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LIQUID CHROMATOGRAPHY WITH CROWN ETHER-CONTAINING MOBILE PHASES

II. RETENTION BEHAVIOUR OF β-LACTAM ANTIBIOTICS IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The retention behaviour of several β-lactam antibiotics in reversed-phase liquid chromatography with mobile phases containing crown ether(18-crown-6 or dicyclohexyl-18-crown-6) has been investigated. The capacity factors were determined with various concentrations of crown ether and methanol in the aqueous mobile phase at different pH values. The observed profiles of capacity factor, k, *vs.* crown ether concentration and k *vs.* pH are discussed with reference to an equation derived from a chromatographic model involving association of β-lactam antibiotics with crown ethers and bindings of free and associated species to the hydrophobic stationary phase. The effect of ionic salts on the capacity factor is also discussed. The applicability of the method is demonstrated by specific separations of ampicillin in urine and cephalexin in plasma.

INTRODUCTION

The specific cation-anchoring ability of crown ethers has been utilized in liquid chromatography of various inorganic and organic ions. Cram and co-workers demonstrated the optical resolution of amino acids and their ester salts through chiral recognition by a crown ether which was contained in the mobile phase[1] or immobilized on silica gel[2] or on macroreticular cross-linked polystyrene *p*-divinylbenzene resin[3]. Blasius and co-workers separated enantiomers of amino acids[4], alkali and alkaline-earth metals[5–7] and several ammonium cations[5,7] using crown ether-based ion exchangers as the stationary phase. Delphin and Horwitz[8] also investigated the effects of crown ethers on the ion exchange behaviour of alkali metals. The associations of metals with several host substances were investigated by Horváth *et al.*[9], and metal–crown ether association constants were measured by using an aqueous cation-containing solution as mobile phase and a non-polar bonded stationary phase.

Anions can also be separated using a common cation associated with a crown ether as a counter ion. Blasius and co-workers[5,7,10,11] demonstrated the separation of various anions with a common cation as well as of various cations with a common

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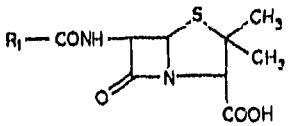
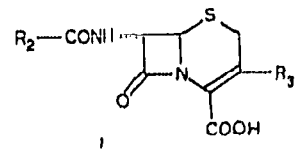
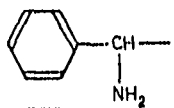
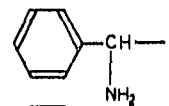
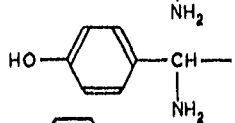
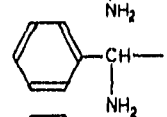
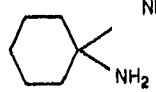
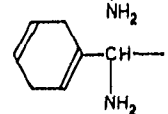
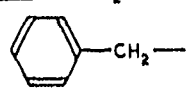
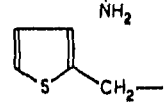
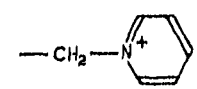
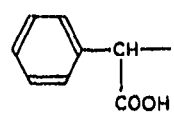
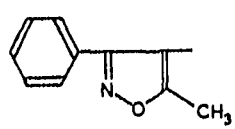
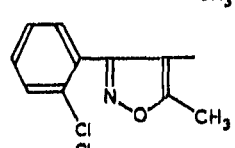
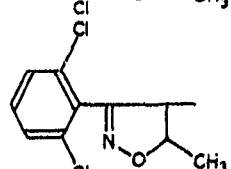
TABLE I

STRUCTURES AND ABBREVIATIONS OF β-LACTAM ANTIBIOTICS





Penicillins	R_1	*Cephalosporins*	R_2	R_3
Ampicillin (ABPC)		Cephalexin (CEX)		$-CH_3$
Amoxicillin (AMPC)		Cephaloglycin (CEG)		$-CH_2OCOCH_3$
Ciclacillin (ACPC)		Cefradine (CED)		$-CH_3$
Benzylpenicillin (PCG)		Cephaloridine (CER)		
Carbenicillin (CBPC)				
Oxacillin (MPIPC)				
Cloxacillin (MCIPC)				
Dicloxacillin (MDIPC)				

anion. Brugman and Kraak[12] achieved normal phase separation of sulphonic acids using potassium associated with a crown ether as a common cation. Recent developments have included the use of crown ether polymers immobilized on silica[13,14] and polyamide crown resin[15,16] for the specific separation of alkali and alkaline-earth metals and various organic ions.

