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INVESTIGATION OF PERCHLORATE, PHOSPHATE AND ION-PAIRING ELUENT MODIFIERS FOR THE SEPARATION OF CEPHALOSPORIN EPIMERS

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SUMMARY

The retention behavior of several pairs of 7α- and 7β-cephalosporin epimers was investigated using perchlorate, phosphate and ion-pairing eluent modifiers. At pH 2.5, sodium perchlorate, sodium phosphate and sodium pentanesulfonate all provided separation of epimers with free 7-amino groups. When the 7-amino group was blocked, as in cephalexin and cefaclor, sodium perchlorate gave the best separation at pH 2.5. A tetrabutylammonium ion-pairing system at pH 7.0 provided separation of all epimer pairs containing a free carboxylic acid at the 3 position. Hydrophobic, residual silanol and ionic interactions were factors in the retention mechanism of the cephalosporins under the conditions investigated. An ionic interaction of perchlorate with the protonated amine of the cephalosporin was postulated as an explanation of the retention and selectivity effects observed with perchlorate as an eluent modifier.

INTRODUCTION

Accurate high-performance liquid chromatographic (HPLC) analysis of a bulk cephalosporin for manufacturing and quality control purposes requires the ability to separate components very similar in structure to the main component. Some possible isomers of the basic cephalosporin structure are shown in Fig. 1. Included are the stereoisomer where the amine group at the 7-position is attached in the alpha configuration (7α-isomer) and the positional isomer in which the double bond of the cephem nucleus is at the 2-position (Δ^2-isomer). Another possible stereoisomer arises when the side chain in the 7-position is optically active. An example of this is cephalexin, where the desired isomer is the D-phenylglycyl side chain rather than the L-isomer.

Many cephalosporins may exist as anions or cations depending on the solution pH. This ionic character is well-suited for methods employing reversed-phase HPLC with ion-suppression[1,2] or ion pairing[3,4] eluent systems. Methods based on ion-pairing of the protonated amine or the carboxylate anion at low and high pH, respectively, are possible.

Salto[5] and Young[6] have described methods using phosphate buffer–methanol
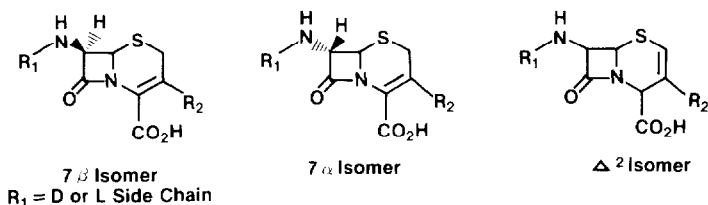
Fig. 1. Cephalosporin isomers.

eluents for the separation of cephalosporin isomers differing in side chain configuration. Kees *et al.*[7] recently reported an ion-pair separation of cefotetan epimers resulting from an asymmetric carbon in the side chain. The only report describing the separation of a 7α-isomer was that of Mason and Tranter[8] who investigated tetrabutylammonium and pentane sulfonate ion-pairing systems for the separation of cephalexin from its 7α, Δ^2 and L-side chain isomers.

It has been our experience that in many cases the 7α- and 7β-epimers of cephalosporins have been difficult to separate and that systems which separate these isomers will usually separate other closely related compounds. This report presents the results of an examination of eluent systems containing inorganic salt modifiers (with emphasis on phosphate and perchlorate) and traditional ion-pairing reagents for the separation of 7α- and 7β-cephalosporin epimers.

EXPERIMENTAL

Reagents

HPLC-grade acetonitrile and methanol were obtained from J. T. Baker (Phillipsburg, NH, U.S.A.). Tetrabutylammonium hydroxide (HPLC-grade, 1 M solu-

TABLE I

COMPOUNDS STUDIED
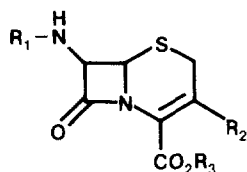
Compound		R_1	R_2	R_3
I	7-Aminodesacetylcephalosporanic acid	H	CH_3	H
II	7-Aminocephalosporanic acid	H	$CH_2O_2CCH_3$	H
III	3-Chloro-cephem nucleus	H	Cl	H
IV	7-Aminodesacetylcephalosporanic PNB ester	H	CH_3	PNB*
V	3-Chloro-cephem ester	H	Cl	PNB*
VI	Cephalexin	PG**	CH_3	H
VII	Cefaclor	PG**	Cl	H

