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SELECTIVITY OF REVERSED-PHASE PACKING MATERIALS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF CEPHALOSPORINS

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SUMMARY

The high-performance liquid chromatographic methods used in recent years for the separation of cephalosporins are briefly discussed. Results obtained by chromatography of sixteen cephalosporins on eight brands of C_8 and C_{18} packing materials are reported. Important differences in selectivity are observed between manufacturers, but also between batches from the same manufacturer.

INTRODUCTION

During the last decade a considerable number of papers dealing with high-performance liquid chromatography (HPLC) of cephalosporins has been published. Earlier work has been reviewed[1,2]. Most of the papers published since 1975 describe the determination of cephalosporins in biological samples, *viz.* cefatrizine[3,4], cefaloglycin[5], cefaclor[6–10], cefalexin[11–16], cefradine[8], cefamandole[17,18], cefalotin[18–21], cefoxitin[18,22–24], cefaloridine[25], cefapirin[24], cefazolin[18,20,26], cefuroxime[15,18, 27–30], cefotaxime[15,18,24,31–36], ceftizoxime[37], cefoperazone[18,38], moxalactam[39–43], cefroxadin[15], cefsulodin[15,44,45], cefotiam[15,44] and cefmenoxime[44,46]. A few of these papers briefly discuss the selectivity of the proposed method for related cephalosporins or for other drugs which may be present in the biological sample, but most do not provide such information, their main concern being the separation of the peak of interest from the peaks of biological origin. Several papers, discussing the stability of cephalosporins in solution, do not give information on the selectivity of the HPLC systems used[47–50]. This is not true for the publications treating the isolation of cephalosporin C from fermentation broth, where interference with other metabolites was checked[51–54]. Most information on the selectivity of HPLC systems for cephalosporins is found in papers reporting on the separation of cephalosporin isomers or mixtures and on the assay or purity control. These systems will be reviewed briefly in the discussion. Usually a particular brand of reversed-phase material is used with a particular mobile phase, which makes it difficult to decide whether differences in selectivity are due to changes in mobile phases or in packing materials. When the literature reports results obtained with C_2, C_8 or C_{18} reversed-phase materials from

different manufacturers and/or batches, and with different mobile phases, one is often tempted to believe that differences in selectivity are mainly caused by the mobile phase. This misconception has already been refuted by studies on HPLC of steroids[55], polycyclic aromatic hydrocarbons[56,57], and benzo[a]pyrene diolepoxidenucleic acid adducts[58], where different columns of the same type were used with the same mobile phase. In this paper we report on the selectivity of C_8 and C_{18} packing materials, used for HPLC of a mixture of sixteen cephalosporins. Results obtained with three batches of C_8 material from the same manufacturer are also reported.
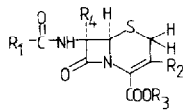
EXPERIMENTAL

Drug samples

The following samples of current production were used: cefatrizine propyleneglycolate, cefadroxil monohydrate, sodium cefapirin (Bristol-Myers, Syracuse, NY, U.S.A.), cefradine anhydrate (Gist Brocades, Delft, The Netherlands), cefazolin (Fujisawa Pharmaceutical, Osaka, Japan), sodium cefuroxime, cefaloridine betaine δ-form (Glaxo, Greenford, U.K.), sodium cefotaxime (Hoechst, Frankfurt, F.R.G.), sodium cefalotin, lithium cefamandole, sodium cefamandole nafate, cefaloglycin dihydrate, cefaclor monohydrate (E. Lilly, Indianapolis, IN, U.S.A.), sodium cefoxitin (Merck, Rahway, NJ, U.S.A.) and monopotassium cephalosporin C (Smith, Kline and French Labs., Philadelphia, PA, U.S.A.). The formulas are given in Fig. 1.

Columns

Columns were packed in the laboratory unless otherwise specified. Column dimensions: 25 cm × 4.6 mm I.D., except for the Hibar column: 25 cm × 4 mm I.D. Packing materials: Nucleosil C_{18} 10 μm (Macherey-Nagel, Düren, F.R.G.) and Partisil-10 ODS (Whatman, Maidstone, U.K.), both packed by Chrompack (Middelburg, The Netherlands), μBondapak C_{18} 10 μm (Waters Assoc., Milford, MA, U.S.A.), RSil C_{18} LL 10 μm (Alltech Europe, Eke, Belgium), Polygosil C_8 10 μm and Nucleosil C_8 10 μm (Macherey-Nagel, Düren, F.R.G.), LiChrosorb RP-8 5 μm and 10 μm, and prepacked LiChrosorb RP-8 10 μm, Hibar (E. Merck, Darmstadt, F.R.G.), Zorbax C_8 7 μm (Du Pont, Wilmington, DE, U.S.A.).

Packing procedure. 2.7 g of packing material (3.2 g for Zorbax) were slurried in 15 ml of a mixture of toluene–cyclohexanol (1:2). The slurry was sonicated for 4 min and quickly introduced into the slurry reservoir: 25 cm × 0.5 in. O.D. stainless-steel tubing, fixed to the column through a 10-cm pre-column, the latter two being already filled with the suspending liquid. The slurry was immediately packed into the column using a Haskel pump, Model DSTV-122 (Haskel, Burbank, CA, U.S.A.), with an inlet pressure of 5 bar and with methanol as the pressurizing liquid. The solvents used were of reagent grade and were glass-distilled before use. Columns were fitted with end fittings of the reducing union type, with zero dead volume. The columns were checked by chromatography of a mixture of benzene, naphthalene, phenanthrene and anthracene with methanol–water (70:30) as the mobile phase, except for Polygosil where 60:40 was used. The flow-rate was 1 ml/min, and the chart speed 0.5 mm/sec. Results are summarized in Table III. None of the columns had ever been used before this experiment, except for Partisil-10 ODS and Nucleosil C_{18}. These had been used for several days in another experiment.

Fig. 1. Cephalosporin structures.

Apparatus

The pump unit consisted of a Milton Roy minipump (Laboratory Data Control, Riviera Beach, FL, U.S.A.) equipped with a flexible hose, Model SS-4HO-6S4 (Swagelok, Crawford Fitting, Solon, OH, U.S.A.), used as a pulse dampener[59], and a Bourdon pressure gauge (Covena, Brussels, Belgium), provided with an adjustable cut-off switch, connected to the power supply in order to protect the equipment against overpressure. The pressure gauge was modified in the laboratory into a flow-through gauge by soldering a piece of HPLC tubing to the pierced end of the Bourdon tube. The gauge was connected in series to the pulse dampener, forming a tee branch on the flow system. A Model 4Z-V6LJ-SSP valve (Parker Hannifin, Huntsville, AL, U.S.A.) was fixed to a tee, inserted in the flow system immediately after the pump, and an identical valve was fixed to the outlet of the pressure gauge. This arrangement facilitated flushing with the mobile phase. When pressures higher than 150 kPa were needed, *viz.*, with the LiChrosorb RP-8 5 μm column, this pump unit was replaced by a Waters pump, Model 6000 A (Waters Assoc., Milford, MA, U.S.A.). The HPLC apparatus further consisted of a Valco injector, Model CV-6-UPHa-N60, equipped with a 10-μl loop (Valco, Houston, TX, U.S.A.), a Waters detector, Model 440 (254 nm) and a Kipp & Zonen recorder, Model BD40 (Kipp & Zonen, Delft, The Netherlands). HPLC tubing was fitted to the columns with the aid of PTFE ferrules in order to protect the end fittings and frits against rapid wear and to avoid dead volumes between the end of the tubing and the stainless-steel frit. The reproducibility of the retention times obtained with the minipump was checked by injecting fifteen times a mixture of five barbiturates[60]. The mean retention times (in min) and the corresponding standard deviations were: 4.48 (0.02), 6.13 (0.03), 10.61 (0.04), 13.24 (0.04), 16.62 (0.05).

