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Optimization of the high-performance liquid chromatographic separation of a mixture of natural and synthetic corticosteroids

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ABSTRACT

Systematic optimization of the HPLC separation of a mixture of natural and synthetic corticosteroids was carried out for screening purposes. The method involves binary, ternary or quaternary mixtures containing water, methanol, acetonitrile and tetrahydrofuran. It was possible to separate thirteen out of fourteen corticosteroids contained in a sample in about 26 min, with a 5-μm Hypersil-C_{18} (250 mm × 4.6 mm I.D.) column thermostated at 30°C, using a mobile phase composed of water–tetrahydrofuran (72:28, v/v). This separation was not improved using other C_8 or C_{18} columns. The effect of temperature on the separation of these compounds was also studied. Calibration graphs were established for each corticosteroid up to 8 μg/ml using indapamide as internal standard. The detection limits were in the range 0.02–0.14 ng. The optimized method was applied to urine samples spiked with corticosteroids and showed potential for future applications.

INTRODUCTION

Corticosteroids are glucocorticoids chemically derived from cyclopentaperhydrophenanthrene (Table I). Cortisol and cortisone are physiologically the most important.

Corticosteroids are normally used to replace steroid hormones in patients lacking these hormones, as anti-inflammatory drugs that also relieve pain, and to reduce the immunological response to a great variety of antigens, including transplanted organs. They are usually metabolized in the liver by reduction and/or by conjugation with glucuronic acid or sulfate, and they are excreted in urine basically as conjugated metabolites as well as in non-metabolized form [1,2].

It is also known that athletes use corticosteroids to improve their performance. Since 1975, the International Olympic Committee Medical Commission has restricted the use of these compounds to legitimate medical purposes. Therefore, the determination of corticosteroids is necessary for both therapeutic monitoring and doping control.

The great majority of the analytical methods used for determining corticosteroids are based on radioimmunoassay (RIA) procedures, characterized by a high sensitivity but also by an important lack of specificity owing to the cross-reactivity of related compounds. GC and GC–MS methods, although highly sensitive and specific, require derivatization prior to injection because of the low volatility of these compounds, which can also be thermally decomposed [3–7]. On the other hand, HPLC offers the possibility of analysing corticosteroids without derivatization showing high sensitivity and selectivity with normal or reversed-phase packings and isocratic or

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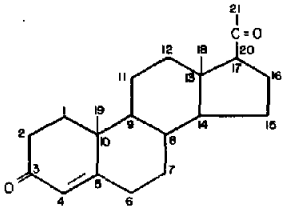
TABLE I

CHEMICAL STRUCTURES OF CORTICOSTEROIDS



Compound	C1–2	C9	C11	C16	C17	C21
Cortisol(CL)			–OH		–OH	–OH
Cortisone(CS)			=O		–OH	–OH
Prednisolone(PL)	Δ		–OH		–OH	–OH
Prednisone(PS)	Δ		=O		–OH	–OH
Triamcinolone(TRI)	Δ	–F	–OH	–OH	–OH	–OH
Corticosterone(CT)			–OH			–OH
Betamethasone(BM)	Δ	–F	–OH	CH_3	–OH	–OH
Triamcinolone acetonide(TRA)	Δ	–F	–OH	[a]	[a]	–OH
11α–Hydroxyprogesterone(HP)					–OH	CH_3
Dexamethasone(DM)	Δ	–F	–OH	CH_3	–OH	–OH
Fluorocortisone(FL)		F	OH		–OH	–OH
Deoxycorticosterone(DC)						–OH
Methylprednisolone(MPL)[b]	Δ		–OH		–OH	–OH
Fluorocortisone acetate(FLA)		–F	–OH		–OH	[c]

[a] Acetonide group: C17 ... O, C16 ... O, $C(CH_3)_2$

[b] CH_3 on C_6.

[c] Acetate group: –O–CO–CH_3.

gradient elutions. Some of these compounds have been determined in several biological fluids for doping control [5] and for clinical and pharmacokinetic studies [8,9]. Usually, prior to analysing corticosteroids by HPLC, pretreatment of the samples, including enzymatic hydrolysis, generally with β-glucuronidase [10], and liquid–liquid extraction (LLE) [11–14] or solid-phase extraction (SPE) [15–18] is necessary. Liquid chromatographic methods for screening of corticosteroids have not been widely employed [5].

