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ACKNOWLEDGMENTS

The authors thank Mrs. Susan Magic and Dr. Paulus Tsui for helpful discussions.

Interactions of Cephalosporins and Penicillins with Nonpolar Macroporous Styrenedivinylbenzene Copolymers

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Received December 1, 1980, from the *Departamento de Química, Facultad de Veterinaria, Universidad de León, León, Spain.* Accepted for publication February 11, 1981.

Abstract □ The conditions of sorption of penicillins and cephalosporins on nonionic macroporous copolymers of styrenedivinylbenzene were evaluated. By increasing the methanol concentration in the eluent, the sorption decreased. Salts exerted little influence on sorption. However, pH exerted a remarkable effect on sorption, and the capacity factor variations according to the pH are quantitatively described. Some typical separations are shown.

Keyphrases □ Cephalosporins—interactions with a nonpolar, macroporous styrenedivinylbenzene copolymer stationary phase □ Penicillins—interactions with a nonpolar, macroporous styrenedivinylbenzene copolymer stationary phase □ Capacity factors—cephalosporins and penicillins, interactions with a nonpolar, macroporous styrenedivinylbenzene copolymer stationary phase □ Antibiotics—cephalosporins and penicillins, interactions with a nonpolar, macroporous copolymer stationary phase

Nonionic polymers have come into use recently as nonpolar stationary phases in high-pressure liquid chromatography (HPLC), primarily because of the work of Pietrzyk and coworkers (1–5). The main advantages of these kinds of packing are their low cost, ability to function at any level, compatibility with most solvents, and high adsorbency properties.

Penicillins and cephalosporins are β-lactam antibiotics produced by acylation with different radicals of the amino groups of 7-aminocephalosporanic, 7-aminodeacetoxycephalosporanic, or 6-aminopenicillanic acids, which give cephalosporins, deacetoxycephalosporins, or penicillins, respectively. They are particularly suitable as models to study the effect of protonic equilibria on retention by nonpolar macroporous copolymers because of their structural similarities and, depending on the medium pH, they can be found in undissociated, anionic, cationic, or zwitterionic form.

In this study, a macroporous styrenedivinylbenzene copolymer[1] adsorbent with an average surface area of 780 m^2/g and an average pore diameter of 50 Å (3) was used. The purposes of this work were to study the variables affecting the retention of penicillins and cephalosporins on resin copolymers and to demonstrate that the equations describing the retention of ionic solutes by the nonpolar stationary phases are applicable to the interactions of penicillins and cephalosporins with the copolymer. The results may assist in the development of new analytical methods and may improve β-lactam antibiotic extraction and purification methods; the optimum conditions of adsorption and elution may then be predicted.

EXPERIMENTAL

7-Aminodeacetoxycephalosporanic acid (I), 7-phenylacetamidodeacetoxycephalosporin (II), cephalexin (III), cephradine (IV), 7-aminocephalosporanic acid (V), cephalosporin C (VI), cephalothin (VII), cephaloridine (VIII), cefazolin (IX), 6-aminopenicillanic acid (X), ampicillin (XI), penicillin G (XII), penicillin V (XIII), phenoxyethylpenicillin (XIV), 7-aminodeacetylcephalosporanic acid (XV), and deacetylcephalothin (XVI) were used as supplied[2].

The macroporous styrenedivinylbenzene copolymer[1] was supplied as spheres with an average size of ~500 μm. The resin particles were washed by extraction (soxhlet) with methanol and allowed to dry; they then were ground and sieved, with the 40–70-μm particles being selected (1). The size distribution of these particles was analyzed[3].

The chemicals were the highest commercial grade available and were used without any further purification. The buffer solutions were 0.05 *M* phosphate, and sodium sulfate was used to obtain the desired ionic strength. The mixtures of methanol–buffer solution were expressed in volume percent.

Chromatographic Conditions—A 2.5 × 600-mm steel column, equipped with suitable fittings and a 10-μm filter, was dry packed with the copolymer between 40 and 70 μm.

