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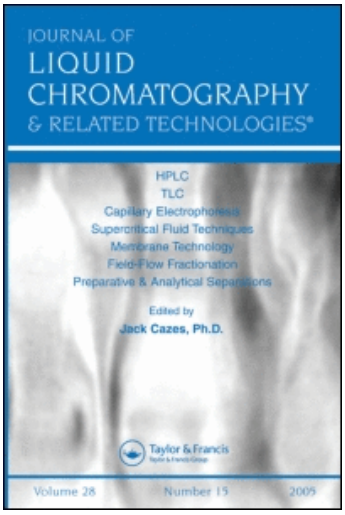

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Separation of Selected Beta Lactam Antibiotic Epimers on Gamma Cyclodextrin, Ion Exchange Ethylvinylbenzene/Divinylbenzene/Copolymer and Poly(Styrene-Divinylbenzene) Copolymer Stationary Phases

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SEPARATION OF SELECTED BETA LACTAM ANTIBIOTIC EPIMERS ON GAMMA CYCLODEXTRIN, ION EXCHANGE ETHYLVINYL-BENZENE/DIVINYLBENZENE/COPOLYMER AND POLY(STYRENE-DIVINYLBENZENE) COPOLYMER STATIONARY PHASES

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ABSTRACT

High performance liquid chromatography (HPLC) stationary phases of gamma cyclodextrin, ion exchange ethylvinylbenzene/divinylbenzene (EVB/DVB) copolymer and poly (Styrene-divinylbenzene) (PRP-1) copolymer were investigated for the separation of beta lactam antibiotic epimers of cephalexin, moxalactam, ticarcillin, and carbenicillin. A combination of ion pair chromatography and inclusion complex formation improved the selectivity of moxalactam epimers on gamma cyclodextrin but had no effect on cephalexin epimers. A 10% increase in resolution was obtained for the moxalactam epimers on gamma cyclodextrin when 3mM tetrapropylammonium bromide was present in the mobile phase. Ion-exchange and reverse phase properties of the ion-exchange EVB/DVB phase coupled with perchlorate or sodium pentane sulfonate ion pair chromatography were also successful in separating some of the epimers. Retention and separation behavior of the analytes could not be easily predicted using this multiphase system. The PRP-1 phase was capable of easily resolving the epimers studied with minor adjustments in the mobile phase pH and organic modifier concentration. The PRP-1 phase would be highly recommended for the separation of antibiotic epimers based on the model compounds studied.

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INTRODUCTION

The beta lactam epimers of cephalosporins and penicillins contain two chiral carbons in the 7-aminocephalosporanic acid or 6-aminopenicillanic acid ring and one chiral carbon in the side chain at the C-7 or C-6 position. The epimers usually possess different biological activity and stability. Moxalactam exists as a racemic mixture of R and S epimers and the R-epimer is two to three times more active in vitro than the S-epimer (1). In vivo stability of a new antibiotic which has a penem structure was found to vary between the epimers with a greater stability to renal dehydropeptidase-I found for the S-epimer (2).

Assays of beta lactam antibiotics were originally performed by microbiological assays. Disadvantages of these assays included relatively long analysis time and inability to detect more than one antibiotic, epimers of the antibiotics, and precursors or decomposition products of the antibiotics simultaneously. Many of the new assay methods use HPLC techniques.

Ion exchange chromatography has been used to separate metabolites, degradation products and precursors of cephalosporins. A mixture of eleven cephalosporins precursors were separated using a aminopropyl column operated in the ion-exchange mode (3). Recently, a new multi-phase stationary phase which combines an ethylvinylbenzene/divinylbenzene (EVB/DVB) macroporous substrate resin with reversed phase properties and a pellicular layer of 60 A diameter latex with ion exchange properties covalently bound to the resin was reported to provide separation of a mixture of cephalosporins and their precursors (4). However, none of these methods were shown to separate the individual epimers.

