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Effect of urea addition in micellar electrokinetic chromatography

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ABSTRACT

The addition of urea to a micellar solution was developed for the separation of hydrophobic compounds by micellar electrokinetic chromatography (MEKC). The logarithm of the capacity factor (k') decreased linearly with increasing concentration of urea. The use of a high concentration of urea allowed the MEKC separation of hydrophobic compounds, which were mostly included in the micelle and could not be resolved by conventional MEKC. The addition of urea also expanded the migration-time window and hence enhanced the resolution. The effect of urea is discussed from the standpoint of the contribution of urea to a diminished water structure around a hydrophobic solute. Two succesful examples of the application of the technique are given for the MEKC separation of a complex mixture and hydrophobic compounds.

INTRODUCTION

Micellar electrokinetic chromatography (MEKC) [1–3], which is also known as micellar electrokinetic capillary chromatography (MECC) [4], is the most popular among various electrokinetic chromatographic (EKC) techniques [5]. The separation principle of EKC is the same as that of chromatography, although the two phases between which the solute is partitioned do not always exist as distinctly separated phases in EKC but mostly constitute a homogeneous solution. The phase which corresponds to the stationary phase in conventional chromatography has to be subject to electrophoresis to migrate with a different velocity from the surrounding medium, which corresponds to the mobile phase.

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An ionic micellar solution, which is easily prepared by dissolving an ionic surfactant in a buffer solution at a concentration higher than the critical micelle concentration (CMC), is employed in MEKC, which has been proved to be useful for the separation of a wide range of compounds including both charged and uncharged molecules [6–8]. Under neutral or alkaline conditions, the electroosmotic flow is stronger than the electrophoretic migration of the ionic micelle, while electroosmosis and electrophoresis oppose their migration directions, and a solute that is totally excluded from the micelle migrates with the fastest velocity equal to the electroosmotic velocity and the micelle migrates with the slowest velocity equal to the difference between the electroosmotic and electrophoretic velocities. The migration velocity and hence the migration time of the solute depend on the distribution of the solute between the micelle and the aqueous phase.

The capacity factor, k', which is the ratio of total moles of the solute in the micelle to the total moles of the solute in the aqueous phase, is calculated from migration time data by the equation [2].

$$k' = \frac{t_R - t_0}{(1 - t_R/t_{mc})t_0} \quad (1)$$

where t_R, t_0 and t_{mc} are the migration time of the sample solute, that of the solute entirely excluded from the micelle and that of the micelle, respectively. The migration time of the micelle is generally measured with a tracer of the micelle, which totally combines with the micelle. Sudan III [1], Sudan IV [9] and timepidium bromide [10] have been conveniently employed as tracers of the sodium dodecyl sulphate (SDS) micelle; in particular, timepidium bromide is suitable in terms of chemical purity [9]. Methanol usually serves as a tracer of the electrosmotic flow, because it is poorly incorporated into the micelle [12] and successfully detected by a UV detector owing to the refractive index change.

The capacity factor is also expressed as [2]

$$k' = K(V_{mc}/V_{aq}) \quad (2)$$

where K is the distribution coefficient and V_{mc} and V_{aq} are the volumes of the micellar and the aqueous phase, respectively. The distribution coefficient is determined by the characteristics of both the micellar and the aqueous phase. For a pertinent micelle, k' will be dependent on the solubility of the solute in the aqueous phase.

In MEKC, the optimum value of the capacity factor is *ca.* 2 for the maximum resolution [2] and a k' range of 0.5–10 is recommended under conventional conditions. However, k' values often exceed 10 for hydrophobic compounds because they tend to be included in the micelle with high partition ratios and the resolution of such compounds is therefore not successful with simple micellar solutions such as SDS, which are widely used in MEKC.

For the separation of hydrophobic compounds by MEKC, three techniques have been developed: (1) the addition of an organic modifier to the micellar solution [11,12]; (2) the use of bile salt micelles [13,14]; and (3) the addition of cyclodextrin (CD) to the micellar solution [15]. The last method, named cyclodextrin-modified MEKC

(CD/MEKC), is promising especially for the separation of isomeric hydrophobic compounds [15] and for chiral separations [16].