Reagents, mobile phases and operating conditions

Methanol, >99% (Janssen Chimica, Beerse, Belgium) and distilled water were glass-distilled before use. HPLC-grade acetonitrile was purchased from Rathburn (Rathburn Chemicals, Walkerburn, U.K.). Potassium monohydrogen phosphate and dihydrogen phosphate pro analysi (E. Merck, Darmstadt, F.R.G.) were used to prepare a 0.2 M buffer, pH 7.0. The mobile phases used for the cephalosporins consisted of acetonitrile–water mixtures, all containing 5% (v/v) of phosphate buffer. The acetonitrile content is specified where necessary. It was adjusted for each column in order to elute within 40–50 min all the cephalosporins, except cefamandole nafate, which was strongly retained on all the columns and therefore needed much higher acetonitrile percentages to be eluted. Mobile phases were degassed by sonication. The flow-rate was adjusted to 1.0 ml/min, the chart speed was set at 5 mm/min and the detector sensitivity at 0.05 a.u.f.s. The cephalosporins were dissolved in water (0.5–1 mg/25 ml) and 10-μl quantities were injected. All separations were carried out at room temperature (18–21°C). The retention times of the cephalosporins were measured manually.

RESULTS AND DISCUSSION

The cephalosporins are an important group of antibiotics and HPLC has frequently been used for their analysis. Tables I and II list HPLC systems used since 1975 for the separation of cephalosporins and their analogues.

Cephalosporins are practically always chromatographed without prior derivatization, only one exception being mentioned[68]. Although HPLC of individual cephalosporins, or of simple mixtures, has been described for most commercial cephalosporins, none of the papers deals with results for very complex mixtures.

Straight-phase chromatography on bare silica has recently been reported for the separation of I and derivatives[80]. Anion-exchange chromatography at pH 2 has also been reported[75] but reversed-phase chromatography on chemically modified silica is used in most cases. A comparison between HPLC on polystyrene divinylbenzene particles and chemically modified silica particles (C_{18}) of about the same dimensions has been described, the latter showing better selectivity[78]. HPLC on silica derivatized with aminoalkyl chains has been reported several times[51,68,75], but most separations were carried out on C_8 or C_{18} packing materials, although some of the more strongly retained cephalosporins were nicely separated on a C_2 column[79].

The diameter of the particles used in recent work was mainly 10 μm or 5 μm and the column dimensions were appropriately adapted, e.g. 30 cm × 4 mm I.D. or 25 cm × 4.6 mm I.D. for the former, and 15 cm × 4.6 mm I.D. for the latter.

All kinds of mobile phases have been used. The organic modifier was generally methanol, or, less frequently, acetonitrile. The application of ion-pair chromatography has been reported several times[63,74,76]. The pH values of the mobile phases vary from low to high, and several papers report the use of ammonium carbonate or borate buffers, which is nowadays generally accepted to be detrimental to the stability of the packing material. Most mobile phases are buffered since the pH influences the retention time and the selectivity[77]. Mobile phases containing only one organic modifier and a phosphate buffer seem to give very satisfactory results in many cases. Weakly acidic mobile phases are used more often than neutral systems. Separations are mainly carried out at room temperature and at flow-rates of 0.5–2 ml/min, although 6 ml/min has been reported[51]. The number of plates per metre, calculated from the figures presented in the papers varies from about 200 to about 42,000, and it is noteworthy that these extreme values were obtained by the same authors[53,72].

This review shows that for most cephalosporins good separations can be obtained on C_{18} or C_8 materials using simple mobile phases by adapting, when necessary, the concentration of the organic modifier, the pH, or, possibly, the flow-rate. No clear evidence of any influence of the nature of the C_{18} or C_8 bonded phase, however, appears from the results cited in Tables I and II. This factor was checked in the following experiment. Sixteen cephalosporins were examined on eight different C_8 and C_{18} packing materials, and for one of them, LiChrosorb RP-8, on material from three different batches. Table III summarizes information on the packing materials, and the columns prepared with them. For most packing materials the loss on ignition (LOI) corresponds quite well with literature values[81] reporting the carbon content (in %), but for Zorbax C_8 the LOI is much lower (about half), while for RSil C_{18} LL a much higher value (about 180%) is obtained. The LOI was determined, after the samples had been dried overnight at 120°C, by heating at 700°C for 4 h. Heating for a longer period did not increase the LOI. The loss is expressed as a percentage of the residue, obtained after ignition, which corresponds approximately to the amount of bare silica involved in the preparation of the reversed phase. Indeed, when a sample of silica is heated in the same conditions, a LOI of about 3% is obtained. The figure reported is the mean of several determinations. For the LOI one

TABLE I

HPLC SYSTEMS DESCRIBED FOR THE SEPARATION OF ISOMERS AND FOR ASSAY AND PURITY CONTROL OF CEPHALOSPORINS

The products examined are cited in the order of elution. ND = not determined.

Ref.	Year	Authors	Products examined	Column packing; particle diameter	Column dimensions L (cm) × I.D. (mm)	Mobile phase	Flow-rate (ml/min)	Plates/metre (peak measured)
61	1978	Salto	VI (isomers)	μBondapak C_{18} 10 μm	30 × 3.9	CH_3OH-0.1 M phosphate buffer pH 3.5 (5:95)	1.5	4000 (D-VI)
62	1978	Young	7-Ureidoacetamido-cephalosporins (isomers)	μBondapak C_{18} 10 μm	30 × 4.0	CH_3OH-0.01 M $(NH_4)_2HPO_4$ (5-20:95-80)	0.8	6300 (D-Δ^3)
63	1981	Mason et al.	VI (isomers)	Hypersil + C_8 5 μm	16 × 5.0	0.2% Na-pentanesulphonate in 1% CH_3CN-HOAc (20:80)	1.0	11,200 (VI)
39	1981	Konaka et al.	Moxalactam (isomers)	Nucleosil C_{18} 10 μm	30 × 4.0	CH_3OH-0.05 M phosphate buffer pH 6.5 (5:95)	2.0	4700 (S-moxalactam)
64	1976	Hartmann et al.	VI, VII	LiChrosorb RP-8 10 μm	25 × 3.0	CH_3OH-0.05 M phosphate buffer pH 7.0 (1:5)	3.2	2450 (VII)
65	1977	Carroll et al.	VI, VII	μBondapak C_{18} 10 μm	30 × 4.0	CH_3OH-0.03% $(NH_4)_2CO_3$ (8:92)	0.9	3000 (VII)

66	1978	Crombez et al.	VI, VII	RSil C_{18} LL 10 μm	25 × 4.6	CH_3OH–0.07 M phosphate buffer pH 6.0 (25:75)	1.0	5700 (VI)
67	1979	Mangia et al.	Cephacetrile	LiChrosorb RP-8 10 μm	25 × 4.5	CH_3OH–0.01 M acetate buffer pH 4.2 (5 30:95–70); 40°C gradient	2.0	ND (gradient)
68	1980	Fabregas et al.	VI as trinitrophenyl derivative	Amino-Sil-X-I 13 μm	25 × 4.6	CH_3OH–H_2O–5% citric acid in H_2O (5:31:9); 50°C	0.75	2200
69	1982	Elrod et al.	Cefsulodin	Zorbax C_8 7 μm	25 × 4.6	CH_3OH–CH_3CN–0.02 M NH_4OAc pH 4.1 (35:15:950)	1.5	6200
70	1982	Coomber et al.	XV	Spherisorb C_6 5 μm	10 × 4.5	CH_3CN–0.1 M acetate buffer pH 3.4 (5:95)	2.0	25,000 (XV)
71	1983	Springolo et al.	XII	LiChrosorb RP-8 10 μm	ND	CH_3OH 0.01 M NaH_2PO_4 (30:70)	1.0	ND
			XIV	LiChrosorb RP-8 10 μm	ND	CH_3OH HOAc–H_2O–CH_3CN (4:3:75:18)	1.0	ND
			XV	LiChrosorb RP-8 10 μm	ND	CH_3CN–0.1 M phosphate buffer pH 7.5 (10:90)	1.2	ND

TABLE II

HPLC SYSTEMS DESCRIBED FOR THE SEPARATION OF CEPHALOSPORINS

The products examined are cited in the order of elution. ND = not determined.

