Pergamon

Journal of Pharmaceutical & Biomedical Analysis, Vol. 12, No. 6, pp. 805–810, 1994

0731-7085/94 $7.00 + 0.00

0731-7085(93)E0029-M

General high-performance liquid chromatographic procedures for the rapid screening of natural and synthetic corticosteroids

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Abstract: A study of a multiple high-performance liquid chromatographic procedure for the separation of 30 different corticosteroids is described. Normal- and reversed-phase general methods operating in a wide polarity range have been developed for the rapid screening of multicomponent mixtures. The complementary application of both normal- and reversed-phase methods could permit clarification of uncertainty deriving from analyses performed by only one method.

Keywords: *Natural and synthetic corticosteroids; reversed-phase HPLC; normal-phase HPLC; corticosteroid mixtures screening.*

Introduction

Natural and synthetic corticosteroids are widely used in the treatment of inflammatory conditions and many pharmaceutical preparations are available in the market. Consequently a large number of HPLC methods have been developed for their detection and assay in biological fluids or in dosage forms. Some of these methods have been used only for a single product [1–9]. Others have been applied to the analysis of solely natural corticosteroid mixtures [10–15]. For mixtures of natural and synthetic cortisteroids, only a few methods have been reported [16, 17].

Because of the large number of therapeutically used corticosteroids and their different physico-chemical properties, it was thought useful to develop HPLC systems suitable for the separation of steroids in a wide polarity range. Studies reported by Tymes [18] on a large number of corticosteroids refer only to retention data obtained separately from each steroid. Therefore, the present study was initiated on complex multicomponent mixtures with the objective of developing general chromatographic procedures for rapid screening.

The methods enable the most suitable procedure for the analysis of each steroid to be chosen and also permit related substances to be identified and evaluated in mixtures. Both normal- and reversed-phase HPLC general methods are reported. The proposed screening methods or the complementary application of some of them, could also be applied for the resolution of components of pharmaceutical preparations as well as for the identification of related foreign compounds. Finally, the described procedures could also be applied to provide valuable information about the presence of undeclared corticosteroids in commercial preparations.

Experimental

Materials

The corticosteroids investigated are listed in Table 1. These were obtained from the European Pharmacopoeia Commission Secretariat (Strasbourg, France), from Merck (Darmstadt, Germany) and from Fluka (Buchs, Switzerland), and were of pharmaceutical grade.

HPLC-grade solvents were: acetonitrile obtained from Merck (Darmstadt, Germany), chloroform (amylene stabilized) from C. Erba

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Table 1
Tested corticosteroids

No.	Chemical name	No.	Chemical name
1	Beclomethasone 17,21-dipropionate	16	9α-Fluoroprednisolone 21-acetate
2	Betamethasone	17	Hydrocortisone
3	Betamethasone 21-acetate	18	Hydrocortisone 21-acetate
4	Betamethasone 17,21-dipropionate	19	Hydrocortisone 21-hemisuccinate
5	Betamethasone 21-disodium phosphate	20	6α-Methylprednisolone
6	Betamethasone 17-valerate	21	6α-Methylprednisolone 21-acetate
7	Cortisone	22	6α-Methylprednisolone 21-sodium succinate
8	Cortisone 21-acetate	23	Prednisolone
9	11-Deoxycorticosterone 21-acetate	24	Prednisolone 21-acetate
10	Dexamethasone	25	Prednisolone 21-disodium phosphate
11	Dexamethasone 21-acetate	26	Prednisolone 21-pivalate
12	Dexamethasone 21-disodium phoshate	27	Prednisolone 21-sodium succinate
13	Fluocinolone acetonide	28	Prednisone
14	9α-Fluorohydrocortisone 21-acetate	29	Triamcinolone
15	9α-Fluoroprednisolone	30	Triamcinolone acetonide

(Milano, Italy); methanol from J.T. Baker B.V. (Deventer, Holland); and tetrahydrofuran from Merck.

Potassium dihydrogen phosphate was obtained from C. Erba; analytical-grade hexylamine was from Fluka.

Water was filtered through a 0.45-μm Nylon-66 membrane on a Millipore Milli-Q device.

Equipment

HPLC analyses were performed using a high-pressure quaternary pump (HP 1050, Hewlett–Packard, Avondale, USA) equipped with an injection valve (model 7125, Rheodyne, Cotati, USA) (20-μl loop), a diode array detector (HP 1040M, Hewlett–Packard) and a computing integrator (HP-9000-300, Hewlett–Packard).