In a previous paper[17], we described the retention behaviour of various amino compounds in reversed-phase liquid chromatography with crown ethers in the aqueous mobile phase.

β-Lactam antibiotics such as penicillins and cephalosporins are widely used in clinical chemotherapy. In investigations of the *in vitro* and *in vivo* properties of these drugs, high-performance liquid chromatography (HPLC) has proved to be a facile assay method. The aim of the present study was to investigate the retention behaviour of β-lactam antibiotics in reversed-phase liquid chromatography with mobile phases containing crown ethers, and to demonstrate the applicability of the method to the analysis of biological samples.

EXPERIMENTAL

Reagents and materials

The β-lactam antibiotics were commercial products available for clinical use. Their structures and abbreviations are shown in Table I. 18-crown-6(18-C-6) and dicyclohexyl-18-crown-6(DC-18-C-6) were products of Nippon Soda Co. (Tokyo, Japan). DC-18-C-6 was used without separation of A,B-isomers. Glass-distilled water and methanol were used to prepare the mobile phases after degassing. Hydrochloric acid of analytical reagent grade was used to adjust the pH of the mobile phase.

Liquid chromatography

Liquid chromatographs (TWINCLE and TRIROTAR-III; Jasco, Tokyo, Japan) each equipped with a variable-wavelength detector (UVIDEC-100 III, Jasco) were used for the measurements of capacity factors. The experimental conditions employed are summarized in Table II. The β-lactam antibiotics were dissolved in water and the minimal amounts required for UV detection (at 220 nm for penicillins and 254 nm for cephalosporins) were used. The flow-rate of the mobile phase was 1.0 ml/min. The capacity factors were calculated according to $k = (t_R - t_0)/t_0$, where t_R is the average retention time of a solute measured repeatedly at the peak of the elution curve and t_0 is that of a non-absorbed substance.

Analysis of experimental data

The non-linear least squares fittings were carried out on a microcomputer (PET 2001, Commodore Co.) specially programmed in BASIC.

THEORETICAL

The β-lactam antibiotics used each have a carboxyl group on the skeletal structure and also in some cases, another carboxyl group or amino group on the lateral chain (see Table I). Therefore, those having an amino group exhibit amphoteric behaviour in their retention on a hydrophobic stationary phase. Since it is known that

TABLE II

HPLC CONDITIONS

Experiment	*Stationary phase*	*Mobile phase*
k vs. [18-C-6]	Develosil ODS-10 packed in stainless-steel tube (25 cm × 4 mm I.D.)	Water–methanol (55:45 v/v), pH 2.5 [18-C-6] = 0–45 m*M*
k vs. [DC-18-C-6]	Develosil ODS-10 packed in stainless-steel tube (25 cm × 4 mm I.D.)	Water–methanol (1:1 v/v), pH 2.5 [DC-18-C-6] = 0–25 m*M*
k vs. pH	Nucleosil 10 C_{18} packed in stainless-steel tube (15 cm × 4 mm I.D.)	Water–methanol (1:1 v/v), pH 2.2–4.4 [18-C-6] = 20 m*M*
k vs. % methanol	Develosil ODS-10 packed in stainless-steel tube (20 cm × 4 mm I.D.)	Water–methanol (3:7–7:3 v/v), pH 4.5 [18-C-6] = 20 m*M*
k vs. [KCl]	Nucleosil 10 C_{18} packed in stainless-steel tube (15 cm × 4 mm I.D.)	Water–methanol (1:1 v/v), pH 4.2 [18-C-6] = 20 m*M* [KCl] = 0–25 m*M*

a solute without an ionized amino group shows no apparent association with crown ethers and the association of an ionized amino group with a crown ether is expected to exhibit 1:1 stoichiometry, the chromatographic processes involved in the present system are as depicted in Fig. 1. The equilibria indicated by the broken lines involve the anionic form of the amphoteric β-lactam antibiotics and are not taken into account in deriving the capacity factor equation since they are not significant in the pH region employed in the present experiments. Thus, the capacity factor of amphoteric β-lactam antibiotics is given by

$$k = \phi \cdot \frac{[LS^+]_s + [LS^\pm]_s + [LCS^+]_s + [LCS^\pm]_s}{[S^+]_m + [S^\pm]_m + [CS^+]_m + [CS^\pm]_m} \quad (1)$$