Ref.	Year	Authors	Products examined	Column packing; particle diameter	Column dimensions L (cm) × I.D. (mm)	Mobile phase	Flow-rate (ml/min)	Plates/metre (peak measured)
72	1975	White et al.	XII, XIV, VII, IV, X	ODS-Sil-X-II	100 × 2.1	CH_3OH-0.05 M $(NH_4)_2CO_3$ (5:95)	0.5	200 (VII)
51	1976	Miller et al.	VI, XII, XIII, IX, VIII, X, XI	μBondapak NH_2 10 μm	30 × 4.0	HOAc-CH_3OH-CH_3CN-H_2O (2:4:50:44)	6.0	17,000 (XI)
73	1977	White et al.	VI, XIV, VII, IV, X	LiChrosorb RP-18 10 μm	30 × 4.6	CH_3OH 0.01 M NaH_2PO_4 (17:83)	1.3	4700 (X)
65	1977	Carroll et al.	I, VI, XIV, VII, X, IV	μBondapak C_{18} 10 μm	30 × 4.0	CH_3OH-0.03% $(NH_4)_2CO_3$ (5:95)	0.9	5000 (X)
74	1979	Crombez et al.	XIV, XIII, X	Vydac RP-18 30 44 μm	50 × 2.1	0.01 M $(Bu)_4NHSO_4$ 0.01 M $(Et)_4NOH$ 0.1 M phosphate buffer pH 7.5· CH_3OH H_2O (10:10:7.5:62.5); 55°C	0.5	600 (X)
75	1979	Quercia et al.	X, XIV, IX	Varian Micropak-NH_2 10 μm	25 × 2.0	[HOAc CH_3OH H_2O(2:4:94)]-CH_3CN (67:33)	1.0	6000 (X)
			III, VI, XII, XV	Partisil 10-SAX 10 μm	25 × 4.6	H_2O 0.05 M $NH_4H_2PO_4$ pH 2 (90:10)	1.6	4000 (X)
76	1979	Barbato et al.	XII, XIV, XIII, VII, X	μBondapak C_{18} 10 μm	30 × 4.0	0.6% cetrimoniumbromide in [0.1 M borate buffer pH 8.5 1-propanol (10:3)]	1.0	5000 (X)
77	1980	Salto et al.	I, VI, VII, XII, XIV, X	Bondapak C_{18} 30-70 μm	60 × 2.5	CH_3OH-0.05 M phosphate buffer pH 6 (10:90)	1.0	ND
78	1981	Salto et al.	XIV, XII, X	Amberlite XAD-4 40 70 μm	60 × 2.5	CH_3OH 0.05 M phosphate buffer pH 6 (50:50)	0.5	ND
79	1981	Okumura et al.	X, VI, IV, XII	LiChrosorb RP-2 10 μm	30 × 4.0	CH_3OH-H_2O (1:4)	0.5	10,000 (X)
53	1981	White et al.	VI, XIV, VII, IV, X	LiChrosorb RP-18 10 μm	30 × 4.6	CH_3OH-0.01 M NaH_2PO_4 (17:83)	1.3	5500 (X)
			VI, XIV, VII, IV, X	LiChrosorb RP-8 5 μm	15 × 4.6	CH_3OH-0.01 M NaH_2PO_4 (15:85)	1.0	42,000 (X)

would expect to find higher figures than for the carbon content since the former includes the loss of hydrogen and of some water from the silica. Moreover the LOI is expressed *versus* the mass of the residue, while the carbon content is expressed *versus* the initial mass. Values of carbon content and LOI, normalized for a surface of 500 m^2/g, are given in parenthesis. Here, the difference between the actual surface of the silica and the value reported in the literature is a potential source of variation. It is observed that the normalized literature value for Zorbax C_8 (25%) is much higher than for other C_8 materials and as high as for well-covered C_{18} materials. This is illogical, since Zorbax C_8 is said to be prepared with octyldimethylsilyl re-agents, which do not polymerize and therefore give a brush-type monolayer, which should give lower carbon contents than the packing materials obtained with trifunc-tional silylating reagents. Therefore, the LOI could be accepted as a more realistic value for Zorbax C_8, since it is slightly lower than that of other well-covered mate-rials. The higher normalized value for LOI of Nucleosil C_8 can be explained by the larger pore diameter, allowing better peneration of the silylating reagent, but it can also be due to a higher degree of polymerization.

The methyl red adsorption values (MRAV) were determined by the previously published method, using a 400 mg/100 ml stock solution of methyl red[82]. The values reported now correspond well with the figures mentioned before; small differences can be explained by the fact that the figures refer to different batches. For Zorbax the low MRAV, together with the low LOI, is an indication for the presence of a monolayer that covers the surface well. Although the normalized carbon content and LOI values for Nucleosil C_8 are higher than for Polygosil C_8, the normalized MRAV is not lower, which may be an indication for polymerization in the organic layer of Nucleosil. The high MRAV value for RSil C_{18} LL corresponds well with the label LL which stands for "low loading", but it is contradictory to the fact that this ma-terial is said to be endcapped to ensure minimum silanol activity. μBondapak C_{18}, showing the same normalized LOI, has a very low MRAV, and is therefore con-sidered to have lower silanol activity.

For reasons of uniformity all the homemade columns were packed following the same procedure. This had an unfavourable effect on the plate number per metre (N/m) of several columns, which was determined on the naphthalene peak. Pre-viously, better columns were prepared with LiChrosorb RP-8 10 μm and μBondapak C_{18}, by using a more adequate slurry. This explains the discrepancy between the LiChrosorb RP-8 10-μm Hibar column and the corresponding homemade column. However, the plate number affects the resolution but not the selectivity, which was our concern here.

The separation factor α (phenanthrene–anthracene) allows a rough distinction between C_8 and C_{18} materials. The figure for Polygosil C_8 is abnormally high because another mobile phase, containing less methanol, had to be used, otherwise no mea-surable separation was observed. No clear correlation exists between α and the other parameters discussed above. Observation of the C_8 columns suggests that α increases with decreasing MRVA, but this is contradicted by the results obtained with the C_{18} columns, where RSil LL and μBondapak C_{18} have the same α but very different MRAV. One could also suggest that for highly apolar structures, like polyaromatic hydrocarbons, the silanol groups play a minor role and the carbon content is more important. In the C_{18} series, however, it is observed that the lower-loaded Partisil

TABLE III

CHARACTERISTICS OF PACKING MATERIALS AND COLUMNS

ND = not determined since columns were prepacked.

