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SEPARATION OF β-LACTAM ANTIBIOTICS BY MICELLAR ELECTROKINETIC CHROMATOGRAPHY

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SUMMARY

The retention behaviour of β-lactam antibiotics in micellar electrokinetic chromatography (EKC) was investigated. Sodium dodecyl sulphate (SDS) and sodium N-lauroyl-N-methyltaurate were used as anionic surfactants at concentrations of 0.05–0.3 *M*. It was found that the retention of ionic substances in micellar EKC is determined by the following three factors: the electrophoretic migration of the ionic substances, the interaction between the ionic substances and ionic surfactants and solubilization of the solute by the micellar phase. A difference in the retention behaviours of cationic substances was observed between the two anionic surfactants, which have different groups neighbouring the charge-bearing groups. The effect of an ion-pairing reagent was also investigated to make the effect of the micelle clearer. All test solutes were successfully separated by micellar EKC at SDS concentrations above 0.1 *M*, with theoretical plate numbers ranging from 70 000 to 260 000.

INTRODUCTION

Micellar electrokinetic chromatography (EKC) is a new type of liquid chromatography based on micellar solubilization and electrokinetic migration[1,2]. Micellar solubilization operates as the partition mechanism, that is, a solute is distributed between the micellar phase and the aqueous phase. A strong electroosmotic flow generated within the capillary tube causes all kinds of solutes to elute at one end of the tube with high column efficiency.

Micellar EKC using surfactants has been successfully applied to the separation of electrically neutral substances[3–5], and was shown to give high resolution within a shorter time than conventional high-performance liquid chromatography (HPLC).

In addition to neutral substances, micellar EKC has brought many advantages for the separation of ionic substances[6–11]. Selectivity and peak shapes have been

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much improved in comparison with capillary zone electrophoresis (CZE). As an example that demonstrates the high resolving power of EKC, we have previously reported the separation of water-soluble vitamins[12].

In this paper, we describe the separation of a mixture of seven penicillin antibiotics (PCs) and a mixture of nine cephalosporin antibiotics (CEPs) by micellar EKC using two different anionic surfactants, sodium dodecyl sulphate (SDS) and sodium N-lauroyl-N-methyltaurate (LMT), in comparison with CZE. We discuss the retention characteristics of the ionic substances, to which PCs and CEPs belong, by considering the electrophoretic effect of the solute and the ion-pair formation between cationic substances and anionic surfactants in addition to micellar solubilization. To make the effect of the micelle clearer, the effect of ion-pairing reagents that do not form a micelle was investigated using sodium pentane sulphonate and sodium octane sulphonate. The effect of an additive to the surfactant solution is also described.

EXPERIMENTAL

Apparatus and procedure

Micellar EKC was performed in a 650 mm × 0.05 mm I.D. fused-silica capillary tube (Scientific Glass Engineering, North Melbourne, Australia) with a Model HJLL-25PO high-voltage d.c. power supply (Matsusada Precision Devices, Otsu, Japan) delivering up to +25 kV. The electric current was monitored between the negative electrode and the negative terminal of the power supply with an ammeter throughout the operation. Detection was carried out by the on-column measurement of UV absorption at 210 or 220 nm at a position 150 mm from the negative end of the tube. For data processing a Shimadzu Chromatopak C-R2AX was used.

A sample solution was siphoned from the positive end into a fused-silica tube filled in advance with a buffer solution, and each end of the tube was dipped into a separate reservoir containing the same buffer solution with which the tube was filled. A d.c. voltage was then applied between the two ends of the tube through platinum electrodes dipped in the reservoirs. Regarding the reproducibility of the system, the coefficient of variation for the retention time of the solute was 0.5–0.9% (n = 5–7) from run to run and 1.5–2.4% (n = 5) from day to day in micellar EKC. When the buffer solution was changed, the new solution was left in the tube for *ca.* 30 min to equilibrate with the capillary walls. The capillary tube was sometimes washed with the same buffer solution during the experiment. Other apparatus and experimental procedures were the same as those described previously[12].

