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SEPARATION OF CORTICOSTEROIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON DYNAMICALLY MODIFIED SILICA

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SUMMARY

High-performance liquid chromatographic methods have been elaborated for the separation of 12 different corticosteroids by reversed-phase chromatography on bare silica, dynamically modified by cetyltrimethylammonium bromide added to the eluent. The selectivity of the systems towards a mixture of the 12 corticosteroids and towards a mixture of hydrocortisone and its related impurities were investigated using different brands of silica. The variations in selectivity were found to be substantially smaller than those of chromatographic systems based on chemically bonded ODS-silicas from the same sources.

INTRODUCTION

The use of corticosteroids in the form of the steroid hormones as well as their synthetic analogues is widespread in the treatment of various deseases. Preparations both for topical application and for systemic treatment are used. The inclusion of monographs on these drug substances in pharmacopoeias has increased during the past two decades. In such monographs chromatographic methods are widely used for identification purposes as well as in purity testing for related foreign steroids. Until now thin-layer chromatography has been the predominant method[1,2] but the use of high-performance liquid chromatography (HPLC) is increasing[3].

The separation of corticosteroids by HPLC was achieved soon after the introduction of this technique and both the straight-phase and the reversed-phase modes were utilized (*e.g.* refs. 4 and 5). The popular column materials for reversed-phase chromatography, which consist of chemically derivatized silica with a coating of apolar carbon chains, suffer for pharmacopoeial purposes from limited possibilities of standardization of the separation methods. Several examples have been published to show that great variabilities in selectivity towards a given test mixture may occur between brands of materials that are claimed to be of the same type (*e.g.* refs. 6–8). Within the corticosteroid field triamcinolone acetonide has been reported as an example[9].

Recently, an alternative approach to performing reversed-phase chromato-

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graphy has been introduced. It is based on the use of bare silica, which is dynamically modified by the addition to the eluent of long-chain quaternary ammonium compounds (*e.g.* ref. 10). When using this approach it has been shown that only minor brand-to-brand variations in selectivity occur[11,12].

The aim of the present work was to investigate the influence of brand-to-brand variations in selectivity on the possibilities of standardizing HPLC methods for the separation of corticosteroids by reversed-phase chromatography using dynamically modified silica and bonded-phase materials, respectively.

EXPERIMENTAL

Chemicals

Corticosteroids were of pharmacopoeial quality except corticosterone, which was obtained from Sigma (St. Louis, MO, U.S.A.). Acetonitrile of HPLC S grade and tetrahydrofuran of HPLC grade were from Rathburn (Walkerburn, U.K.). All other reagents were of analytical grade from E. Merck (Darmstadt, F.R.G.).

Chromatography

A liquid chromatograph consisting of a Kontron Model 410 LC pump, a Pye-Unicam LC UV spectrophotometer detector operated at 254 nm, and a Rheodyne Model 7125 injection valve was used. Chromatograms were recorded on a Kipp & Zonen Model BD-8 recorder and retention data were measured by means of a Hewlett-Packard Model 3353 A laboratory data system.

All experiments were performed on 120 × 4.6 mm I.D. columns from Knauer (Berlin, F.R.G.), packed by the dilute slurry technique with silica or chemically bonded octadecylsilyl (ODS) silica (*cf.* Table II). For chromatography on dynamically modified silica the eluents were mixtures of methanol, acetonitrile or tetrahydrofuran and water with the addition of 2.5 m*M* of cetyltrimethylammonium (CTMA) bromide and 5% (v/v) of a 0.2 *M* phosphate buffer (pH 7.0). The pH value stated is that measured in the buffer before dilution in the final eluent. The buffer was prepared from potassium dihydrogen phosphate by titration to pH 7.0 with 5 *M* potassium hydroxide, followed by dilution to the final concentration of 0.2 *M*. During chromatography the columns were protected by a silica pre-column situated between the pump and the injection valve to saturate the eluent. The chromatographic system was equilibrated by elution overnight. Following each chromatographic testing the column was brought to its initial status by eluting with methanol–0.05 *M* nitric acid (1:1) and finally with methanol. For bonded-phase chromatography the eluents were mixtures of methanol, acetonitrile or tetrahydrofuran and water.

For identification purposes 10 μl of 0.05% solutions were injected and for purity testing 20 μl of 0.1% solutions. The solvent was in each case the eluent without any buffer or CTMA.

