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Optimization of separation of a complex mixture of natural and synthetic corticoids by micellar liquid chromatography using sodium dodecyl sulphate Application to urine samples

A. Santos-Montes, R. Izquierdo-Hornillos*

Departamento de Química Analítica, Facultad de Química, Universidad Complutense, 28040 Madrid, Spain

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Abstract

A systematic optimization of the separation of a mixture of corticoids by micellar liquid chromatography, using sodium dodecyl sulphate as surfactant, a Hypersil (250 mm×3.2 mm I.D.) C_{18} column, a flow-rate of 0.5 ml min^{-1}, and UV absorbance detection at 245 nm has been carried out. Several mobile phases consisting of sodium dodecyl sulphate and different organic modifiers were tested of which tetrahydrofuran, PrOH and BuOH were finally selected. On the basis of analysis time, resolution and number of compounds separated, a mobile phase containing 36 m*M* sodium dodecyl sulphate and 1.91% butanol allowed the separation of thirteen corticoids out of sixteen in about 27 min. Under these conditions the optimal concentration of sodium dodecyl sulphate was found to be 36 m*M*. A bivariant optimization method for the mobile phase BuOH–sodium dodecyl sulphate corrobored these results. The effects of temperature, ionic strength and flow-rate effect have also been studied. The most important analytical figures of merit were assessed and compared with those obtained using conventional mobile phases. The optimized method was applied to human urine samples of subjects administered with Dezacor® (tablets containing 30 mg of the active ingredient deflazacort) with and without sample preparation.

Keywords: Corticoids

1. Introduction

Corticoids (CC) are steroid hormones normally used as anti-inflammatory drugs that also relieve pain. They are also used to replace hormones in patients lacking these hormones, and to reduce the immunological response to a great variety of antigens, including transplanted organs. Their degradation usually takes place in the liver by reduction (UV adsorption is eliminated) and/or by conjugation with glucuronic acid or sulphate, and they are excreted in urine basically as conjugated metabolites as well as in parent form [1,2]. These compounds have been recently forbidden in sport by the International Olympic Committee (IOC) by oral and parenteral administration, being their use authorized only at therapeutic dose, under specific applications and always with medical consent.

Micellar liquid chromatography (MLC) has been widely used since the first application was reported

*Corresponding author.

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[3]. One of the reasons for the popularity of MLC is its unique separation selectivity [4]. In addition MLC has other advantages [5], such as simultaneous separation of ionic and nonionic compounds, rapid gradient capability, possibility of direct injection of biological fluids, enhanced luminescence detection [6–9] and low cost and toxicity, as compared with hydroorganic mobile phases.

The most important drawback of the MLC technique is the decrease in chromatographic efficiency [10] as compared to that obtained in conventional liquid chromatography (CLC) using hydroorganic mobile phases. The main reasons for this efficiency reduction are poor wetting of the stationary phase and restricted mass transfer [4]. Available columns with inner diameter smaller than the conventional ones (4.6 mm) can improve the wetting of the stationary phase since a lower flow-rate is required. Another way of improving the latter is by adding small amounts of organic modifiers [11] which reduce the net charge density in the micelle surface [12]. The efficiency in MLC seems to be related to the rigidity of the organic layer covering the silica surface. The organic modifiers reduce this rigidity, increasing diffusion of solutes and mass transfer [13]. In addition, the efficiency is also improved by increasing the column temperature [11].

Different HPLC methods for the separation of single compounds and complex mixtures of CC using conventional mobile phases have been recently developed and applied to urine samples and pharmaceutical formulations, Usually, prior to analyzing corticoids, a pretreatment of urine samples including enzymatic hydrolysis, generally with β-glucuronidase, and liquid–liquid extraction or solid-phase extraction is necessary [14–19]. These works reviewed also different methods and applications related to the determination of CC in different samples. A review involving CC and including different topics, such as HPLC methods in CLC and MLC, different kinds of samples and sample preparations, has also been reported [20]. However, little attention has been payed to MLC methods, especially for complex mixtures [21–22].

In this paper, a systematic optimization of the separation of a mixture of natural and synthetic corticoids by MLC is described using a Hypersil 5 μm (250 mm×3.2 mm I.D.) C_{18} column and mobile phases containing SDS. A similar optimization method to that reported by Glajch et al. [23] has been extended to MLC, after selection of mobile phases of SDS and THF, BuOH and PrOH. In this way, BuOH was finally selected. The effect of other variables such as SDS concentration, ionic strength, column temperature and flow-rate have also been discussed. This method has been applied to urine samples (with and without sample preparation) of subjects administered with Dezacor® tablets.

