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SEPARATION OF SOME CEPHALOSPORIN DERIVATIVES BY ION-PAIR REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Cephapirin, its major metabolite desacetylcephapirin, 7-aminocephalosporanic acid and some other cephalosporin derivatives are separated on a chemically bonded octadecylsilane reversed-phase column. The selectivity between cephapirin and desacetylcephapirin on the reversed-phase column is too high, resulting in a poor separation. The effect of several variations of the chromatographic conditions on the selectivity has been examined. The *in situ* ion-pair formation of the cephalosporins with several counter ions and the influence of the ion-pair formation on the capacity factor and on the selectivity have been investigated. Other factors, such as the temperature, combination of counter ions and nature of the organic modifier and their influence on the selectivity have also been studied.

INTRODUCTION

Desacetylcephapirin, the major metabolite of cephapirin (Fig. 1), can be separated from the latter by liquid–solid chromatography on silica gel or by ion-exchange chromatography on a strong basic anion exchanger[1]. Although there is a sufficient selectivity between cephapirin and desacetylcephapirin, there is little or no selectivity between cephapirin and other cephalosporin derivatives such as cephalothin and cefazolin. We have found that liquid–liquid partition chromatography, using a ternary liquid system with the organic phase of a dichloromethane–methanol–water mixture as mobile phase on a microparticulate silica gel support, offers sufficient selectivity between cephapirin, cefazolin and desacetylcephapirin. However, the chromatographic system is not very stable and reproducible. The "normal" liquid–liquid chromatography with an apolar mobile phase also has the disadvantage of incompatibility between the organic mobile phase and the aqueous cephalosporin samples.

Reversed-phase thin-layer chromatography of cephalosporins on silanized silica gel[2] or on silica gel impregnated with silicone oil[3,4] showed good selectivity between several cephalosporin derivatives. Our experiments on silica gel plates impregnated with paraffin oil and using water as the mobile phase have also given good

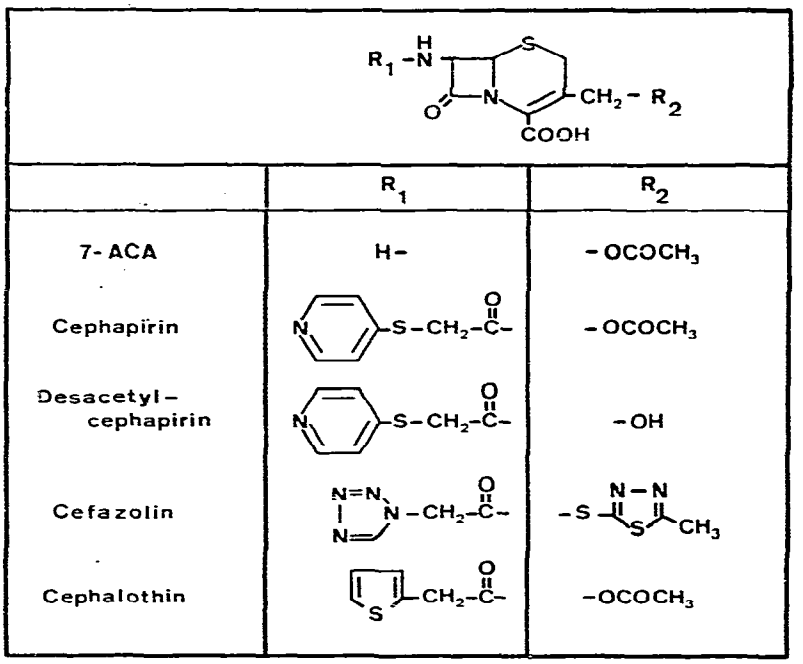

Fig. 1. Formulas of the cephalosporins used. 7-ACA = 7-Aminocephalosporanic acid.

selectivity. These experiments also showed that ion-pair formation took place when a counter ion was added either to the hydrophobic stationary phase or to the aqueous mobile phase. This ion-pair formation, with tetrabutylammonium ion or trioctylmethylammonium ions, resulted in a decrease of the R_F values and a change in selectivity. These experiments, and the compatibility of the aqueous nature of the sample with the hydrophilic mobile phase, prompted us to choose the reversed-phase liquid chromatographic approach for the separation of cephapirin, desacetylcephapirin and some other cephalosporin derivatives. A chemically bonded octadecylsilane pellicular stationary phase was employed, and the selectivity of the separation and the influence of ion-pair formation on the selectivity have been investigated.