In this paper, we describe the addition of urea to micellar solutions for the separation of hydrophobic compounds. Although urea is not often employed in high-performance liquid chromatography (HPLC), it has been proved to be a useful additive for aqueous phase modification in MEKC. Urea is a well known protein-denaturing agent and the solubilities of some hydrocarbons [17], amino acids [18] and related compounds [19] in high-concentration urea solutions have been examined to clarify the denaturing mechanism. The effect of urea on micelle formation has also been investigated [20,21] in terms of the contribution of the water structure to micelle formation.

EXPERIMENTAL

Apparatus

Capillary electrophoresis (CE) systems used in three different laboratories (Kyoto University, Tanabe Seiyaku and Osaka Prefectural College) were essentially the same as the system described previously [1,2,22]. Fused silica tubing of *ca.* 50 μm I.D. (Polymicro Technologies, Phoenix, AZ, U.S.A. and Scientific Glass Engineering, Ringwood, Victoria, Australia) was used as separation capillaries without any inside coatings. The CE instruments consisted of regulated high-voltage d.c. power supplies (HepLL-30P0.08, HepLL-30N0.08 and HJLL-25-PO; Matsusada Precision Devices, Kusatsu, Shiga, Japan), variable-wavelength UV detectors for HPLC (Uvidec 100-III and 100-V, Jasco, Tokyo, Japan; and SPD-6A, Shimadzu, Kyoto, Japan), the cell holders of which were made from plastic or aluminium blocks with 0.7 mm $\times$ 50 μm stainless-steel apertures to accommodate the 50 μm I.D. fused-silica capillaries for on-column detection, and Chromatopac C-R3A and C-R6A data processors (Shimadzu). The detectors were operated at 210 or 220 nm. Sample solutions were introduced manually into the capillaries by the siphoning method, as described previously [1]. The capillaries were left at ambient temperature without any temperature control in the case of the laboratory-made instruments. A commercial CE system, P/ACE 2000 (Beckman, Palo Alto, CA, U.S.A.), with a 57 cm $\times$ 52 μm I.D. fused-silica capillary was also used for the temperature-controlled experiments.

Reagents

SDS and urea of protein-research grade purchased from Nacalai Tesque (Kyoto, Japan) were used as received. Urea of analytical-reagent grade was obtained from Katayama Kagaku (Osaka, Japan). Chemicals employed as test solutes were of analytical-reagent grade or equivalent and used as received. Six corticosteroids used as test samples were purchased from Sigma (St. Louis, MO, U.S.A.). All buffer solutions were prepared from analytical-reagent grade reagents and water purified with a Milli-Q or Milli-RO system (Millipore Japan, Tokyo, Japan).

Surfactant solutions were prepared by dissolving SDS and urea in 50 m*M* phosphate–100 m*M* borate buffer (pH 7.0), 20 m*M* phosphate–20 m*M* borate buffer (pH 9.0) or 50 m*M* borate buffer (pH 9.0) and passed through membrane filters of 0.45 μm pore size (Gelman Science Japan, Tokyo, Japan). Test solutes were dissolved in 20% aqueous or pure methanol at concentrations of 0.2–1 mg ml^{-1} to give adequate peak heights.

Solubility measurement

The solubilities of corticosteroids in urea solutions were determined by HPLC. About 5 mg of a sample were added to 1 ml of each solution (20 m*M* phosphate–20 m*M* borate buffer, pH 9.0) containing a different amount of urea in a test-tube. The test-tube was then sonicated for 30 min with an ultrasonic cleaner. The solution was passed through the membrane filter of 0.45 μm pore size and 20 μl of each filtrate were injected into the HPLC system (Shimadzu LC-5A), which was equipped with a Rheodyne Model 7125 loop injector, a Shimadzu SPD-2A UV detector (operated at 220 nm) and a Shimadzu CTO-2A column oven (40°C). The peak areas were measured with a Shimadzu Chromatopac C-R5A. The solubility of each sample was calculated from the peak area observed for the standard solution and that for the sample solution. The column employed was an Inertsil ODS-2 (5 μm) (150 mm × 4.6 mm I.D.) purchased from Gaskuro Kogyo (Tokyo, Japan). The retention times of fluocinolone acetonide, hydrocortisone acetate and fluocinonide were 3.3, 3.8 and 6.4 min, respectively, using a mobile phase of acetonitrile–water (50:50) at a flow-rate of 1.0 ml min^{-1}.