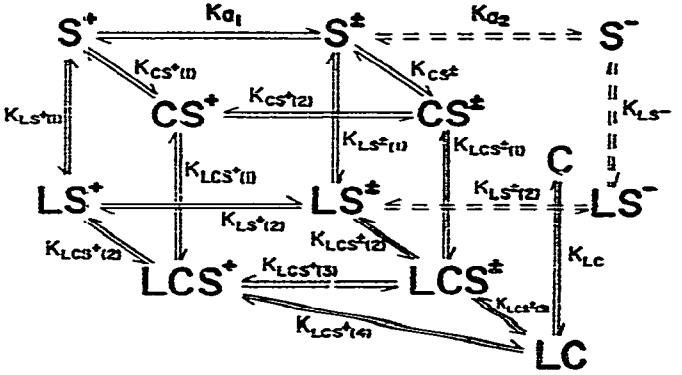


Fig. 1. The equilibria involved in reversed-phase liquid chromatography with a crown ether-containing mobile phase. C = Crown ether; S^+, $S^\pm$, S^- = cationic, zwitterionic and anionic forms of amphoteric β-lactam antibiotics; L = hydrophobic stationary phase.

where the subscripts m and s specify the mobile and stationary phases, respectively, and ϕ denotes the phase ratio. Introducing the equilibrium constants, K, in Fig. 1 we obtain

$$k = \phi[L]_s \cdot \frac{(A + B[C]) + (D + E[C])[H^+]}{(1 + K_{CS^\pm}[C])K_{a_1} + (1 + F[C])[H^+]} \quad (2)$$

where [C] is the concentration of crown ether in the mobile phase and $A = K_{LS^\pm(1)}K_{a_1}$, $B = K_{LCS^\pm(1)}K_{CS^\pm}K_{a_1} = AK_{LCS^\pm(2)} = K_{LCS^\pm(3)}K_{LC}K_a$, $D = K_{LS^+(1)} = AK_{LS^-(2)}$, $E = FK_{LCS^-(1)} = DK_{LCS^-(2)} = BK_{LCS^-(3)} = K_{LC}K_{LCS^+(4)}$ and $F = K_{CS^+(1)} = K_{CS^+(2)}K_{CS^\pm}K_{a_1}$. Since the amount of stationary phase bound to the solute represents only a small part of the total, $[L_t]$:

$$[L_t] = [L]_s + [LC]_s \quad (3)$$

Introducing K_{LC} into eqn. 3:

$$[L]_s = \frac{[L_t]}{1 + K_{LC}[C]} \quad (4)$$

Substituting for $[L]_s$ in eqn. 2, we finally obtain:

$$k = \phi \cdot \frac{[L_t]}{1 + K_{LC}[C]} \cdot \frac{(A + B[C]) + (D + E[C])[H^+]}{(1 + K_{CS^\pm}[C])K_{a_1} + (1 + F[C])[H^+]} \quad (5)$$

Eqn. 5 yields several expressions for the capacity factor depending on the choice of the constants B to F. These constants reflect the processes of crown ether–cation complexation and of binding of the complex with the hydrophobic stationary phase. Eqn. 5 predicts that the k *vs.* $[H^+]$ profile is rectangular hyperbolic at a constant concentration of crown ether. When the pH of the mobile phase is low enough for the amphoteric β-lactam antibiotics to be in the cationic form, the capacity factor is expressed by

$$k = \frac{k_0 + \phi[L_t]G[C]}{(1 + K_{LC}[C])(1 + K_{CS^+(1)}[C])} \quad (6)$$

where k_0 is the capacity factor for $[C] = 0$ and $G = K_{LCS^+(1)}K_{CS^+(1)} = K_{LCS^+(2)}K_{LS^+(1)} = K_{LCS^+(4)}K_{LC}$. Eqn. 6 indicates that the capacity factor at low pH initially increases with increase in the concentration of crown ether, reaches a maximum and then decreases at higher concentrations. For a small value of K_{LC}, *i.e.*, weak retention of the crown ether on the hydrophobic stationary phase

$$k = \frac{k_0 + k_{CS^+}K_{CS^+(1)}[C]}{1 + K_{CS^+(1)}[C]} \quad (7)$$

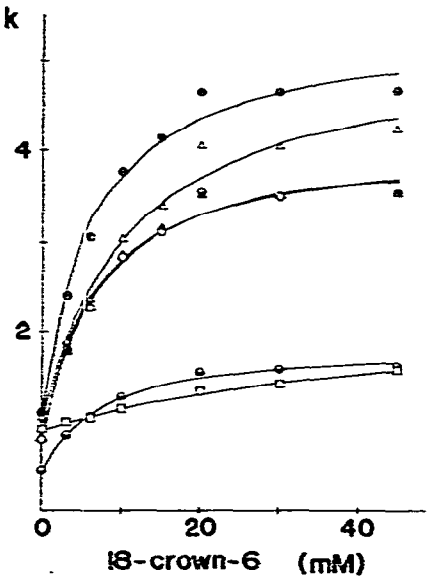
where k_{CS^+} is the capacity factor of the cationic form of the amphoteric β-lactam antibiotics, which is equal to $\phi[L_t]K_{LCS^+(1)}$.