Packing materials	Shape: irregular or spherical silica	Surface area of the parent silica (m²/g)	Pore diameter (nm)	Carbon*·§§ content (%)	Loss**·§§ on ignition (LOI) (%)	Methyl red***·§§ adsorption value (MRAV) (mg/g)	Plates§ per m determined on naphthalene (N/m)	Separation§ factor α phenanthrene-anthracene	Capacity factor k', anthracene	Plates per m (N/m), determined on cephalospor. (Roman numeral) from figure (Arabic numeral)	
LiChrosorb RP-8 10 µm (Hibar)	I	500	6	13-14	ND	ND	19,600	1.09	6.2	X	2
LiChrosorb RP-8 10 µm	I	500	6	13 14	14.5	2	10,300	1.08	5.7	X	4
LiChrosorb RP-8 5 µm	I	500	6	13 14	16.7	2	26,800	1.09	7.1	X	5
Zorbax BP-C₈ 7 µm	S	300	6 8	15 (25)	7.6 (12.7)	0.5	33,600	1.10	9.9	IV	6
Polygosil C₈ 10 µm	I	500	6	10-11	9.8	15	11,600§§§	1.08§§§	6.8§§§	XII	7
Nucleosil C₈ 10 µm	S	300	10	10-11 (16.7 18.3)	10.6 (17.7)	13 (22)	15,800	1.05	4.4	XII	8
RSil C₁₈ LL 10 µm	I	550	6	10 (9)	17.9 (16.3)	92 (84)	18,400	1.12	7.4	VII	9
µBondapak C₁₈ 10 µm	I	350	10	10 (14.3)	11.5 (16.4)	1	7200	1.12	9.2	X	10
Nucleosil C₁₈ 10 µm	S	300	10	15-16 (25-26.7)	ND	ND	12,000	1.19	6.0	X	11
Partisil ODS 10 µm	I	360	6	5 (6.9)	ND	ND	13,600	1.17	2.1	XIII	12

* These values are taken from the literature[81].

** 4 h at 700°C.

*** Determined following a method previously published[82].

§ See Experimental for chromatographic conditions.

§§ Figures in parentheses are calculated for a surface of 500 m²/g.

§§§ A different mobile phase was used, see Experimental.

column shows better α values than several higher-loaded columns, while in the C_8 series the highest α value is observed for the Zorbax column, showing the lowest loss on ignition. Nor was any clear correlation observed between the capacity factor for anthracene and the column parameters discussed so far. It is not even possible to distinguish C_8 from C_{18} materials. These results confirm some recently published observations[83].

Acetonitrile was chosen as the organic modifier for this experiment since in preliminary experiments it was observed that it generally gave better separations than methanol. The mobile phase was buffered since the cephalosporins have acid–base properties. The influence of pH on the selectivity was not examined in detail, but a limited number of experiments showed that at pH 7 good results were obtained and that small deviations from this pH did not influence the separation.

When the cephalosporins were chromatographed on the different columns, the percentage of organic modifier was adapted in order to obtain complete elution within about 40 min, which corresponds to k' values of about 15–20. From the beginning it was observed that IX was retained very strongly on all the columns and if it had to be eluted within 40 min the other cephalosporins were practically not separated, which would render comparison of selectivities almost impossible. It was then decided to exclude IX from further experiments, and therefore no chromatographic results are reported. The strong retention must be due to a particular interaction between the formyl group and the column material. This phenomenon was not observed (Table III) for chromatography with acidic mobile phases on silica derivatized with aminoalkyl chains, where IX eluted even faster than the corresponding VIII[51,75]. Strong retention of IX was also observed in reversed-phase thin-layer chromatography on silanized silica gel with mobile phases buffered at pH 6.2[84]. Replacement of the buffer by acetic acid did not markedly increase the R_F value. It is therefore concluded that the faster elution of IX is merely due to the alkylamine bonded phase and not to the lower pH of the mobile phase.

Results obtained for the other fifteen cephalosporins are summarized in Table IV. It is observed that acetonitrile contents of 1 to 11.5% are needed to elute the cephalosporins within about 40 min. Here again, no correlation with the parameters mentioned in Table III is seen, and no distinction can be made between C_8 and C_{18} materials. For several columns results obtained with mobile phases containing increasing amounts of organic modifier are reported. The most representative separations are shown in Figs. 2–13. The dead volume of the columns was determined by injecting sodium nitrite, but on Zorbax C_8, I and II eluted faster than sodium nitrite. This once again emphasizes the problem of dead volume determination[84]. Sodium nitrite as well as sodium nitrate have repeatedly been cited as very useful for dead volume determination[86–88]. One paper reports the contrary, but the method used raises questions as to its validity[89].

It is impossible to see any relationship between the structure of the cephalosporins and their elution order, which is different on all the columns, except for the very polar I and II, which are always eluted first. For the LiChrosorb columns it is observed that the elution order is influenced by the amount of organic modifier. This phenomenon is not observed for the other columns. For the same acetonitrile content, there are also differences in elution order between the prepacked Hibar column and the home-packed 10-μm column. However, Figs. 3 and 5 demonstrate that LiChro-