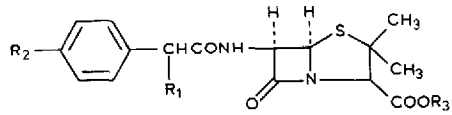
Reagents

Benzylpenicillin (PCG), ampicillin (ABPC), carbenicillin (CBPC), sulbenicillin (SBPC), piperacillin (PIPC), amoxicillin (AMPC), cefotaxime (CTX), cefoperazone (CPZ), cefmenoxime (CMX), cefpiramide (CPM), ceftriaxone (CTRX), cefpimizole (CPIZ) and cefminox (CMNX) were obtained from commercial sources or the National Institute of Hygienic Sciences. Aspoxicillin (ASPC), ceftazidime (CAZ) and C-TA were obtained from our laboratory. These test samples are summarized in Tables I and II. All samples were used as received and dissolved in water at concentrations of 0.5–2 mg/ml to give satisfactory peak heights.

SDS from Nacalai Tesque (Kyoto, Japan) and LMT from Nikko Chemicals

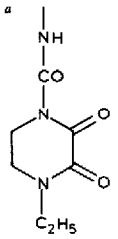
TABLE I

STRUCTURES OF THE SEVEN PENICILLIN ANTIBIOTICS



Penicillin (PC)	*Abbreviation*	*Symbol*	R_1	R_2	R_3
Benzylpenicillin	PCG	1	H	H	K
Ampicillin	ABPC	2	NH_2	H	Na
Carbenicillin	CBPC	3	COONa	H	Na
Sulbenicillin	SBPC	4	SO_3Na	H	Na
Piperacillin	PIPC	5	[a]	H	Na
Aspoxicillin	ASPC	6	[b]	OH	H
Amoxicillin	AMPC	7	NH_2	OH	H

[a]



[b] $-NH-CO-CH(NH_2)-CH_2CONHCH_3$.

(Tokyo, Japan) were used as anionic surfactants. Sodium pentanesulphonate and sodium octanesulphonate were purchased from Wako (Tokyo, Japan) as ion-pairing reagents. These were dissolved in a buffer solution prepared by mixing 0.02 *M* sodium dihydrogenphosphate solution with a 0.02 *M* sodium tetraborate solution to give appropriate pH values, and these solutions were filtered through a 0.45-μm membrane filter, degassed by sonication prior to use.

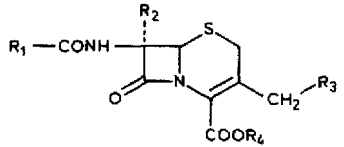
RESULTS AND DISCUSSION

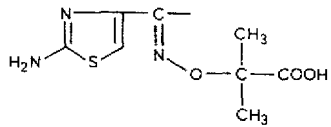
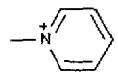
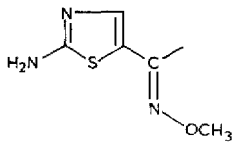
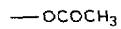
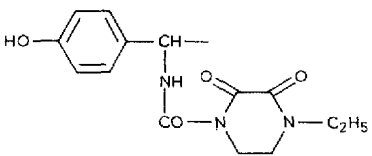
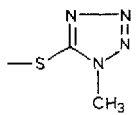
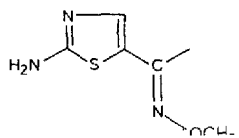
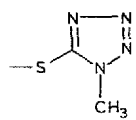
CZE separation

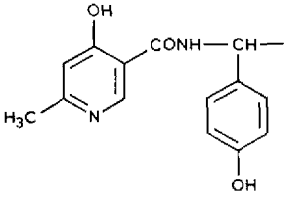
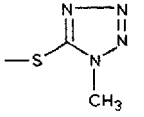
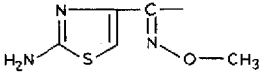
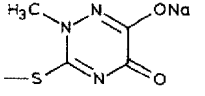
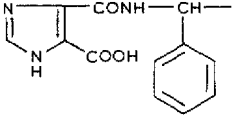
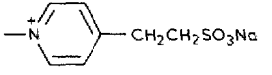
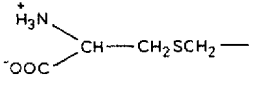
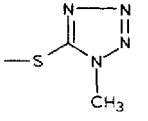
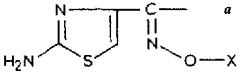
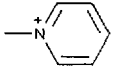
We first investigated the separation of a mixture of seven PCs and a mixture of nine CEPs with a buffer solution containing no surfactant. In this CZE mode, one of the experimental parameters for improving the resolution is the pH of the buffer solution, and we consequently examined the effect of pH in the range 6–9. The elution order in CZE is almost explained in terms of the electric charge and polarity of the solute. That is, under the experimental conditions mentioned above, cationic solutes elute first because both the electroosmotic flow and the electrophoretic migration of the solute are in the same direction to the negative end of the tube, electrically neutral solutes migrate after cationic solutes with the same velocity as the electroosmotic flow and negatively charged solutes migrate slowest because they are pulled back strongly by the electrophoretic effect.

