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CHIRAL SEPARATION BY ELECTROKINETIC CHROMATOGRAPHY WITH BILE SALT MICELLES

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SUMMARY

Various bile salt solutions were employed for micellar electrokinetic chromatography. Many racemic dansylated amino acids were successfully resolved, especially with a taurodeoxycholate solution under acidic conditions. Apparent capacity factors and apparent separation factors of racemic Dns-amino acids were determined from retention times of the solute, the micelle and methanol. Retention parameters of charged solutes and the effect of electroosmosis on resolution are discussed and a possible chiral separation mechanism is suggested.

INTRODUCTION

Electrokinetic chromatography (EKC)[1–3] has been developed for the separation of electrically neutral compounds by means of capillary zone electrophoresis (CZE)[4–6]. In micellar EKC, an ionic surfactant solution is employed instead of a buffer solution in CZE at a higher concentration than the critical micelle concentration (CMC). While the ionic micelle migrates with a different velocity from that of the surrounding aqueous medium by electrophoresis, an injected solute solute distributes itself between the micelle and the surrounding aqueous phase. Although the micelle may be classified as a pseudo-phase, the difference in distribution coefficients and the differential migration of the two phases satisfy the chromatographic separation principle.

As the bulk solution in the capillary is generally transported by electroosmosis, even the aqueous phase also migrates under CZE conditions with a different velocity from that of the surrounding aqueous medium by electrophoresis, an injected solute distributes itself between the micelle and the surrounding aqueous phase. Although the micelle may be classified as a pseudophase, difference in distribution coefficients and the differential migration of the two phases satisfy the chromatographic separation principle.

Since the bulk solution in the capillary is generally transported by electroosmosis, even the aqueous phase also migrates under CZE conditions with a different velocity from that of the ionic micelle. In addition to the distribution of the solute between the two phases that constitute a homogeneous solution, plug-like flow profiles of both electroosmosis and electrophoresis make micellar EKC highly efficient in

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comparison with high-performance liquid chromatography (HPLC). Accordingly, plate heights of about 2 μm can be easily obtained under favourable conditions[7].

Most separation studies on micellar EKC have been performed with sodium dodecyl sulphate (SDS)[8–16]. Some other surfactants such as cetyltrimethylammonium bromide[8] and sodium N-lauroyl-N-methyltaurate[16] have also been successfully employed. Although micellar EKC is undoubtedly useful for the separation of electrically neutral compounds, it is also effectively applicable to the separation of ionic compounds, especially when they cannot be separated sufficiently by CZE alone[9,11–14,16].

In this paper, we describe chiral separations by micellar EKC with a chiral micelle, as the use of a chiral micelle has not previously been reported. Bile salts are one type of the most common biological surfactants among various kinds of chiral surfactants. In order to function as the micellar phase in EKC, the surfactant must be ionic, as mentioned above, and thus some readily available bile salts are employed for chiral separations by micellar EKC.

Recently, Cohen *et al.*[17] reported the use of a mixed micelle of SDS with N,N-didecyl-L-alanine in the presence of copper(II) for the chiral separation of some amino acid derivatives. The L-alanine derivative itself will not form a micelle, and the reported chiral separation may be based on differential metal chelate complexation on the surface of the micelle, which is similar to the chiral separation with an amino acid–copper(II) complex described by Gozel *et al.*[11]. The chiral separation described in this paper is based purely on differential micellar solubilization and provides an example of a chiral separation in an aqueous solution.

EXPERIMENTAL

Apparatus

Micellar EKC was performed in a 700 mm × 0.05 mm I.D. fused-silica capillary tube (Polymicro Technologies, Phienix, AZ, USA, or Scientific Glass Engineering, Ringwood, Victoria, Australia), of which 500 mm was the effective length for separation, *i.e.*, from the injection end to the detection cell. Apparatus essentially the same as that described previously[2] was employed and a spctrophotometric detector (UVIDEC-100-II, Jasco, Tokyo, Japan) together with the capillary tube and electrode vessels were placed in an air oven thermostated at 35 or 40°C. The detector was usually operated at 210–220 nm. A regulated high-voltage power supply of an IP-2A isotachophoretic analyser (Shimadzu, Kyoto, Japan), which worked only under constant-current conditions up to 500 μA and 25 kV, was used. Sample injection was accomplished by the hydrostatic method or by siphoning[1].