The liquid chromatograph was fitted with a pump[4], a 20-μl sample valve injector[5], a 250-nm UV detector[6], and a strip-chart recorder[7].

The chromatographed substances were prepared in the mobile phase at a concentration of 0.5 mg/ml, except for the penicillins, which were dissolved at a concentration of 3 mg/ml. In each case, a 20-μl aliquot was injected. The flow was maintained at 0.5 ml/min, and the sensitivity of the UV detector was set between 0.004 and 0.128 aufs, according to the absorptivity of the analyzed product.

The capacity factors (k') were calculated in accordance with:

$$k' = \frac{V_R - V_0}{V_0} \qquad \text{(Eq. 1)}$$

[1] Amberlite XAD-4, Rohm & Haas Chemical Co.

[2] Courtesy of Antibióticos, S.A., Madrid, Spain.
[3] Sharples Micromerograph, Franklin Electronics, Bridgeport, Pa.
[4] Constametrik 00, Laboratory Data Control.
[5] Model 7120, Rheodyne.
[6] UV III monitor, Laboratory Data Control.
[7] Model XER, Sargent-Welch Scientific Co., Skokie, Ill.

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Table I—Apparent pK'a Values of Investigated Substances Determined Potentiometrically at 20°, an Ionic Strength of 0.15, and 50% (v/v) Methanol

Compound	$pK'a_1$	$pK'a_2$
I	3.63[a]	5.16[a]
II	4.41	—
III	4.51	6.97
IV	4.63	7.19
V	3.49[a]	4.71[a]
VII	4.32	—
IX	3.85	—
X	3.04[a]	4.90[a]
XI	4.11	6.84
XII	4.36	—
XIII	4.40	—
XIV	4.49	—

[a] Zero percent methanol (Ref. 6).

where V_R is the elution volume of the chromatographic peak and V_0 is the column void volume, determined as the elution volume of a nonretained peak.

Analysis of Experimental Data—The numerical analysis of experimental data was carried out on a calculator[8] programmed for a nonlinear least-squares fit.

Determination of pKa—The apparent pKa values were potentiometrically determined previously (6) under the conditions of the mobile phases used, except for an ionic strength of 0.15, 50% water–methanol, and room temperature, which were determined in the present study.

RESULTS AND DISCUSSION

The structures of the β-lactam substances studied were indicated previously (6).

The apparent pKa values for the penicillins and cephalosporins were studied in a 50% (v/v) methanol solution at an ionic strength of 0.15 and at room temperature (Table I).

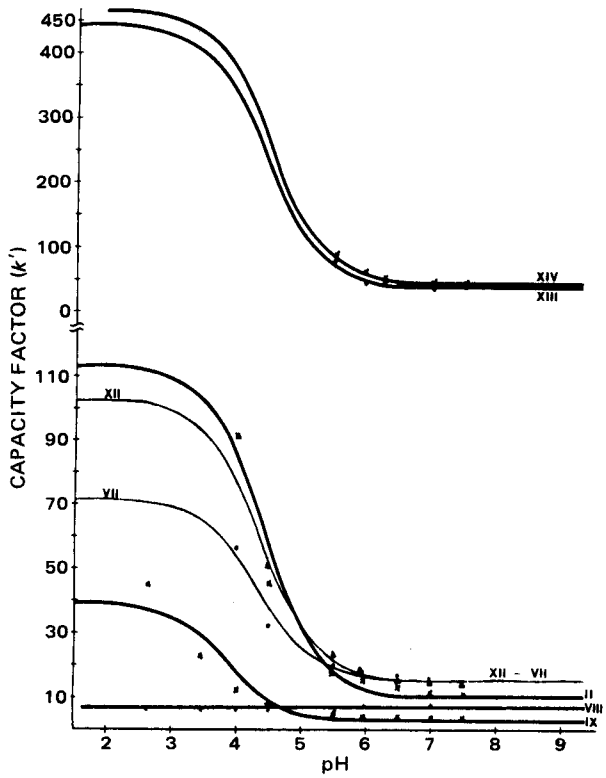

Figure 1—*Capacity factors, k', of acidic penicillins and cephalosporins on resin columns as a function of pH using mobile phases of 50% (v/v) methanol–0.05 M phosphate buffer at an ionic strength of 0.15. The continuous lines are the result of the nonlinear least-squares fit of the pairs of experimental k'/pH values to Eq. 2.*

[8] Texas Instruments TI-59.