* PNB = *p*-Nitrobenzyl.
** PG = Phenylglycyl.

tion) was obtained from Fisher Chemical (Fair Lawn, NJ, U.S.A.). Sodium pentane sulfonate (HPLC-grade) was obtained from Eastman (Rochester, NY, U.S.A.). Reagent-grade perchlorate salts were obtained from Fluka Chemical (Nauppauge, NY, U.S.A.). All other chemicals were reagent grade. Water for eluent and sample solutions was purified with a Milli-Q system from Millipore (Bedford, MA, U.S.A.).

All of the cephalosporin isomers studied were prepared at Eli Lilly and Company. These compounds are given in Table I. Sample solutions were prepared by dissolving 4–6 mg of the 7α- and 7β-isomers in 25 ml of 0.5 M hydrochloric acid–acetonitrile (1:1) for p-nitrobenzyl (PNB) esters or 0.25 M hydrochloric acid for all other compounds.

Apparatus

The chromatographic system consisted of a Model 8100 HPLC pump and autoinjector with a Model 8400 variable wavelength detector (Spectra Physics, San Jose, CA, U.S.A.). A 250 mm × 4.6 mm I.D. stainless-steel column prepacked with 5-μm Zorbax™ C_8 (DuPont, Wilmington, DE, U.S.A.) was used. Chromatograms were recorded with a Model 4100 computing integrator (Spectra Physics). The column temperature was maintained at 40°C and detection was at 254 nm. Flow-rates between 1.5 and 2.0 ml/min were used.

RESULTS AND DISCUSSION

The cephalosporins which were examined may be divided into three groups depending on the substituents at the 3 and 7 positions (Table I). Compounds I–III have free amine and carboxylic acid groups whereas IV and V both have the carboxylic acid blocked as an ester. Cephalexin (VI) and cefaclor (VII) have the 7-amino group blocked although a free amine is present in the phenylglycyl side chain.

TABLE II

SELECTIVITY (α) VALUES FOR THE SEPARATION OF CEPHALOSPORIN EPIMERS UNDER DIFFERENT MOBILE PHASE CONDITIONS

NaClO$_4$: 100 mM sodium perchlorate, pH 2.5 with sulfuric acid; TBA HPO$_4$: 10 mM tetrabutylammonium hydroxide, pH 7.0 with orthosphoric acid; NaPS: 100 mM sodium pentane sulfonate, pH 3.0 with sulfuric acid. NaH$_2$PO$_4$: 100 mM orthophosphoric acid, pH 2.5 with sodium hydroxide. Eluent strength: I–III, 5% methanol; IV–V, 40% methanol; VI–VII, 20% acetonitrile.

Compound	α			
	$NaClO_4$	$TBA\ HPO_4$	$NaPS$	NaH_2PO_4
I	1.83	1.35	1.29	1.42
II	1.64	1.26	—	—
III	1.34	1.11	1.37*	1.18
IV	1.22	—	1.15	1.09
V	1.18	—	—	—
VI	1.16	1.32	1.03**	1.00
VII	1.10	1.24	—	1.00

* 400 mM NaPS.
** 10 mM NaPS.
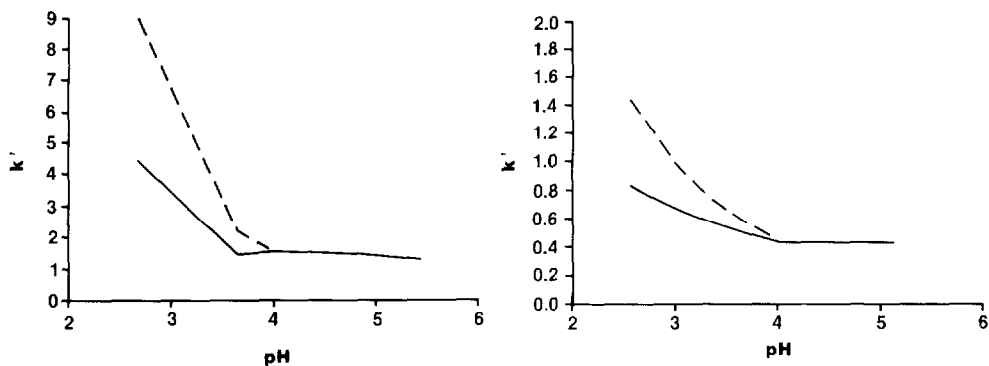
Fig. 2. k' versus pH for 7-aminodesacetylcephalosporanic acid epimers. Eluent: methanol–0.1 M sodium pentane sulfonate (10:90), (——) 7β-epimer; (– – –) 7α-epimer.