RESULTS AND DISCUSSION

Fig. 1 shows the dependence of ln k' of test samples on the concentration of urea in MEKC with 50 m*M* SDS solutions. All the ln k' values decreased linearly with increase in the concentration of urea. Similar dependences of ln k' on the concentration of urea were observed for aromatic compounds (Fig. 2), corticosteroids and alkyl *p*-hydroxybenzoates. These linear relationships mean that the free energy of transfer from the aqueous phase to the micelle decreased linearly with increase in urea concentration, provided urea did not cause a substantial alteration of CMC. The linear dependence of ln k' on the percentage of an organic modifier in the mobile phase has been well documented in reversed-phase HPLC [23].

It has been reported that the free energies of transfer of hydrocarbons from water to 7 *M* urea are negative and that the transfer process is spontaneous owing to

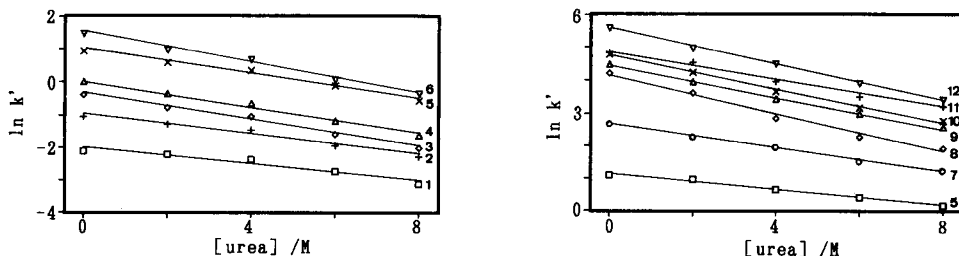

Fig. 1. Dependence of ln k' on the concentration of urea. 1 = Resorcinol; 2 = phenol; 3 = *p*-nitroaniline; 4 = nitrobenzene; 5 = toluene; 6 = 2-naphthol. Capillary, 70 cm × 52 μm I.D. (50 cm to the detector); separation solution, 50 m*M* SDS in 100 m*M* borate–50 m*M* phosphate buffer; applied voltage, 20 kV.

Fig. 2. Dependence of ln k' on the concentration of urea. 5 = Toluene; 7 = naphthalene; 8 = 9-fluorenone; 9 = fluorene; 10 = xanthene; 11 = dibenzyl; 12 = phenanthrene; 13 = stilbene; 14 = fluoranthene. Capillary, 65 cm × 50 μm I.D. (50 cm to the detector); separation solution, 50 m*M* SDS in 20 m*M* borate–20 m*M* phosphate buffer (pH 9.0); applied voltage, 20 kV.

a large positive entropy change, sufficient to override an opposing enthalpy change [17]. This effect is explained in terms of a diminished water structure (so-called "iceberg") around the hydrocarbon by the addition of urea to the aqueous solution [18,21].

We measured the thermodynamic quantities of micellar solubilization for the test solutes given in Fig. 1 and for *p*-alkylphenols in 50 m*M* SDS dissolved in 50 m*M* phosphate–100 m*M* borate buffer [24] and found that although the free energy and enthalpy changes were negative for all the solutes, the entropy changes were positive for *p*-alkylphenols and became more positive with increasing alkyl chain length. Micellar solubilization is a transfer process in a homogeneous solution and different from the above-mentioned system in this regard. However, the two processes are the same from the standpoint of the transfer of the solute from water to the other phase. The positive entropy change found in micellar solubilization can also be interpreted by the major contribution of the water structure around the alkyl group to the transfer process from the aqueous phase to the micelle.