2. Experimental

2.1. Chemicals

Triamcinolone (TRI) (9α-fluoro-11β,16α,17,21-tetrahydroxy-1,4-pregnadiene-3,20-dione), prednisone (PS) (17α,21-dihydroxy-l,4-pregnadiene-3,11,20-trione), cortisone (CS) (17α,21-dihydroxy-pregn-4-ene-3,11,20-tri-one), prednisolone (PL) (1,4-pregnadiene-11β,17α,21-triol-3,20-dione), cortisol (CL) (11β,17α,21-trihydroxypregn-4-ene-3,20-dione), dexamethasone (DM) (9α-fluoro-16α-methyl-prednisolone), betamethasone (BM) (9α-fluoro-16β-methyl-11β,17α,21-trihydroxypregn-1,4-diene-3,20-dione), corticosterone (CT) (4-pregnene-11β,21-diol-3,20-dione), 11α-hydroxy-progesterone (HP) (4-pregnen-11α-ol-3,20-dione), fludrocortisone (FL (9α-fluoro-11β,17α,21-trihydroxy-4-pregnene-3, 20-dione), fludrocortisone acetate (FLA) (9α-fluoro-11β,17α,21-trihydroxy-4-pregnone-3,20-dione acetate), deoxy-corticosterone (DOC) (4-Pregnen-21-ol-3,20-dione), methyl-prednisolone (MPL) (6α-Methyl - 11β, 17α, 21 - trihydroxy - 1, 4 - pregnadiene-3,20-dione), and triamcinolone acetonide (TRA) (9α-fluoro - 11β, 16α, 17α, 21 - tetrahydroxypregn - 1, 4-diene-3,20-dione-16,17-acetonide) were purchased from Sigma (St. Louis, MO, USA) and deflazacort (DF) (11β,16β)-21-(acetyloxy)-11-hydroxy-2′-methyl-5′H-pregna-l, 4-diene[17, 16-d]oxazole-3, 20-dione and its metabolite 21-hydroxy-DF (MDF), was a gift of Marion Merrel Dow España (Madrid, Spain). Sodium dodecyl sulphate, $Na_2HPO_4 \cdot 2H_20$ and anhydrous Na_2SO_4 of analytical-reagent grade were from Merck (Darmstadt, Germany). HPLC-grade methanol, 1-propanol, 1-butanol, 1-pentanol,

acetonitrile and terahydrofuran were purchased from Promochem (Wesel, Germany). Water was purified with a Milli-Q system (Millipore, Moisheim, 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 (FL, USA): ConstaMetric 4100 solvent delivery system, SpectraMonitor 5000 photodiode-array detector 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 reversed-phase Hypersil 5 μm (250 mm×3.2 mm I.D.) C_{18} and a vortex mixer Mixo-Tub-30 from Crison (Barcelona, Spain) and a Visiprep vacuum manifold system from Supelco (Bellefonte, PA, USA), were also used.

2.3. Mobile phase

The mobile phase was prepared daily by mixing aqueous solutions of SDS (prepared with Milli-Q water) with PrOH, BuOH, PenOH, acetonitrile (AcCN) 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. Drug administration

One male volunteer aged 43 years, having given informed consent, received 30 mg of Dezacor® (tablets containing 30 mg of the active ingredient deflazacort) (Merrel Dow Spain). Several urine samples from the subject were collected during 24 h and stored at 4°C until analysis.

2.5. Sample preparation

After drug administration, human urine samples (3 ml) were spiked with MPL (0.33 μg ml^{-1}) and processed according to a previously described solvent extraction procedure in which recoveries (±RSD) were found to be (101.3±4.3), (91.3±4.5) and (94.4±5.2) for MPL, MDF and DF, respectively [15,16]. In brief, NaCl was added to the samples in order to avoid the production of emulsions and the pH was adjusted using Na_2HPO_4. Next, 4 ml dichloromethane was added. The mixture was shaken and centrifuged. The organic phase was removed and dried over anhydrous Na_2SO_4. A 3 ml aliquot was evaporated to dryness and the dried residue was reconstituted with 200 μl of MeOH and 20 μl were injected into the HPLC system.

3. Results and discussion

3.1. Preliminary experiments using THF in CLC and MLC

In a previous paper, a systematic optimization of the HPLC separation of a mixture of CC based on the Glajch's triangle was reported using CLC, which allowed the separation of 13 CC out of 14, using a Hypersil C_{18} (250×4.6 mm I.D.) column (30°C), water–THF (72:28, v/v) as mobile phase, a flow-rate of 1.0 ml min^{-1} and UV absorbance detection at 245 nm [14]. DF and MDF were introduced into the above mixture and a refined study of the separation of these compounds was carried out using the above described Hypersil column (30°C) and THF as organic modifier. In this way, 15 CC out of 16 were separated using water–THF (77:23, v/v) as mobile phase [16].