In this paper a systematic HPLC study, based on the method developed by Snyder *et al.* [19] for the separation and determination of corticosteroids in samples containing natural and synthetic corticosteroids (structures in Table I), is reported. The studied variables were: type of C_{18} packing, composition of binary, ternary or quaternary mobile phases (water, methanol, acetonitrile, tetrahydrofuran) and column temperature. Detection was performed with UV–diode-array detector (UV–DAD), which is useful for the identification of the separated components and/or comparison of peak purity. In this way the separation of thirteen corticosteroids out of fourteen in a mixture was performed in about 26 min, with a water–tetrahydrofuran mixture as mobile phase and a bonded-silica Hypersil 5-ODS column (250 mm × 4 mm I.D.). The method was applied to urine samples spiked with corticosteroids and showed potential for future applications.

EXPERIMENTAL

Chemicals

Triamcinolone (TRI) (9α-fluoro-11β,16α,17,21-tetrahydroxy-1,4-pregnadiene-3,20-dione), prednisone (PS) (17α,21-dihydroxy-1,4-pregnadiene-3,11,20-trione), cortisone (CS) (4-pregnene-17α,21-diol-3,11,20-trione), 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-methylprednisolone), betamethasone (BM) (1,4-pregnadiene-3,20-dione-9α-fluoro-16β-methyl-11β,17α,21-triol), 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-pregnene-3,20-dione acetate), deoxycorticosterone (DC) (4-pregnen-21-ol-3,20-dione), methylprednisolone (MPL) (6α-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione), triamcinoloneacetonide (TRA) (1,4-pregnadiene-9α-fluoro-3,20-dione-11β,16α,17α,21-tetrol-16,17 acetonide) and indapamide (IP) were purchased from Sigma (St. Louis, MO, USA). $Na_2HPO_4 \cdot 2H_2O$ and anhydrous Na_2SO_4 of analytical reagent grade from Merck (Darmstadt, Germany). HPLC-grade methanol, acetonitrile and tetrahydrofuran 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 chemicals used were of analytical reagent grade.

Apparatus

The chromatographic system consisted of the following components, all from LDC Analytical (Riviera Beach, FL, USA): a Constametric 4100 solvent-delivery system, a Spectromonitor 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 Selecta (Barcelona, Spain) thermostated bath was used to maintain the column temperature below 30°C. The following reversed-phase columns were used: bonded-silica Hypersil 5-ODS (250 mm × 4.6 mm I.D.); C_8 5 μm (200 mm × 4.0 mm I.D.), Ultracarb C_{18} 5 μm (150 mm × 4.6 mm I.D.); Nucleosil C_{18}, 5 μm (150 mm × 4.6 mm I.D.); and Spherex C_{18}, 5 μm (150 mm × 4.6 mm I.D.) from Phenomenex (Torrance, CA, USA). A Mixo-Tub-30 vortex mixer from Crison (Barcelona, Spain) and a Visiprep vacuum manifold system from Supelco (Bellefonte, PA, USA) were also used.

Mobile phase

The mobile phase was prepared daily by mixing Milli-Q water with methanol, acetonitrile, or tetrahydrofuran (THF) at the required volume ratio, programming the pump. All solvents and mobile phases were first filtered under vacuum through 0.45-μm nylon filters and degassed using helium sparge.

Chromatographic analysis

Once the column had been conditioned with the mobile phase, chromatograms were obtained at the programmed temperature (range 20–60°C). A methanolic solution containing a single corticosteroid or an appropriate mixture of them (4 μg/ml) was injected (20 μl). The flow-rate was 1.0 ml/min and UV–DAD detection in the range 190–360 nm was used. Corticosteroids were identified by comparing the UV spectra of the chromatographic peaks with UV spectra previously registered. After identification, subsequent confirmation was performed by injection of each one. Analysis was carried out at 245 nm.