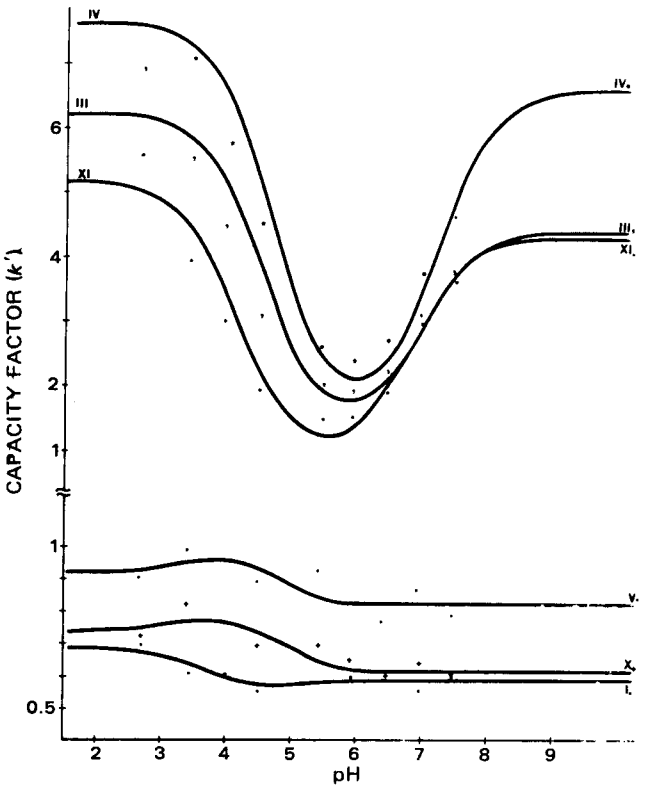

Figure 2—*Capacity factors, k', of amphoteric pencillins and cephalosporins on resin columns as a function of pH using mobile phases of 50% (v/v) methanol–0.05 M phosphate buffer at an ionic strength of 0.15. The continuous lines are the result of the nonlinear least-squares fit of the pairs of experimental k'/pH values to Eq. 3.*

Under the microscope, the resin particles were irregular in shape, with no dimension predominating over any other. The average size was ~55 μm. The size distribution analysis showed the particles to be 20–70 μm, with 50% (w/w) being <45 μm. The dry-packed column of resin showed an efficiency in which the height values of the theoretical plate were ~0.06 cm.

The peak tailings shown with the asymmetry factor calculated as 10% of the height of the peaks (7) were similar to those obtained with 30–70-μm octadecylsilica columns. Column permeability was relatively low. The values found for the permeability coefficient (8) were $\sim 4 \times 10^{-10}$ cm^2, considerably lower than those estimated by the Kozenzy–Carmen expression (8). This result is probably due to the unevenness of the resin particles since the smallest particles occupy the voids of the largest ones, thus giving a tighter packing than exists when all particles are of equal size.

Effect of pH—Although macroporous styrenedivinylbenzene copolymer permits the use of mobile phases at any pH, the instability of penicillins and cephalosporins in highly acidic or alkaline media limited this study to pH levels of 2.7–7.5.

The capacity factors of the penicillins and cephalosporins were studied on the resin column using 0.05 *M* phosphate buffer–methanol 50% (v/v) at different pH as the mobile phase (Figs. 1 and 2).

For nonpolar stationary phases, general equations have linked (6, 9) the variations of capacity factors of each solute ionic form. These general equations, worked out for nonpolar stationary phases and based on hydrophobic interactions, also are valid for describing variations in the capacity factors of penicillins and cephalosporins on macroporous styrenedivinylbenzene resins as a function of the mobile phase pH, as will be shown.

