, UV detection at 245 nm. Limits of detection for a signal-to-noise ratio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

the intercept values ($\Delta S/R + \ln\phi$ in the Van't Hoff plots) and consequently with ΔS.

3.8. Effect of the flow-rate

The flow-rate has a relevant influence on the chromatographic efficiency in MLC [24]. This effect has been studied in the range 0.4–0.6 ml min^{-1} under optimum working conditions for AAS. t_{tot} and peak width decreased as increased flow-rate, showing a tendency to coelute at higher flow-rates. A slight increase of resolution at lower flow-rates was also observed. In addition, the achieved separations yielded always $n = 12$. 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) [25]. 50 mM Na_2HPO_4 (pH 6) or 50 mM $(NH_4)_2HPO_4$ (pH 6), 5% PrOH and 40 mM SDS mobile phases were used to study this effect. When sodium salt was used no significant changes in retention and resolution were observed. However, when ammonium salt was used, retention and resolution was affected. For those compounds which are retained more (B, T, AOO and BLS) retention increases and resolution decreases (e.g. BLS/ET), coeluting OM and DZ. However, for the remainder compounds, 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 [15,16]. Consequently, the presence of salts in MLC for AAS is not recommended to improve the separation previously obtained.

3.10. MLC versus HPLC

The chromatograms obtained from the optimum RP-HPLC separation [16] of steroids and those obtained with the optimum micellar separation were compared. The data from RP-HPLC [16] illustrate a much superior separation with HPLC than with MLC. Plate number for these compounds are generally 10-fold greater in HPLC versus MLC (e.g. the resolution, R_s, between HT and DMT at 40 °C is larger in HPLC (2.0) than in MLC (1.6)). This inconvenience can be overcome, in part, increasing temperature, because a wider temperature range can be used in MLC versus HPLC. Fig. 2A–D show the influence of temperature in MLC. As can be seen, resolution increases with temperature for compounds 1–2 (HT/KT), 8–9 (T/MT) and 10–11 (AOO/BLS). Some differences have been found when t_{tot} and n are compared. In HPLC, the complex mixture of steroids was separated in about 38 min (and in 23 min when DZ is excluded) whereas in MLC 12 out 14 were separated in about 20 min. However, MLC is cheaper and less toxic and contaminant 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 (45% ACN in HPLC and 5% PrOH and 40 mM SDS in MLC). Moreover, the optimal separation achieved in MLC (see Sections 3.6 and 3.7) does not change in a significant way in the range 50–70 °C (n was always 12). However, significant changes were observed in HPLC (in the temperature range 10–40 °C, n varies from 11 to 14). Differences in selectivity are also found. In summary, the micellar separation can be considered as an alternative to HPLC separation and perhaps suitable for a subset of the tested compounds.

An additional difference in the mass transfer process of solutes in HPLC (negative enthalpy values) versus MLC (positive) has also been observed. These enthalpic differences can be understood taking into account that HPLC and MLC have different retention mechanisms. In MLC the entropic factor ($T\Delta S$) may overcome the enthalpy one (ΔH), explaining then the retention factor variation with temperature.

4. Conclusions

Several micellar mobile phases were prepared with different organic modifiers and SDS and used for the separation of a complex sample containing natural and synthetic anabolic steroids by applying an experimental design using a partial version of the 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 mM SDS mobile phase, a 3.0 mm i.d. Hypersil C18 column (60 °C)), 12 out of 14 steroids (natural and synthetic) were separated in about 20 min. Moreover, this separation presents a certain robustness since a slight variation of the SDS/PrOH ratios (range 2–8) using SDS 28–40 mM and PrOH 5–8% in the mobile phase, and column temperature (50–70 °C) does not change the separation performances in a significant way. Additionally, these studies have been considered as the starting point in the method development for single mixtures of AAS [9].

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