Sample preparation

A 3-ml urine sample spiked with corticosteroids (0.33 μg/ml) and IP (0.33 μg/ml) was placed in a stoppered centrifuge tube. Afterwards, the pH of urine was adjusted by adding 0.5 g of di-

sodium hydrogenphosphate, the solution was vortex-mixed and 4 ml of dichloromethane were added. The tube was shaken by vortex-mixing for 1 min. The mixture was centrifuged for 3 min at 5000 rpm and the organic phase was removed and dried with anhydrous sodium sulphate. A 3-ml aliquot was evaporated to dryness under a vacuum system (see *Apparatus* section). The dried residue was reconstitued with 200 μl of methanol and 20 μl were injected into the HPLC system [20].

RESULTS AND DISCUSSION

Preliminary observations

The chromatographic approach chosen for developing a HPLC separation for samples containing all the fourteen corticosteroids was the method reported by Snyder *et al.* [19], also called "Glajch's triangle". This method requires a systematic optimization of mixtures of methanol, acetonitrile, THF and water to be used as mobile phase, by plotting resolution *versus* the mobile phase composition. Usually, the optimization process is accomplished starting with a binary mixture and increasing the number of components up to a quarternary mobile phase. Although k' (capacity factor) should be within the range 1–10 for all components, it may be increased depending upon the separation complexity. In order to develop the method, a Hypersil column, thermostated at 30°C, was used.

Optimization of mobile phases

The A, B and C vertices of the ABC triangle (Fig. 1) were determined using different H_2O–methanol, H_2O–acetonitrile and H_2O–THF mixtures. The optimum separation was achieved with H_2O–methanol (45:55, v/v) (A vertex, Fig. 1). Under these conditions eight corticosteroids were separated: TRI, PS, CS (PL, FL and CL co-eluted), (BM and DM co-eluted), (TRA, MPL, FLA and CT co-eluted), HP and DC.

The B vertex was achieved with H_2O–acetonitrile (75:35, v/v). Under such conditions eight corticosteroids were separated: TRI, PS (PL, CL, FL and CS co-eluted), (BM and DM co-eluted), (CT and TRA co-eluted), MPL (HP and FLA co-eluted) and DC.

The C vertex was achieved with H_2O–THF (72:28, v/v). Under such elution conditions, thirteen corticosteroids were separated: TRI, PS, CS, PL, CL, FL, CT (MPL and HP co-eluted), TRA, BM, DM, DC and FLA. When MPL and HP are to be separated, A and B phases are recommended.

A, B and C were mixed appropriately to give the separations D, E and F and G. The D point was obtained with H_2O–methanol–acetonitrile (58:28:14, v/v/v) and under these conditions eight corticosteroids were separated: TRI, PS, CS (PL, FL and CL co-eluted), (BM, DM, TRA, MPL co-eluted) (FLA and CT co-eluted), HP and DC. The E point was obtained with the mixture H_2O–methanol–THF (58:28:14, v/v/v). Under such elution conditions eight corticosteroids were separated: TRI (PS and CS co-eluted), (PL, FL and CL co-eluted), CT, (BM and MPL coeluted); (DM, TRA and HP coeluted), FLA and DC. The F point was obtained with the mixture H_2O–acetonitrile–THF (68.5:17.5:14, v/v/v). Under such elution conditions twelve corticosteroids were separated: TRI, PS, CS, PL, CL, FL, MPL, (BM and CT co-eluted), DM, TRA, HP and (FLA and DC co-eluted).

The G point (triangle centroid) was obtained with the mixture H_2O–methanol–acetonitrile–THF (61:18:12:9, v/v/v/v). In this way eleven corticosteroids were separated. The elution order was TRI, PS, CS, (PL and CL coeluted), FL, (CT and MPL co-eluted), DX, (TRA, BM co-eluted), HP, FLA, and DC.

Other phases very close to this point such as H_2O–methanol–THF (67:11:22, v/v/v) (middle point between C and F) and H_2O–acetonitrile–THF (71:7:22, v/v/v) (middle point between C and E), were also tried. This new phases did not improve the separation.