TABLE II

STRUCTURES OF THE NINE CEPHALOSPORIN ANTIBIOTICS



Cephalosporin (CEP)	*Abbreviation*	*Symbol*	R_1	R_2	R_3	R_4
Ceftazidime	CAZ	A		H		–
Cefotaxime	CTX	B		H		Na
Cefoperazone	CPZ	C		H		Na
Cefmenoxime	CMX	D		H		H

Cefpiramide	CPM	E		H		Na
Ceftriaxone	CTRX	F		H		Na
Cefpimizole	CPIZ	G		H		–
Cefminox	CMNX	H		OCH_3		Na
C-TA	– –	I		H		–

[a] X = pyrrolidone derivatives (no ionic substituent).

The separation of the mixtures of antibiotics in CZE with a buffer solution at pH 9.0 is shown in Fig. 1. Some solutes eluted very closely or overlapped. Complete separation was not achieved in the CZE mode over the experimental pH range, indicating insufficient selectivity in the CZE separation of these antibiotics.

The solutes having cationic groups, such as a dioxopiperazinyl group (PIPC), an N-methylasparaginyl group (ASPC) or a pyridinium group (CAZ, C-TA), migrated relatively faster than the others and the solutes that have two carboxyl groups (CBPC, CMNX) and a sulphonate group (SBPC, CPIZ) in addition to a carboxyl group eluted late. This retention behaviour agrees well with the above-mentioned explanation.

In addition to the elution order, column efficiency is also influenced by the pH or composition of the buffer solution[11]. Electrostatic interactions between solutes, in particular cationic solutes, and negatively charged capillary walls are indicated by peak tailing. An asymmetric peak appears when an unsuitable buffer solution is used. In the pH range examined above (6–9), the peak shapes were improved with increasing pH, particularly for the separation of the mixture of seven PCs. This improvement is probably a consequence of ion suppression of cationic groups of the solutes. From these observations, we decided to use a buffer solution of pH 9.0 in the subsequent experiments.

Micellar EKC with SDS solutions

We next investigated the effect of surfactant concentration on the retention times of solutes. The results obtained in micellar EKC with buffer solutions containing SDS are summarized in Fig. 2. Typical chromatograms for the seven PCs and nine CEPs obtained by micellar EKC with 0.15 and 0.3 *M* SDS solutions at pH 9.0 are

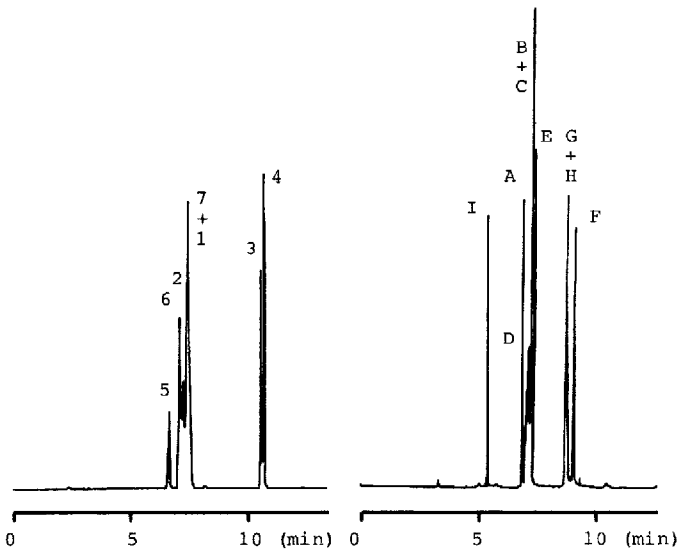


Fig. 1. Separation of seven PCs and nine CEPs by CZE. Conditions: buffer, 0.02 *M* phosphate–borate buffer (pH 9.0); separation tube, 650 mm × 0.05 mm I.D.; length of the tube used for separation, 500 mm; applied voltage, 20 kV; temperature, ambient; detection wavelength, 210 nm; attenuation, 0.04 a.u.f.s. Solutes are indicated by the symbols given in Tables I and II.