Taking into account the commented on above results, a reference separation was carried out in the present work using THF as organic modifier and a Hypersil column (40°C) with the same length and 3.2 mm I.D. This column requires a lower flow-rate and allows a better mass transfer of solutes between the mobile and stationary phases (especially in MLC), improving the separation efficiency. Therefore, the separation of 13 CC has been allowed

Table 1
CC separation characteristics obtained using mobile phases containing 36 m*M* SDS and different solvents. SCR and OPT are the solvent concentration range used and optimum selected; NSC is the number of separated compounds and RTA the run time analysis involved

Solvent	SCR (%)	OPT (%)	NSC	RTA (min)
THF	5.09–7.63	5.73	12	35
PrOH	5.09–7.63	5.73	13	28
BuOH	1.91–6.36	1.91	13	27
PenOH	0.36–3.61	0.63	11	25

working at a flow-rate of 0.5 ml $min^{-1}$and water THF (76:24, v/v) as mobile phase.

MLC was initiated using mobile phases of THF–SDS. In order to have a suitable SDS concentration to afford retention factors, *k*, in the range 1–15, 6% THF was selected as organic modifier. Under such conditions, several SDS concentrations over its critical micellar concentration (cmc=8.1 m*M*) were tested. Satisfactory result were achieved using 36 m*M* SDS. In addition, UV absorption spectra obtained using these mobile phases did not show significant differences with respect to those obtained using THF–water mobile phases [14,16]. Therefore, the chromatographic detection using the THF–SDS mobile phases was carried out at the same wavelength as that used in CLC (245 nm).

3.2. Separation characteristics of CC using different organic modifiers in MLC

In order to evaluate the separation characteristics of CC in MLC, mobile phases containing 36 m*M* SDS (previously selected) and variable composition of several solvents of different nature, commonly used in MLC as organic modifiers, such as acetonitrile (AcCN), tetrahydrofuran (THF), PrOH, BuOH or PenOH, were tested. In Table 1, the solvent concentration range studied and optimum composition achieved according to the number of separated compounds and run time involved, are summarized. Table 2 lists the retention factors, *k*, for these compounds under optimum solvent conditions (*k* values in CLC using THF are also given in Table 2). As can be observed from the data in Table 1, good results were obtained when using BuOH and PrOH, whereas if PenOH and THF are used, reasonable results are achieved. However, poor results were obtained when AcCN was used because in this case a concentration over 15% was required and the run time for the separation of 11 CC was of 45 min (these results are not included in Table 2).

Table 2
Retention factors, *k*, for CC using different organic modifiers in CLC and MLC (36 m*M* SDS). Conditions: Hypersil C_{18} (150 mm×3.2 mm I.D.) 5 μm column (50°C). Flow-rate 0.5 ml min^{-1}

CC	CLC THF 24%	MLC			
		THF 5.73%	PrOH 5.73%	BuOH 1.91%	PenOH 0.63%
TRI	0.92	1.69	1.51	1.40	1.88
PS	2.44	5.04	3.98	4.06	4.23
MDF	2.27	7.62	6.17	6.03	6.19
CS	2.74	5.19	4.04	4.06	4.23
CL	3.33	6.08	4.54	4.40	4.61
PL	3.05	6.49	5.00	4.96	5.21
FL	4.05	5.85	4.17	4.06	4.23
CT	4.58	9.08	7.04	6.42	6.44
MPL	5.13	6.71	7.54	7.16	6.79
BM	5.51	9.45	7.23	6.53	6.79
DH	6.42	9.80	7.25	7.16	6.79
DF	6.79	9.80	11.68	10.61	10.41
HP	5.85	10.16	8.74	7.69	7.34
TRA	5.85	10.59	7.75	7.16	7.34
DOC	8.10	12.27	10.24	9.12	8.78
FLA	12.43	14.80	5.84	5.48	5.46

The *k* data corresponding to CLC (using THF) and MLC, shown in Table 2, indicate changes in selectivity. In other words, different elution orders were achieved when these values are compared (differences between MLC and CLC). This behavior was also observed in MLC when comparing alcohols with THF (Table 2). However, solutes followed the same elution order using alcohols even though coelution of CC occurred: for PrOH (coeluted BM and DM), for BuOH (DM and MPL) and for PenOH (BM, DM and MPL). From data listed in Table 1 and comparing the different organic modifier performances (number and peak shape of separated compounds and run time analysis involved), THF, PrOH and BuOH were finally selected to build the Glajch's triangle extended to MLC.