From the above discussion, the effect of the urea addition to micellar solutions is straightforward for *p*-alkylphenols. Urea will increase the solubility of the alkylphenols in the aqueous phase by diminishing the water structure around the alkyl group as described above. Urea will concurrently prevent the alkylpheols from transferring to the micelle from the aqueous phase, by reducing the positive entropy of the transfer from water to the micelle. In fact, we observed in a preliminary experiment that entropy changes of the transfer process from 6 *M* urea to the SDS micelle (50 m*M* SDS at pH 7.0) were substantially reduced close to zero or even to small negative values for *p*-alkylphenols [24].

The above discussion is successfully applicable to the alkylphenols, but for the other solutes the effect of urea is more complicated, judging from the observed thermodynamic quantities of micellar solubilization. It is not easy to find a simple explanation for the observed effect of urea. However, it is obvious that urea increases the solubility of most solutes in water [17–19] and consequently that the distribution coefficient to the micelle is significantly reduced. We confirmed the solubility enhancement by urea for some steroidal compounds as shown in Fig. 3. The solubility of these compounds in 8 *M* urea increased 12–20 times as much as that in the absence of urea. A detailed discussion of the thermodynamic effect on MEKC will be presented elsewhere after obtaining more refined data.

In addition to the free energy changes of the transfer process, we have to take into account the effect of urea on micelle formation [20,21], because the SDS concentration was kept constant in this study. Schick [21] measured the CMC of SDS at different concentrations of urea up to 6 *M* and reported that the CMC of SDS in 6 *M* urea at 25°C increased 1.67 times higher than that in water. The increase in CMC indicates that micelle concentrations for 50 m*M* SDS will decrease from 43 m*M* in water (given in monomer concentration) to 38 m*M* in 6 *M* urea at 25°C [21]. Eqn. 2 suggests that low micelle concentrations produce small k' value, that is, 38 m*M* SDS micelle gives 12% lower k' values than 43 m*M* micelle for every solute. The observed k' at 6 *M* urea were reduced to 40–70% of those in the absence of urea and therefore, the contribution of the change in CMC to the reduced k' values should be less significant than that of the solubility change discussed above.

The migration time of the SDS micelle, t_{mc}, increased with increasing

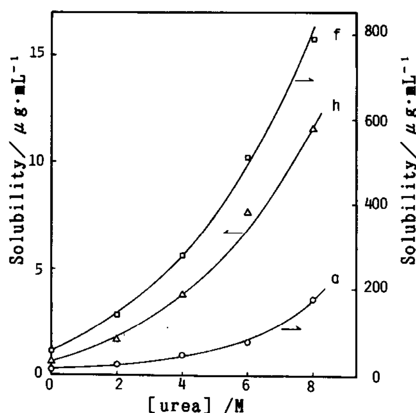

Fig. 3. Solubility of three corticosteroids as a function of the concentration of urea in a phosphate–borate buffer (pH 9.0): (a) Hydrocortisone; (f) fluocinolone acetonide; (h) fluocinonide.

concentration of urea, whereas the electroosmotic velocity did not alter significantly, as indicated in Table I. The ratio t_0/t_{mc} was reduced considerably and hence the migration-time window between t_0 and t_{mc} was considerably expanded. A wide migration-time window is favourable for high resolution, although it requires a long analysis time [2,25]. Similar tendencies of t_0/t_{mc} were also found under three different conditions, as shown in Fig. 4. The differences in the t_0/t_{mc} values among the three were mainly ascribed to the difference in electroosmotic velocities.

The data in Table I suggest that the electrophoretic mobility of the SDS micelle increased with increase in the concentration of urea. We can suggest two possibilities to explain the enhanced electrophoretic mobility: either diminished hydration around the micelle on the addition of urea or an increase in the effective charge on the micelle. At present, we have no preference for either explanation.