Table II shows the capacity factors (k') obtained for each corticosteroid with each mobile phase used.

As expected, different selectivities were obtained, which were dependent on the mobile phase involved.

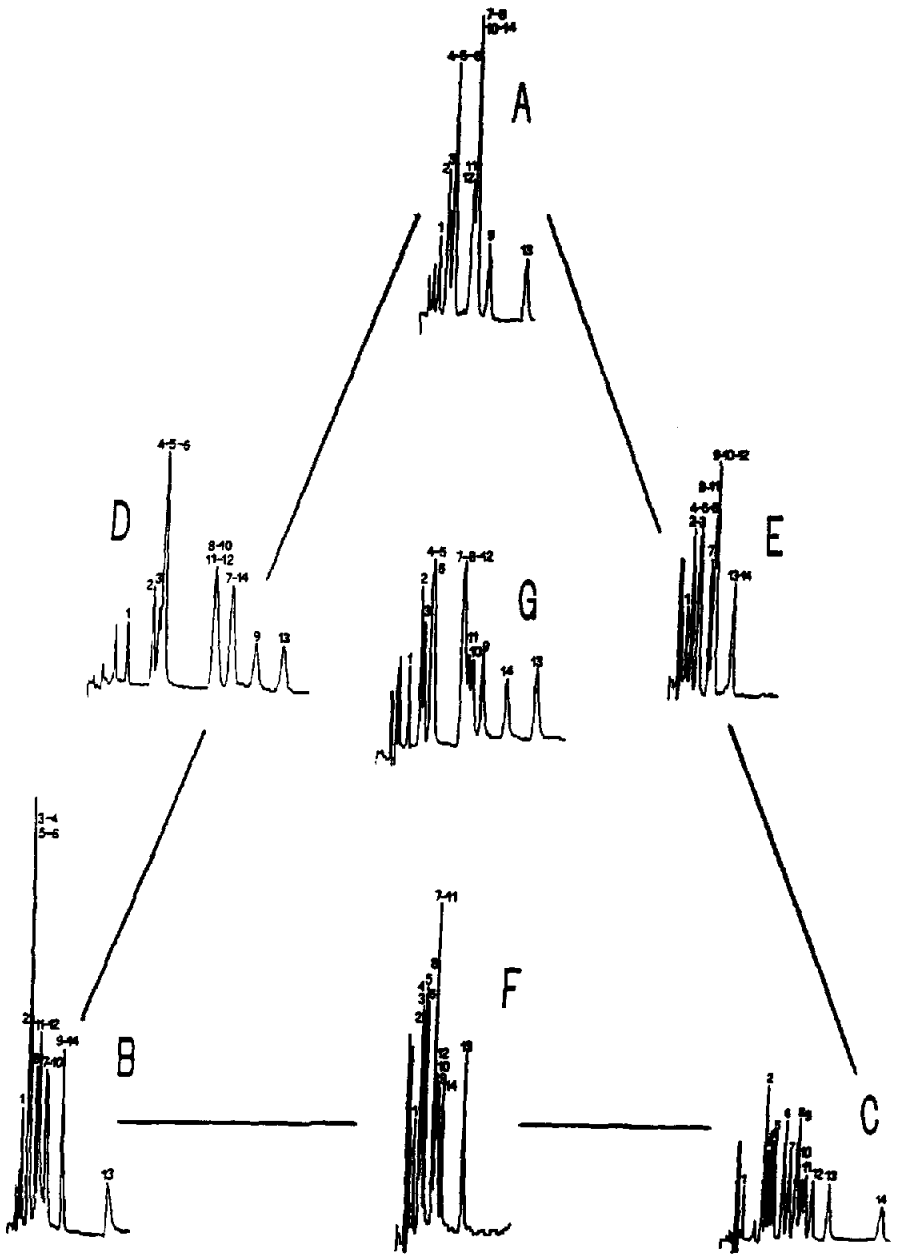


Fig. 1. HPLC of a standard mixture containing corticosteroids (10 μg/ml). Conditions: composition of mobile phases used and peak numbers as in Table II. Column: Hypersil 5-ODS (250 mm × 4.6 mm I.D.). Flow-rate: 1.0 ml/min. UV detection at 245 nm. Injection volume: 20 μl. Run time 25–30 min.