3.3. Organic modifier optimization in MLC based on Glajch's method

Briefly, Glajch's method (applied to complex mixtures in CLC) requires different mixtures of three different organic modifiers and water as mobile phases. The optimum results can be achieved by mapping resolution versus the mobile phase composition. Usually, the optimization process is accomplished starting with a binary mixture are increasing the number of components up to a quaternary mobile phase [23]. Although retention factors, *k*, should be within the range 1–15 for all components, they may be increased depending on the complexity of the separation. This method has been extended to MLC for CC using mobile phases of aqueous SDS and PrOH, BuOH and THF as organic modifiers. In order to build the triangle and taking into account the results presented above (see Tables 1 and 2), micellar solutions containing 36 m*M* SDS and THF (5.73%), PrOH (5.73%) and BuOH (1.91%) were selected (A, B and C triangle vertex, respectively) (Fig. 1). For simplicity, water and SDS compositions will not be included in further mobile phases nomenclature unless indicated otherwise. The above mobile phases: A (THF), B (PrOH) and C (BuOH), were mixed appropriately to give the separations D, E and F (middle side point B of the triangle) and G (triangle centroid). The D point was obtained with PrOH–THF (2.9:2.9, v/v). Under these conditions 12 CC were separated and coeluted: (PS, CS), (CT, BM), (MPL, DM) and (HP, TRA). E point was obtained with the mixture BuOH:THF (0.96:2.9 v/v) and 12 steroids were separated and compounds (PS, CS, FL), (BM, DM), (MPL, TRA) were coeluted. F point was obtained, with the mixture PrOH–BuOH (2.9:0.96, v/v). Under these conditions 12 CC were separated and compounds (PS, CS); (BM, MPL, DM), (HP, TRA) were coeluted.

G point was obtained with the mixture PrOH:BuOH:THF (1.9:1.9:0.64, v/v/v). In this way, 12 CC were separated and coeluted (PS, CS), (BM, MPL, DM).

Table 3 shows the retention factors, *k*, obtained for CC with each mobile phase used. As expected, different selectivities were obtained, and they were dependent on the different mobile phases involved.

The mobile phase SDS:BuOH (36 mM:1.91%) was finally selected on the basis of the number of corticoids separated.

3.4. Effect of SDS concentration

Once the organic modifier (BuOH) was selected, a study of the effect of SDS concentration on the CC separation was carried out. SDS concentration was varied in the range 36–108 m*M*. The retention factors, *k*, obtained at 50°C are shown in Table 4. As expected, shorter retention times for each CC [24] were obtained as SDS concentration increased. In addition, when log *k* for CC is plotted versus log [SDS] (Fig. 2), linear plots were obtained (different slopes). Thus, not only the retention factor, *k*, but also the selectivity is dependent on the SDS concentration. Consequently, variation of the SDS concentration provides a means of controlling both the selectivity and the resolution achievable.

3.5. Bivariant optimization method for the SDS–BuOH system

In order to optimize an adequate composition of the mobile phase SDS–BuOH for CC separation, a bivariant method using a continuous variation of the concentrations of the system SDS–BuOH was performed (SDS was decreased when BuOH increased). The ranges of BuOH and SDS were 1–4% and 20–80 m*M*, respectively. A mobile phase SDS:BuOH (35 m*M*:3.25%) allowed the separation