Fig. 5 shows the dependence of current on the concentration of urea at a constant applied voltage at a constant temperature. The current decreased linearly with an increase in urea concentration. With 8 *M* urea, which consisted of 43% urea

TABLE I

MIGRATION TIMES OF THE AQUEOUS PHASE AND THE MICELLE AT DIFFERENT UREA CONCENTRATIONS

Conditions in Fig. 1.

Migration time	Urea concentration (*M*)				
	0	2.0	4.0	6.0	8.0
t_0 (min)	3.92	3.92	4.65	5.46	6.38
t_{mc} (min)	14.57	16.10	22.76	30.11	36.45
t_0/t_{mc}	0.269	0.243	0.204	0.181	0.175

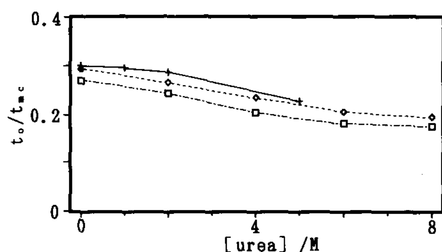

Fig. 4. Dependence of t_0/t_{mc} on the concentration of urea. Conditions: □ = as in Fig. 1; ◇ = as in Fig. 2; + = capillary, 65 cm × 50 μm I.D. (50 cm to the detector). Separation solution, 20 mM SDS in 50 mM borate buffer (pH 9.0); applied voltage, 15 kV.

and 57% buffer [26], the current decreased to about 50% of that observed in the absence of urea, as shown in Fig. 5. As the current depends not only on the concentration of electrolytes but also on the viscosity of the solution, the results in Fig. 5 seem reasonable, because a high concentration of urea does not seriously increase the viscosity of the solution; *e.g.*, the viscosity of 8 M urea in water is 1.66 times higher than that of water at 25°C [26]. In addition, the mobilities of ions will also be affected by a high concentration of urea.

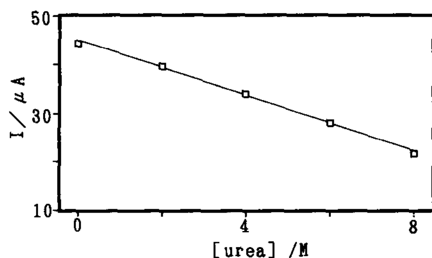

Fig. 5. Dependence of current on the concentration of urea. Capillary, 57 cm × 52 μm I.D. (50 cm to the detector); temperature, 20°C. Other conditions as in Fig. 1.

Two examples which successfully take advantage of the addition of urea in MEKC separation are presented in this paper in contrast to the separation in the absence of urea. Fig. 6 shows the separation of 23 phenylthiohydantoin (PTH)-amino acids using SDS solution with and without urea. We previously reported the separation of 22 PTH-amino acids by MEKC using SDS and dodecyltrimethylammonium bromide (DTAB) solutions [6]. Although the separation was successful in that study, some pairs of PTH-amino acids were not completely resolved from each other under single conditions. In Fig. 6a, where urea was not added, five pairs of PTH-amino acids were poorly or not resolved. The use of 100 mM SDS solution containing 4.3 M urea allowed a complete separation of 23 PTH-amino acids in one run, as shown in Fig. 6b. The optimum concentration of urea was critical in this

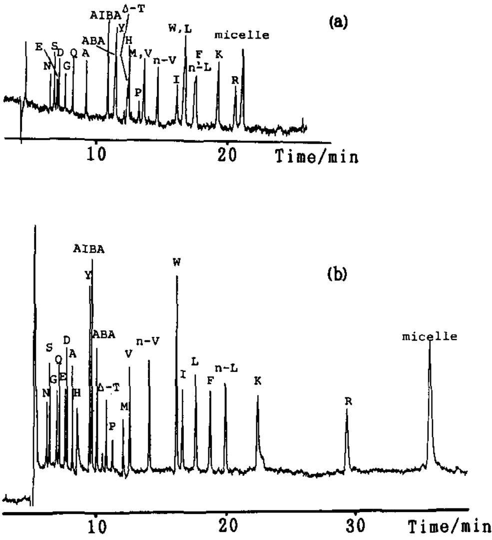

Fig. 6. Electrokinetic chromatogram of a mixture of 23 PTH-amino acids. The peaks are identified with one-letter abbreviations for the amino acids; AIBA = 2-aminoisobutyric acid; ABA = 2-aminobutyric acid; ΔT = PTH-dehydrothreonine. The micelle is traced with timepidium bromide. (a) Conditions as in Fig. 1, except for capillary length (50 cm, 30 cm to the detector) and applied voltage (10.5 kV); (b) separation solution, 100 mM SDS and 4.3 M urea in the same buffer as in (a); other conditions as in (a).