Fig. 3. k' versus pH for 7-aminodesacetylcephalosporanic acid epimers. Eluent: methanol–0.1 M sodium perchlorate (10:90). Key as in Fig. 2.

In Table II, the separation factors (α) for the cephalosporin epimers using four types of eluent modifiers are given. The phosphate buffer system and the pentane sulfonate and tetrabutylammonium ion-pairing systems were examined as examples of eluents commonly used for cephalosporin separations. The choice of perchlorate was prompted by its use as an ion-pairing reagent in normal-phase separations[9] and as an eluent modifier for controlling the retention of amines[10,11].

Of the compounds with free 7-amino and 3-carboxyl groups, the retention and separation of the epimers of 7-aminodesacetylcephalosporanic acid (I) were examined as functions of pH and modifier concentration. Fig. 2 illustrates the effect of pH on the retention of 7-aminodesacetylcephalosporanic acid epimers using sodium pentane sulfonate as a modifier. Retention and selectivity increased at pH values less than 4.0. This was in contrast to the constant k' values obtained by Salto et al.[2] for 7-aminodesacetylcephalosporanic acid using phosphate buffers between pH 2 and 8. The increase in retention is indicative of ion-pairing separations. The retention of this pair of epimers also increased at low pH with a sodium perchlorate eluent (Fig. 3). The magnitude of the retention increase was not as large as with pentane sulfonate but good separation of the epimers was obtained.

The separation of 7-aminodesacetylcephalosporanic acid epimers using perchlorate was compared to that obtained with sodium phosphate at pH 2.5. Chromatograms illustrating longer retention times and better separation obtained with sodium perchlorate are shown in Fig. 4.

The effects of varying the concentration of pentane sulfonate and perchlorate are shown in Figs. 5 and 6, respectively. With pentane sulfonate, the retention changes differently for the two isomers, giving a net improvement in α values with increasing concentration. With sodium perchlorate, k' and α were relatively constant. At modifier concentrations lower than 50 mM, retention increased and separation decreased for both systems. Also, poor peak shapes were obtained at low concentrations.

The free carboxylic acid group of 7-aminodesacetylcephalosporanic acid makes
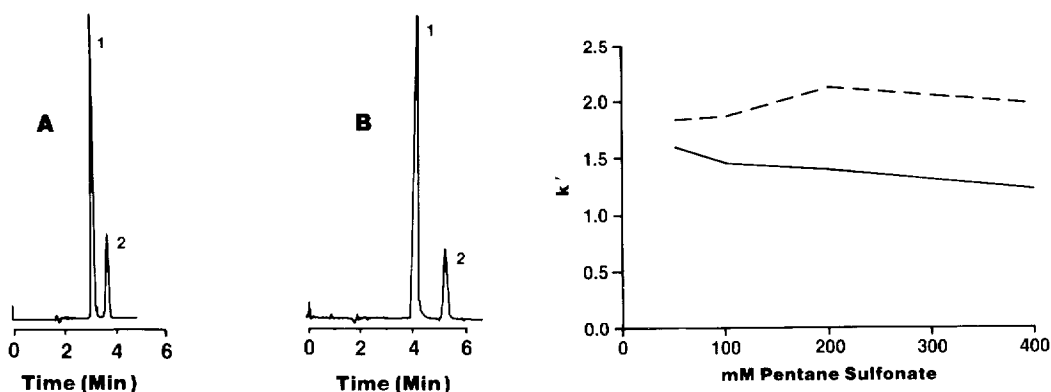
Fig. 4. Separation of 7-aminodesacetylcephalosporanic acid epimers using sodium perchlorate and sodium phosphate. (A) Eluent: methanol–0.1 M NaH$_2$PO$_4$ (5:95), pH 2.5 with sulfuric acid; (B) methanol–0.1 M sodium perchlorate (5:95), pH 2.5 with sulfuric acid. Peaks: 1 = β-isomer; 2 = α-isomer.