RESULTS AND DISCUSSION

The corticosteroids investigated, twelve in all, represent the majority of those described in the European Pharmacopoeia[2] plus the genuine hormonal steroid corticosterone. For identification purposes it was attempted to devise a single isocratic

system which was able to separate all the steroids. As a starting point was used an eluent consisting of methanol–water–0.2 *M* potassium phosphate buffer (pH 7.0) (50:45:5) with the addition of 2.5 m*M* of CTMA bromide. With it a mean retention (capacity factor k') for the twelve corticosteroids of 4.0 was achieved, but several of the solutes were not separated from each other.

In an attempt to improve the separation, acetonitrile or tetrahydrofuran was substituted for methanol as the organic solvent in the eluent. It was found that 32% of acetonitrile or 26% of tetrahydrofuran was needed to obtain eluents that exhibited approximately the same elution strength towards the mixture of corticosteroids as did the 50% methanol (mean retentions of 4.0 and 4.5, respectively, were found). However, none of the three systems was sufficiently effective.

It was further investigated whether the use of a mixture of two organic solvents might improve the separation. According to the optimization procedure described previously by Schoenmakers *et al.*[13] for reversed-phase chromatography on bonded-phase materials, and which has also proven useful in HPLC on dynamically modified silica[14], calculations for the three possible combinations showed a mixture of 25% of methanol and 13% of tetrahydrofuran to be the best choice. Table I lists the retention data for eluents each containing one of the three organic solvents and for the one containing a mixture of methanol and tetrahydrofuran. It appears from the table that for the separation of certain corticosteroid pairs, the superior eluents may differ from the optimized mixture chosen to exhibit the best overall separation. If hydrocortisone or predniolone, for example, are to be separated from their acetate

TABLE I

RETENTION OF TWELVE CORTICOSTEROIDS WHEN CHROMATOGRAPHED ON DYNAMICALLY MODIFIED SILICA WITH VARIOUS ORGANIC MODIFIERS IN THE ELUENTS

Column, LiChrosorb Si 60, 120 × 4.6 mm I.D.; eluents, 2.5 m*M* CTMA and 5% of phosphate buffer (pH 7.0), organic modifier as specified.

Solute	k'			
	50% Methanol	*32% Acetonitrile*	*26% Tetrahydrofuran*	*25% Methanol plus 13% tetrahydrofuran*
1 Cortisone acetate	2.32	3.25	4.51	3.19
2 Hydrocortisone	2.39	2.07	2.49	2.68
3 Hydrocortisone acetate	2.46	2.99	4.55	3.40
4 Prednisolone	2.46	2.08	2.29	2.60
5 Prednisolone acetate	2.52	2.92	3.99	3.28
6 Corticosterone	4.13	4.22	3.99	4.58
7 Triamcinolone acetonide	4.19	3.59	3.52	4.05
8 Methylprednisolone	4.30	3.58	3.66	4.42
9 Betamethasone	4.90	3.44	3.83	4.65
10 Fluocinolone acetonide	5.73	4.12	6.29	6.55
11 Dexamethasone	6.19	4.12	4.65	5.99
12 Desoxicortone acetate	6.52	11.54	10.16	8.18
Mean retention	4.01	3.99	4.49	4.46

esters the eluent containing 26% of tetrahydrofuran is the most suitable.

For purity testing a sample of hydrocortisone was investigated and it was found that the eluent containing 50% of methanol was the most suitable. This eluent gave rise to a better separation of impurities from the main peak than the above mentioned with a mixture of organic solvents.

In order to compare the two separation systems described above with a system utilizing bonded-phase materials a corresponding procedure was followed to elaborate a separation method using ODS-silica. Three isoeluotropic eluents were found to contain 60% of methanol, 37% of acetonitrile or 34% of tetrahydrofuran, respectively. Using the optimization calculations the eluent containing 37% of acetonitrile was found to be the best choice for the separation of the twelve corticosteroids. Also in this case methanol as the organic solvent was the most suitable for the purity testing of hydrocortisone. Fig. 1 shows chromatograms of an aged sample of hydrocortisone on a dynamically modified silica column and on an ODS-silica column.