Reagents

Sodium cholate and sodium taurocholate were purchased from Nacalai Tesque (Kyoto, Japan), sodium dehydrocholate from Wako (Osaka, Japan) and sodium taurolithocholate, sodium deoxycholate, sodium taurodeoxycholate and Dns-amino acids from Sigma (St. Louis, MO, USA), and were used as received. Other reagents used for the preparation of buffer solutions or as the solutes were of analytical-reagent grade and were used without further purification. Water was purified through a Milli-Q system (Nihon Millipore, Tokyo, Japan). All the micellar solutions were filtered through a membrane filter of 0.5-μm pore size prior to use.

Procedure

In order to obtain good reproducibility of retention data, the capillary tube was cleaned according to the following procedure each time when the solution was replaced: the capillary was continuously flushed with water delivered from an HPLC pump at 0.05 ml min^{-1} for 15 min, swept with 0.15 *M* hydrochloric acid from a manually operated syringe for 5 min, flushed again with water for 15 min as described above, swept with 0.1 *M* sodium hydroxide solution with a syringe for 5 min as mentioned above, flushed with water again for 30 min and finally filled with the working solution and allowed to stand in the thermostated oven for 30 min prior to operation.

RESULTS AND DISCUSSION

Chiral separation with bile salts

Sodium taurolithocholate and sodium deoxycholate solutions in phosphate buffer solutions (pH 7.0) were gelated and hence could not be used in this study. When 50 m*M* solutions of sodium cholate and sodium dehydrocholate in 50 m*M* phosphate buffer (pH 7.0) were employed as micellar solutions, most Dns-amino acids were not separated from one another or from the enantiomers.

The chiral separation of Dns-DL-amino acids was investigated with sodium taurocholate solute at pH 7.0 and 3.0. Only racemic Dns-Trp was partially resolved under neutral pH conditions, where the electroosmotic flow was strong and hence all the Dns-amino acids migrated toward the negative electrode. Eight Dns-amino acids were separated from one another under the above conditions in the following elution order: Dns-Ser, Dns-Thr, Dns-Val, Dns-Met, Dns-Leu, Dns-Trp, Dns-Glu and Dns-Asp. This order is explained in terms of the hydrophobicity of the amino acid residue, except for Dns-Glu and Dns-Asp, each of which have an additional carboxyl group.

When a 50 m*M* sodium taurocholate solution in 50 m*M* phosphate buffer (pH 3.0) was employed instead of the neutral pH solution, the electroosmotic velocity decreased considerably; consequently, the direction of migration of the solute was reversed and hence the elution order of Dns-amino acids was significantly altered (see Fig. 1), e.g., Dns-Trp, which was eluted late at pH 7.0, was now eluted first. In this instance, a more hydrophobic solute, which is more incorporated by the micelle, will migrate faster than a less hydrophobic solute. Only Dns-DL-Nle was partially resolved, as shown in Fig. 1. Although the other Dns-DL-amino acids shown in Fig. 1 were not optically resolved explicitly, most Dns-DL-amino acids, especially Dns-DL-Met and Dns-DL-Nva, seemed, likely to be optically resolved.

The partial enantiomeric separation of Dns-DL-Trp and Dns-DL-Nle was also observed with a 30 m*M* sodium taurodeoxycholate solution in 50 m*M* phosphate buffer (pH 7.0) and the elution order of the eight Dns-amino acids mentioned above was the same as that with sodium taurocholate solution (pH 7.0).

One of the best chiral separations accomplished is shown in Fig. 2, where micellar EKC was performed with a 50 m*M* sodium taurodeoxycholate solution in 50 m*M* phosphate buffer (pH 3.0) at 40°C. The theoretical plate number is about 70 000. It should be noted that the relative elution order of Dns-Leu and Dns-Phe is reversed between Figs. 1 and Fig. 2. Enantiomers of Dns-DL-Phe and Dns-DL-Met were

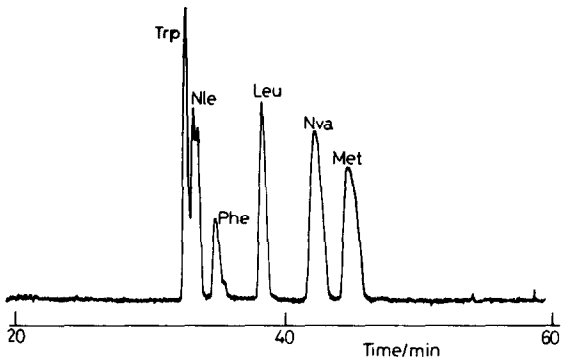

Fig. 1. Micellar EKC separation of Dns-DL-amino acids with 50 m*M* taurocholate in 50 m*M* phosphate buffer (pH 3.0) in a 500 mm (total length, 700 mm) × 0.05 mm I.D. tube at 40°C. Current, 50 μA.