Chromatographic conditions

For the reversed-phase chromatography two stainless-steel columns were used: 5-μm Hypersil ODS, 250 × 4.6 mm i.d. (r_1; HPLC Technology, Cheshire, UK); 5-μm LiChrospher 100 RP-18, 250 × 4 mm i.d. (r_2; Merck, Darmstadt, Germany). The following mobile phases for the isocratic elution were developed: acetonitrile–potassium dihydrogen phosphate [pH 4.5; 0.01 M; 50:50, v/v (R_1); 35:65, v/v (R_2); and 21:79, v/v (R_3)]; and acetonitrile–potassium dihydrogen phosphate (pH 4.5; 0.05 M)–hexylamine (26:74:0.3, v/v/v; R_H).

The mobile phase selected for the linear gradient elution was 30–70% (v/v) acetonitrile in potassium dihydrogen phosphate (pH 4.5; 0.01 M) in 40 min (R_G).

For the normal-phase chromatography two stainless-steel columns were used: 7-μm LiChrosorb Si 60, 250 × 4 mm i.d. (n_1; Merck, Darmstadt, Germany); 5-μm Viosfer Si, 250 × 4 mm i.d. (n_2; Violet, Rome, Italy). The mobile phases for the isocratic elution were: chloroform–methanol–water (967.9:30:2.1, v/v/v; N_1) and chloroform–tetrahydrofuran–methanol–water (878.6:100:20:1.4, v/v/v/v; N_2).

All the measurements were made at room temperature at a flow rate of 1 ml min^{-1}; the monitoring wavelengths were 240 and 254 nm for reversed- and normal-phase, respectively.

Sample preparation

A weighed quantity of each of the 30 reported corticosteroids was dissolved in methanol at a concentration of 1 mg ml^{-1}. Different mixtures (M_R, M_H, M_N) containing 20 μg ml^{-1} of each component were prepared. Their composition is reported in Tables 2, 3 and 4, respectively.

A fixed volume of M_N mixture was evaporated and the residue dissolved in the same volume of the mobile phase. M_R and M_H mixtures were injected directly.

Results and Discussion

Reversed-phase HPLC

The results obtained under the different experimental conditions for the corticosteroid mixtures are shown in Tables 2 and 3. In

Table 2
Corticosteroids mixture (M_R). Reversed-phase HPLC. Capacity factors by different methods (R_1, R_2, R_G) and columns (r_1, r_2)

Mixture components‡	Capacity factor (K') Method* R_1 Column† r_1	r_2	R_2 Column† r_1	r_2	R_G Column† r_1	r_2
Prednisolone 21-disodium phosphate (25)	0.18	0.33	0.30	0.56	0.43	0.66
Prednisolone 21-sodium succinate (27)	0.18	0.33	0.30	0.56	0.43	0.66
Betamethasone 21-disodium phosphate (5)	0.18	0.33	0.30	0.56	0.43	0.66
Dexamethasone 21-disodium phosphate (12)	0.18	0.33	0.30	0.56	0.43	0.66
Triamcinolone (29)	0.43	0.61	0.73	0.92	1.01	1.33
Prednisolone (23)	0.43	0.61	1.43	1.82	1.85	2.55
Hydrocortisone 21-hemisuccinate (19)	0.43	0.61	1.48	1.87	1.90	2.61
Prednisone (28)	0.43	0.61	1.54	1.95	1.96	2.69
9α-Fluoroprednisolone (15)	0.43	0.61	1.54	1.95	1.96	2.69
Hydrocortisone (17)	0.43	0.61	1.54	1.95	1.96	2.69
Cortisone (7)	0.43	0.61	1.73	2.28	2.16	3.02
6α-Methylprednisolone 21-sodium succinate (22)	0.58	0.85	2.04	2.61	2.50	3.35
6α-Methylprednisolone (20)	0.58	0.85	2.52	3.10	2.86	3.84
Betamethasone (2)	0.73	0.97	2.76	3.61	3.17	4.24
Dexamethasone (10)	0.73	0.97	3.03	3.78	3.27	4.37
Triamcinolone acetonide (30)	0.93	1.28	3.92	5.12	3.86	5.24
Fluocinolone acetonide (13)	1.04	1.41	4.70	6.19	4.34	5.83
Prednisolone 21-acetate (24)	1.18	1.64	5.04	6.83	4.53	6.19
9α-Fluoroprednisolone 21-acetate (16)	1.18	1.64	5.34	7.19	4.71	6.34
Hydrocortisone 21-acetate (18)	1.29	1.83	5.51	7.49	4.78	6.53
9α-Fluorohydrocortisone 21-acetate (14)	1.29	1.83	5.98	8.12	5.02	6.79
Cortisone 21-acetate (8)	1.53	2.15	7.21	9.82	5.54	7.49
6α-Methylprednisolone 21-acetate (21)	1.71	2.42	8.81	11.55	6.05	8.07
Betamethasone 21-acetate (3)	1.71	2.42	9.48	12.24	6.26	8.25
Dexamethasone 21-acetate (11)	2.01	2.75	11.27	14.49	6.76	8.89
Betamethasone 17-valerate (6)	4.12	5.85	not eluted	not eluted	9.79	12.82
Prednisolone 21-pivalate (26)	4.36	6.10	not eluted	not eluted	9.96	12.95
11-Deoxycorticosterone 21-acetate (9)	5.59	9.35	not eluted	not eluted	10.79	15.18
Betamethasone 17,21-dipropionate (4)	7.66	10.73	not eluted	not eluted	12.25	15.95
Beclomethasone 17,21-dipropionate (1)	9.85	14.23	not eluted	not eluted	13.24	17.41