A few reports on the separation of the epimers of moxalactam and other selected antibiotics have utilized non-polymeric reversed

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phase stationary phases. Due to differing physico-chemical properties of the antibiotics, a variety of stationary phases had to be employed to resolve the epimers and their precursors (5-9). The use of alkyl bonded phases has been shown to contribute to increased epimer retention and separation. Also the addition of amine modifiers to the mobile phase has caused loss of epimer resolution on these phases (9).

Cyclodextrin HPLC stationary phases have been reported to separate D,L Norgestrel epimers (10). Ion pairing in combination with cyano and octadecylsilane stationary phases have been shown to improve enantiomer separations when cyclodextrins are also present in the mobile phase (11). Polymeric stationary phases of the poly(styrene-divinylbenzene) type have also shown a selectivity for geometrical isomers by combining pi-pi interactions with reversed phase properties. A study has demonstrated that this phase allows the separation of the Z and E isomers of the beta lactam antibiotic aztreonam along with its precursors (12).

The objective of this study was to investigate the applicability of using selected polymeric stationary phases for the separation of the beta lactam antibiotic epimers. Phases studied included gamma cyclodextrin, a multi-phase ion exchange ethylvinylbenzene/divinylbenzene copolymer, and a poly (styrene-divinylbenzene) copolymer. It was thought that these phases might provide better resolution and faster elution compared to non-polymeric stationary phases currently in use and that a single stationary phase might be satisfactory for most, if not all, of the epimer separations. Where possible, the nature of the analyte/stationary phase interactions was studied. The epimers of cephalexin and moxalactam and R,S-mixtures of ticarcillin and carbenicillin were obtained and used as model compounds in this study (see Figure 1).

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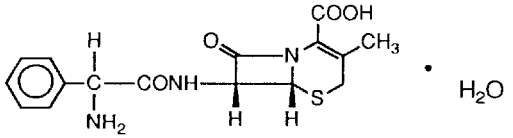
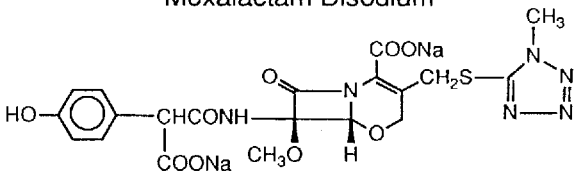
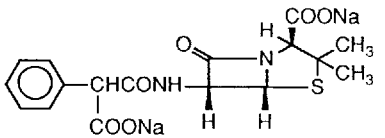
Cephalexin



Moxalactam Disodium



Carbenicillin Disodium



Ticarcillin Disodium

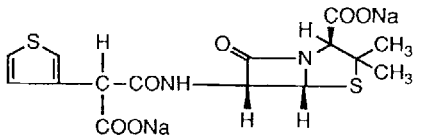


FIGURE 1 Structures of Selected Beta-Lactam Antibiotics

EXPERIMENTAL

Reagents and Chemicals

R and S epimer enriched moxalactam disodium were supplied by Shionogi Research Labs (Osaka, Japan). D and L cephalexin epimers were supplied by Kanazawa University (Kanazawa, Japan) and additional L- cephalexin epimer was supplied by Eli Lilly and Co. (Indianapolis, Indiana). R,S-Ticarcillin disodium was obtained from Beecham Labs. (Bristol, TN) and R,S-Carbenicillin disodium was obtained from Pfizer Inc. (New York, NY). HPLC grade acetonitrile, methanol and water for ion exchange chromatography were obtained from J. T. Baker Inc. (Phillipsburg, NJ). Double distilled water obtained in house was used for all other chromatography. HPLC grade sodium pentane sulfonate was obtained from Eastman Kodak Co. (Rochester, NY) and tetrapropylammonium bromide from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of highest quality and obtained from J. T. Baker Inc. (Phillipsburg, NJ).

Instrumentation

The HPLC system consisted of a Beckman pump Model 110B (Fullerton, CA), a Rheodyne injector Model 7150 (Cotati, CA) equipped with a 20 ul loop, a Beckman variable wavelength UV detector Model 163 set at 254 nm, and a Hewlett Packard integrator Model 3390 (Avondale, PA). A Fiatron HPLC temperature control system (Oconomovoc, WI) was used to adjust column temperature for the gamma cyclodextrin stationary phase.