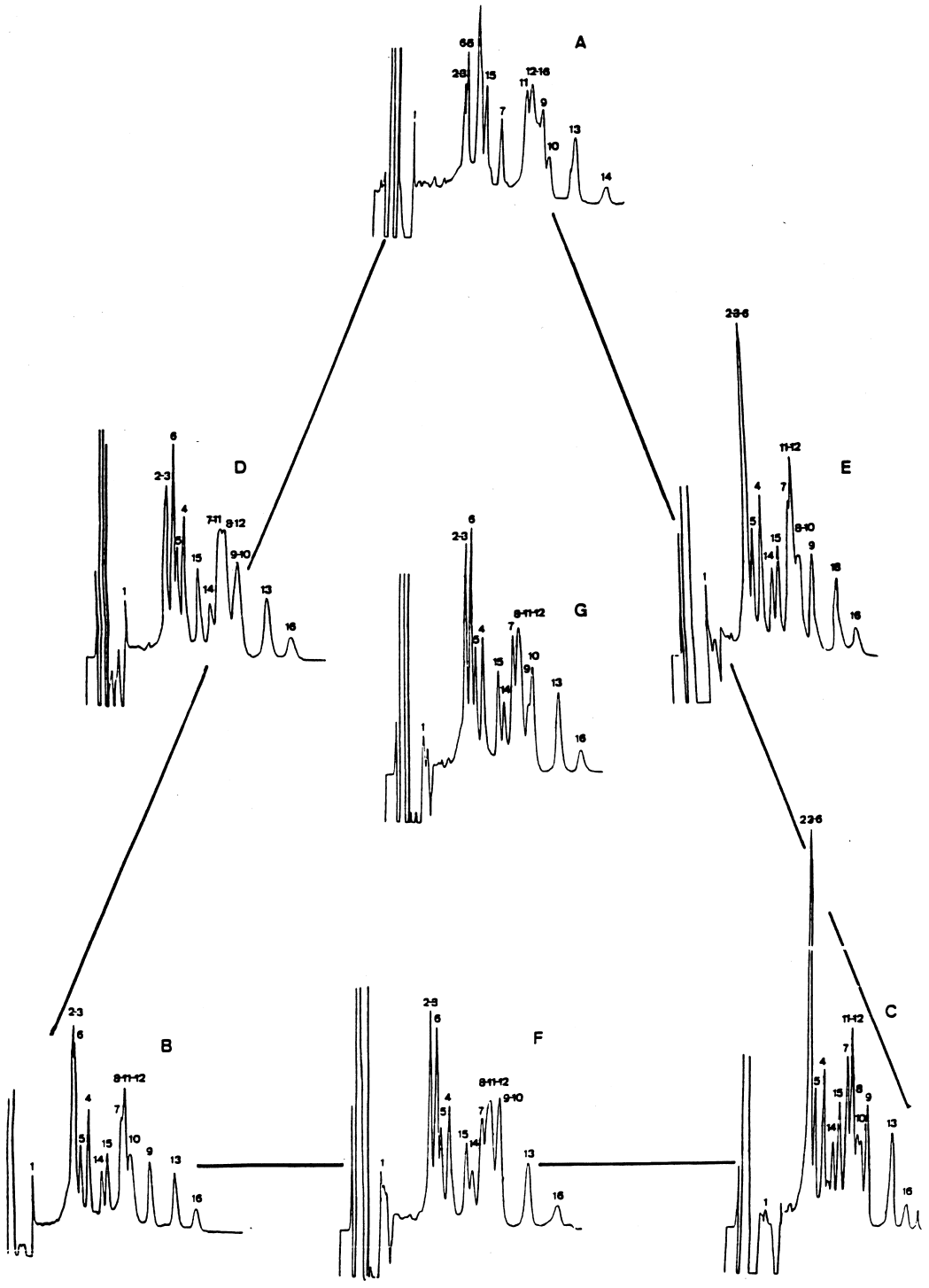

Fig. 1. Glajch's triangle applied to CLM using a standard mixture of corticoids. Conditions as in Table 3. Peak numbers: 1 (TRI), 2 (PS), 3 (CS), 4 (PL), 5 (CL), 6 (FL), 7 (CT), 8 (MPL), 9 (HP), 10 (TRA), 11 (BM), 12 (DM), 13 (DOC), 14 (FLA), 15 (MDF), 16 (DF).

Table 3
k values of CC for different mobile phases (A–G) used to build the Glajch's triangle. Mobile phases (A–G) composition (see text). Conditions: Hypersil C_{18} (150 mm×3.2 mm I.D.) 5 Min column (50°C). Flow-rate 0.5 ml min^{-1}

CC	A	B	C	D	E	F	G
TRI	1.69	1.5.	1.40	1.69	1.47	1.61	1.58
PS	5.04	3.98	4.06	4.41	4.01	4.68	4.35
MDF	7.62	6.17	6.03	6.54	6.06	6.97	6.48
CS	5.19	4.04	4.06	4.41	4.01	4.68	4.35
CL	6.08	4.54	4.40	5.16	4.46	5.38	4.99
PL	6.49	5.00	4.96	5.37	4.98	5.89	5.45
FL	5.85	4.17	4.06	4.86	4.01	5.08	4.71
CT	9.08	7.04	6.42	8.02	6.77	7.95	7.46
MPL	6.71	7.54	7.16	8.30	7.36	8.47	7.84
BM	9.45	7.23	6.53	8.02	7.00	8.47	7.84
DM	9.80	7.25	6.53	8.30	7.00	8.47	7.84
DF	9.80	11.68	10.61	12.64	11.03	12.8	11.94
HP	10.16	8.74	7.69	9.17	8.24	9.07	8.49
TRA	10.59	7.75	7.26	9.17	7.36	9.07	8.75
DOC	12.27	10.24	9.12	11.05	9.81	10.91	10.47
FLA	14.80	5.84	5.78	7.31	5.67	7.34	6.89

of 11 CC in 18 min. However, BuOH concentration was decreased since no changes in the elution order of CC were observed. In this way, 13 compounds were separated in a run time of 27 min using a BuOH concentration of 1.91%. The latter results corroborate those presented above and a SDS concentration over its cmc was guaranteed.