Fig. 5. k' *versus* sodium pentane sulfonate concentration for 7-aminodesacetylcephalosporanic acid epimers. Eluent: methanol–sodium pentane sulfonate solution (10:90), pH 3.5 with citric acid. Key as in Fig. 2.

possible ion-pairing at pH 7 with tetrabutylammonium ion. The k' values for 7-aminodesacetylcephalosporanic acid epimers as the tetrabutylammonium concentration was increased from 2.5 to 20 mM are shown in Fig. 7. Concentrations lower than needed with pentane sulfonate provided adequate separation with good peak shape. Above 5 mM, the k' and α values did not change significantly.

Effects of the 3-carboxyl group on the chromatography of cephalosporins are removed when this group is esterified. This is the case with 7-aminodesacetylcepha-losporanic PNB ester (IV) in which the acid is blocked as the *p*-nitrobenzyl ester. The retention of 7-aminodesacetylcephalosporanic PNB ester increased with pH using
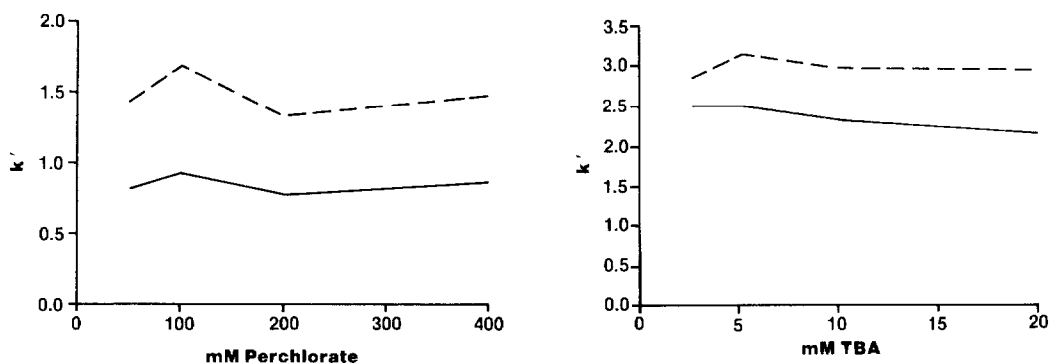

Fig. 6. k' *versus* sodium perchlorate concentration for 7-aminodesacetylcephalosporanic acid epimers. Eluent: methanol–sodium perchlorate (10:90), pH 2.5 with sulfuric acid. Key as in Fig. 2.

Fig. 7. k' *versus* tetrabutylammonium concentration for 7-aminodesacetylcephalosporanic acid epimers. Eluent: methanol–tetrabutylammonium hydroxide (20:80), pH 7.0 with orthophosphoric acid. Key as in Fig. 2.
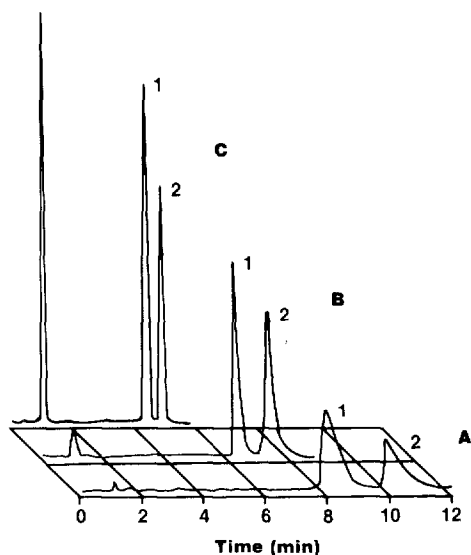
Fig. 8. Effect of sodium perchlorate concentration on retention and peak shape of 7-aminodesacetylceph-
alosporanic PNB ester epimers. Eluent: methanol–sodium perchlorate (40:60), pH adjusted to 2.5 with
sulfuric acid. (A) No sodium perchlorate, (B) 2.5 mM sodium perchlorate, (C) 25 mM sodium perchlorate.
Peaks: 1 = β-isomer; 2 = α-isomer.

sodium perchlorate eluent as expected for solvophobic interactions. At higher pH
values the amino group is not protonated and the compound is more hydrophobic.
Similar increases in retention with increased pH were observed with pentane sulfon-
ate.