The two chromatographic systems for separating the corticosteroids were tested on eight different silica materials and on six different ODS-silicas. For each of the individual chromatographic systems the separation factors between hydrocortisone and each of the remaining eleven solutes were calculated and the results are shown in Table II. It appears from the table that the variations in selectivity observed in the chromatographic system based on dynamically modified silica are substantially smaller than those in the system based on ODS-silica. The relative standard deviation (R.S.D.) for the eleven sets of separation factors range between 1.0 and 4.6 for the first system and between 2.4 and 18.6 for the latter.

Similarly, the two systems for purity testing of hydrocortisone were tested on

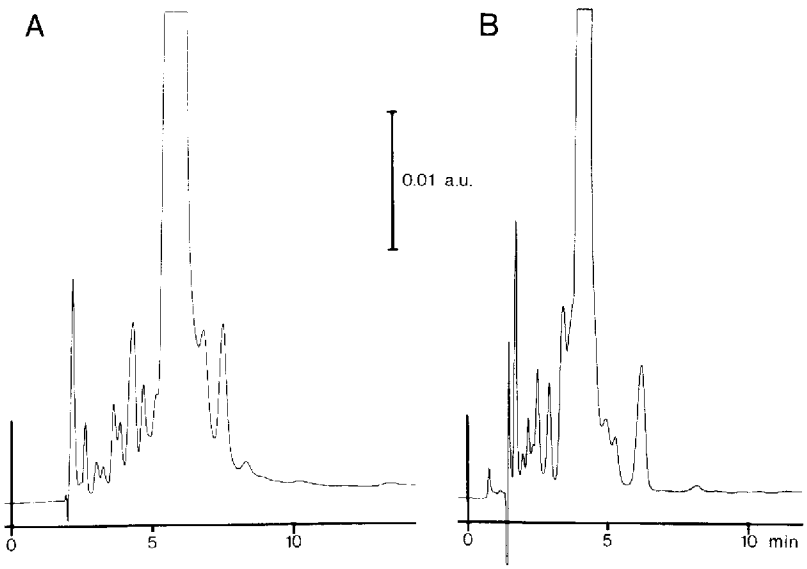

Fig. 1. Separation of impurities in hydrocortisone on dynamically modified silica (A) and on ODS-silica (B). Columns: (A) LiChrosorb Si 60 120 × 4.6 mm I.D.; (B) LiChrosorb RP-18 120 × 4.6 mm I.D. Eluents: (A) methanol–water–0.2 *M* phosphate buffer (pH 7.0) (50:45:5) containing 2.5 m*M* CTMA; (B) methanol–water (6:4). Flow-rate, 1 ml/min; detection, UV 254 nm.

TABLE II

SEPARATION FACTORS BETWEEN HYDROCORTISONE AND ELEVEN OTHER CORTICOSTEROIDS MEASURED ON EIGHT DIFFERENT SILICA COLUMNS AND SIX DIFFERENT ODS-SILICA COLUMNS

Eluents: for silica, methanol–tetrahydrofuran–water–0.2 *M* phosphate buffer (pH 7.0) (25:13:57:5) containing 2.5 m*M* CTMA; for ODS-silica, acetonitrile–water (37:63). The numbers in the column headings refer to solutes as in Table I.

	Separation factor from hydrocortisone										
	1	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
Silica											
LiChrosorb Si 60	1.21	1.29	0.97	1.24	1.69	1.49	1.65	1.71	2.39	2.15	3.07
Nucleosil 50-5	1.20	1.28	0.96	1.22	1.69	1.46	1.61	1.67	2.33	2.06	3.06
Zorbax SIL	1.30	1.39	0.96	1.31	1.67	1.55	1.59	1.70	2.46	2.14	3.30
Partisil 5	1.17	1.24	0.94	1.18	1.71	1.44	1.58	1.65	2.34	2.10	3.00
Spherisorb S5 W	1.33	1.42	0.97	1.37	1.63	1.50	1.59	1.63	2.42	2.05	3.30
LiChrosorb Si 100	1.25	1.35	0.97	1.27	1.60	1.43	1.62	1.66	2.29	1.98	3.06
Nucleosil 100-5	1.22	1.31	0.96	1.27	1.61	1.40	1.57	1.57	2.21	1.87	3.00
Hypersil	1.26	1.35	0.96	1.30	1.60	1.41	1.58	1.61	2.28	1.94	3.06
ODS-Silica											
LiChrosorb RP-18	5.07	3.90	0.91	3.52	2.40	2.62	1.57	1.85	3.21	1.95	27.12
Nucleosil 5 C_{18}	5.07	3.93	0.91	3.58	2.41	2.68	1.54	1.80	3.25	1.89	27.85
Hypersil ODS	4.61	3.59	0.93	3.33	2.35	2.50	1.57	1.74	2.94	1.86	24.45
Zorbax ODS	3.88	3.24	0.95	2.87	3.04	2.10	1.60	1.45	2.07	1.49	25.27
Partisil ODS-3	5.02	3.88	0.97	3.71	2.35	2.83	1.55	1.81	3.25	1.89	26.34
Spherisorb S5 ODS	3.85	3.06	0.92	2.88	3.54	2.33	1.49	1.82	2.59	1.66	23.69