Table 4
k values of CC using 1.91% BuOH and different SDS concentrations. Hypersil C_{18} (250 mm×3.2 mm I.D.) 5 μm column (50°C). Flow-rate 0.5 ml min^{-1}

CC	36 m*M*	75 m*M*	108 m*M*
TRI	1.40	0.84	0.59
PS	4.06	2.09	1.52
MDF	6.03	2.96	2.12
CS	4.06	1.71	1.54
CL	4.40	2.20	1.54
PL	4.96	2.48	1.76
FL	4.06	2.08	1.48
CT	6.42	3.16	2.22
MPL	7.16	3.46	2.38
BM	6.53	3.34	2.21
DM	7.16	3.37	2.35
DF	10.61	5.07	3.67
HP	7.69	3.67	2.55
TRA	7.16	3.67	2.61
DOC	9.12	4.49	3.14
FLA	5.48	2.72	1.91

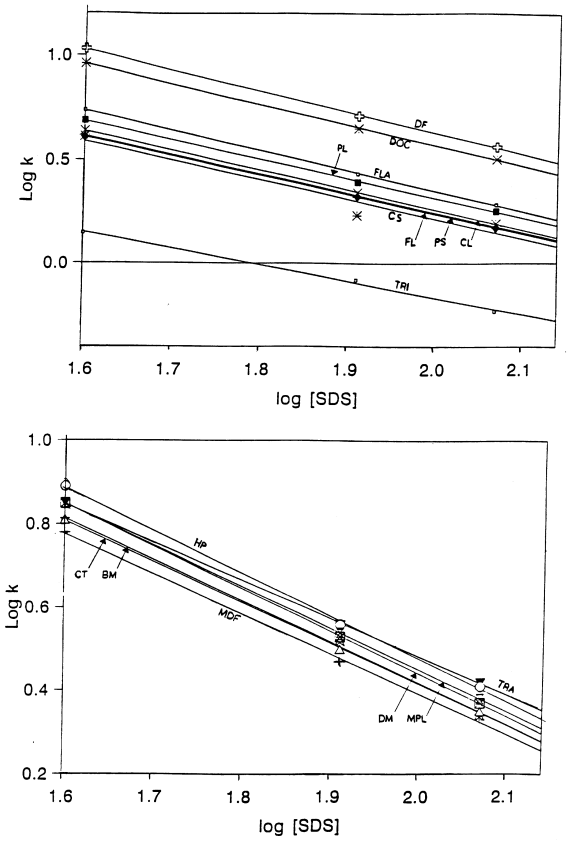

Fig. 2. Effect of SDS concentration on retention factors of CC.

3.6. Effect of temperature

The effect of the temperature on CC retention was studied in the range 30–60°C under the optimum working conditions (C mobile phase). Fig. 3 shows the chromatograms obtained at different temperatures. As can be seen, as the temperature increases from 30°C to 50°C, the chromatographic resolution improves. However, at the highest temperature of 60°C the resolution decreases. In this way, 8, 11, 12 and 11 CC were separated at 30, 40 50 and 60°C, respectively, but never up to base line. From these experiments, a temperature of 50°C was finally selected. In addition, the data shown in Table 5 (k values obtained at different temperatures) show a progressive decrease of the k values as temperature increases. An exception to this behavior is found for the latter four compounds eluted (TRA, HP, DOC and DF) for which retention increases when temperature increases. Van't Hoff plots (in k vs. $1/T$) were obtained from the retention data of Table 5, showing good linearity ($r \geq 0.99$). Enthalpy values, ΔH, were derived from the slopes. The observed behavior is in good agreement with the data reported in the literature and evidences that the integrity of the micelle structure is maintained over the temperature range studied [11,25]. The negative enthalpy values obtained for the compounds firstly eluted indicate that

Table 5
k Values of CC at different temperatures using 1.91% BuOH and 36 mM SDS. Conditions: Hypersil C_{18} (250 mm×3.2 mm I.D.) 5 μm column. Flow-rate 0.5 ml min^{-1}

CC	30°C	40°C	50°C	60°C
TRI	2.30	2.39	2.45	2.50
PS	4.13	4.06	4.06	3.99
CS	4.13	4.06	4.06	3.99
FL	4.13	4.06	4.06	3.99
CL	4.13	4.36	4.40	4.37
PL	5.09	4.97	4.96	4.84
FLA	6.06	5.22	5.48	5.82
MDF	6.39	5.91	6.03	5.82
CT	6.39	6.35	6.42	6.62
BM	6.94	6.73	7.16	6.62
DM	6.94	6.73	7.16	6.62
MPL	6.94	7.05	7.16	7.07
TRA	6.94	7.05	7.26	7.30
HP	6.94	7.25	7.69	8.03
DOC	8.86	8.93	9.12	9.37
DF	9.98	10.07	10.61	10.51