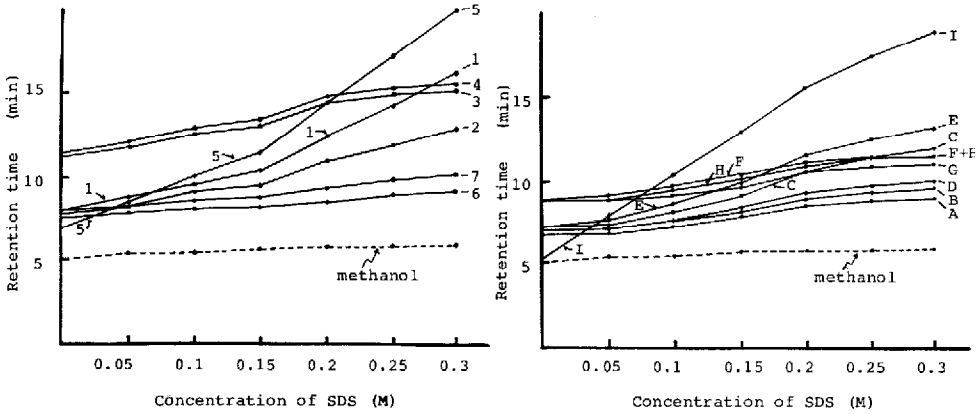


Fig. 2. Effect of SDS concentration on the retention times of PCs and CEPs. Applied voltage, 20 kV. Other conditions as in Fig. 1.

shown in Figs. 3 and 4. The retention times of the solutes increased with an increase in SDS concentration even if the solute was ionized, although the electroosmotic flow was not changed significantly over the whole SDS concentration range.

This trend of the retention times is especially remarkable with PCs. The retention times of PCG and PIPC were delayed more than those of CBPC and SBPC in 0.3 *M* SDS on increasing the SDS concentration, probably because PCG and PIPC were more easily incorporated into SDS micelles owing to their high lipophilicity. On the

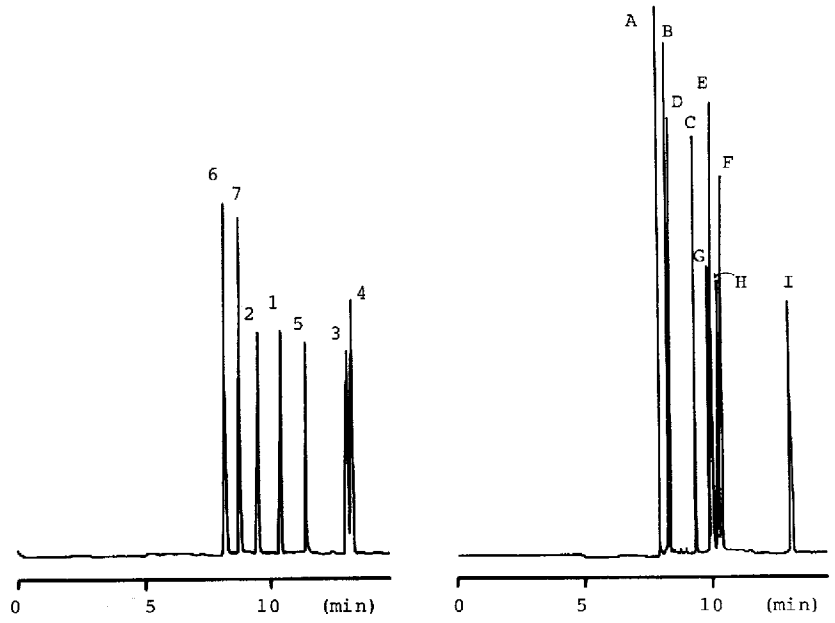


Fig. 3. Micellar EKC of PCs and CEPs. Conditions: applied voltage, 20 kV; SDS concentration, 0.15 *M*. Other conditions as in Fig. 1.