completely resolved and the L-isomers were eluted faster than the corresponding D-isomers in both Dns-amino acids. This relative elution order means that the L-isomers are incorporated by the micelle more than the D-isomers. Dns-DL-Nle, Dns-DL-Leu and Dns-DL-Nva were also optically resolved, as shown in Fig. 2. Dns-DL-Trp, Dns-DL-Val, Dns-DL-Thr and Dns-DL-Ser were also optically resolved under different electroosmotic flow conditions; *e.g.*, the chiral separation of Dns-DL-Trp is shown in Fig. 3, where the electroosmotic flow is slightly faster than that in Fig. 2.

As the separations shown in Figs. 2 and 3 needed a long time, we tried to reduce the analysis time without a serious decrease in resolution. An example is illustrated in Fig. 4, where 10 m*M* SDS was added to a 50 m*M* sodium taurodeoxycholate solution. The analysis time was almost halved but the resolution was not much impaired. The addition of SDS to the solution probably increased the electrophoretic velocity of the micelle owing to the formation of a mixed micelle having a larger charge than the original micelle.

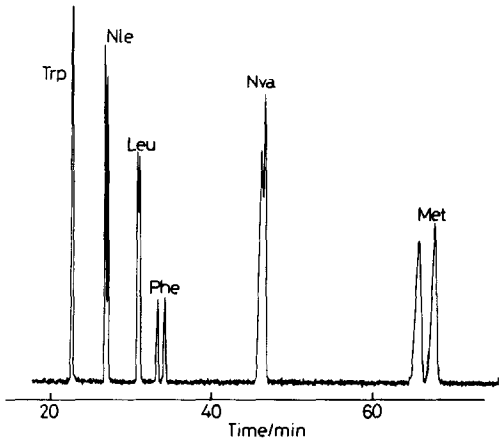

Fig. 2. Micellar EKC separation of Dns-DL-amino acids with 50 m*M* taurodeoxycholate in 50 m*M* phosphate buffer (pH 3.0). Other conditions in Fig. 1.

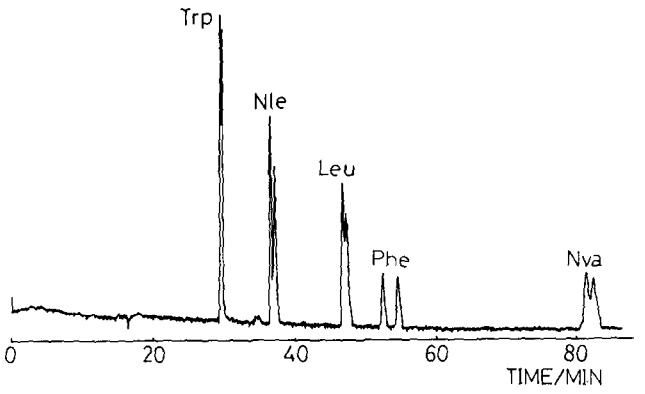

Fig. 3. Chiral separation of Dns-DL-amino acids. Conditions as in Fig. 2 but the electroosmotic velocity is slightly faster.

Retention times, capacity factors and separation factors of Dns-amino acids

In micellar EKC, if the solute is electrically neutral, the relationship between the retention time, t_R, and the capacity factor, $\tilde{k}'$, is given by[1,2]

$$t_R = \frac{1 + \tilde{k}'}{1 + (t_0/t_{mc})\tilde{k}'} \cdot t_0 \tag{1}$$

or

$$\tilde{k}' = \frac{t_R - t_0}{t_0(1 - t_R/t_{mc})} \tag{2}$$

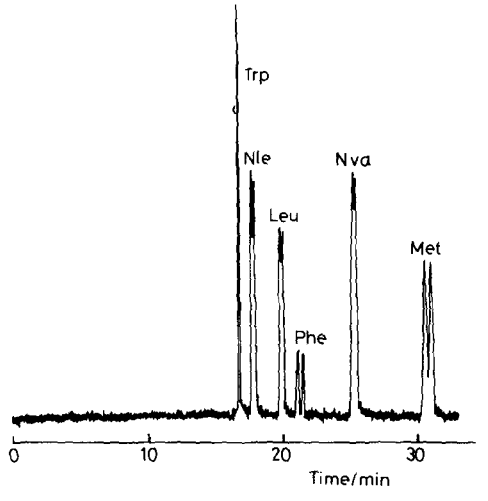

Fig. 4. Effect of addition of SDS. SDS (0.01 *M*) was added to the same solution as used in Fig. 2. Other conditions as in Fig. 2.

where t_0 and t_{mc} are retention times of an unincorporated solute and the micelle, respectively, and $\tilde{k}'$ is defined by the ratio n_{mc}/n_{aq}, where n_{mc} and n_{aq} are the total numbers of moles of the solutes in the micelle and in the aqueous phase, respectively. It should be noted that t_{mc} is required in addition to t_R and t_0 in order to calculate $\tilde{k}'$ according to eqn. 2.