TABLE IV

ORDER OF ELUTION AND RETENTION TIMES OF CEPHALOSPORINS

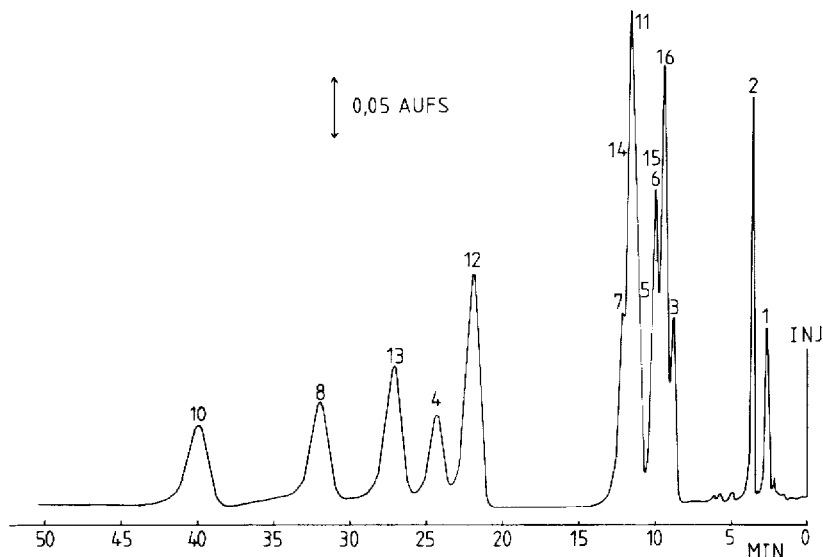| Column	CH₃CN in mobile phase (%)	Fig.	Retention time of NaNO₂ (min)	Order of elution of cephalosporins with the retention time (min)														
LiChrosorb RP-8 Hibar 10 μm	8.5	2	2.04	I 2.8	II 3.6	III 8.8	XVI 9.4	VI 10.0	XV 10.0	V 11.0	XI 11.4	XIV 11.6	VII 12.2	XII 22.0	IV 24.4	XIII 27.2	VIII 32.0	X 40.2
	10			II	III	XVI	VI	XV	XIV	V	VII	XI	XII	IV	XIII	VIII	X	
	12	3		I 2.6	II 3.4	III 6.6	XVI 7.2	VI 7.8	XV 8.1	V 8.1	XIV 9.0	VII 9.2	XI 9.6	XII 17.2	IV 17.4	XIII 8.2	VIII 25.2	X 29.8
LiChrosorb RP-8 10 μm	8.5	4	2.50	I 2.2	II 3.2	III 4.8	XVI 5.2	VI 5.8	III 5.8	V 6.2	XI 6.6	XIV 6.6	VII 7.6	XII 11.4	IV 11.6	XIII 12.4	VIII 15.2	X 20.0
LiChrosorb RP-8 5 μm	11.5	5	2.86	I 3.0	II 4.6	III 10.6	XVI 10.8	VI 11.2	VI 11.4	V 12.6	XI 12.6	XIV 12.8	VII 14.8	XII 27.0	IV 28.4	XIII 32.2	VIII 33.6	X 41.4
Zorbax C₈ 7 μm	1.0	6	2.80	I 3.0	II 4.0	XI 6.8	V 8.6	VI 9.8	VI 9.8	V 11.6	XVI 10.6	VII 11.2	XII 13.4	IV 19.4	XIII 20.2	VIII 22.0	X 30.8	XIII 42.0
	2.0			I 2.0	II 2.2	XI 4.6	V 5.0	VI 5.4	XV 5.8	III 7.2	XVI 7.4	VII 7.6	XII 10.6	XIV 11.0	IV 18.6	XIII 25.0	VIII 25.2	X 35.2
	5.0			I 2.0	II 2.2	XI 3.6	V 3.8	VI 4.0	XV 4.2	III 4.6	XVI 4.8	VII 5.2	XII 7.0	XIV 7.0	IV 10.6	XIII 14.6	VIII 15.4	XII 16.6
Polygosil C₈ 10 μm	5.0	7	2.46	I 3.0	II 4.8	XI 6.8	XV 7.2	VI 7.8	XV 8.2	V 8.8	XI 10.6	III 11.4	VII 12.2	XII 16.6	X	XIII 18.0	VIII 25.0	XII 47.8
	7.0			I 2.8	II 4.1	XI 6.0	XV 6.0	VI 6.0	XV 6.8	VI 7.0	XIV 7.4	VII 7.8	XII 9.0	IV 11.2	XIII 12.2	VIII 12.2	XII 17.2	X 34.6
	5.0			I 3.6	II 4.6	III 10.0	XV 10.6	VI 11.0	XV 10.8	V 11.4	XI 14.4	III 15.6	VII 15.6	IV 25.2	XIII 27.0	X 27.8	XIII 42.8	XII 50.2
Nucleosil C₈ 10 μm	7.0	8		I 3.0	II 4.8	III 8.4	XVI 8.4	VI 8.8	XV 8.8	V 9.0	XI 10.4	III 11.2	VII 11.2	IV 17.8	XIII 19.6	X 21.0	XIII 28.8	XII 35.6
	9.0		2.52	I 2.8	II 4.2	III 7.2	XVI 7.2	VI 7.4	XV 7.4	V 7.8	VI 7.8	VII 8.4	XII 8.8	IV 13.2	XIII 14.0	VIII 15.8	XIII 18.8	XII 25.2
	11.0			I 2.8	II 4.2	III 5.4	XV 5.8	VI 6.2	VI 6.2	VI 6.2	VI 6.2	VII 6.4	VIII 7.2	IV 9.8	XIII 11.0	XIII 12.2	XIII 13.4	XII 19.0
RSil C₁₈ LL 10 μm	6.0	9	2.20	I 3.2	II 5.8	XI 10.6	XV 10.8	VI 12.0	V 14.8	VI 15.8	III 19.4	XIV 15.6	VII 24.0	VIII 25.2	XIII 34.0	X 15.6	IV 37.2	XII 39.8
	8.0			I 3.0	II 5.2	III 8.8	XVI 8.6	VI 9.0	XV 11.6	VI 12.0	XI 13.2	XIV 13.6	VII 17.2	IV 23.4	XIII 25.4	X 25.6	VIII 25.6	XII 91
	10.0			I 2.8	II 4.2	III 7.2	XVI 6.6	VI 6.6	XV 9.0	VI 9.2	XI 9.2	XIV 9.2	VII 12.4	IV 16.0	XIII 17.2	XII 17.2	VIII 17.6	XII 63.2
μBondapak C₁₈ 10 μm	10.0	11	2.84	I 3.2	II 4.2	XVI 9.4	III 9.6	VI 10.4	V 10.4	XIV 11.8	XI 12.0	VII 13.0	XIV 13.0	XIII 22.6	IV 23.4	XII 27.4	VIII 30.6	X 38.2
Nucleosil C₁₈ 10 μm	7.0	12	2.54	I 3.2	II 4.4	XVI 8.6	XV 8.6	VI 8.8	XI 8.8	V 10.6	III 10.6	XI 10.6	VII 14.0	IV 22.8	XIII 23.2	VIII 26.4	X 30.0	XII 41.4
	10.0			I 2.8	II 4.0	XI 5.6	XV 5.8	VI 6.4	V 6.4	VI 7.0	VI 7.0	VII 7.0	VIII 8.6	IV 11.6	XIII 12.0	XIII 13.2	XII 16.2	XII 25.0
Partisil ODS 10 μm	5.0	13	2.46	I 2.8	II 4.2	XI 4.8	XV 5.0	VI 5.4	VI 7.6	VI 7.6	XIV 9.0	VIII 9.4	VII 10.8	VIII 11.4	X 11.4	IV 13.8	XIII 16.6	XII 59.8
	7.0			I 2.6	II 3.8	III 4.2	XV 4.0	VI 4.2	V 6.2	VI 5.8	XIV 6.4	VII 6.4	VIII 8.2	VIII 8.2	X 8.2	IV 9.8	VIII 10.8	XII 39.2

Fig. 2. Chromatogram obtained on LiChrosorb RP-8 10 μm Hibar with mobile phase acetonitrile–water–0.2 *M* phosphate buffer pH 7.0 (8.5:86.5:5).