Effect of temperature

The effect of temperature on corticosteroid retention was studied in the 10–40°C range under the optimum working conditions (C mobile phase). A water bath was used to maintain the temperature below room temperature, while the columns were thermostated in a block-heated system for higher than ambient temperatures.

TABLE II

k' VALUES OF CORTICOSTEROIDS SHOWN IN TABLE I FOR DIFFERENT MOBILE PHASES

Binary phases: A, B and C; ternary phases D, E and F; quaternary phase: G (for identification see text). Column: Hypersil C_{18}, 5 μm (250 mm × 4.6 mm I.D.); flow-rate: 1.0 ml/min; temperature, 30°C.

Compound	Mobile phase						
	A	B	C	D	E	F	G
1 (TRI)	1.35	0.92	1.74	1.90	1.36	1.28	1.45
2 (PS)	2.31	1.73	2.36	3.86	1.96	1.95	2.35
3 (CS)	2.57	1.86	2.67	4.28	1.96	2.15	2.59
4 (PL)	3.07	1.86	2.96	4.62	2.16	2.30	3.22
5 (CL)	3.23	1.86	3.25	4.62	2.16	2.34	3.22
6 (FL)	3.23	1.86	4.02	4.62	2.16	2.75	3.36
7 (CT)	5.99	4.30	4.59	8.65	4.46	4.27	5.64
8 (MPL)	5.99	2.92	5.12	10.05	4.92	3.90	5.64
9 (HP)	7.67	6.68	5.12	11.97	5.20	5.23	7.11
10 (TRA)	5.99	4.52	5.53	10.05	5.20	4.68	6.34
11 (BM)	5.45	3.27	5.92	10.05	4.92	4.27	6.34
12 (DM)	5.45	3.39	6.52	10.05	5.20	4.49	6.03
13 (DC)	12.41	13.07	8.46	14.16	7.75	8.17	11.31
14 (FLA)	5.99	6.68	13.06	11.97	7.75	8.27	8.98

TABLE III

INFLUENCE OF TEMPERATURE ON k' VALUES OF CORTICOSTEROIDS SHOWN IN TABLE I

Mobile phase: H_2O–THF (72:28, v/v); column: Hypersil C_{18}, 5 μm (250 mm × 4.6 mm I.D.); flow-rate: 1.0 ml/min.

Compound	Temperature			
	10°C	20°C	30°C	40°C
1 (TRI)	3.36	2.27	1.74	1.68
2 (PS)	4.52	3.08	2.36	2.27
3 (CS)	5.18	3.51	2.67	2.59
4 (PL)	6.35	4.07	2.96	2.85
5 (CL)	6.73	4.40	3.25	3.16
6 (FL)	8.85	5.60	4.02	3.89
7 (CT)	10.16	6.42	4.59	4.45
8 (MPL)	11.83	7.54	5.12	5.02
9 (HP)	12.52	7.54	5.12	5.02
10 (TRA)	13.64	8.33	5.53	5.23
11 (BM)	14.24	8.56	5.92	5.72
12 (DM)	16.64	9.66	6.52	6.24
13 (DC)	19.58	12.08	8.46	8.13
14 (FLA)	>20	18.86	13.06	12.73

Table III shows the k' values at different temperatures. A progressive decrease in k' values and a loss of the chromatographic resolution were observed with increasing temperature. At a temperature of 10°C separation of the fourteen corticosteroids was possible with a better resolution than that observed at 30°C. However, a run time of about 80 min was necessary. Consequently, a temperature of 30°C was finally chosen as a compromise between selectivity and run time.