Acidic Penicillins and Cephalosporins—Cephalosporins and penicillins, inasmuch as they are weak acids, may be found in neutral, undissociated, or anionic form depending on the medium pH. The hydrophobic interactions of undissociated cephalosporins and penicillins with the nonpolar resin phase will be greater than for the corresponding ionic forms.

At high pH, distant from pKa, capacity factors are small and constant.

Table II—Capacity Factors of Anionic (k'_{-1}) and Undissociated (k'_0) Forms of Penicillins and Cephalosporins in 50% (v/v) Methanol

	Compound					
	II	VII	IX	XII	XIII	XIV
k'_0	113	71.5	39.7	103	444	467
k'_{-1}	10.0	15.6	2.66	15.2	39.5	43.3

Table III—Capacity Factors of Anionic (k'_{-1}), Zwitterionic (k'_0), and Cationic (k'_{+1}) Forms of Amphoteric Penicillins and Cephalosporins in 50% (v/v) Methanol

	Compound		
	III	IV	XI
k'_0	0.93	1.58	0.92
k'_{-1}	4.69	6.59	4.32
k'_{+1}	6.29	7.63	5.17

As the pH is lowered and approaches the pKa values, the anionic and the undissociated forms begin to coexist and the capacity factors increase. At acidic pH, where only the undissociated form may be found, the capacity factors are again constant and higher than those for high pH levels.

The quantitative description of the variations in the capacity factors with the concentration of hydronium ions may be expressed by (6, 9):

$$k' = \frac{k_0 + \frac{K_a}{[H^+]} k_{-1}}{1 + \frac{K_a}{[H^+]}} \qquad \text{(Eq. 2)}$$

where k_0 and k_{-1} are the capacity factors of the dissociated and anionic forms, respectively; K_a is the dissociation constant of penicillins or cephalosporins at 50% (v/v) methanol at an ionic strength of 0.05; and $[H^+]$ is the concentration of hydronium ions in the mobile phase.

In Fig. 1, the continuous line is the result of the nonlinear least-squares fit of experimental values of k'/pH to Eq. 2. An acceptable agreement exists between the theoretical provisions of Eq. 2 and the experimental values. All of the curves obtained have the same shape. The pH value at the inflection point is that of the pKa, and the pKa values found potentiometrically coincide favorably with those obtained from the curves in Fig. 1.

By applying the experimental data (k'/pH) to Eq. 2 using nonlinear least-squares fit, the capacity factors for the undissociated and anionic forms of each penicillin and cephalosporin studied were calculated (Table II).

Amphoteric Penicillins and Cephalosporins—The variations in the capacity factors of amphoteric cephalosporins and penicillins on stationary phases of nonpolar resins can be described by (6, 9):

$$k' = \frac{k_0 + k_{-1}\frac{K_{a_2}}{[H^+]} + k_{+1}\frac{[H^+]}{K_{a_1}}}{1 + \frac{K_{a_2}}{[H^+]} + \frac{[H^+]}{K_{a_1}}} \qquad \text{(Eq. 3)}$$

where k_0, k_{-1}, and k_{+1} represent the capacity factors of zwitterionic, anionic, and cationic forms, respectively; and K_{a_1} and K_{a_2} represent the first and the second dissociation constants of penicillins or cephalosporins at 50% (v/v) methanol at an ionic strength of 0.05, respectively.

In Fig. 2, the continuous lines are obtained from the nonlinear least-squares fit of the experimental k'/pH values to Eq. 3. All of the curves obtained have the same shape, with the minimums coinciding with the pH value at the isoelectric point. The minimum of retention is due to the fact that the zwitterion form has a double charge (positive and negative), so the hydrophobic interactions with the resin are less than those of the anionic and cationic forms.

By applying the experimental k'/pH values to Eq. 3 by nonlinear least-squares fit, the capacity factors of the anionic, cationic, and zwitterionic forms of the cephalosporins and penicillins were calculated (Table III).

As expected, the capacity factors for the zwitterionic form are considerably smaller than those of the other molecular forms. The capacity factors of cationic forms are somewhat greater than those of anionic ones, which could imply that the undissociated carboxylic acid group contributes to adsorption more than does the uncharged amine group.