sorb is very useful for the separation of cephalosporins. The separation of the individual cephalosporins will not be discussed in detail, the comparison of the selectivity of the column packings being what is emphasized. The home-packed 10-μm column shows poorer resolution (Fig. 4) as is expected from the lower plate number (Table
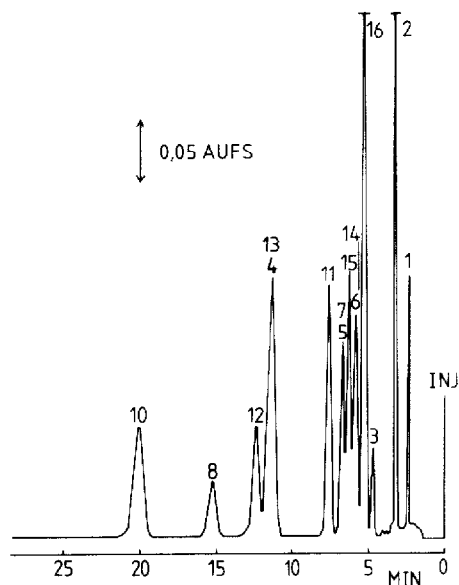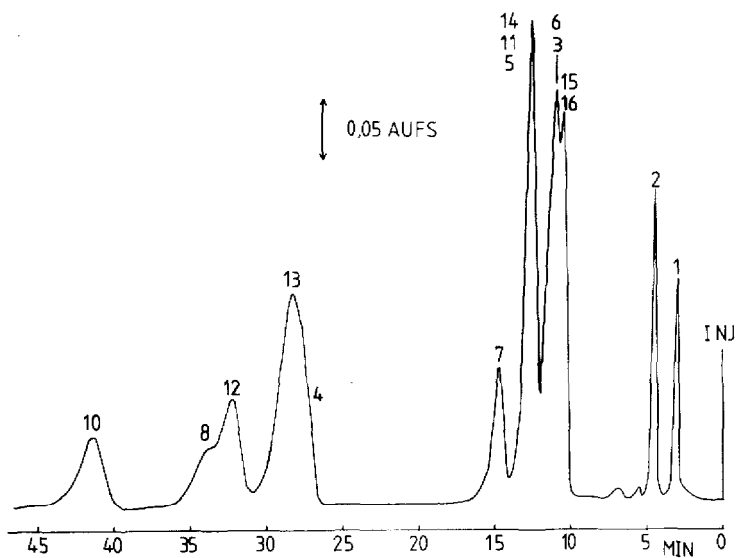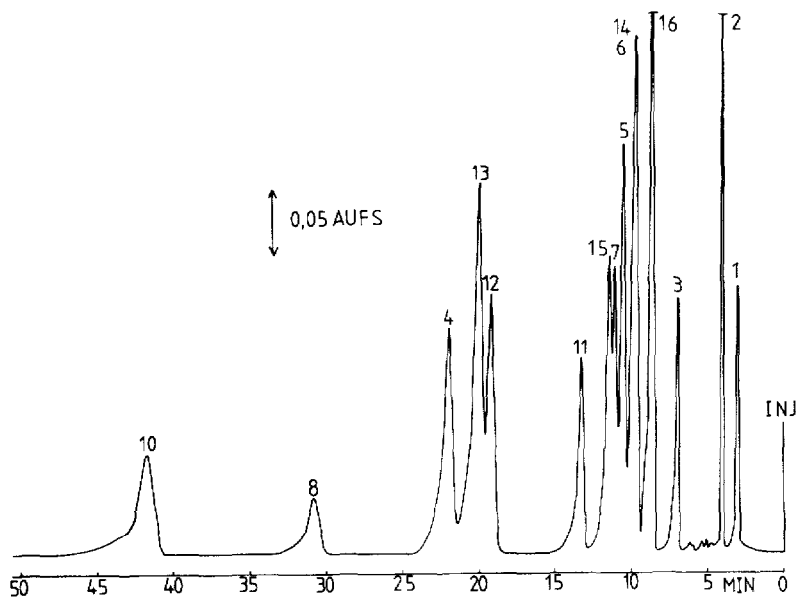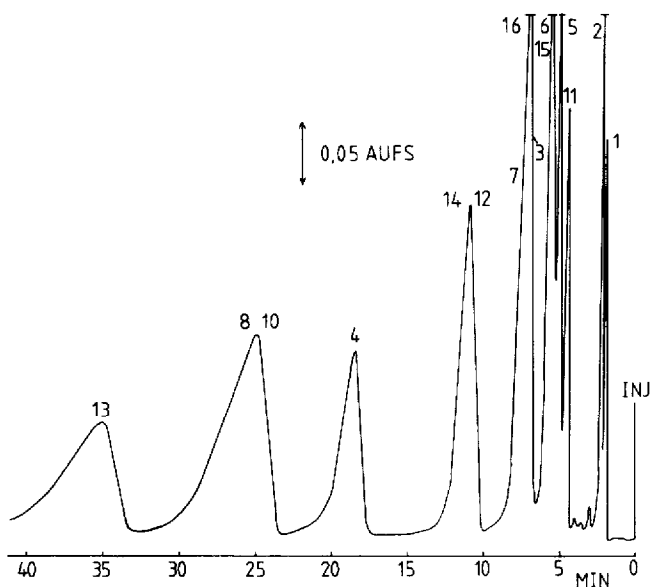

Fig. 3. Chromatogram obtained on LiChrosorb RP-8 10 μm Hibar with mobile phase acetonitrile–water–0.2 *M* phosphate buffer pH 7.0 (12:83:5).

Fig. 4. Chromatogram obtained on LiChrosorb RP-8 10 μm with mobile phase acetonitrile–water–0.2 *M* phosphate buffer pH 7.0 (8.5:86.5:5).

III). The 5-μm column gives better resolution. Fig. 5 shows also that HPLC can be successfully used for the identification of cephalosporins, although simpler techniques such as colour reactions and TLC will generally be preferred for this purpose[84].

Table III also lists plate numbers, calculated for each column on a well sepa-

Fig. 5. Chromatogram obtained on LiChrosorb RP-8 5 μm with mobile phase acetonitrile–water–0.2 *M* phosphate buffer pH 7.0 (11.5:83.5:5).

Fig. 6. Chromatogram obtained on Zorbax C$_8$ 7 μm with mobile phase acetonitrile–water–0.2 M phosphate buffer pH 7.0 (1:94:5).

rated cephalosporin. These calculations were performed on chromatograms obtained at low chart speed and the results are therefore less reliable. The general tendency is that for cephalosporins lower N/m are recorded, with striking extremes for Partisil ODS and, particularly, Zorbax. On this column the cephalosporins are practically
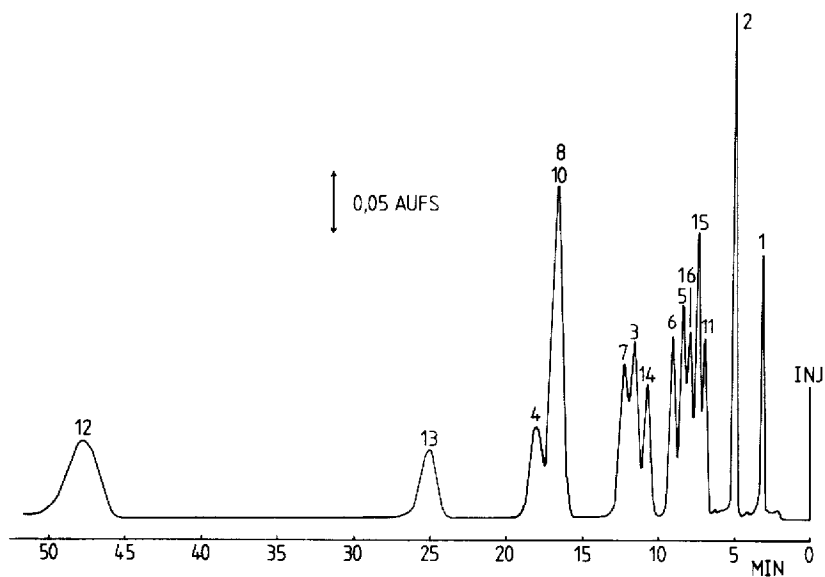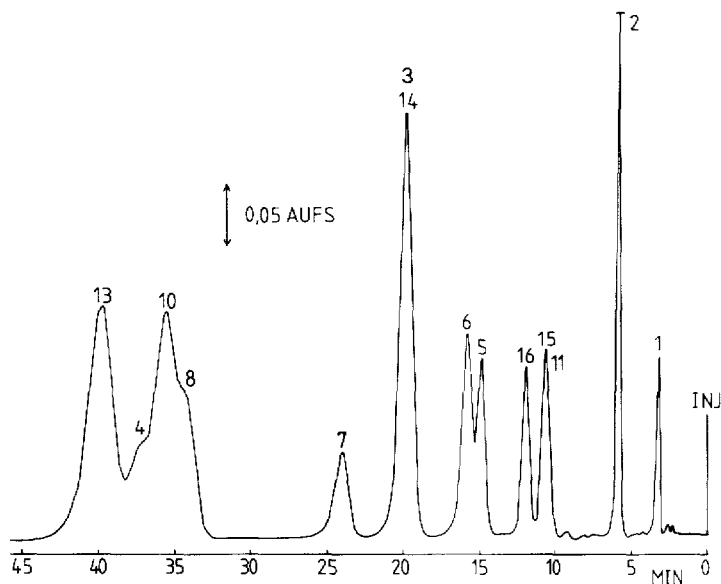

