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SEPARATION OF WATER-SOLUBLE VITAMINS BY MICELLAR ELECTROKINETIC CHROMATOGRAPHY

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SUMMARY

The retention behaviour of eleven water-soluble vitamins in micellar electrokinetic chromatography (micellar EKC) was investigated in comparison with capillary zone electrophoresis. Sodium dodecyl sulphate (SDS) and sodium lauroylmethyl taurate were used as the anionic surfactants at concentrations of 0.05–0.2 *M* in micellar EKC. The retention times of cationic substances increased more rapidly with increasing concentration of the anionic surfactant than those of other substances. This result suggests that ion-pair formation between cationic substances and anionic surfactants contributes to the retention of the former. The difference in the structures of the two surfactants affects the retention behaviour of solutes, especially cationic substances. To clarify the effect of the micelle, an ion-pairing agent that does not form the micelle structure was employed. All solutes were successfully separated within 15 min by using a 650 mm × 0.05 mm I.D. fused-silica tube with a 0.05 *M* SDS solution (pH 9.0) to give theoretical plates ranging from 100 000 to 350 000.

INTRODUCTION

Capillary zone electrophoresis (CZE) has many advantages for the separation of ionic substances[1–5]. In CZE, ionic substances are separated according to their electrophoretic mobilities under the influence of an high potential field which creates a strong electroosmotic flow within a tube. This strong electroosmotic flow which has a flat velocity profile causes every kind of solute including cationic, neutral and anionic substances to elute at one end of the tube with high resolution. On-column detection also allows microanalysis of a sample without any loss of column efficiency.

Although CZE has been shown to be a very powerful separation technique for ionic substances, it is lacking in ability to separate non-ionic substances. Because the migration velocity of such non-ionic substances is not affected by the electric field and

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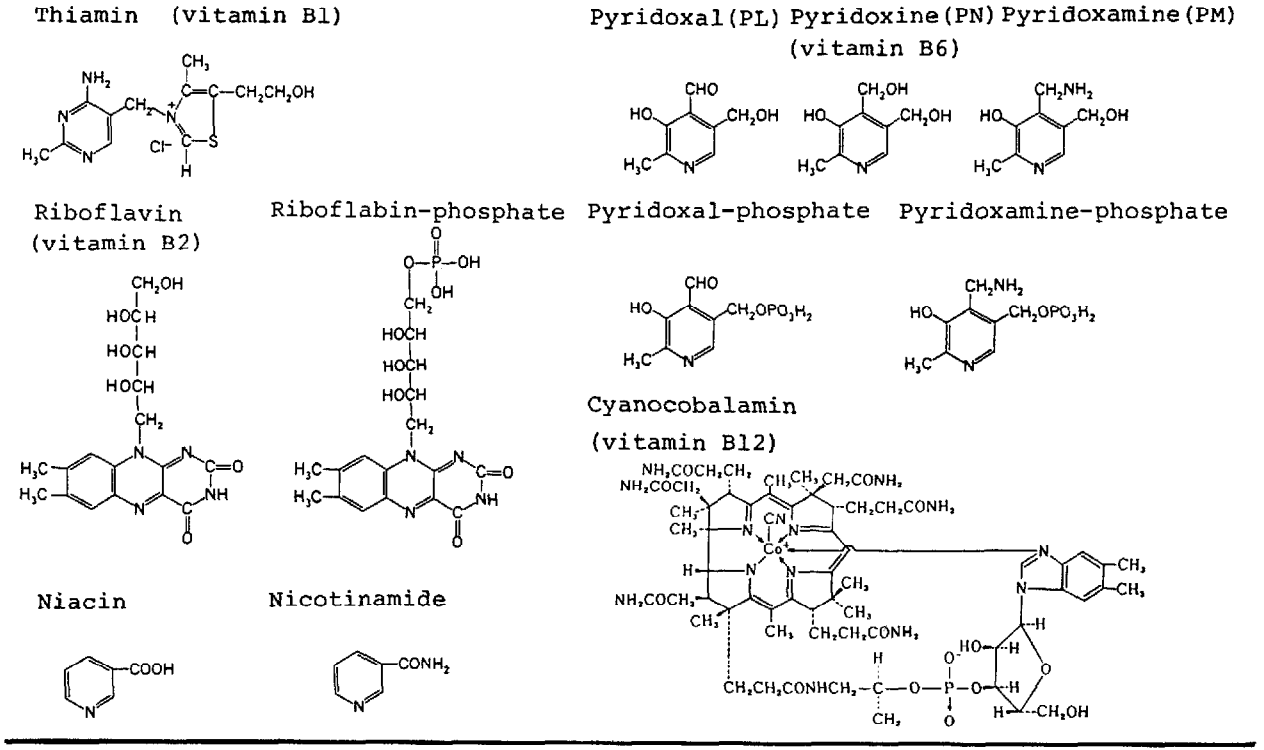