Being more polar then penicillins and cephalosporins, I, V, and X are less retained by the nonpolar column. The differences observed in the capacity factors with pH are small so the relative errors are large, which

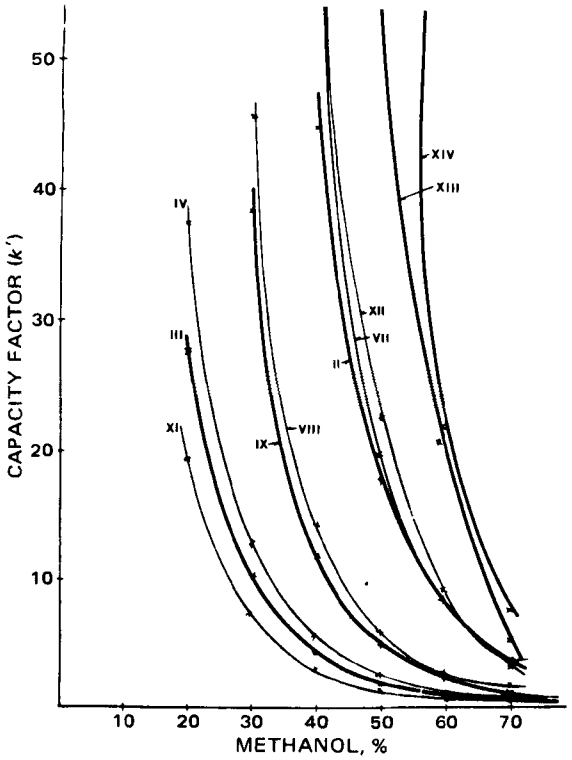

Figure 3—*Capacity factors, k', of penicillins and cephalosporins on resin columns using mobile phases of 0.05 M phosphate buffer at pH 5.5 and various methanol concentrations.*

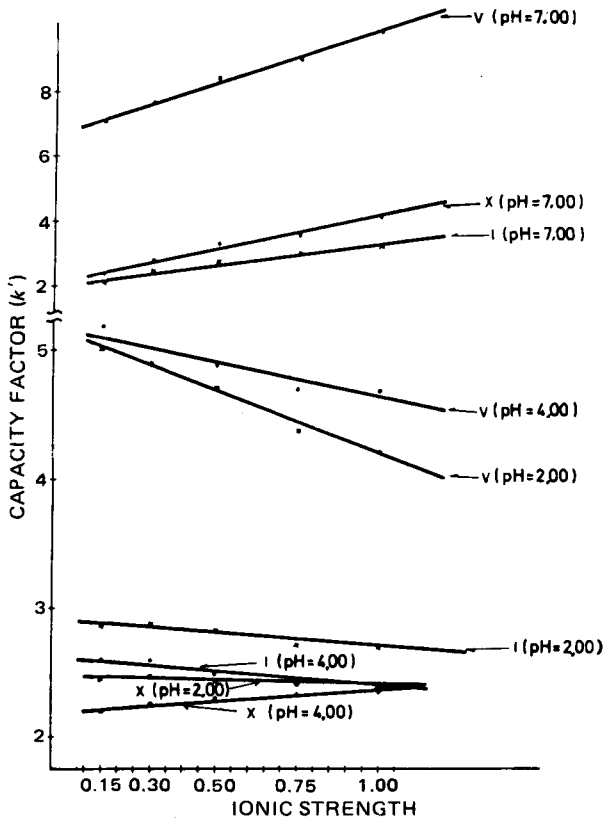

Figure 4—*Capacity factors, k', of I, V, and X on resin columns using mixtures of 0.05 M phosphate buffer at the pH indicated with 20% (v/v) methanol and variable ionic strengths as the mobile phases.*

Table IV—Capacity Factors of Anionic (k'_{-1}), Zwitterionic (k'_0), and Cationic (k'_{+1}) Forms of 7-Aminocephalosporanic (V), 6-Aminopenicillanic (X), and 7-Aminodeacetoxycephalosporanic (I) Acids in 50% (v/v) Methanol

	Compound		
	V	X	I
k'_0	1.0	0.78	0.56
k'_{-1}	0.82	0.61	0.59
k'_{+1}	0.92	0.73	0.69

implies that the application of Eq. 3 may be doubtful (Fig. 2). The results obtained for the capacity factors of the various forms are given in Table IV.