Fig. 7. Chromatogram obtained on Polygosil C$_8$ 10 μm with mobile phase acetonitrile–water–0.2 M phosphate buffer pH 7.0 (5:90:5).
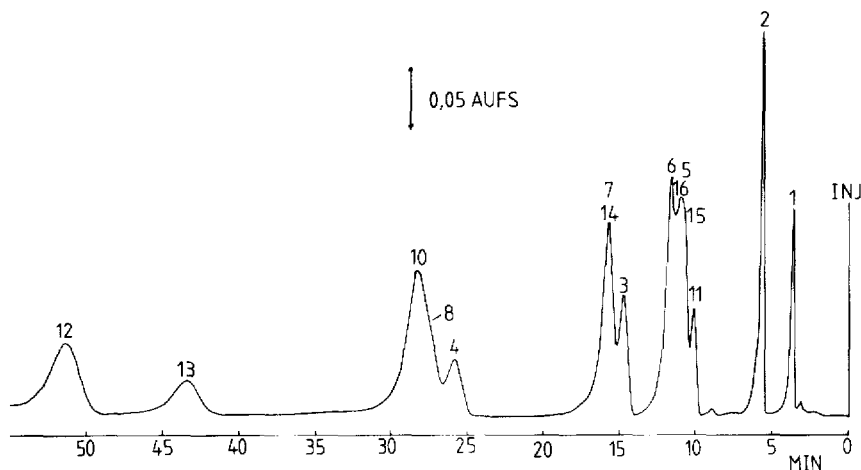
Fig. 8. Chromatogram obtained on Nucleosil C$_8$ 10 μm with mobile phase acetonitrile–water–0.2 M phosphate buffer pH 7.0 (5:90:5).

not retained and are badly resolved (Fig. 6). However, from the N/m value for anthracene and the MRAV, one would expect this column to be the best. For other drugs the authors obtained very nice separations on Zorbax[60,90]. This illustrates that columns with the highest claimed N/m values are not necessarily the best for a particular separation problem.

The elution order on Polygosil and Nucleosil is somewhat different although both materials are of the same origin, but Polygosil, with the lower N/m values,

Fig. 9. Chromatogram obtained on RSil LL C$_{18}$ 10 μm with mobile phase acetonitrile–water–0.2 M phosphate buffer pH 7.0 (6:89:5).
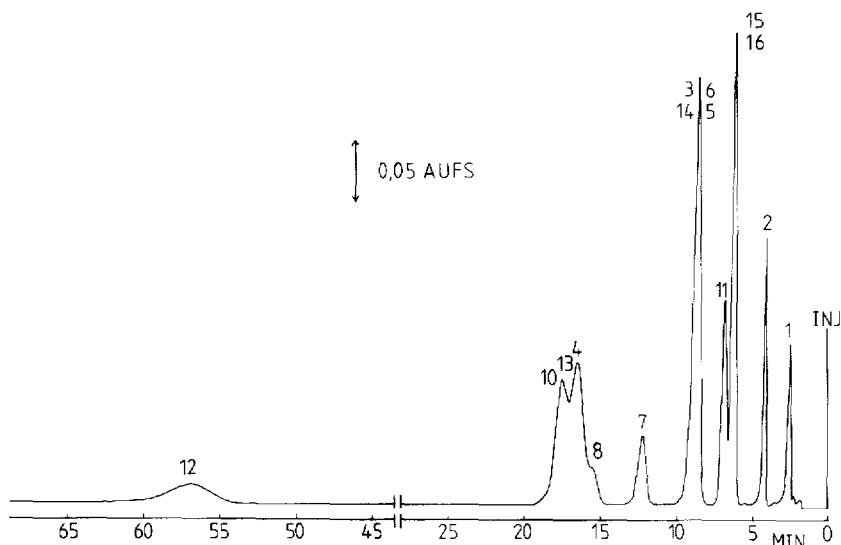
Fig. 10. Chromatogram obtained on RSil LL C$_{18}$ 10 μm with mobile phase acetonitrile–water–0.2 *M* phosphate buffer pH 7.0 (10:85:5).

shows a better resolution (Fig. 7). RSil LL is very useful for the separation of the more polar cephalosporins (Fig. 9), probably because there is a better interaction with the less covered silica. This also explains the strong retention of XII. When mobile phases with higher acetonitrile contents are used in order to elute XII, poor resolution of the cephalosporins is observed (Fig. 10). The same elution pattern is obtained with the low-loaded Partisil column (Fig. 13). On the whole, there seems to be very good correlation between the retention of XII and the MRAV which is explained by the presence of a permanent positive charge on the pyridinium moiety, interacting with free silanol groups. The use of cefaloridine to check for residual silanols would have the advantage that the test is performed on the packed column.
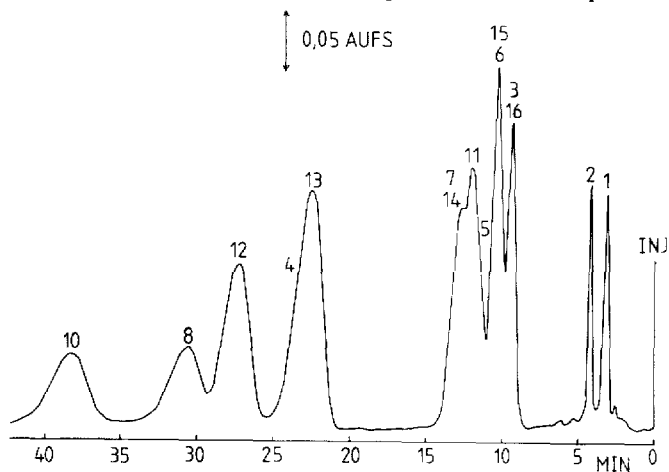

Fig. 11. Chromatogram obtained on μBondapak C$_{18}$ 10 μm with mobile phase acetonitrile–water–0.2 *M* phosphate buffer pH 7.0 (10:85:5).
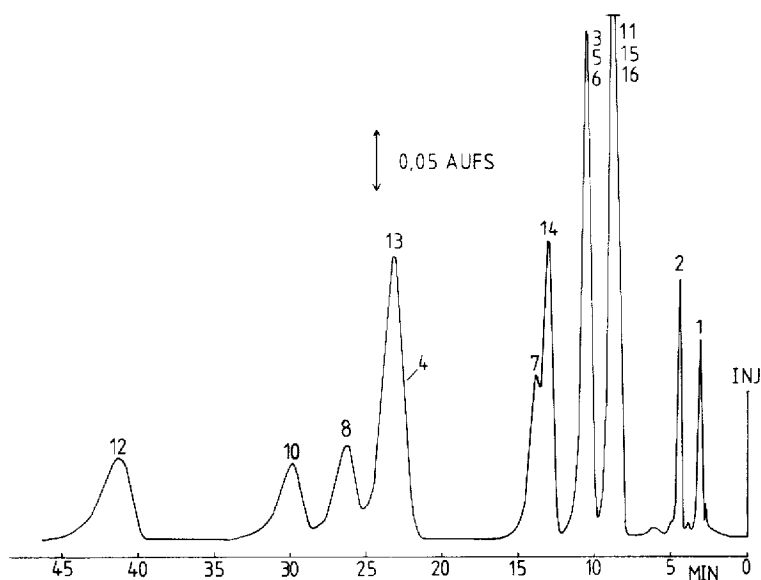
Fig. 12. Chromatogram obtained on Nucleosil C_{18} 10 μm with mobile phase acetonitrile–water–0.2 M phosphate buffer pH 7.0 (7:88:5).