Retention time and α values for the 7-aminodesacetylcephalosporanic PNB
ester epimers were constant as perchlorate concentration was varied between 50 and
400 mM. At lower concentrations of perchlorate, k' was larger. Fig. 8 illustrates the
decrease in k' as the perchlorate concentration was increased from 0 to 25 mM. At
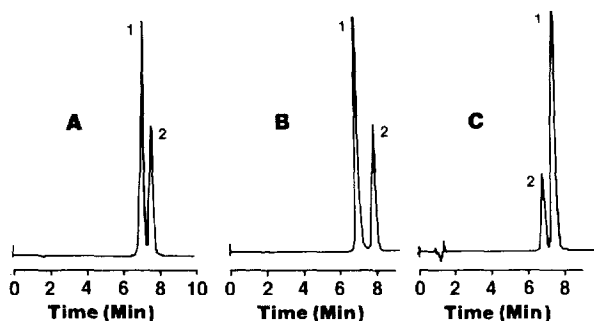concentrations higher than 25 mM, k' did not change but the band shape and reso-

Fig. 9. Comparison of inorganic salt modifiers for the separation of 7-aminodesacetylcephalosporanic
PNB ester epimers. Eluent: (A) methanol–0.10 M sodium bromide (40:60), pH 2.5 with sulfuric acid; (B)
methanol–0.10 M sodium perchlorate (40:60), pH 2.5 with sulfuric acid; (C) methanol–0.10 M ortho-
phosphoric acid (40:60), pH 2.5 with sodium hydroxide. Peaks: 1 = β-isomer; 2 = α-isomer.

lution improved. The improvement in band shape may be due to a decrease in occurrence of kinetically different retention mechanisms.

Sodium pentane sulfonate resulted in longer retention of 7-aminodesacetylcephalosporanic PNB ester than perchlorate at pH 2.5. When the methanol concentration of the eluent was increased to 50%, the pentane sulfonate system gave a separation equivalent to that obtained with sodium perchlorate with 40% methanol.

A comparison of separations of 7-aminodesacetylcephalosporanic PNB ester epimers obtained with sodium perchlorate, sodium bromide and sodium phosphate is shown in Fig. 9. Papp and Vigh[11] found no difference in the retention of aromatic amines using potassium perchlorate and potassium bromide modifiers. The k' of the 7β-epimer was the same with perchlorate and bromide, however, the 7α-epimer was retained longer with perchlorate. This produced baseline resolution between the compounds that was not achieved with sodium bromide. The retention order of the epimers was reversed with the sodium phosphate eluent.

Cephalexin (VI) and cefaclor (VII) have the 7-amino group blocked but contain a free carboxylic acid and an amino group in the side chain. These epimer pairs proved to be the most difficult to separate. Separation was not achieved with pentane sulfonate or phosphate buffer at pH 2.5. Fig. 10 shows separations that were obtained for cephalexin epimers using perchlorate at pH 2.5 and tetrabutylammonium at pH 7.0. For cephalexin and cefaclor, the tetrabutylammonium system provided a slightly better separation. As noted by Mason and Tranter[8], acetonitrile gave better band shapes than methanol for cephalexin.