If the solute is charged, it will migrate with a different velocity from the aqueous phase even when it is not incorporated into the micelle. Accordingly, we need to employ another equation to calculate $\tilde{k}'$ for a charged solute[9]. Thus,

$$\tilde{k}' = \frac{v^*_{ep}(s) - v_{ep}(s)}{v_{ep}(mc) - v^*_{ep}(s)} \tag{3}$$

where $v_{ep}(s)$ and $v_{ep}(mc)$ are electrophoretic velocities of the solute in the absence of the micelle and electrophoretic velocity of the micelle, respectively, and $v^*_{ep}(s)$ is the apparent electrophoretic velocity of the solute in the micellar solution, which is the difference between the electroosmotic velocity and the migration velocity of the solute in the micellar solution.

Dns-amino acids are zwitterionic compounds and they may have a positive or negative charge depending on whether the pH is lower or higher than the isoelectric point. Although neither pK_a values nor isoelectric points have been reported, most Dns-amino acids may be positively charged under the conditions given in Fig. 2.

It is not possible to calculate the capacity factors of Dns-amino acids from the chromatograms shown in Figs. 1–4, because either t_0 *or* t_{mc} is not evident. We employed methanol or formamide as a tracer of the electroosmotic flow and Sudan III as that of the micelle to obtain t_0 *and* t_{mc}. Retention times, capacity factors calculated according to eqn. 2 and separation factors, $\alpha = \tilde{k}'_2/\tilde{k}'_1$, are summarized in Table I. It should be noted that the conditions in Table I are approximately the same as those in Fig. 2, but are not identical. Under acidic conditions, the electroosmotic flow was not

TABLE I

RETENTION TIMES, APPARENT CAPACITY FACTORS AND APPARENT SEPARATION FACTORS (α) OF DNS-AMINO ACIDS IN MICELLAR EKC

Micellar solution, 50 m*M* sodium taurodeoxycholate in 50 m*M* phosphate buffer (pH 3.2); capillary tube, 700 mm (effective length, 500 mm) × 0.05 mm I.D.; current, 40 μA; applied voltage, *ca.* 19 kV; temperature, 35°C.

Solute	t_{R1} *(min)*[a]	t_{R2} *(min)*[a]	$\tilde{k}'_1$	$\tilde{k}'_2$	α
Formamide	18.3 (t_0)		0		
Sudan III	−12.7 (t_{mc})		∞		
Dns-Trp	−20.7		3.40		1.01[b]
Dns-Nle	−25.3	−25.0	2.40	2.44	1.02
Dns-Leu	−30.0	−29.8	1.94	1.95	1.01
Dns-Phe	−33.0	−32.0	1.75	1.81	1.03
Dns-Nva	−45.4	−45.1	1.35	1.36	1.01
Dns-Met	−62.2	−60.4	1.13	1.15	1.02

[a] A negative sign means the migration from negative to positive electrode.
[b] Calculated from a different chromatogram where Dns-DL-Trp was resolved.

likely to be exactly reproducible, probably because the zeta potential of the tube wall was low and hence unstable.

Capacity factors of Dns-amino acids under acidic conditions must be accurately calculated according to eqn. 3, because Dns-amino acids are not electrically neutral as described above. It was, however, not easy to obtain a reliable value of the electrophoretic mobility of Dns-amino acids under the conditions given in Table I. As the purpose of this study was to demonstrate possible chiral separations by micellar EKC with a chiral surfactant, we compromised with the apparent values of capacity factors and separation factors. The exact capacity factors will be larger than those in Table I owing to the effect of the electrophoretic migration of unincorporated Dns-amino acids, as mentioned above. Accordingly, the separation factors in Table I will be smaller than the actual values.