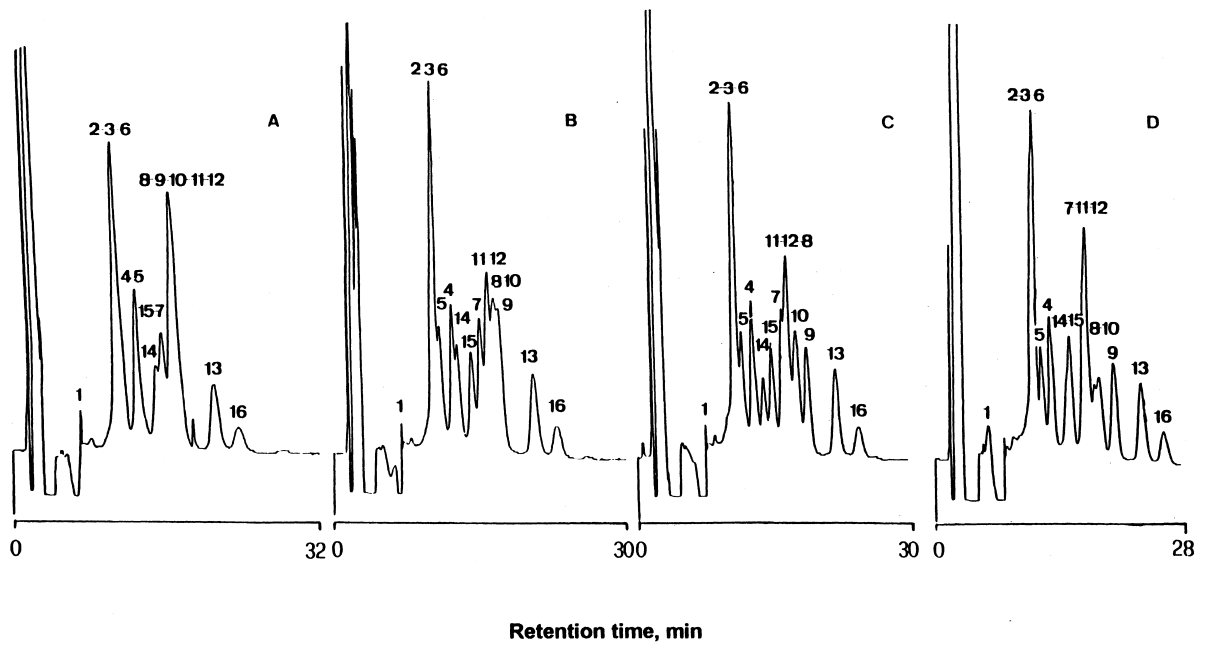

Fig. 3. Effect of temperature on the separation of CC. (A) 30°C, (B) 40°C, (C) 50°C and (D) 60°C. Peak numbers as in Fig. 1.

the mass transfer process is exothermic for these compounds. However, for the four latter eluted compounds the behavior is just the opposite. For these latter compounds the entropic factor (TΔS values) in the Gibb's equation probably overcomes the enthalpy one, changing in this way the sign of the slope.

3.7. Effect of flow-rate

Given the importance of the mobile phase flow-rate on the chromatographic efficiency in MLC [4], its effect on the CC separation was studied in the range 0.4–0.6 ml min^{-1} under optimum working conditions. Using a flow-rate of 0.4 ml min^{-1}, eleven peaks were observed (30 min run time) and twelve peaks were obtained using flow-rates of 0.5 ml min^{-1} or 0.6 ml min^{-1} (25 or 20 run time min respectively). However, it was observed that the resolution decreases when a flow-rate of 0.6 ml min^{-1} is used. This indicates that column efficiency was optimum working at a flow-rate of 0.5 ml min^{-1}.

3.8. Effect of salts added to the mobile phase

The influence of the salts on the ionic surfactants cmc and on the aggregation number of micelles is enormous since the cmc is greatly decreased, the degree of counterion binding is affected and the micelle size is increased [26]. Consequently, the separation characteristics of CC can also be modified by adding salts to the mobile phases. In order to check that, 50 m*M* ammonium acetate (pH 6) and 50 m*M* disodium hydrogen phosphate (pH 6) were added to the mobile phase. This addition produced a decrease of the retention factors, affecting thus to the resolution and separation characteristics. In CLC, however, the addition of salts to the mobile phase did not show a significant effect for these compounds [14]. This is an indication that it is not convenient to add salts to the mobile phases and that micelles are the only ones affected by the addition of salts.

3.9. Calibration graphs

Standards containing mixtures of the CC were prepared at five different concentrations in the range 2.0–8.0 μg ml^{-1} using 4.0 μg ml^{-1} MDF as internal standard (IS). When MDF was evaluated PS was used as I.S. These solutions were analyzed with a mobile phase composed of 36 m*M* SDS containing 1.91% BuOH, a flow-rate of 0.5 ml min^{-1}, and UV–DAD detection at 245 nm. The results were analyzed by linear regression. Plotting each corticoid peak area to MDF or PS (IS) ratio (PAR) versus the concentration (x) of each corticoid, the calibration equations, $PAR = A + Bx$(mg ml^{-1}), were obtained. In Table 6 the parameters A (intercept), B (slope) and r (regression coefficient) are shown. In all cases the intercepts were not significantly different from zero.