EXPERIMENTAL

Apparatus

Liquid chromatography was carried out on a Varian 4100 liquid chromatograph equipped with a Varian fixed-wavelength UV 254 monitor containing an 8-μl flow cell. Samples were injected with a Hamilton syringe directly on top of the column through a Varian stop-flow septumless injector. A Whitey three-way valve was placed before the injector to cut off the liquid stream during the injection and to maintain the pressure in front of the injector. After the injection and closure of the injector the valve was opened and the pump turned on again. In this way the pressure on the column could be restored immediately after the injection of the sample. The column was constructed from a stainless-steel tube (500 $\times$ 2.1 mm I.D.) and fitted at the column outlet with a 2-μm filter union at low dead volume. The column was filled with

the pellicular packing material by the "tap-and-fill" procedure, a dry packing technique[5]. A homemade filling apparatus with a rotating motor and cam was used. The top of the column was sealed with a porous PTFE disc and connected to the septumless injector. The column was thermostated by means of a homemade water jacket, connected to a temperature-controlled bath.

Chemicals and reagents

Vydac RP, an octadecylsilane chemically bonded, pellicular packing material (30–44 μm) from Macherey, Nagel w Co. (Düren, G.F.R.), was used as the chromatographic support. Methanol, sodium dihydrogen phosphate and sodium hydroxide were p.a. grade. Trioctylmethylammonium chloride (Adogen 464) was obtained from Serva (Heidelberg, G.F.R.); tetrabutylammonium hydrogen sulphate (TBA HSO_4), chemical pure from Aldrich Europe (Beerse, Belgium) and 25% tetraethylammonium hydroxide (TEA OH), aqueous solution from UCB (Drogenbos, Belgium). Cephapirin, desacetylcephapirin, cefazolin sodium and 7-aminocephalosporanic acid were supplied by Bristol Laboratories (Syracuse, N.Y., U.S.A.) and cephalothin by Eli Lilly Benelux (Brussels, Belgium).

Liquid chromatography conditions

These mobile phases and conditions are referred to in Results and discussion.

(I) Mobile phase, water; flow-rate, 1 ml/min. Temperature, 25°.

(II) Mobile phase, sodium phosphate buffer, 10 m*M*, pH 7.5: a 10 m*M* sodium dihydrogen phosphate solution was adjusted to pH 7.5 with sodium hydroxide (mobile phase IIa). Less concentrated buffer solutions (1 m*M*, mobile phase IIb; 0.5 m*M*, mobile phase IIc) were prepared by dilution of the 10 m*M* buffer. Flow-rate, 1 ml/min. Temperature, 25°.

(III) Mobile phase, aqueous solution containing 1 m*M* sodium phosphate buffer and 1 m*M* TBA HSO_4; flow-rate, 1 ml/min. Temperature, 25°.

(IV) Mobile phase, aqueous solution containing 1 m*M* TBA HSO_4 in 10 m*M* sodium phosphate buffer; flow-rate, 1 ml/min. Temperature, 25°.

(V) Mobile phase, aqueous solution containing 1 m*M* TBA HSO_4 and 7.5% methanol in 10 m*M* sodium phosphate buffer; flow-rate, 1 ml/min. Temperature, 25°.

(VI) Mobile phase, aqueous solution containing 1 m*M* TBA HSO_4, 1 m*M* TEA OH and 7.5% methanol in 10 m*M* sodium phosphate buffer; flow-rate, 1 ml/min. Temperature as given in Table IV.

(VII) Mobile phase, aqueous solution containing 1 m*M* TBA HSO_4, 1 m*M* TEA OH and 5% acetonitrile in 10 m*M* sodium phosphate buffer; flow-rate, 0.8 ml/min. Temperature, 55°.

RESULTS AND DISCUSSION

Pure reversed-phase chromatography

With water as eluent (mobile phase I), there was a complete separation between cephapirin and desacetylcephapirin, but the capacity factor, k', of the compounds was very low. The addition of a salt to the mobile phase in the form of a sodium phosphate buffer (mobile phase II) resulted in enhanced capacity factors, increasing with

the buffer concentration. A mobile phase consisting of 0.5 m*M* sodium phosphate buffer yielded sufficient retention of the compounds and permitted analysis in a reasonable time (Table I). The separation between cephapirin and desacetylcephapirin, with a high selectivity factor of $\alpha = 11.65$, was too good. This was a disadvantage for the simultaneous determination of both substances. When the retention time for cephapirin was reasonable, the desacetylcephapirin was eluted very close to the solvent peak. An increase in the sodium phosphate concentration in the eluent gave a better retention for desacetylcephapirin but resulted in a rather retarded elution of the strongly tailing cephapirin peak. Cefazolin was eluted in between both compounds and could be used as the internal standard.