In order to further investigate the separation of cephalosporin epimers using perchlorate as an eluent modifier, the effect of different cations was examined. At a concentration of 100 mM, the retention of 7-aminodesacetylcephalosporanic acid decreased slightly in going from lithium to potassium as the perchlorate counterion. A further reduction was observed with divalent cations. These observations support
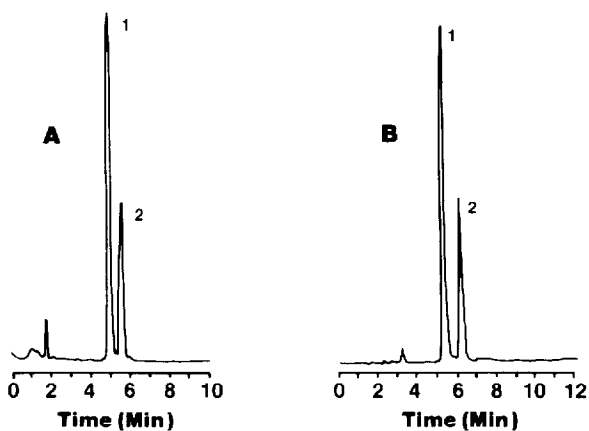

Fig. 10. Cephalexin epimer separation. Eluent: (A) acetonitrile–0.10 M sodium perchlorate (20:80), pH 2.5 with sulfuric acid; (B) acetonitrile–10 mM tetrabutylammonium hydroxide (20:80), pH 7.0 with orthophosphoric acid.

earlier work that demonstrated ion-exchange interactions between protonated amine and silanol groups on reversed-phased packings[10,11].

Bases such as triethylamine or morpholine are often used as modifiers to suppress interactions of basic compounds with silanol groups. Addition of triethylamine (10 mM) to pH 2.5 eluent for epimer separation resulted in lower k' values and loss of resolution.

The retention of cephalosporins appears to be controlled by a combination of three processes: hydrophobic interaction with the stationary phase, interaction with silanol groups on the stationary phase and interaction with ionic components of the mobile phase. The relative importance of these interactions is determined by properties of both the solute and the eluent.

The hydrophobicity of the solute will determine the eluent strength required for elution in a appropriate amount of time. For example, 7-aminodesacetylcephalosporanic PNB ester which contains a hydrophobic ester group, required 40% methanol for elution, whereas 7-aminodesacetylcephalosporanic acid which has a polar carboxyl group, required only 5% methanol.

Ion exchange with silanol groups has been used to explain the decrease in retention of protonated amines with the addition of inorganic salts[10,11]. This also occurred for cephalosporins at low pH, although at high salt concentrations (> 50 mM) retention was not changed significantly.

Differences in retention behavior observed when using different salts with the same cation in the eluent is evidence for interaction of cephalosporin epimers with the anion of the added salt. This was most evident in the separation of the isomers of 7-aminodesacetylcephalosporanic PNB ester where the retention order changed when going from phosphate to perchlorate. The formation of ion pairs between protonated amines and inorganic anions has been described by Schill *et al.*[12], who found that phosphate did not form extractable ion pairs with basic drugs. In the case of 7-aminodesacetylcephalosporanic PNB ester, where differences between phosphate and perchlorate were most apparent, the eluent contained 40% methanol. This may have caused ionic interactions to be more important than in highly aqueous eluents where ionic species are hydrated to a greater extent.

Whether ionic interaction occurs in the mobile phase or next to the stationary phase is uncertain. A stronger interaction of perchlorate and the protonated amine of the 7α-isomer at the surface could explain a retention time of the 7α-isomer longer than that of the 7β-isomer. Computer-calculated minimum energy structures for each epimer of 7-aminodesacetylcephalosporanic acid indicated that the amino group of the 7β-isomer is closer to and perhaps more shielded by the β-lactam and dihydrothiazine ring systems than the 7α-amine. The greater accessibility of the protonated 7α-amine could facilitate ionic interactions occurring at the stationary phase surface by removing steric hindrance.

CONCLUSIONS

Sodium perchlorate as an eluent modifier was shown to be superior to sodium phosphate and equivalent to pentane sulfonate and tetrabutylammonium modifiers for the separation of cephalosporins. As an eluent modifier, sodium perchlorate has advantages in addition to providing good selectivity and peak shape. It is a suitable

supporting electrolyte for electrochemical detection, allows low-wavelength UV detection and is significantly less expensive than traditional ion-pairing reagents.

Hydrophobic, residual silanol and ionic interactions were factors in the retention mechanism of the cephalosporins under the conditions investigated. An ionic interaction of perchlorate with the protonated amine of the cephalosporin was postulated as an explanation of the retention and selectivity effects observed with perchlorate as an eluent modifier.

ACKNOWLEDGEMENT

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