*Isocratic eluents (R_1, R_2); linear gradient eluent (R_G). See experimental part of text.
†See experimental part of text.
‡The identification numbers in parentheses are those listed in Table 1.

Table 3
Corticosteroid phosphates, succinates and corresponding alcohols mixture (M_H). Reversed-phase HPLC. Capacity factors by different methods (R_3, R_H) and columns (r_1, r_2)

Mixture components†	Capacity factor (K') Method* R_3 Column* r_1	r_2	R_H Column* r_1	r_2
Prednisolone 21-disodium phosphate (25)	1.30	1.89	2.48	3.12
Prednisolone 21-sodium succinate (27)	2.26	3.05	3.69	4.53
Betamethasone 21-disodium phosphate (5)	3.26	4.17	4.87	6.24
Dexamethasone 21-disodium phosphate (12)	3.74	4.55	5.52	7.13
6α-Methylprednisolone 21-sodium succinate (22)	>25	>25	7.26	9.29
Prednisolone (23)	>25	>25	3.01	4.21
6α-Methylprednisolone (20)	>25	>25	6.65	8.69
Betamethasone (2)	>25	>25	7.83	9.89
Dexamethasone (10)	>25	>25	8.30	10.70

*See experimental part of text.
†The identification numbers in parentheses are those listed in Table 1.

Table 4
Corticosteroids mixture (M_N). Normal phase HPLC. Capacity factors by different columns (n_1, n_2)

Mixture components†	Capacity factor (K'), N_1 method Column*	
	n_1	n_2
11-Deoxycorticosterone 21-acetate (9)	0.03	0.05
Beclomethasone 17,21-dipropionate (1)	0.15	0.15
Betamethasone 17,21-dipropionate (4)	0.15	0.15
Cortisone 21-acetate (8)	0.72	0.60
Prednisolone 21-pivalate (26)	0.80	0.65
Dexamethasone 21-acetate (11)	1.03	0.85
Hydrocortisone 21-acetate (18)	1.03	0.97
9α-Fluorohydrocortisone 21-acetate (14)	1.19	1.17
6α-Methylprednisolone 21-acetate (21)	1.19	1.17
Betamethasone 17-valerate (6)	1.39	1.27
Betamethasone 21-acetate (3)	1.49	1.34
Prednisolone 21-acetate (24)	1.49	1.34
9α-Fluoroprednisolone 21-acetate (16)	1.75	1.41
Triamcinolone acetonide (30)	2.50	2.06
Fluocinolone acetonide (13)	2.94	2.45
Cortisone (7)	3.16	2.66
Prednisone (28)	4.08	3.14
Hydrocortisone (17)	8.58	6.58
Dexamethasone (10)	9.79	7.64
Betamethasone (2)	11.87	8.18
6α-Methylprednisolone (20)	11.87	8.18
Prednisolone (23)	12.95	8.90
9α-Fluoroprednisolone (15)	17.34	11.20
Triamcinolone (29)	not eluted	not eluted
Hydrocortisone 21-hemisuccinate (19)	not eluted	not eluted

*See experimental part of text.
†The identification numbers in parentheses are those listed in Table 1.

particular, capacity factors for all the mixture components are reported. The analyses were performed on two differnt columns (r_1, r_2) in order to verify the reproducibility of the results. The separation pattern was similar for both the columns although some negligible differences in the resolution factors were found.