RESULTS AND DISCUSSION

Effect of crown ether concentration

The dependence of capacity factor on the concentration of crown ether was investigated by using mobile phases containing 0–45 m*M* 18-C-6 or 0–25 m*M* DC-18-C-6 at pH 2.5. Figs. 2 and 3 show the *k vs.* [18-C-6] profiles for β-lactam antibiotics with and without an amino group, respectively. Figs. 4 and 5 show the *k vs.* [DC-18-C-6] profiles for the same substances. The capacity factors of β-lactam antibiotics without an amino group remained constant despite the addition of 18-C-6 (Fig. 3), but decreased significantly upon addition of DC-18-C-6 (Fig. 5). On the contrary, the capacity factors of amphoteric β-lactam antibiotics initially increased markedly with increase in the concentrations of both crown ethers followed by a gradual approach to a maximum (Figs. 2 and 4), and then decreased with further increase in the concentration of DC-18-C-6 (Fig. 4).

These results suggest that crown ethers exert different effects on the retention of β-lactam antibiotics on the hydrophobic stationary phase; an increase in the capacity factor by complex formation with the amino group, and a decrease in the capacity factor by competing with β-lactam antibiotics in binding to the stationary phase. The former effect is predominant in the retention of amphoteric β-lactam antibiotics, resulting in the enhancement of the hydrophobicity of the cation. β-Lactam antibiotics without an amino group are subject solely to the latter effect. Figs. 2–5 indicate that degree of such effects depends on the hydrophobicity of the crown ether, that is, an increase in the hydrophobicity results in an increase in the capacity factors of amphoteric β-lactam antibiotics, but a decrease in those of β-lactam antibiotics



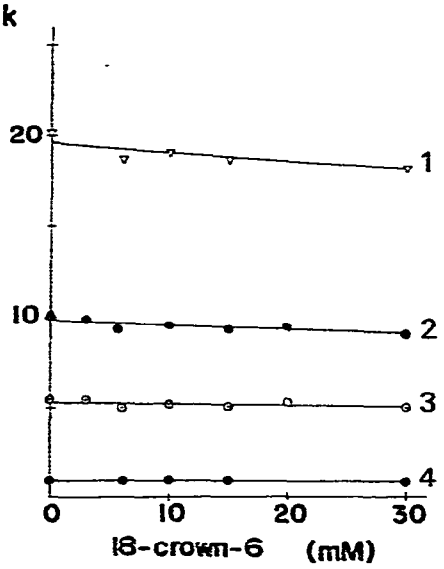
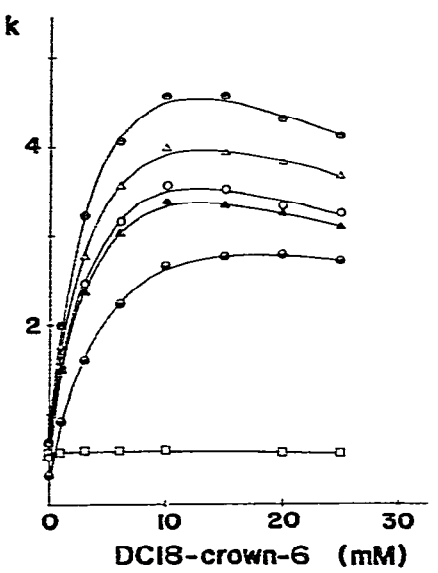


Fig. 2. Effect of 18-crown-6 concentration on the capacity factor of amphoteric β-lactam antibiotics (pH 2.5). Key: ⊖, ABPC; △, CED; ○, CEX; ▲, CEG; ⊖, AMPC; □, ACPC.

Fig. 3. Effect of 18-crown-6 concentration on the capacity factor of β-lactam antibiotics without an amino group (pH 2.5). Key: 1, MPIPC; 2, PCG; 3, CBPC; 4, CER.