Effect of different reversed-phase packings

Under the optimum working conditions (C mobile phase and 30°C), a comparative study using different reversed-phase packings was carried out. Table IV shows the columns used, and the values of k' and the R.S.D. obtained for different packings, which lie in the 19.6–27.1% range. All reversed-phase packings gave similar results: thirteen corticosteroids were separated, the elution order and the retention times being very similar. However, the thirteen separated compounds

TABLE IV

INFLUENCE OF DIFFERENT REVERSED-PHASE PACKINGS ON k' VALUES OF CORTICOSTEROIDS SHOWN IN TABLE I

Columns: (i) Hypersil C_{18}, 5 μm (250 mm × 4.6 mm I.D); (ii) Nucleosil C_{18}, 5 μm (150 mm × 4.6 mm I.D); (iii) Spherex C_{18}, 5 μm (150 mm × 4.6 mm I.D); (iv) Ultracarb C_{18}, 5 μm (150 mm × 4.6 mm I.D); (v) (Hypersil C_8, 5 μm (200 mm × 4.6 mm I.D); mobile phase: H_2O–THF (72:28, v/v); flow-rate: 1.0 ml/min; temperature, 30°C.

Compound	Reversed-phase packing					
	i	ii	iii	iv	v	R.S.D. (%)
1 (TRI)	1.74	1.20	2.24	2.30	1.44	27.1
2 (PS)	2.36	3.37	2.62	2.65	1.93	20.3
3 (CS)	2.67	3.83	2.62	3.00	2.15	21.9
4 (PL)	2.96	4.07	2.62	3.13	2.45	20.8
5 (CL)	3.25	4.51	2.97	3.51	2.63	21.2
6 (FL)	4.02	5.55	3.69	4.31	3.18	21.4
7 (CT)	4.59	6.27	4.12	4.87	3.60	21.5
8 (MPL)	5.12	6.71	4.28	5.21	4.25	19.6
9 (HP)	5.12	7.42	4.87	5.74	4.25	22.1
10 (TRA)	5.53	7.95	5.26	6.16	4.51	22.1
11 (BM)	5.92	7.95	5.26	6.68	4.69	20.9
12 (DM)	6.52	8.60	5.67	8.75	5.12	24.1
13 (DC)	8.46	11.47	7.84	8.75	5.99	23.2
14 (FLA)	13.06	17.56	12.30	13.73	9.15	23.0

varied: MPL and HP co-eluts using columns (i) or (v); TRA and BM co-eluts using columns (ii) and (iii); and DM and DC coeluts using column (iv).

Calibration graphs

Standards containing mixtures of the corticosteroids mentioned in Table I and indapamide (IP) as internal standard (I.S.) were prepared at five different concentrations in the range 0.2–8.0 μg/ml using 4.0 μg/ml IP. These solutions were analysed with a mobile phase composed of H_2O–THF (72:28, v/v), a flow-rate of 1.0 ml/min, and UV–DAD detection at 245 nm. The results were analysed by linear regression. Plotting each corticosteroid peak area to IP (I.S.) ratio (PAR) *versus* the concentration (x) of each corticosteroids, the calibration equations, $PAR = A + Bx$ (μg/ml), were obtained. In Table V the parameters A (intercept), B (slope) and r (regression coefficient) are shown. In all cases the intercepts were not significantly different from zero.

Precision and accuracy

The precision was examined by analysing ten different samples of corticosteroids containing 5 μg/ml each by means of the calibration graphs. The R.S.D. for each corticosteroid is shown in Table V.

The accuracy was assessed in the 2–20 μg/ml range for each corticosteroid. The Barlett and Harley test [21] was applied to the results to corroborate the randomness of the variances. A linear regression analysis on the values obtained for known concentrations was carried out and the corresponding calculated values obtained. The t-test was applied to the results and the value of the intercept was obtained. This study confirmed that the present chromatographic method does not present a systematic error (*i.e.* it has a slope value equal to unity) and does not require a blank correction (*i.e.* it has an intercept equal to zero).

TABLE V

LINEAR REGRESSION EQUATIONS (PAR = $A + Bx$) OF CORTICOSTEROIDS SHOWN IN TABLE I

PAR is the peak area ratio of corticosteroids to IP(I.S.) = 4 μg/ml; x = μg/ml corticosteroids; r = correlation coefficient.