3.10. Precision and detection limits

The precision was examined by analyzing ten different samples of CC containing 5 μg ml^{-1} each using the calibration graphs. The C.V. for each corticoid is shown in Table 6.

The detection limits (LDs) for each CC were assessed for a signal-to-noise ratio (S/N) of three by means of the calibration graphs (Table 6). These values are lower than those obtained in CLC for CC since the noise was lower.

Table 6
Linear regression equations ($PAR = A + Bx$), detection limits (*DL*s) and C.V. of CC. PAR is the peak area ratio of CC to MPL or PS (IS) 4 μg ml^{-1}; x = μg ml^{-1} of CC and r the correlation coefficient

CC	A	B	r	DLs (ng)	C.V. (%)
TRI	0.041	0.151	0.9991	0.049	3.1
Ps	0.051	0.132	0.9996	0.052	2.2
CS	−0.012	0.214	0.9994	0.047	2.6
FL	−0.052	0.176	0.9992	0.049	1.7
CL	−0.133	0.197	0.9990	0.042	2.7
PL	0.020	0.131	0.9998	0.053	2.1
FLA	−0.192	0.224	0.9995	0.045	3.2
MDF	−0.089	0.154	0.9992	0.066	2.5
CT	0.099	0.143	0.9994	0.055	3.3
BM	0.022	0.121	0.9998	0.057	3.5
DM	−0.069	0.132	0.9990	0.052	3.4
MPL	−0.141	0.130	0.9991	0.049	2.9
TRA	0.045	0.130	0.9996	0.057	3.7
HP	0.039	0.105	0.9998	0.066	3.2
DOC	0.060	0.160	0.9996	0.043	3.4
DF	−0.031	0.093	0.9990	0.074	3.5

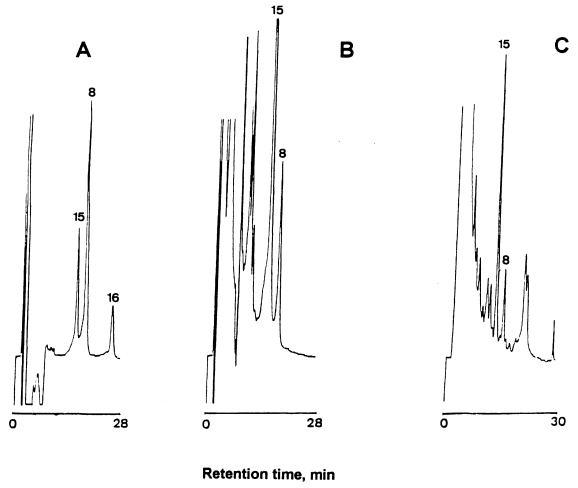

Fig. 4. Chromatograms obtained under optimal conditions: (A) standard mixture of CC, (B) urine sample with previous solvent extraction and (C) urine sample with direct injection. Peak numbers as in Fig. 1.

3.11. Application to urine samples.

MLC using SDS allows the direct injection of several untreated biological samples onto the chromatographic column [27]. On this basis and taking into account a previous pharmacokinetic study carried out in human urine samples of subjects administrated with Dezacor® [16], the above developed method was applied to urine samples with and without sample preparation of one subject administered with Dezacor®. In Fig. 4 are shown the chromatograms obtained for different samples spiked with 4 $\mu g\ ml^{-1}$ MPL (IS): (A) a standard solution of DF and MDF (5 $\mu g\ ml^{-1}$), (B) urine sample prior to solvent extraction and (C) direct injection of urine sample. The chromatographic peaks in Fig. 4B and C were analyzed and identified using UV absorbance–DAD detection [18]. In this way, deflazacort metabolite (MDF) was only detected. The peaks following MPL (IS) in Fig. 4C correspond to endogenous compounds from urine samples.

4. Conclusions

Mobile phases containing SDS and THF, BuOH or PrOH, and a 3.2 mm inner diameter Hypersil C_{18} column were used for the separation of a complex sample containing CC. Satisfactory results were obtained when the Glajch's method was applied. Different separations with different selectivities were obtained depending on the nature of organic modifier, SDS concentration and temperature. Thirteen CC out of sixteen were separated in 27 min using a SDS–BuOH mobile phase. MLC constitutes an alternative to CLC, and presents several advantages, such as a decrease of analysis time and LDs, the possibility of direct injection of human urine samples and the use of cheaper and less toxic mobile phases.

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