Columns

A 250 x 4.6 mm i.d. Cyclobond II column packed with gamma cyclodextrin bonded to silica gel of 5 micron particle size was obtained from Applied Separation Technologies Inc. (Whippany,NJ). A 250 x 4 mm i.d. Omnipac PCX-500 column packed with 60 angstrom pellicular cation exchange latex bound to ethylvinylbenzene / divinylbenzene macroporous substrate resin of 8 micron particle size was supplied by Dionex Corp. (Sunnyvale, CA). A 150 x 4 mm i.d.

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PRP-1 column packed with poly(styrene-divinylbenzene) of 5 micron particle size was obtained from Hamilton Co. (Reno, NV).

Sample preparation

An approximate 0.5 mg/ml aqueous solution of each antibiotic epimer or 1.0 mg/ml of the R,S-mixture was prepared in one ml volumetric flasks. Aliquots (20ul) of each solution were mixed in another one ml volumetric flask and diluted to volume with HPLC water. These solutions were prepared fresh daily, filtered and sonicated for 5 mins. prior to injection.

Mobile phases

The mobile phase solutions were filtered through a 0.45 micron nylon 66 membrane filter from MSI Inc. (Westboro, MA) and then sonicated prior to use. The following mobile phases were prepared for use in this study : mobile phase A = 10:90 methanol-0.05M ammonium acetate buffer adjusted to pH 4.5 with glacial acetic acid. B = mobile phase A adjusted to pH 6.0 with glacial acetic acid. C = 25:75 acetonitrile-HPLC water containing 2.5 mM sodium pentane sulfonate. D = 25:20:55 acetonitrile-0.06M $HCLO_4$-HPLC water. E = mobile phase D with 15:15:70 composition. F = mobile phase D with 20:18:62 composition. G = 12:88 acetonitrile-0.05M KH_2PO_4 adjusted to pH 3.0 with concentrated phosphoric acid. H = 3:97 acetonitrile-0.05M KH_2PO_4 adjusted to pH 5.5 with KOH.

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RESULTS AND DISCUSSION

Gamma Cyclodextrin Stationary Phase

The gamma cyclodextrin stationary phase was initially selected to investigate resolution of the model beta lactam epimers since optimal candidates for chiral recognition on the gamma cyclodextrin phase are those analytes whose structures contain fused ring substituents connected to a chiral center (13). The cyclodextrin phase operates via an inclusion complexation mechanism for the