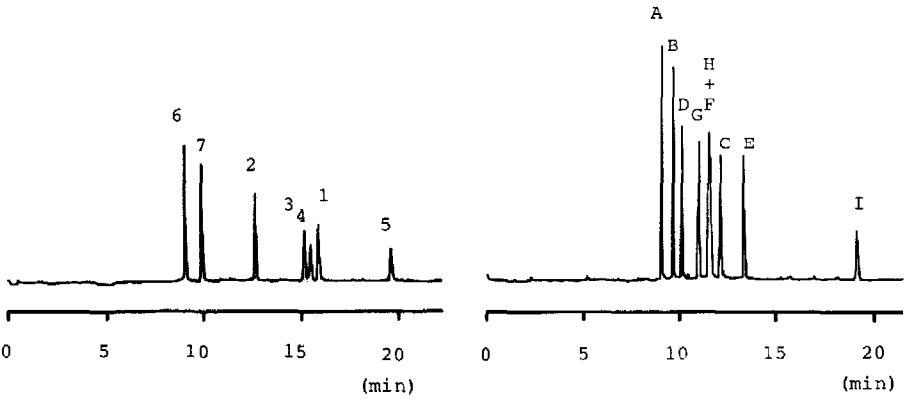


Fig. 4. Micellar EKC of PCs and CEPs. SDS concentration, 0.3 *M*. Other conditions as in Fig. 1.

other hand, the retention behaviour of CBPC and SBPC did not change as much as that of the others with variation in SDS concentration. As these solutes have an anionic group such as a sulphonic or a carboxyl group in their structure, the electrostatic repulsion between the ionized solutes and SDS micelles will suppress the micellar solubilization of the solutes.

The retention times of CEPs also gradually increased with increasing SDS concentration similarly to those of PCs. The migration velocities of CPM, CPZ and, in particular, C-TA were more retarded than those of the others with increasing SDS concentration. CPM and CPZ have relatively more lipophilic groups in their structure and the micellar solubilization effect will consequently act more strongly than with the others. The change in retention time was the largest for C-TA among the CEPs. This may be due to the presence of a pyridinium group, which has a positively charged nitrogen atom. Hence an ion pair may be formed between the cationic group of the solute and the polar group of the anionic surfactant and its formation in addition to the micellar solubilization effect may contribute to the large variation in the retention time. The retention time of CAZ, which also has a pyridinium group, however, did not change as much as that of C-TA. This result is explained in terms of the extra carboxyl group in CAZ compared with C-TA. The marked differences described above show that the electrophoretic mobility of the solute itself is still the most effective parameter in micellar EKC for the separation of ionic solutes.

The factors that affect the elution times of ionic solutes in micellar EKC in our system can be summarized as follows: (1) the number of negatively charged groups (a carboxyl group and a sulphonate group in the solute), (2) the distribution ratio of the solute in the micellar phase to that in the aqueous phase (lipophilicity of the solute) and (3) ion-pair formation between the cationic group of the solute and the polar group of the anionic surfactant. The elution of the solutes will be determined mainly by these three factors, depending on the physico-chemical properties of the solutes.

In addition to the improvement in selectivity, the peak shapes were also improved by addition of SDS even at lower pH, where asymmetric peaks were observed in the CZE mode for the separation of these antibiotics. The same result was observed in the separation of water-soluble vitamins, indicating one of the advantages of micellar EKC.

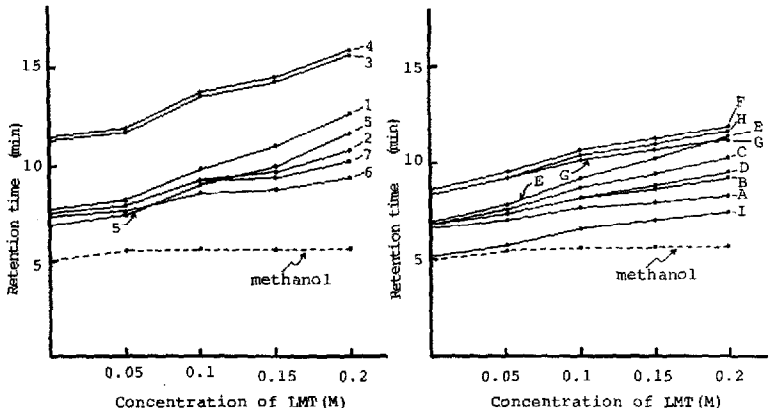


Fig. 5. Effect of LMT concentrations on the retention times of PCs and CEPs. Detection wavelength, 220 nm. Other conditions as in Fig. 1.