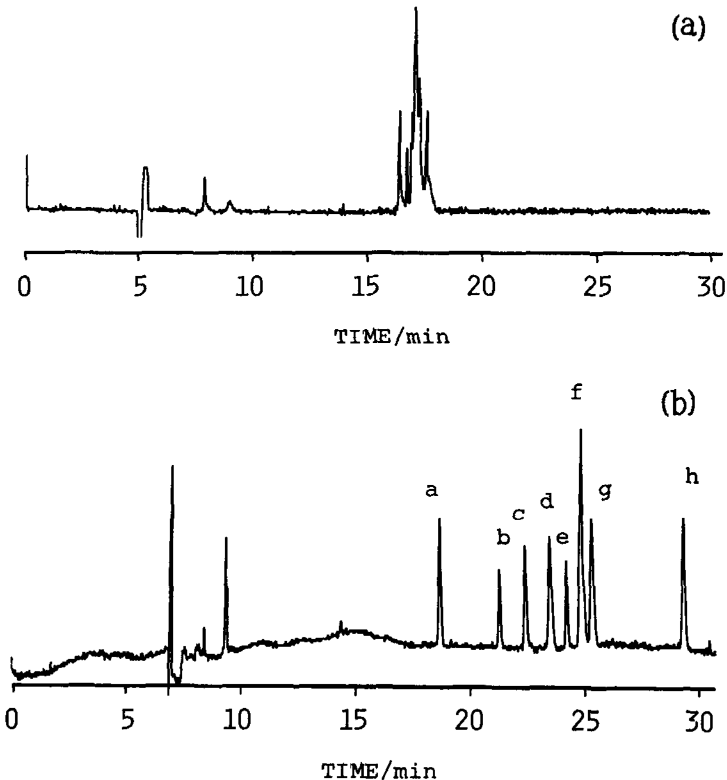

Fig. 7. Separation of eight corticosteroids: (b) hydrocortisone acetate; (c) betamethasone; (d) cortisone acetate; (e) triamcinolone acetonide; (g) dexamethasone acetate; (a, f and h) see Fig. 3. (a) Conditions are as in Fig. 2; (b) separation solution, same SDS solution as in (a) but containing 6 M urea; other conditions as in (a).

experiment, that is, the total selectivity was very sensitive to the concentration of urea for the complex mixture of such closely related compounds. PTH-threonine was not added in Fig. 6, because it gave a seriously tailed peak at a migration time close to PTH-serine (S). The pH of the separation solution was kept constant at 7.0 throughout the experiment, but optimization of pH will permit the complete separation of these PTH-amino acids in a shorter time.

Fig. 7 shows another successful application of the addition of urea to the MEKC separation of eight corticosteroids. As these solutes are poorly soluble in water, as mentioned above, they tend to be mostly incorporated into the micelle. Therefore, they were detected at migration times close to t_{mc} and their capacity factors were higher than 10, although no tracer of the micelle was added in Fig. 7. The use of 6 *M* urea dramatically improved the resolution, as shown in Fig. 7b. In this example, the concentration of urea was not critical and it was easy to obtain a complete separation.

CONCLUSIONS

Although the effect of urea on micellar solubilization is not completely understood, the addition of urea to micellar solutions causes considerable decreases in the capacity factors for most solutes in MEKC. The technique is especially useful for the separation of hydrophobic compounds from the standpoint of the extended applicability of MEKC. The expanded migration-time window generated by the addition of urea is also convenient for high resolution.

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