Influence of Penicillin and Cephalosporin Structure on Capacity Factors—Penicillin and cephalosporin nuclei are retained little by the nonpolar stationary phase of the resin. 7-Aminocephalosporanic acid (V), which has an acetoxy group in position 3 of the thiazine ring, is somewhat more retained than I and X. Acids I and X have very similar retention times, with that of the latter being somewhat greater.

Amphoteric α-aminophenylacetamide penicillins and cephalosporins have substantially higher capacity factors than their respective nuclei. Nevertheless, these factors are remarkably lower than those found for the acidic penicillins and cephalosporins. Within the group of amphoteric penicillins and cephalosporins, the least retained is ampicillin.

Within the deacetoxycephalosporins, cephradine is more retained than cephalexin, probaly due to the fact that the aromatic ring of cephalexin contributes to adsorption less than does the 2,4-cyclodihexene ring of cephradine.

Of the penicillins and cephalosporins studied that possess only one carboxylic group, the least retained is cephaloridine. In position 3 of the cephaloridine thiazine ring, the substitution of the pyridinic cation by the acetoxy group produces cephalothin, which, being much less polar, is retained considerably longer than cephaloridine. Cefazolin has intermediate capacity factors.

7-Aminocephalosporanic acid (V) and 6-aminopenicillanic acid (X), which have the same lateral chain and differ only as regards the nucleus, have very similar capacity factors. However, both V and X possess higher capacity factors than their respective nonacylated nuclei.

The introduction of phenoxy groups in the lateral chain of penicillins produces much higher capacity factors. Phenoxymethylpenicillin and phenoxyethylpenicillin are the most retained of those studied, the latter being somewhat retained owing to its slightly lesser polarity (Fig. 1).

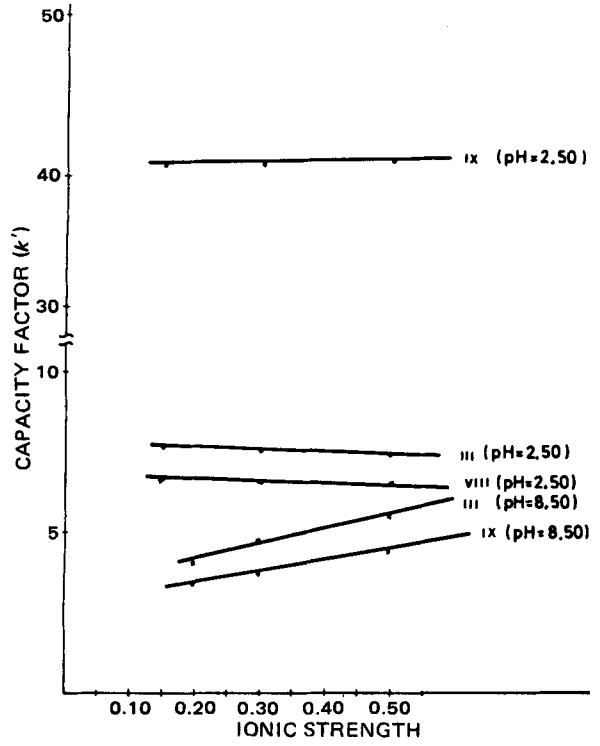

Figure 5—*Capacity factors, k', of some penicillins and cephalosporins on resin columns using mixtures of 0.05 M phosphate buffer at the pH indicated with 20% (v/v) methanol and variable ionic strengths as the mobile phases.*