Micellar EKC with LMT solutions

LMT was also employed as an anionic surfactant and the effect of LMT concentration on the retention times of the PCs and CEPs were examined. The results are shown in Fig. 5. Typical chromatograms obtained with a 0.15 *M* LMT solution at pH 9.0 are shown in Fig. 6. The effect of LMT concentration was not as great as that of

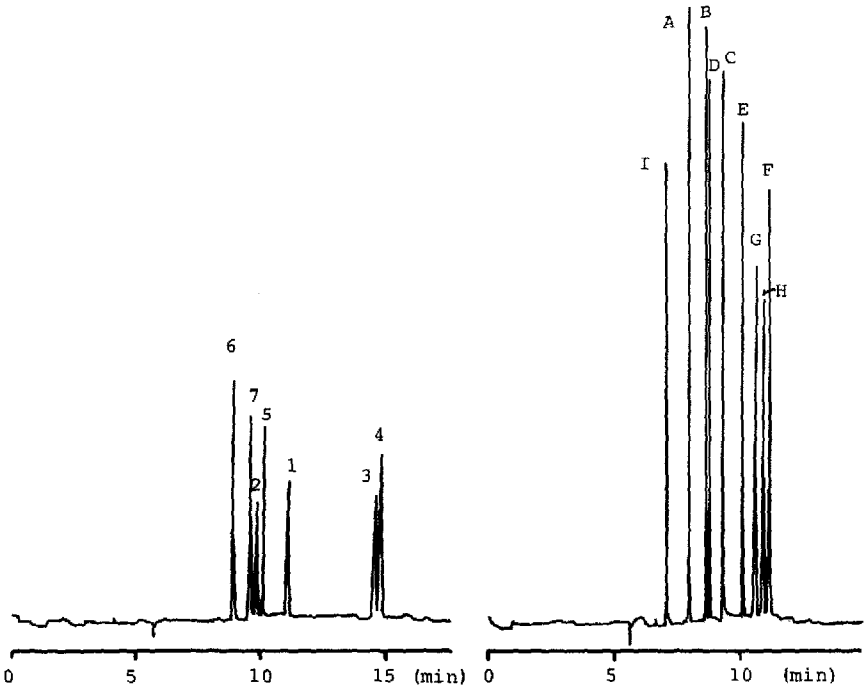


Fig. 6. Micellar EKC of the PCs and the CEPs. LMT concentration, 0.15 *M*; detection wavelength, 220 nm. Other conditions as in Fig. 1.

SDS concentration, although the elution behaviour of the solutes was almost the same as that obtained with SDS.

The variation of the retention time of PIPC was relatively large among the PCs. However, PIPC in a 0.2 *M* LMT solution eluted between PCG and ABPC, although its retention time was longer than that of PCG in a 0.2 *M* SDS solution.

The effect of LMT concentration on the retention times of CPM and CPZ was almost the same as that of SDS concentration. On the other hand, the retention time of C-TA in LMT solutions varied much less than that in SDS solutions.

These marked differences in the retention behaviour of PCs and CEPs, in particular of PIPC and C-TA, between in SDS and LMT solutions can be attributed to the differences in the physico-chemical properties of the two surfactants. We have already observed the same results in the separation of water-soluble vitamins[12]. These differences are probably due to differences in the ionic groups, *i.e.*, a sulphate group in SDS and a sulphonate group in LMT. In addition to the charge-bearing groups, the groups adjacent to them are also different. LMT has an $-N(CH_3)-C(=O)-$group near the ionic group in comparison with a linear hydrocarbon chain in SDS. Consequently, the ion-pair formation between the solutes and the ionic group of LMT might be blocked by steric hindrance. The physico-chemical properties of the two surfactants are given in Table III.