TABLE III

SEPARATION FACTORS BETWEEN HYDROCORTISONE AND IMPURITIES MEASURED ON EIGHT DIFFERENT SILICA COLUMNS AND DIFFERENT ODS-SILICA COLUMNS

Eluents: for silica, methanol–water–0.2 *M* phosphate buffer (pH 7.0) (50:45:5) containing 2.5 m*M* CTMA; for ODS-silica, methanol–water (60:40). The numbers in the column headings refer to arbitrarily numbered impurity peaks.

	*Separation factor from hydrocortisone**									
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Silica										
LiChrosorb Si 60	0.10	0.22	0.33	0.39	0.49	0.55	0.67	0.76	1.33	1.51
Nucleosil 50-5	0.08	0.21	0.32	0.38	0.48	0.54	0.65	0.76	1.36	1.50
Zorbax SIL	0.10	0.22	0.33	0.40	0.49	0.56	0.66	0.77		1.52
Partisil 5	0.09	0.22	0.32	0.39	0.49	0.55	0.66	0.76	1.35	1.51
Spherisorb S5 W	0.11	0.23		0.41	0.52	0.57	0.71	0.84	1.37	1.56
LiChrosorb Si 100	0.13	0.25		0.42		0.56		0.80		1.52
Nucleosil 100-5	0.11	0.24	0.33	0.42	0.51	0.55	0.69	0.80	1.31	1.53
Hypersil	0.13	0.23		0.42		0.54	0.72		1.32	1.54
ODS-Silica										
LiChrosorb RP-18	0.17	0.34	0.46	0.61		0.79	1.38	1.46	1.81	
Nucleosil 5 C_{18}	0.17	0.33	0.46	0.61	0.72	0.81	1.40	1.47	1.85	
Hypersil ODS	0.12	0.29	0.39	0.52	0.74				1.62	
Zorbax ODS	0.12	0.29	0.41	0.57	0.69			1.62	2.27	
Partisil ODS-3	0.16	0.32	0.45	0.61	0.69			1.43	1.92	
Spherisorb S5 ODS	0.19	0.33	0.50	0.67			1.45	1.63	2.05	

* Values omitted imply that the corresponding peak coincided with the main peak or with closely adjacent peaks from other impurities due to a less effecient column and/or a less retentive column.

the same column materials as mentioned above (Table III). In this case the separation factors between hydrocortisone and each of the impurities were calculated. When disregarding peak No. 1 in the two systems, which is close to the solvent front and thus less well-defined, the sets of separation factors exhibit R.S.D.s ranging between 1.3 and 5.6 for the dynamically modified silica system and between 3.5 and 11.6 for the ODS-silica based system.

Although it is obvious that the variations in selectivity are substantially smaller for the dynamically modified silica system than for one based on a chemically bonded phase, the differences are not quite as large as found in a previous investigation[12]. The smaller variations for the bonded-phase system observed in this case are probably due to the fact that the compounds investigated are very closely related in structure and thus differences in the chromatographic conditions will to a large extent affect all the solutes in a similar way.

CONCLUSION

HPLC methods based on the use of dynamically modified silica have been elaborated for the separation of twelve different corticosteroids and for the separation of related impurities from one of the corticosteroids, hydrocortisone. A comparison was made with corresponding methods based on the use of chemically bonded ODS-silica materials. It was found that the variations in selectivity of the dynamically modified silica systems were substantially smaller than those of the bonded-phase systems.

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