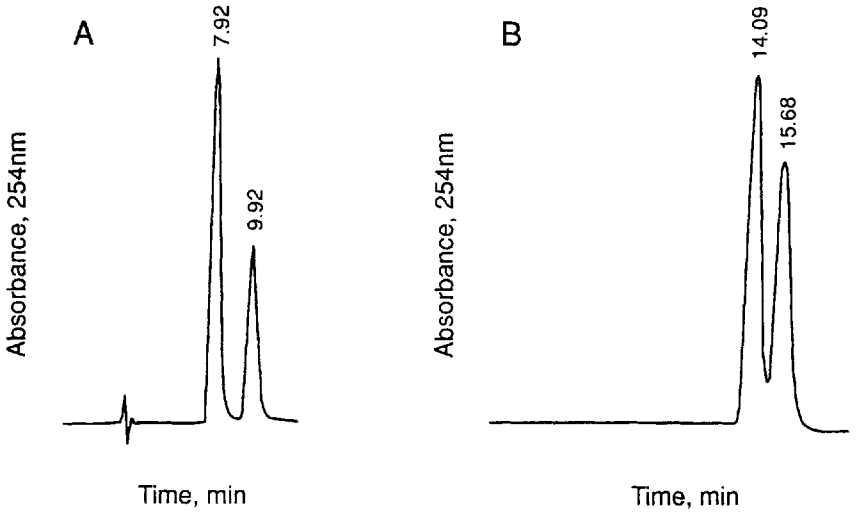

FIGURE 2

Chromatograms of cephalexin and moxalactam epimers on gamma cyclodextrin column (Cyclobond II, 5μm). A: L-epimer of cephalexin at 7.92 min and D-epimer at 9.92 min. Mobile Phase: 5/95 absolute methanol - 0.05M ammonium acetate, pH 4.5 at flow rate 0.8 ml/min. B: R-epimer of moxalactam at 14.09 min and S-epimer at 15.68 min. Mobile Phase: 10/90 absolute methanol - 0.05 M ammonium acetate, pH 6.0 at 1.0 ml/min flow rate.

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separation of analytes. Separation of the cephalexin epimers (Rs 2.0) was readily obtained on this stationary phase as shown in Figure 2 . Using a similiar mobile phase containing only 10:90 absolute methanol-pH 6.0 buffer, the separation of moxalactam epimers (Figure 2) (Rs 1.2) was also achieved.

Since inclusion complexation along with ion pairing techniques have been shown to produce an improvement in selectivity of enantiomer separations (11), tetrapropylammonium bromide (TPAB) was added to the mobile phase as an ion pair reagent to investigate its effect on epimer resolution. The presence of the TPAB reagent did not cause a significant increase in resolution of the cephalexin epimers as indicated by the Rs values in Table 1 for mobile phase A

TABLE 1

Separation Parameters for Selected Antibiotic Epimers on Gamma-Cyclodextrin, Ion Exchange EVB/DVB Copolymer, and Poly(Styrene-Divinylbenzene) Copolymer

Stationary Phase	Drug	k'$_1$[a]	Rs	alpha	Mobile[b] Phase
Gamma Cyclodextrin	Cephalexin	1.09	1.51	1.33	A *
		1.15	1.56	1.33	A
	Moxalactam	3.83	1.35	1.15	B *
		3.17	1.20	1.15	B
Ion Exchange EVB/DVB,	Cephalexin	7.58	2.72	1.31	C
		4.27	2.10	1.25	D
	Moxalactam	9.20	1.20	1.14	E
		4.49	0.85	1.13	F
Poly(Styrene-Divinyl-benzene)	Cephalexin	0.57	2.90	3.10	G
	Moxalactam	7.23	1.51	1.29	H
	Ticarcillin	3.39	1.23	1.30	H
	Carbeni-cillin	4.54	1.53	1.35	H

[a] k'$_1$ is the capacity factor of the first eluted epimer.
[b] Mobile phase compositions are described in the experimental section. The flow rate for mobile phase A and B was 0.8 ml/min; the flow rate for C-H was 1.0 ml/min.An asterick (*) means that 3 mM TPAB ion pair reagent was present in the mobile phase.

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with or without the TPAB, but TPAB did affect retention of these epimers while maintaining adequate resolution of the peaks. This effect could potentially be useful for the simultaneous separation of a series of antibiotic epimers. The use of a cyclodextrin phase coupled with ion-pair formation for enhanced separations of epimers has not been reported previously in the litrature. Resolution of the moxalactam epimers did improve with TPAB ion pairing (Rs 1.35 vs 1.20) as seen in Table 1. The retention of these epimers on this gamma cyclodextrin phase was very similar to that expected when conventional reversed phase ion-pairing techniques are used.

The best separation of the cephalexin epimers on the gamma cyclodextrin column was achieved when the pH of the buffer in the

TABLE 2

Effect of Variation of Tetrapropylammonium Bromide (TPAB) Concentration on Separation Parameters for Cephalexin and Moxalactam Epimers on Gamma Cyclodextrin

Drug	k'_1[a]	Rs	alpha	TPAB (mM)	Mobile Phase[b]
Cephalexin	0.63	0.96	1.29	3	A
	0.64	1.03	1.27	5	
	0.58	1.00	1.27	10	
Moxalactam	3.17	1.35	1.15	3	B
	3.76	1.05	1.14	5	
	5.01	1.10	1.15	10	

[a] k'_1 is the capacity factor of the first eluted epimer
[b] The flow rate was 1.0 ml/min.