All solutes were successfully separated within 15 min by micellar EKC with SDS or LMT solutions at concentrations of 0.1–0.3 *M*. The numbers of theoretical plates observed in Figs. 3 and 6 are in the range 70 000 (ABPC)–260 000 (PIPC) for PCs and 120 000 (CMNX)–250 000 (CPM) for CEPs, as calculated according to the equation $N = 2\pi(t_R h/A)^2$, where t_R, h, and A are retention time, peak height and peak area, respectively.

Effect of the micelle and additives

To make the effect of the micelle clearer, we investigated the effect of ion-pairing reagents that are often used in reversed-phase HPLC, *i.e.*, sodium pentanesulphonate (C_5) and sodium octanesulphonate (C_8) in a buffer solution at pH 9.0. The effect of C_5 concentration on the retention times of PCs and CEPs was investigated with 0.1 and 0.2 *M* C_5 solutions. Typical chromatograms obtained with 0.2 *M* C_5 are shown in Fig. 7. The retention times of all the solutes increased with increasing concentration of the ion-pairing reagent because of the decrease in the electroosmotic flow (Fig. 8). The selectivity in separation was slightly improved in comparison with CZE. However, the change in the elution order observed in micellar EKC was not observed in this system. C_8 gave almost the same results as C_5, although the elution

TABLE III

PHYSICO-CHEMICAL PROPERTIES OF SDS AND LMT

Surfactant	*MW*	*Kraft point (°)*	*Critical micellar concentration (mmol/l)*
SDS	288.38	9–10	8.1
LMT	343.46	<0	8.7

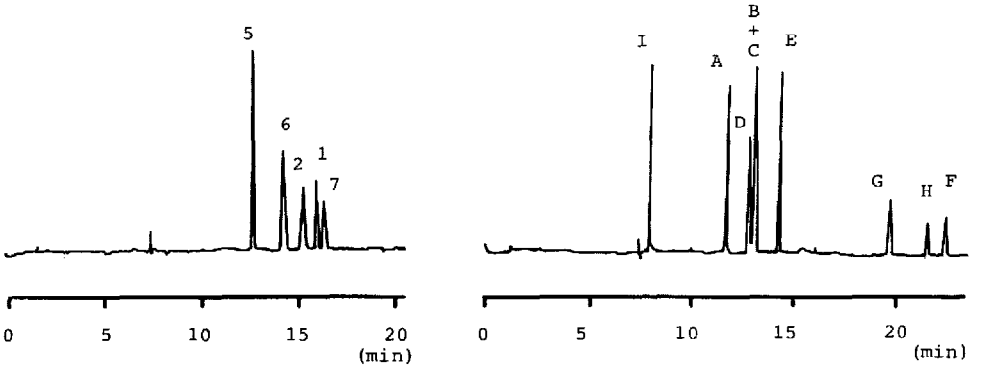


Fig. 7. Separation of PCs and CEPs with a buffer solution containing 0.2 *M* sodium pentanesulphonate. Other conditions as in Fig. 1.

orders of some solutes (PIPC and ASPC, CPIZ and CMNX) altered slightly in 0.1 and 0.2 *M* C_8 solutions. The addition of an ion-pairing reagent was not as effective as the addition of the micelle in improving the resolution, although the separation was slightly improved over the poor resolution given by CZE. The drastic change in the elution order of PCs and CEPs observed in micellar EKC was due to the effect of the micelle because the ion-pairing reagents used in this study do not form a micelle. The charge of the ionic solutes may be neutralized by the ion-pair formation between the solute and the surfactant, and this might consequently accelerate the micellar solubilization and cause the drastic change in the retention behaviour of the ionic solutes. The effect of ion-pair formation is probably superimposed on the effect of micellar solubilization in the presence of the micellar phase.