In order to achieve a good resolution of compounds included in a wide polarity range, different ratios of the mobile phase components (acetonitrile–potassium dihydrogen phosphate) were chosen. In particular, three different systems (R_1, R_2, R_3) at decreasing eluting strength were used.

As shown in Table 2, the isocratic R_1 eluent permitted a good resolution of rather long chain esters (C_5) and diesters as betamethasone 17-valerate (No. 6), prednisolone 21-pivalate (No. 26), betamethasone 17,21-dipropionate (No. 4) and beclomethasone 17,21-dipropionate (No. 1) as well as 11-deoxycorticosterone 21-acetate (No. 9). On the other hand, the strength of this mobile phase did not allow satisfactory separation of corticosteroid phosphate esters or of succinates, alcohols, acetonides and short chain monoesters (acetates), which presented short retention times, up to about 7 min. Consequently the R_1 eluent permitted only a discriminating qualitative analysis of these products. An improvement of the separation was obtained with the isocratic R_2 eluent (Table 2) which, owing to its lower eluting power gave good results for many of the products. On the other hand, this eluent was not suitable for some of the examined products. In particular, the phosphates (No. 5, 12, 25) and one succinate (No. 27) were eluted in a too short time (<3 min); three alcohols (No. 28, 15, 17) were again unresolved and the compounds well resolved with the R_1 mobile phase (No. 6, 26, 4, 1, 9) showed too long retention times (>90 min). However, the linear gradient eluent (R_G) permitted the decrease of the retention time of the latter products (No. 6, 26, 4, 1, 9; Table 2).

Satisfactory separation of the phosphates

(No. 5, 12, 25) and the succinate (No. 27), unresolved by the described systems, was obtained by decreasing the eluting strength of the isocratic mobile phase (R_3 eluent; Table 3). Moreover, the addition of hexylamine to the eluent [19] permitted the simultaneous separation of the corresponding alcohols, which otherwise took too long to elute (>50 min; Table 3). This effect can be ascribed to the formation of ion pairs between hexylammonium and phosphate or succinate groups resulting in a decrease in polarity of the esters.

Finally, resolution of the co-eluting alcohols (No. 28, 15, 17) was achieved using normal-phase systems, as described below.

Normal-phase HPLC

As for the described reversed-phase methods, the developed normal-phase procedures were suitable for general application, with the obvious exception of the very polar compounds (phosphates, succinates, triamcinolone and hydrocortisone 21-hemisuccinate).

The analyses were performed on two different columns (n_1 and n_2) in order to verify the reproducibility of the results. As for the reversed-phase methods, the separation pattern was generally similar for both the columns (Table 4).

A satisfactory separation of the components of the mixture was obtained with a mobile phase (N_1) comprising an eluent system that had been previously applied to some acetates and their corresponding alcohols; this eluent was chloroform–methanol–water (946.5: 50.0:3.5, v/v/v) [20], whose eluting power has been reduced to improve the resolution of the multicomponent mixture. In fact, whereas the previous eluent system [20] was suitable only for a restricted number of substances, the N_1 mobile phase permitted the resolution of a larger number of the components and in particular of the three alcohols (Nos 28, 15, 17) unresolved by the reversed-phase methods (Table 4). The solvents ratio of the N_1 eluent in the adopted experimental conditions was such as to guarantee satisfactory stability of the mobile phase. In fact, only when the water content in the mobile phase was not more than 7% of methanol did no phase separation occur in the time.

In order to obtain a still more stable eluent, a further modification in its composition was performed by means of the addition of tetrahydrofuran which is miscible with water and simple or chlorinated hydrocarbons, without any noticeable modification of the chromatographic behaviour. In fact, the latter mobile phase (N_2) caused only negligible variations in selectivity (inverted elution order of betamethasone–hydrocortisone) and resolution power (unsatisfactory resolution of betamethasone and dexamethasone).

Conclusions

An extensive study of a multiple chromatographic procedure was conducted. Different HPLC methods which can be generally applied to the analysis of the most widely used corticosteroids were developed. Thirty corticosteroids which can be found in pharmaceutical preparations either as active principles and/or related impurities were tested. A reversed-phase linear gradient technique was the most suitable method for analysing many corticosteroids with quite different polarity features. Other reversed and normal methods were developed and were successfully applied to those corticosteroids unresolved by the linear gradient system.

Acknowledgements — The authors are grateful to Professor G. Cavina for his advice and assistance during this study. We thank also Mr R. Alimenti for his contribution to the experimental work.

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[*Received for review 14 June 1993; revised manuscript received 1 November 1993*]