Resolution and separation mechanism

The resolution (R_s) equation in micellar EKC is[2]

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{\tilde{k}'_2}{1 + \tilde{k}'_2}\right)\left[\frac{1 - t_0/t_{mc}}{1 + (t_0/t_{mc})\tilde{k}'_1}\right] \quad (4)$$

As already discussed previously[18,19], the last term on the right-side of eqn. 4 is additional to that in usual chromatography and hence R_s is seriously dependent on the last term, particularly if t_0/t_{mc} is large or negative. The electroosmotic flow had the opposite sign to the velocity of the micelle under the conditions shown in Table I, that is, t_0/t_{mc} was negative. Under this circumstance, when the capacity factor approaches the value $-t_{mc}/t_0$, we can expect a great enhancement in resolution at the expense of a long analysis time[18,19]. For example, Dns-DL-Nle and Dns-DL-Met have almost equal α values but the resolution of the latter is much better than that of the former because the capacity factor of Dns-Met is closer to $-t_{mc}/t_0 = 0.69$ than that of Dns-Nle.

Bile salts differ significantly in molecular structure from the long-chain alkyl surfactants such as SDS. They consist of a relatively flat-shaped steroid portion, in which the A ring is *cis* with respect to the B ring, and a side-chain having a carboxyl group, which may be conjugated by taurine or glycine (see Fig. 5A for the structure of taurodeoxycholate). All the hydroxyl groups bonded to the steroid portion are oriented in the same direction nearly perpendicular to the steroidal frame, and consequently the bile salts possess a hydrophobic and a hydrophilic face. They are generally considered to form small or primary micelles which are composed of up to ten monomers by the hydrophobic interaction between the non-polar faces of the monomers[20]. A possible structure of a bimolecular aggregate of sodium taurodeoxycholate is shown diagrammatically in Fig. 5B[20].

Deoxycholic acid differs from cholic acid only in the number of hydroxyl groups. The 7α-hydroxyl group in the latter is replaced by a hydrogen atom in the former molecule. Remarkably different results are observed in the chiral separation of Dns-amino acids between the use of taurodeoxycholate and taurocholate, as can be seen in Figs. 1 and 2. However, it is difficult to explain this difference in terms of the separation mechanism. A remarkable difference in the chiral separation is also

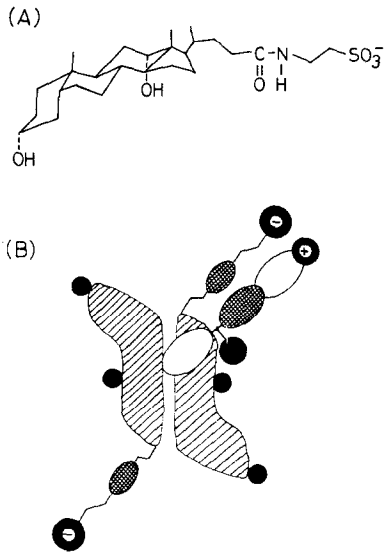

Fig. 5. (A) Structural formula of taurodeoxycholate and (B) a possible spatial arrangement of configuration of Dns-L-Ala interacting with a bimolecular aggregate of taurodeoxycholate. Filled parts indicate hydroxyl, carboxyl, sulphonate and ammonium groups, meshed parts amide and sulphonamide groups and the other parts (open and hatched) hydrophobic moieties.

observed between pH 3.0 and 7.0 with taurodeoxycholate solutions. In acidic media, the dimethylamino group on the Dns group may be partly protonated and therefore the positively charged amino group will interact strongly with the negatively charged sulphonyl group of the taurine moiety on the side-chain through electrostatic attraction. The amino acid residue, if it is hydrophobic, will also be combined concurrently with the non-polar portion of the bile salt micelle by the hydrophobic interaction as shown in Fig. 5B, where Dns-L-Ala is employed. If Dns-D-Ala is displayed in Fig. 5B instead of the L-isomer whilst maintaining the ionic and hydrophobic interactions shown, the carboxyl group has to be far from the 11α-hydroxyl group and come close to a hydrophobic part of the micelle. Dns-L-amino acids may thus be incorporated by the taurodeoxycholate micelle at the two different positions. The interactions at more than two positions are more favourable for chiral recognition than those without the electrostatic attraction under neutral pH conditions.

CONCLUSIONS

Although most chiral separations of Dns-amino acids described in this study are not satisfactory from the viewpoints of the expected high efficiency of micellar EKC and also many papers reporting successful chiral separations of Dns-amino acids by HPLC, the results presented suggest that micellar EKC will become a promising technique for chiral separations. It should be stressed that only a micelle consisting of a chiral surfactant in the separation system can recognize the enantiomers in the absence of any other additives such as a metal to form a chelate complex. Even if the

surfactant is not chiral, a mixture of an ionic surfactant and a chiral compound that tends to form a mixed micelle will be applicable to chiral separations by micellar EKC.

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