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mobile phase was 4.5. Interestingly, the isoelectric point for these epimers is reported to be pH 4.5 . The retention of these epimers decreased as shown in Table 2 when the TPAB concentration was increased from 3 to 10 mM in the 10:90 methanol-0.05M aqueous ammonium acetate mobile phase, pH 4.5 . This observed decrease in retention may be due to repulsion of the epimers from inclusion cavities where the ion pairs have competitively become a guest. However, the retention of the moxalactam epimers increased with increasing TPAB concentration indicating that other factors are affecting the retention of the cephalexin epimers on this column.

The mechanism of inclusion complexation can be controlled by temperature thereby allowing for enhancement of resolution. When the gamma cyclodextrin column was heated to 55° C, complete resolution of the moxalactam epimers was observed. However, heated columns are not practical for these epimer assays since heat has been shown to accelerate epimerization of racemic beta lactam antibiotics.

The best resolution of the Carbenicillin epimers (Rs 0.91) was achieved on the gamma cyclodextrin phase using a mobile phase

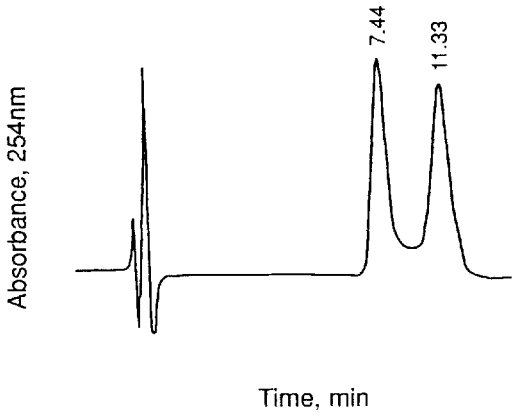

FIGURE 3

Chromatogram of cephalexin epimers on ion exchange - EVB/DVB copolymer column (Omnipac PCX-500, 8.5 μm). D-epimer at 7.44 min and L-epimer at 11.33 min. Mobile Phase: 20/25/55 0.6 M perchloric acid - acetonitrile - HPLC grade water at a 1 ml/min flow rate.

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consisting of 4:96 acetonitrile/0.1 M dibasic ammonium phosphate pH 6.0 containing 3mM TPAB. However, little or no success was obtained separating the ticarcillin epimers on the stationary phase using similar HPLC conditions successful for the other model beta lactam compounds.

Ion Exchange EVB/DVB Copolymer Stationary Phase

The multiphase ion exchange and EVB/DVB copolymer column contains both a pellicular ion exchange latex and an adsorptive macroporous substrate resin to allow both ion exchange and reversed phase mechanisms to occur. Each mechanism can be used simultaneously or independently according to the mobile phase composition. Figure 3 shows a chromatogram of the resolved cephalexin epimers on this stationary phase using an aqueous acetonitrile mobile phase containing perchlorate as ion-pair reagent. Perchlorate has been reported to be of value in a gradient separation of a cephalosporin mixture (4). In this lab, we have also found that resolution of the

TABLE 3

The Effect of Sodium Pentane Sulfonate (SPS) Ion Pair Concentration in the Mobile Phase[a] on Separation and Capacity Factors of Cephalexin Epimers with Ion Exchange EVB/DVB Copolymer

SPS concentration (mM)	k'_1[b]	k'_2[c]	alpha
2.5	7.58	9.96	1.31
5.0	2.46	3.33	1.35
10.0	3.96	5.54	1.40

[a] Mobile phase C composition is listed in Experimental Section
[b] k'_1 is the capacity factor of the first eluted epimer
[c] k'_2 is the capacity factor of the second eluted epimer

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cephalexin epimers is enhanced (Rs 2.72 vs 2.10) when perchlorate is replaced by sodium pentane sulfonate (SPS) (see Table 1) (9). Therefore, ion pairing with SPS was used to further examine the effect of simultaneous ion exchange and reverse phase mechanisms of this stationary phase on epimer resolution. The effects of SPS ion pairing on the retention and separation of the cephalexin epimers are shown in Table 3.