TABLE I

INFLUENCE OF THE SALT CONCENTRATION OF THE MOBILE PHASE ON CAPACITY FACTOR

Mobile phase	*k'*		
	Desacetylcephapirin	*Cefazolin*	*Cephapirin*
Water (I)	0.03	—	1.55
0.5 m*M* Sodium phosphate buffer, pH 7.5 (IIc)	0.97	4.30	11.30
1 m*M* Sodium phosphate buffer, pH 7.5 (IIb)	1.33	5.11	16.5
10 m*M* Sodium phosphate buffer, pH 7.5 (IIa)	5.66	13.2	±80

Ion-pair reversed-phase partition chromatography

Trioctylmethylammonium chloride (TOMA Cl) as counter ion. With water or phosphate buffer as the mobile phase, a drastic increase of the capacity factors of cephapirin and desacetylcephapirin was obtained after a unique injection of 15 µl of a 10% TOMA Cl solution in methanol. Cephapirin was no longer eluted as a detectable peak. This drastic enhancement of the capacity factors is due to the highly lipophilic nature of the ion pairs formed. The hydrophobic counter ion is also insoluble in the aqueous mobile phase and cannot be added in a known concentration. Therefore a less lipophilic counter ion had to be chosen.

Tetrabutylammomium hydrogen sulphate (TBA HSO_4) as counter ion. This water-soluble salt can be added to the mobile phase in an exactly known and readily adjustable concentration. The addition of 10 m*M* TBA HSO_4 to the 0.5 m*M* phosphate buffer (mobile phase IIc) caused an unexpected decrease of the capacity factors (Fig. 2). Even an increase of the salt concentration by use of a 10 m*M* sodium phosphate buffer did not result in higher capacity factors. However, a decrease of the counter ion concentration to 1 m*M* TBA HSO_4 (mobile phase III) caused a drastic increase of the capacity factors. These were brought back to reasonable values by a decrease of the sodium phosphate concentration to 1 m*M* .These experiments demonstrate the existence of an interaction between the counter ion and the phosphate ion. The decrease of the capacity factors at higher counter ion concentrations can be explained by micelle formation of the ion pair with the excess of counter ion, resulting in a higher water solubility and hence a lower retention on a reversed-phase packing[6]. This effect was also reported for other compounds and other counter ions[6,7].

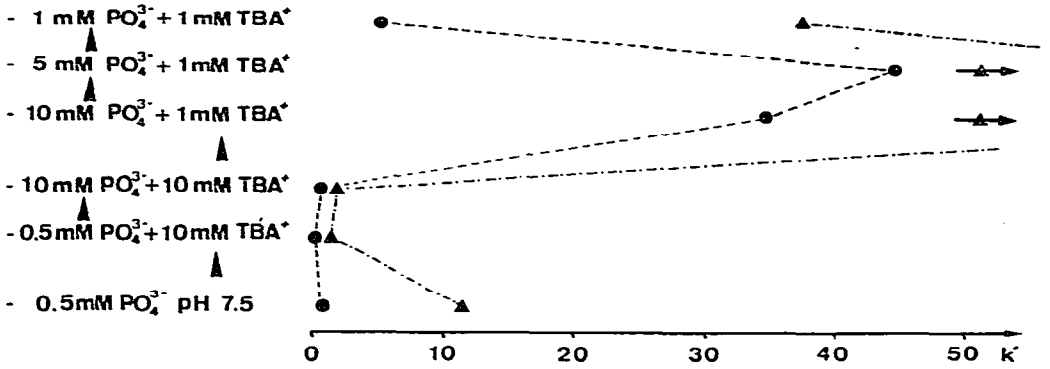

Fig. 2. Influence of the concentration ratio of sodium phosphate to TBA HSO_4 on k' of desacetylcephapirin (●) and cephapirin (▲). Column: Vydac RP, 500 × 2.1 mm I.D. Mobile phase: as indicated in the figure; flow-rate, 1 ml/min. Temperature, 25°.