The results obtained with the C_{18} columns (Figs. 10–13) are very different. The use of packing materials with a longer chain does not necessarily improve the separation of cephalosporins. For 7% acetonitrile the retention times on Nucleosil C_8 and C_{18} are not very different, which can be explained by the polar character of the cephalosporins. For the apolar anthracene the difference between the capacity factors of the two columns is noticeable (Table III). The results in Table IV show that the position in the elution order is more variable for some cephalosporins; it can be seen,
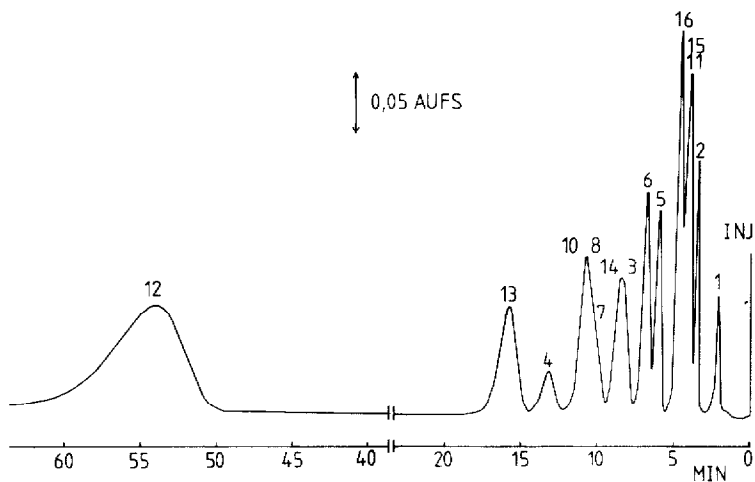

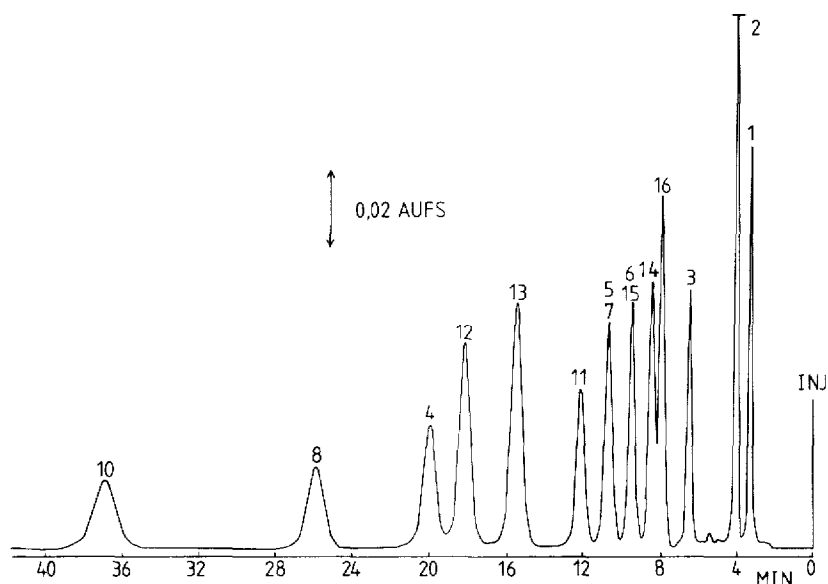Fig. 13. Chromatogram obtained on Partisil 10 ODS 10 μm with mobile phase acetonitrile–water–0.2 M phosphate buffer pH 7.0 (5:90:5).

Fig. 14. Chromatogram obtained previously on LiChrosorb RP-8 10 μm with mobile phase acetonitrile–water–0.2 M phosphate buffer pH 7.0 (12:83:5).

for instance, that XI moves from the third place to the tenth, while VII is always found at the ninth or tenth place.

It is clear that it is impossible to make valid predictions about the selectivity of a column towards cephalosporins and even for materials from the same manufacturer the differences are not negligible. It would even appear that over the years chromatographic materials with quite different properties have been manufactured under the same label, as is illustrated by Figs. 14 and 15, showing chromatograms
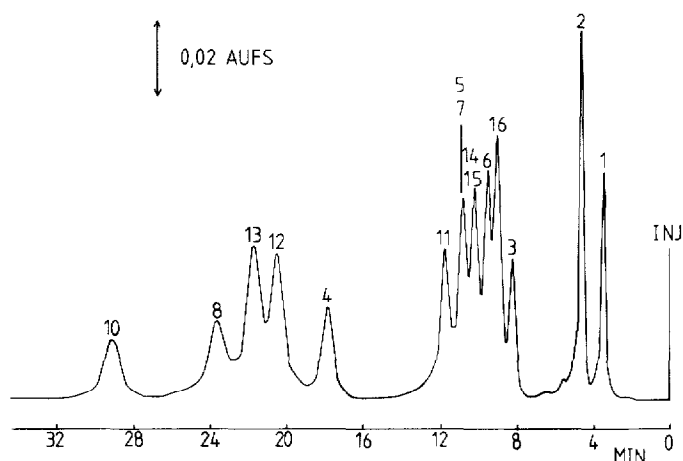

Fig. 15. Chromatogram obtained previously on Nucleosil C$_8$ 10 μm with mobile phase acetonitrile–water–0.2 M phosphate buffer pH 7.0 (10:85:5).

presented by the authors about five years ago[91]. The chromatograms were obtained at a somewhat lower flow-rate (0.8 ml/min) and another chart speed and should be compared with Figs. 2 and 8, respectively. Distinct differences in selectivity are undeniable. It is the old packing material that gives the best results, which is probably due to a somewhat lower coverage and better interaction between polar solutes and free silanols; in other words, the best covered materials do not necessarily give the best separations. Differences in properties between batches from the same manufacturer were not deduced from chromatographic results only. It was observed that at a certain moment LiChrosorb material could no longer be packed by the same procedure as before, although it was still sold under the same label.

It can be concluded that chromatographic materials classified under the general label of, *e.g.* C_8 or C_{18}, can behave in very different ways, providing a wide range of unique selectivities indeed[92]. Not all the manufacturers are yet able to produce chromatographic materials with sufficient reproducibility. A particular separation cannot always be reproduced on another column of the same type or from the same manufacturer. The information given by the manufacturers, such as the plate number obtained in a test chromatogram, is often insufficient or irrelevant.

Therefore, great care is to be recommended when a high-performance liquid chromatographic method is planned for application in several laboratories. The proposed chromatographic system should be used with a series of different packing materials of the same type to check for possible influence of the packing material on the selectivity. If no significant influence is observed, as for example for the phenanthrene–anthracene separation in Table III, the simple indication of the column type, *e.g.* C_2, C_8 or C_{18}, may be sufficient. But if there is an influence, as is the case with the cephalosporins, a selectivity test has to be provided. This necessitates the use of reference materials, which will render the method less attractive in cases where the reference products are not commonly available. The citation in official methods of the manufacturing brand(s) of packing material(s) known to give good results is often impossible for administrative reasons, but for scientific reasons, too, it is not justifiable, as long as manufacturers do not prepare packing materials with better reproducibility.

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