As SPS concentration increased, k'values for each epimer first decreased and then increased while alpha only increased. In conventional reverse phase chromatography, retention and hence separation of an analyte generally increase as a result of an increase in ion pair concentration. This observed chromatographic behavior of the cephalexin epimers on this multiphase column at 2.5 and 5.0 mM SPS ion pair concentration may be due to the sodium ion functioning as an competing cation with respect to ion exchange sites present on the stationary phase. However, at 10 mM SPS concentration, an increase in retention of the cephalexin epimers was obtained despite the presence of sodium ions in the mobile phase.

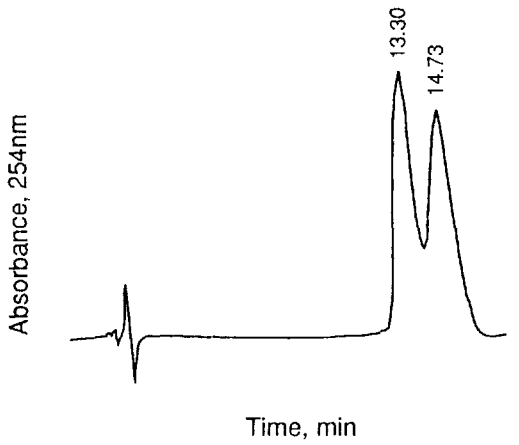

FIGURE 4

Chromatogram of moxalactam epimers on ion exchange - EVB/DVB copolymer column (Omnipac PCX-500, 8.5 μm). R-epimer at 13.3 min and S-epimer at 14.73 min. Mobile Phase: 20/18/62 0.6 M perchloric acid - acetonitrile - HPLC grade water at a 1 ml/min flow rate.

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Some studies with perchlorate ion as ion-pair reagent were also performed on this multiphase column. Ion pairing of the moxalactam epimers with perchlorate ion only resulted in a partial separation of the epimers (Rs 0.85)(Figure 4). Resolution of the epimers increased (Rs 1.20) when the acetonitrile concentration was lowered to 15 % v/v, but the retention time for the first eluted moxalactam epimer increased from 13 min to 19 min.

Poly (Styrene Divinylbenzene) Copolymer Stationary Phase (PRP-1)

Figure 5 shows the resolution of cephalexin epimers on the PRP-1 phase using a mobile phase of acetonitrile and 0.05 M phosphate buffer pH 3.0. The moxalactam epimers containing a carboxyl group on the chiral carbon in the C-7 side chain were also resolved (Figure 5) (Rs 1.5) using a similiar mobile phase that contained less acetonitrile and more pH 5.5 phosphate buffer. The pKa values for the C-4 COOH and the side chain COOH functional