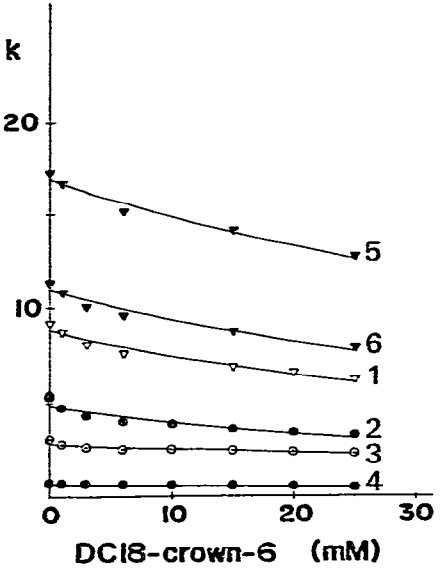


Fig. 4. Effect of dicyclohexyl-18-crown-6 concentration on the capacity factor of amphoteric β-lactam antibiotics (pH 2.5). For key see Fig. 2.

Fig. 5. Effect of dicyclohexyl-18-crown-6 concentration on the capacity factor of β-lactam antibiotics without an amino group (pH 2.5). Key: 5, MDIPC; 6, MCIPC; others, see Fig. 3.

without an amino group. The decrease in the capacity factor shown in Fig. 5 is ascribable to the stronger hydrophobicity of DC-18-C-6 than 18-C-6. Therefore, the k *vs.* crown ether concentration profiles in Fig. 5 can be expressed by

$$k = \phi[L_t] \cdot \frac{K_{LS}}{1 + K_{LC}[C]} \tag{8}$$

where K_{LS} is the equilibrium constant for β-lactam antibiotics without an amino group to bind to the stationary phase. The values of K_{LC} were estimated by non-linear least squares fittings of the k *vs.* [DC-18-C-6] data using eqn. 8. The curves in Fig. 5 are those best fitted thereby. The results for K_{LC} of DC-18-C-6 given in Table III, which are essentially independent of the β-lactam antibiotics, are scattered in a relatively narrow range with an average value of 15.3 M^{-1}.

The decrease in the capacity factor at higher concentrations as shown in Fig. 4 may also be due to the strong hydrophobicity of DC-18-C-6, since the capacity

TABLE III

PARAMETERS IN EQN. 8 FOR k *vs.* [DC-18-CROWN-6] OF β-LACTAM ANTIBIOTICS WITHOUT AN AMINO GROUP

	PCG	*CBPC*	*CER*	*MPIPC*	*MCIPC*	*MDIPC*
$\phi[L_t]K_{LS}$	4.87	2.81	0.634	8.81	11.0	16.8
K_{LC} (M^{-1})	23.3	9.06	11.7	17.7	17.0	13.1

TABLE IV

PARAMETERS IN EQN. 7 FOR k vs. [18-CROWN-6] OF AMPHOTERIC β-LACTAM ANTIBIOTICS

	ABPC	*AMPC*	*ACPC*	*CEX*	*CEG*	*CED*
k_0	1.06	0.444	0.909	0.781	0.775	1.00
k_{CS^+}	5.42	1.87	2.36	4.17	4.11	5.18
k_{CS^+}/k_0	5.11	4.21	2.60	5.34	5.30	5.18
$K_{CS^+(1)}$ (M^{-1})	158	138	19.0	148	158	94.3

factors of the same substances in the corresponding concentration range of 18-C-6 (Fig. 2) increase gradually toward a maximum. Thus, the k *vs.* [DC-18-C-6] profiles in Fig. 4 were approximated by eqn. 6 and the k *vs.* [18-C-6] profiles in Fig. 2 by eqn. 7. The computer fittings obtained using these equations are also shown in Figs. 2 and 4, and the parameters derived therefrom are listed in Tables IV and V, respectively. Although the average value of K_{LC} in Table V is a little larger than that in Table III, the computer fitting achieved by putting the latter value (15.3 M^{-1}) into eqn. 6 gave almost the same results as those in Table V.

It is notable in Figs. 2 and 4 that ACPC exhibits a peculiar behaviour upon addition of crown ether. The slight increase in the capacity factor in Fig. 2 suggests weak association with 18-C-6. This is possibly due to a steric effect of the amino group of ACPC, which unlike the others is located at a quaternary carbon atom on a cyclohexyl ring (see Table I). The stronger hydrophobic effect of DC-18-C-6, as mentioned above, compensates this effect, resulting in no apparent change in the capacity factor (Fig. 4).