The separation obtained with mobile phase III gave a change in selectivity between desacetylcephapirin and cephapirin compared to the same eluent system, but without counter ion (mobile phase IIb) (Table II). However, this chromatographic system had a practical disadvantage. The retention time of the compounds was influenced by the ionic strength of the sample, due to the low ionic strength of the mobile phase. This disadvantage could be eliminated by using a more concentrated sodium phosphate buffer as mobile phase (mobile phase IV). The very high capacity factors, obtained with this mobile phase (Table II), could be reduced by addition of methanol as organic modifier to the mobile phase. The content of the organic modifier was varied from 3 to 7.5% (mobile phase V) without great influence on the selectivity factor between cephapirin and desacetylcephapirin. Only a slight decrease of the selectivity factor α from 6.5 to 5.5 was observed when the methanol concentration was increased from 3 to 7.5%. Cefazolin was eluted in between both compounds and can be used as the internal standard. Table III shows the changed selectivity for these substances and especially for cefazolin relative to the other compounds with mobile phase V as compared to mobile phase IIa, where no methanol nor counter ion were used.

TABLE II

INFLUENCE OF ION-PAIR FORMATION ON THE CAPACITY FACTOR AND SELECTIVITY BETWEEN CEPHAPIRIN AND DESACETYLCEPHAPIRIN

Mobile phase	*Parameter*	*Desacetylcephapirin*		*Cephapirin*
1 m*M* Sodium phosphate buffer, pH 7.5 (IIb)	k'	1.33		16.5
	α		12.4	
1 m*M* TBA HSO_4 in 1 m*M* Sodium phosphate buffer, pH 7.5 (III)	k'	5		37.5
	α		7.5	
1 m*M* TBA HSO_4 in 10 m*M* Sodium phosphate buffer, pH 7.5 (IV)	k'	34.7		very high
	α			

TABLE III

INFLUENCE OF THE ION-PAIR FORMATION ON THE SELECTIVITY FACTOR BETWEEN CEPHAPIRIN, CEFAZOLIN AND DESACETYLCEPHAPIRIN

Mobile phase	*Parameter*	*Desacetylcephapirin*		*Cefazolin*		*Cephapirin*
10 m*M* sodium phosphate buffer, pH 7.5 (IIa)	k'	5.66		13.2		±80
	α		2.33		6.06	
1 m*M* TBA HSO_4, 7.5% methanol in 10 m*M* sodium phosphate buffer, pH 7.5 (V)	k'	5.39		20.7		29.5
	α		3.85		1.42	

The temperature plays an important role in partition chromatography and can have a big influence on the selectivity in ion-pair chromatography[8]. The temperature of the column was varied from 25° to 40°. Besides the decrease of the capacity factors, there was an increased selectivity factor between cephapirin and cefazolin at higher temperatures, because the decrease of the capacity factors was not in proportion. The selectivity factor between cefazolin and desacetylcephapirin decreased at higher temperatures.

Combination of counter ions. A combination of several counter ions with identical charge can have an effect on the selectivity, such that the elution order of the compounds may even be reversed[9]. From our experiments on ion-pair extraction of cephalosporins, it became evident that the addition of a second less lipophilic counter ion had a negative influence on the extraction. The decrease of the extraction yield was not in proportion for all cephalosporin derivatives. For these reasons, a second less lipophilic counter ion, tetraethylammonium hydroxide, was added to mobile phase V in a concentration of 1 m*M*. With this mobile phase VI the capacity factors of all the compounds decreased slightly, but the selectivity between the compounds remained unchanged. An increase of the column temperature caused an expected decrease of the capacity factors, but again an increased selectivity between cephapirin and cefazolin, and also between cephapirin and cephalothin, was observed with increasing temperature (Table IV). Fig. 3A shows a chromatogram obtained at 55°

TABLE IV

INFLUENCE OF TEMPERATURE ON k' AND α

Modifier, methanol; eluent, mobile phase VI.

Temperature (°C)	*Parameter*	*Desacetylcephapirin*		*Cefazolin*		*Cephapirin*		*Cephalothin*
20	k'	3.97		15.4		20.9		29.7
	α		3.87		1.36		1.42	
25	k'	3.42		11.3		15.5		23.1
	α		3.31		1.37		1.49	
30	k'	2.94		9.8		14.2		21.3
	α		3.33		1.45		1.50	
55	k'	1.15		3.09		5.08		8.86
	α		2.69		1.64		1.74	