Compound	Linear regression parameters				
	A	B	r	DL (ng)[a]	R.S.D. (%)
1 (TRI)	−0.036	0.139	0.9990	0.14	3.4
2 (PS)	0.022	0.195	0.9990	0.10	0.8
3 (CS)	0.105	0.121	0.9990	0.13	2.9
4 (PL)	−0.044	0.130	0.9995	0.12	2.2
5 (CL)	−0.042	0.172	0.9999	0.10	3.8
6 (FL)	−0.026	0.160	0.9990	0.10	2.7
7 (CT)	−0.026	0.171	0.9990	0.10	4.8
8 (MPL)	−0.035	0.122	0.9990	0.13	3.0
9 (HP)	−0.099	0.215	0.9990	0.08	3.8
10 (TRA)	−0.010	0.137	0.9996	0.02	3.1
11 (BM)	−0.030	0.128	0.9990	0.12	4.1
12 (DM)	−0.070	0.166	0.9997	0.10	3.1
13 (DC)	−0.060	0.151	0.9990	0.10	4.9
14 (FLA)	0.105	0.118	0.9990	0.13	1.0

[a] DL = detection limit.

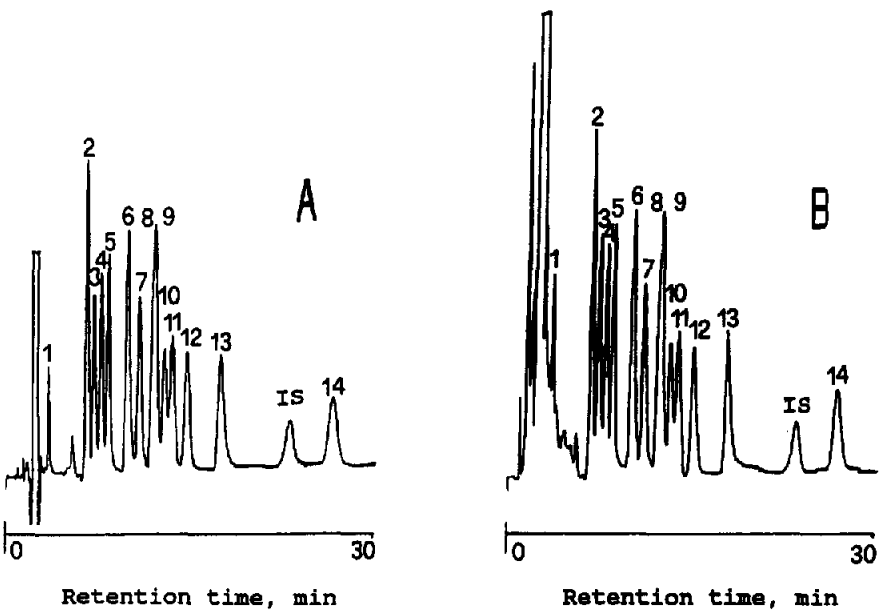


Fig. 2. HPLC of a mixture of corticosteroids. (A) Standard mixture of corticosteroids (4 μg/ml) containing IP (5 μg/ml) as I.S. (B) Urine sample spiked with a mixture of standard corticosteroids (0.33 μg/ml) and IP (0.33 μg/ml) with subsequent extraction. Mobile phase: water–THF (72:28, v/v). Other conditions as in Fig. 1.

Detection limits

The detection limits (DLs) for each corticosteroid were assessed for a signal-to-noise ratio (S/N) = 3 by means of the calibration graphs (Table V).

Spiked urine samples analysis

In Fig. 2 typical chromatograms obtained from a human urine sample spiked with corticosteroids with subsequent liquid–liquid extraction and a solution containing standards of corticosteroids are shown.

CONCLUSIONS

The optimization method reported by Snyder *et al.* [19] was applied to the separation of fourteen corticosteroids. Thirteen out of fourteen of them were separated in 26 min using a H_2O–THF isocratic binary mobile phase and a Hypersil 5-ODS (250 mm × 4.6 mm I.D.) column (30°C). Under optimum mobile phase conditions different selectivities were obtained using different reversed-phase packings and different temperatures. This separation was applied to spiked urine samples and showed the possibility for solving different problems related to the separation and determination of these corticosteroids in biological fluids or other materials, with good analytical performances.

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