Effect of pH

The dependence of capacity factor on pH between 2.2 and 4.4 was investigated using a mobile phase containing a constant concentration (20 mM) of 18-C-6. The results are shown in Fig. 6. The initial increase in proton concentration gave rise to a marked decrease in the capacity factors of amphoteric β-lactam antibiotics, and to a slight increase in those antibiotics without an amino group. Further increase in

TABLE V

PARAMETERS IN EQN. 6 FOR k *vs.* [DC-18-CROWN-6] OF AMPHOTERIC β-LACTAM ANTIBIOTICS

	ABPC	*AMPC*	*ACPC*	*CEX*	*CEG*	*CED*
k_0	0.721	0.323	0.580	0.547	0.547	0.702
$\phi[L_L]G$ (M^{-1})	1600	689	30.6	1240	1220	1330
K_{LC} (M^{-1})	29.5	23.1	19.4	28.5	24.8	27.1
$K_{CS^+(1)}$ (M^{-1})	185	123	23.1	188	206	180
k_{CS^+}*	8.65	5.60	1.32	6.60	5.92	7.39
k_{CS^+}/k_0	12.0	17.3	2.28	12.1	10.8	10.5

* Calculated from $\phi[L_L]G/K_{CS^+(1)}$.

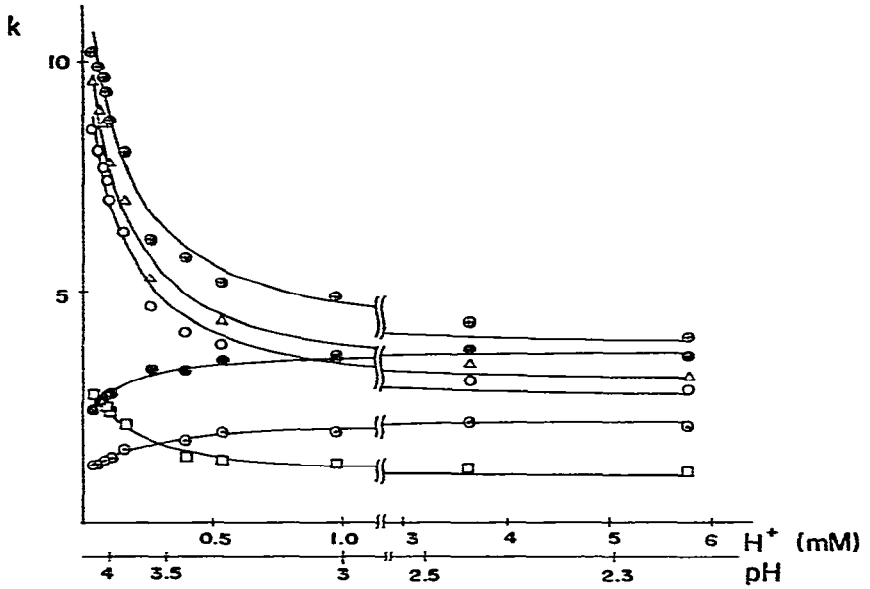


Fig. 6. Effect of proton concentration on the capacity factor of β-lactam antibiotics ([18-C-6] = 20 m*M*). Key: see Figs. 2 and 3.

proton concentration (pH < 3.3), however, had no effect in both cases. The behaviour of the β-lactam antibiotics without an amino group is similar to the pH dependence of the capacity factor of carboxylic acids in reversed-phase systems.

The *k vs.* pH profiles of amphoteric β-lactam antibiotics in Fig. 6 were analyzed according to

$$k = \frac{P_2 + P_3[H^+]}{P_1 + [H^+]} \tag{9}$$

which is the simplified form of eqn. 5 for a constant concentration of crown ether, where:

$$P_1 = \frac{1 + K_{CS^\pm}[C]}{1 + F[C]} \cdot K_{a_1} \tag{10}$$

Assuming that association of the protonated amino group with the crown ether is not affected by the dissociation of the carboxyl group, *i.e.*, $K_{CS^\pm} = K_{CS^-(I)}$, P_1 approximates to K_{a_1}. The non-linear least squares fittings of the *k vs.* pH data in Fig. 6 using eqn. 9 allowed the estimation of K_{a_1} values. The pK_{a_1} values thus obtained are 3.89(3.93) for ABPC, 3.94(4.02) for CEX, 3.98(4.37) for CED and 3.87 for ACPC, the values in parentheses being measured potentiometrically by Salto *et al.*[18] in 30% aqueous methanol with ionic strength 0.15 at 20°C. It is seen that these values are similar and also to the literature values.