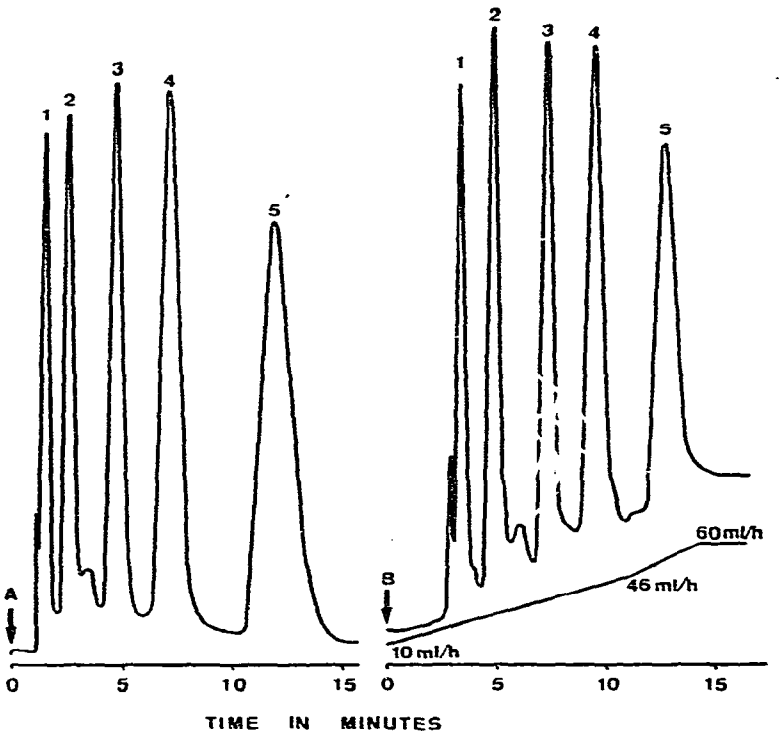

Fig. 3. Isovolumetric (A) and flow-programmed (B) separation of a few cephalosporin derivatives. Column: Vydac RP, 500 × 2.1 mm I.D. Temperature, 55°. Mobile phase: 1 m*M* TBA HSO_4, 1 m*M* TEA OH, 7.5% methanol in 10 m*M* sodium phosphate buffer, pH 7.5; flow-rate, 30 ml/h (A) and flow-programming (B). Peaks: 1 = 7-ACA; 2 = desacetylcephapirin; 3 = cefazolin; 4 = cephapirin; 5 = cephalothin.

under isovolumetric conditions and Fig. 3B a separation at 55° by flow programming. There was also a remarkable increase in column efficiency with increasing temperature (Table V). The enhanced efficiency and selectivity factor at higher temperatures resulted in a separation with a very good resolution.

TABLE V

INFLUENCE OF TEMPERATURE ON PLATE HEIGHT (*H*) AND RESOLUTION (*R*)
Eluent: mobile phase VI; flow-rate, 1 ml/min (except at 55°, 0.5 ml/min).

Temperature (°C)	*Parameter*	*Cefazolin*		*Cephapirin*
20	*H* (mm)	3.76		4.62
	R		0.8	
30	*H* (mm)	2.96		2.96
	R		1.08	
55	*H* (mm)	1.45		1.45
	R		1.90	

* The reduction of the plate height at 55° is partly due to the lower flow-rate.

The influence of the nature of the organic modifier on the selectivity was checked by replacing methanol by the less polar acetonitrile. The use of 5% acetonitrile as modifier (mobile phase VII) resulted in an increased selectivity between cephapirin and cephalothin, while the selectivity factors between the other compounds remained unchanged (Table VI). The influence of the temperature on the selectivity factor in this system was not as pronounced as with methanol as organic modifier. The efficiency of the system was also much improved at higher temperatures. The use of methanol–acetonitrile mixtures had only one advantage: the selectivity factor between cephapirin and cephalothin could be reduced to an intermediate value.

TABLE VI

INFLUENCE OF THE ORGANIC MODIFIER ON SELECTIVITY

Mobile phases: VI and VII.

Modifier	*Parameter*	*Desacetylcephapirin*		*Cefazolin*		*Cephapirin*		*Cephalothin*
7.5% Methanol (VI)	k'	1.15		3.09		5.08		8.86
	α		2.69		1.64		1.74	
5% Acetonitrile (VII)	k'	1.26		3.40		5.69		13.26
	α		2.70		1.67		2.33	

CONCLUSIONS

A separation of cephalosporin derivatives on an octadecylsilane reversed-phase packing is possible and several factors can be used to influence the selectivity of the system. The largest variations in selectivity could be achieved by ion-pair formation of the cephalosporins with tetrabutylammonium hydrogen sulphate as counter ion. Smaller variations of the selectivity could be obtained by changing the column temperature and by the choice of the organic modifier. The separation of the cephalosporins as ion pairs was obviously more efficient at elevated temperatures.

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