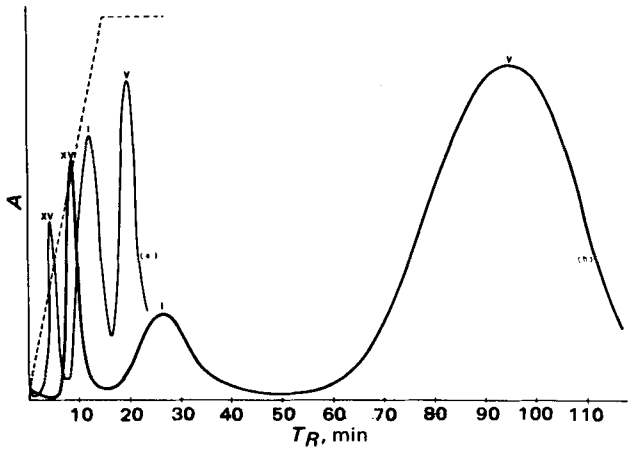

Figure 6—*Separation of 7-aminodeacetylcephalosporanic acid (XV), 7-aminocephalosporanic acid (V), and 7-aminodeacetoxycephalosporanic acid (I) on 2.5 × 600-mm resin columns. Key: a, 0.05 M phosphate buffer (pH 4.5) with 5% (v/v) methanol; and b, linear gradient elution (- - -) with 0.05 M phosphate buffer (pH 4.5) with from 5 to 30% (v/v) methanol.*

Effect of Mobile Phase Methanol Content—The retention of β-lactam substances by nonpolar macroporous styrenedivinylbenzene copolymers was studied using a mobile phase of 0.05 *M* phosphate buffer (pH 5.5), an ionic strength of 0.15, and different methanol contents.

As the methanol content of the mobile phase rose, the capacity factors decreased (Fig. 3). For the amphoteric substances I, III–V, X, and XI, the effect of the methanol content of the mobile phase on retention was not great, especially when compared with that of methanol on the retention of penicillins and cephalosporins having a single carboxyl group.

At pH 5.5, at which this study was conducted, amphoteric penicillins and cephalosporins are mainly in zwitterionic form whereas acidic penicillins and cephalosporins are principally anionic, so the polarity effect of the mobile phase is much greater on the latter than on the former.

In any event, III, IV, and XI had higher polarities than II and VII. Thus, it seems natural that mobile phase polarity should have a greater effect on the retention of less polar penicillins and cephalosporins.

Effect of Mobile Phase Ionic Strength—The effect of the ionic strength on the capacity factors of I, V, and X on resin was studied using mobile phases of 0.05 *M* phosphate buffer at pH 2.0, 4.0, and 7.0 with 20% (v/v) methanol. These pH levels were chosen to study the influence of the ionic strength on each ionic form (anionic at pH 7.0, zwitterionic at pH 4.0, and cationic at pH 2.0).

As shown in Fig. 4, the ionic strength had little influence on the capacity factors of I and X. A slight increase was observed in the retention of these acids as the ionic strength increased at alkaline pH, where both acids are anionic. For V, at pH 2.0 and 4.0, where it exists in cationic or zwitterionic form, respectively, the capacity factors were practically constant, with only a slight decrease as the ionic strength increased. At pH 7.0, the retention increased as the saline concentration increased. This phenomenon may be explained by the formation of ionic pairs between the carboxylate anion and the positive ions of the medium, which could alter their polar nature and/or the orientation of the molecules on the adsorbent surface.

The effect of the ionic strength on the retention of certain cephalosporins on nonpolar copolymers also was studied using mobile phases of 0.05 *M* phosphate buffer at pH 2.5 and 8.5 and a methanol content of 50% (v/v). The capacity factors of the cephalosporins studied varied little with the ionic strength of the mobile phase (Fig. 5). At pH 2.5, where the cefazolin is undissociated and cephalexin and cephaloridine are cationic, the capacity factors did not vary with increasing ionic strengh. At pH 8.5, where the cephalosporins are anionic, the representation k'/μ gives straight lines with a slightly positive slope. As in the previous case, a possible explanation could be the formation of ion-pairs as the ionic strength is increased, which could entail a rise in the contribution of the carboxylate anion on retention.

Applications—The results previously obtained were used to select the conditions for penicillin and cephalosporin separations.