It is interesting to compare the *k vs.* pH profiles of the amphoteric β-lactam antibiotics in Fig. 6 with those given by Salto *et al.*[18], who found that the capacity

factors of the zwitterion forms of amphoteric β-lactam antibiotics in a reversed-phase system (*i.e.*, stationary phase of ODS and mobile phase of phosphate buffer–methanol) were consistently lower than those of the cationic and anionic forms. Thus, the k *vs.* pH curves shown therein are convex toward the pH axis with minimal k values at around pH 5–6. This is in contrast to the k *vs.* pH profiles of ABPC, CEX and CED shown in Fig. 6. However, the profile of ACPC exhibits a slight decrease in the capacity factor. This suggests that the weaker association of the amino group with the crown ether is less dependent on proton concentration.

Effect of solvent

The solvent effect on capacity factor was investigated using mobile phases of water–methanol (7:3 to 3:7 v/v) containing 20 m*M* 18-C-6 at pH 4.5. The results are shown in Fig. 7, where it is seen that β-lactam antibiotics with and without an amino group exhibit linear decreases in log k values with similar slopes upon increasing concentration of methanol expressed in volume percent. This result is consistent with those found in other reversed-phase systems without crown ethers.

Effect of salt

The effect of inorganic electrolyte added to the crown ether-containing mobile phase is expected to be quite different from that in usual reversed-phase systems, because alkali metal cation from the salt associates with the crown ether more strongly than does the cationic amino group. As a result, the capacity factors of amphoteric β-lactam antibiotics in the present system must be decreased upon addition of such a salt. Fig. 8 shows the dependences of the capacity factors of several β-

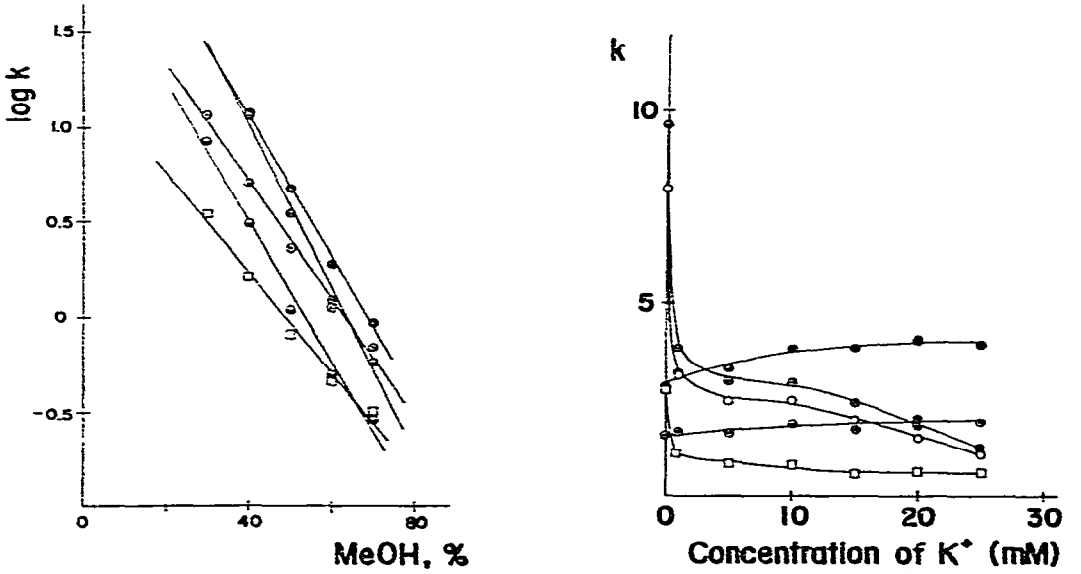


Fig. 7. Effect of methanol (MeOH) concentration on the capacity factor of β-lactam antibiotics (pH 4.5, [18-C-6] = 20 m*M*). Key: see Figs. 2 and 3.

Fig. 8. Effect of KCl concentration on the capacity factor of β-lactam antibiotics (pH 4.2, [18-C-6] = 20 m*M*). Key: see Figs. 2 and 3.

lactam antibiotics on the concentration of KCl dissolved in a mobile phase containing 20 m*M* 18-C-6 at pH 4.2. It is seen that the capacity factors of amphoteric β-lactam antibiotics initially decrease markedly with increasing KCl concentration followed by a gentle decline at higher concentrations. In contrast, the capacity factors of PCG and CBPC show a slight increase over the whole [KCl] region, in accord with previous results in reversed-phase systems. The profiles of the amphoteric β-lactam antibiotics, as expected, differ from those in usual reversed-phase systems, where an increase of ionic strength increases the retention of charged species on a hydrophobic stationary phase.