We would also expect an improvement in selectivity by mixing an additive with

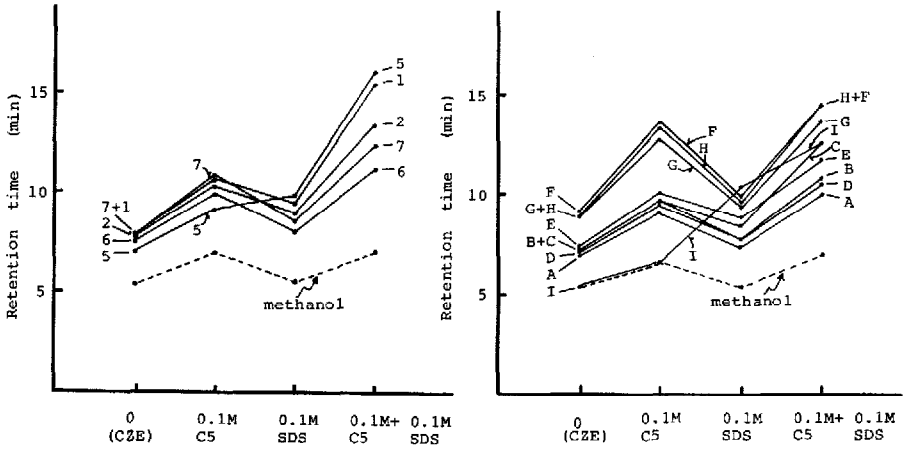


Fig. 8. Effect of the ion-pairing reagent on the separation of PCs and CEPs by mixing with a surfactant. 0, 0.02 *M* phosphate–borate buffer (pH 9.0), CZE separation; C_5, sodium pentanesulphonate. Applied voltage, 20 kV. Other conditions as in Fig. 1.

a surfactant to separate a complex mixture. The results obtained with a solution containing 0.1 *M* SDS and 0.1 *M* C_5 are compared in Fig. 8 with the results given by the other separation modes. Almost the same results were obtained with either C_8 or C_5. When a mixture of an ion-pairing reagent and a surfactant was used, a different retention behaviour was observed to that with either reagent alone. We intend to investigate separations by this mixing method.

We also intend to apply this micellar EKC to the determination of antibiotics in plasma and urine on the basis of above study. Recently, Nakagawa *et al.*[13] reported the determination of CPM in plasma by micellar EKC. A direct plasma injection method was applied and pretreatment of the plasma sample was not necessary. This also demonstrates the capability of micellar EKC.

In conclusion, micellar EKC has brought many advantages for the separation of ionic substances, including penicillin and cephalosporin antibiotics. Selectivity and peak shapes were much improved in comparison with CZE. Satisfactory separations of the seven PCs and the nine CEPs were achieved within 15 min with high theoretical plate values. As very small amounts of sample can also be detected by this method, in addition to the excellent separation capability, EKC will become a powerful analytical method, with further development of the detection system and the injection system, in clinical or biological analysis where ultra-small-scale separations are demanded.

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REFERENCES

1 S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya and T. Ando, *Anal. Chem.*, 56 (1984) 111.
2 S. Terabe, K. Otsuka and T. Ando, *Anal. Chem.*, 57 (1985) 834.
3 K. Otsuka, S. Terabe and T. Ando, *J. Chromatogr.*, 348 (1985) 39.
4 K. Otsuka, S. Terabe and T. Ando, *J. Chromatogr.*, 332 (1985) 219.
5 K. Otsuka, S. Terabe and T. Ando, *Nippon Kagaku Kaishi*, 7 (1986) 950.
6 D. E. Burton, M. J. Sepaniak and M. P. Maskarinec, *J. Chromatogr. Sci.*, 24 (1986) 347.
7 K. H. Row, W. H. Griest and M. P. Maskarinec, *J. Chromatogr.*, 409 (1987) 193.
8 D. E. Burton, M. J. Sepaniak and M. P. Maskarinec, *Chromatographia*, 21 (1986) 583.
9 A. S. Cohen, S. Terabe, J. A. Smith and B. L. Karger, *Anal. Chem.*, 59 (1987) 1021.
10 S. Fujiwara and S. Honda, *Anal. Chem.*, 59 (1987) 2773.
11 R. A. Wallingford and A. G. Ewing, *J. Chromatogr.*, 441 (1988) 299.
12 H. Nishi, N. Tsumagari, T. Kakimoto and S. Terabe, *J. Chromatogr.*, 465 (1989) 331.
13 T. Nakagawa, Y. Oda, A. Shibukawa and H. Tanaka, *Chem. Pharm. Bull.*, 36 (1988) 1622.