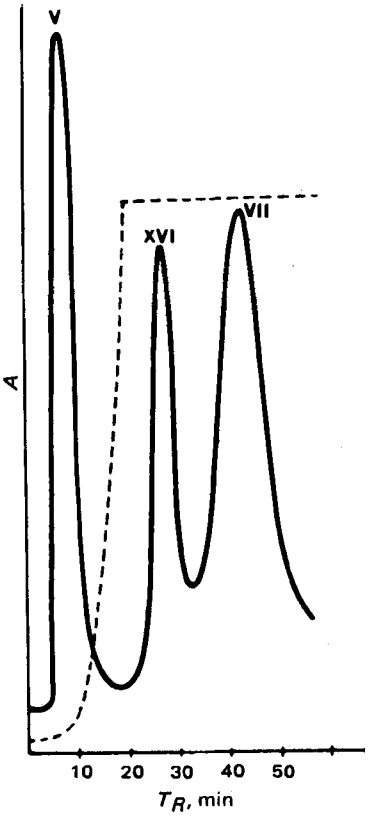

Figure 7—*Separation of 7-aminocephalosporanic acid (V), deacetylcephalothin (XVI), and cephalothin (VII) on resin columns; linear gradient elution (- - -) with 0.05 M phosphate buffer (pH 4.5) with from 35 to 60% (v/v) methanol.*

Figure 6 shows the separation from 7-aminocephalosporanic acid of two of its most frequent impurities, 7-aminodeacetoxycephalosporanic acid and deacetyl-7-aminocephalosporanic acid. The results obtained in an isocractic system and with gradient elution are shown in Figs. 6*a* and 6*b*, respectively.

Other examples of separation are shown in Figs. 7 and 8. In the former, cephalothin is separated from some of its possible impurities, 7-aminocephalosporanic acid and deacetylcephalothin. In the latter, cephalexin is separated from 7-aminodeacetoxycephalosporanic acid.

Comparison of Stationary Phases of Nonionic Styrenedivinylbenzene Copolymers with Octadecylsilica—The chromatographic interactions of penicillins and cephalosporins with the stationary phase of octadecylsilica were studied previously (6). From comparison of these results with those in the present work using polystyrenedivinylbenzene resins, the following conclusions can be drawn:

1. Polystyrenedivinylbenzene behaves as a reversed phase in a similar manner as the stationary phase of octadecylsilica. The same equations describe the capacity factor variations with pH for each stationary phase.

2. Even if the particle size for each column is similar, that of octadecylsilica has a theoretical plate height of about three times less than resin columns under the same conditions.

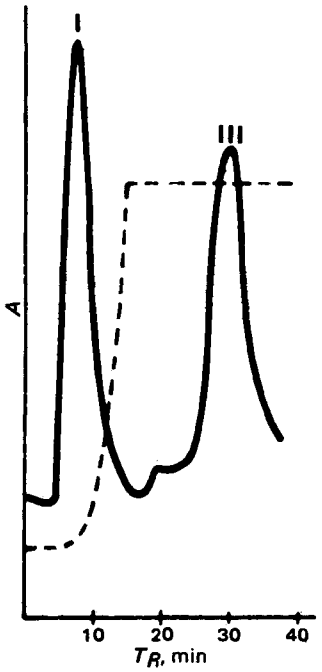

Figure 8—*Separation of 7-aminodeacetoxycephalosporanic acid (I) and cephalexin (III) on resin columns; linear gradient elution (- - -) with 0.05 M phosphate buffer (pH 4.5) with from 20 to 45% (v/v) methanol.*

3. The interactions among β-lactam antibiotics are more intense with resins than with octadecylsilica. Therefore, when using the resin column, a greater methanol content in the mobile phase is required than when the octadecylsilica column is used.

The octadecylsilica column is more appropriate for chromatographic separations of penicillins and cephalosporins than are columns of styrenedivinylbenzene copolymer. However, given the low cost of nonionic resins, adsorption–elution conditions may have many applications in extraction and purification processes of penicillins and cephalosporins.

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