Anions are also expected to affect the retention of cationic substances in the present system, since the crown ether–ammonium complex can associate with the counter anion to form an ion pair. The degree of ion pairing and consequent enhancement of the capacity factor are dependent on the dielectric constant of the medium and on the hydrophobicity of the counter ion. The mobile phase used in this experiment includes Cl^- as a common anion in 50% aqueous methanol. However, the solvation of chloride ion in this medium, although perhaps weaker than in aqueous solution, seems unlikely to favour the formation of a hydrophobic ion pair with amphoteric β-lactam antibiotics. The addition of organic acid to the crown ether-containing mobile phase may further enhance the capacity factor.

Application

In order to demonstrate the applicability of the present method, biological samples were chromatographed on an ODS stationary phase. Fig. 9 shows chromato-

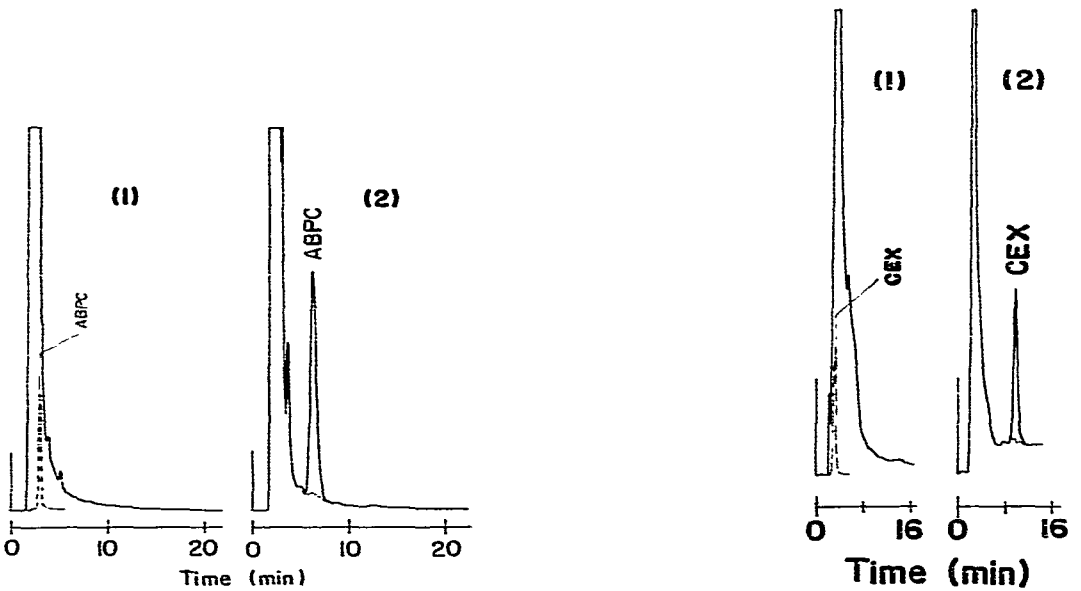


Fig. 9. Separation of ampicillin in human urine. HPLC conditions: stationary phase, Develosil ODS-10 (20 cm × 4 mm I.D.); mobile phase, (1) methanol–water (1:1 v/v), pH 5.2, (2) methanol–water (1:1) containing 20 m*M* 18-crown-6, pH 5.2; flow-rate, 1.0 ml/min; detection, UV 220 nm.

Fig. 10. Separation of cephalexin in human plasma. HPLC conditions: stationary phase, Develosil ODS-10 (20 cm × 4 mm I.D.); mobile phase, (1) methanol–water (1:1), pH 4.7, (2) methanol–water (1:1) containing 6 m*M* dicyclohexyl-18-crown-6, pH 4.7; flow-rate, 1.0 ml/min; detection, UV 254 nm.

grams of urine excreted after oral administration of ABPC to a man, and Fig. 10 indicates the separation of CEX from regular plasma components. Compared with the chromatograms obtained by using a mobile phase without crown ether, the elutions of these amphoteric β-lactam antibiotics are markedly delayed by addition of crown ether with complete separation from endogenous substances, while the retention times of the background peaks remained amost unchanged.

The present method is easily accessible by a simple modification of the mobile phase in conventional reversed-phase HPLC, although special care should be taken because of the toxicity of the crown ether monomer.

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