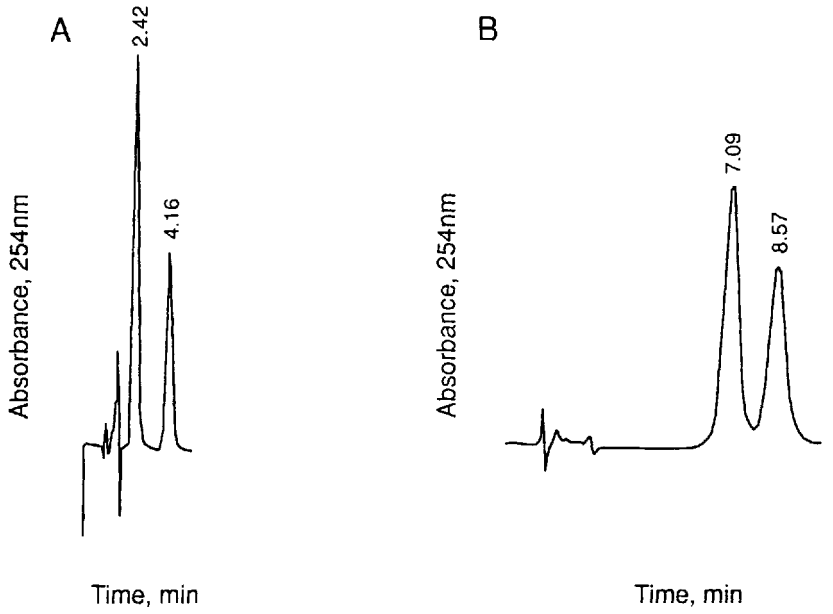

FIGURE 5

Chromatograms of cephalexin and moxalactam epimers on poly (styrene-divinylbenzene) copolymer column (PRP-1, 5 μm). A: D-epimer of cephalexin at 2.42 min and L-epimer of cephalexin at 4.16 min. Mobile Phase: 12/88 acetonitrile - 0.05 M potassium diacid phosphate, pH 3.0 at 1.0 ml/min flow rate. B: R-epimer of moxalactam at 7.09 min and S-epimer at 8.57 min. Mobile Phase: 3/97 acetonitrile - 0.05 M potassium diacid phosphate, pH 5.5 at a 1.0 ml/min flow rate.

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groups in moxalactam are 2.17 and 3.38, respectively (14). The selectivity of this stationary phase for an analyte(s) results from adsorption, partitioning, and pi-pi interactions. In a study of elution behavior of substituted benzoic acids, it was found that the selectivity of PRP-1 for these acids was enhanced by varying the pH of the mobile phase (15).

The Rs values shown in Table 1 obtained on the PRP-1 phase indicated that epimers with either an amino or carboxyl group

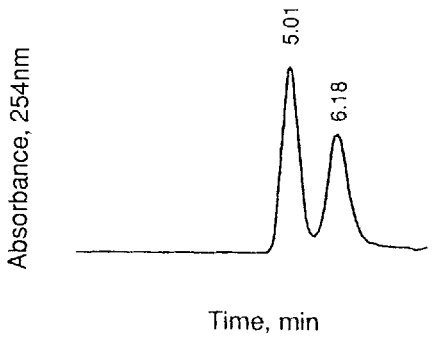

FIGURE 6

Chromatogram of ticarcillin epimers on poly(styrene-divinylbenzene) copolymer column (PRP-1, 5 μm). R-epimer at 5.01 min and S-epimer at 6.18 min using a mobile phase of 3:97 acetonitrile - 0.05 M potassium diacid phosphate, pH 5.5 and a flow rate of 1.0 ml/min.

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substitution on the C-7 side chain can be easily separated by adjusting the pH and composition of the mobile phase. Since pH greatly influences retention and resolution of these antibiotic epimers on conventional reversed phase stationary phases, the adjustment of mobile phase pH to 3.0 and 5.5 for cephalexin and moxalactam, respectively, also favorably affected retention and resolution of the epimers on the PRP-1 phase. PRP-1 displayed excellent selectivity for these antibiotic epimers as seen in the chromatogram for ticarcillin (Figure 6). This antibiotic showed the smallest Rs value (Rs 1.23) among the antibiotics resolved on this column (see Table 1). The extreme hydrophobic nature of the PRP-1 phase and the ionic nature of cephalexin at pH 3.0 probably account for the short retention times of 2.4 and 4.2 min for D and L cephalexin, respectively.

Summary

The gamma cyclodextrin stationary phase is capable of separating the epimers of the model beta lactam antibiotics studied.

However, the addition of TPAB as an ion pair reagent to the mobile phase appears to be necessary to modify retention while maintaining adequate resolution for these epimers. The multiphase ion exchange EVB/DVB copolymer phase shows selectivity for the epimers, but epimer separation is highly dependent upon the mobile phase composition and the physicochemical nature of the ion pair reagent used. The poly (styrene divinylbenzene) copolymer phase shows good selectivity and fast elution for these model epimers with only pH and mobile phase composition changes required. Thus, it appears that the PRP-1 phase would be the best overall stationary phase for the separation of beta lactam antibiotic epimers based on the model compounds studied.

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