1. pyridoxamine (PM)
2. nicotinamide
3. pyridoxal (PL)
4. vitamin B6
5. vitamin B2
6. vitamin B12
7. vitamin B2 phosphate
8. PM-5'-phosphate
9. niacin
10. vitamin B1
11. PL-5'-phosphate

Fig. 1. Structures of eleven water-soluble vitamins. Solute numbers are also indicated.

pH, they migrate with the same velocity as that of the electroosmotic flow and no separation is achieved.

Recently, a new technique was introduced to improve the selectivity in the separation of non-ionic substances in CZE. This technique is based on micellar solubilization and was first reported in 1984[6]. A wide variety of applications to the separation of non-ionic substances are well documented[7–9]. In this system, the separation of non-ionic substances is based on the difference in the solute distribution between the aqueous phase and the micellar phase. So this technique belongs to a branch of chromatography which is probably similar to liquid–liquid partition chromatography. We term this kind of technique "electrokinetic chromatography (EKC)", in which electrokinetic migration of the ionic carrier that interacts with the solute is employed. For example, distribution mechanisms such as solubilization by micelles, host–guest interaction by cyclodextrins[10] and ionic interaction[11,12] have been explored. In the use of surfactants mentioned in this paper, we term this mode "micellar EKC".

A wide variety of applications of micellar EKC will be possible other than the separation of non-ionic substances, and it is expected to improve the selectivity in the separation even of ionic substances. We are interested in small scale analysis of pharmaceutical drugs. So we will first demonstrate the capabilities of micellar EKC for the separation of water-soluble vitamins in comparison with CZE. The effect of the structural differences of surfactants and the effect of adding an ion-pairing agent will be also discussed. There are two reports on micellar EKC separation of water-soluble vitamins[13,14]. We have found that ion-pair formation occurs in some cases and partition also participates in distribution even for ionic substances.

EXPERIMENTAL

Apparatus

A schematic diagram of the EKC system employed has been shown previously[3]. Separations were performed in a 650 mm × 0.05 mm I.D. fused-silica capillary tube (Scientic Glass Engineering), with a Model HJLL-25PO high-voltage d.c. power supply (Matsusada Precision Devices, Otsu, Japan) delivering up to +25 kV. Each end of the capillary tube was dipped into a solution in a small glass beaker (10 ml) with a silicone-rubber stopper having two small-bore holes, one for a platinum electrode and the other for the capillary tube. The electric current was monitored between the negative terminal of the power supply and the negative electrode with an ammeter throughout the operation. At 150 mm from the negative end of the tube, the on-column measurement of UV absorption at 210 or 220 nm was carried out with an UVIDEC-100-VI spectrophotometric detector (Jasco, Tokyo, Japan). Peak areas and peak heights were measured by a Shimadzu Chromatopak C-R2AX data processor.

Reagents

Water-soluble vitamins were obtained from Katayama (Osaka, Japan), Nakarai (Kyoto, Japan), Wako (Tokyo, Japan) or Tokyo Kasei Kogyo (Tokyo, Japan). These test samples are listed in Fig. 1. As anionic surfactants, sodium dodecyl sulphate (SDS) from Nakarai (Kyoto, Japan) and sodium lauroylmethyl taurate (LMT) from Nikko Chemicals (Tokyo, Japan) were used as recieved. Sodium pentanesul-

phonate used as an ion-pairing agent was from Katayama. All other reagents were of analytical reagent grade from Katayama. Test samples were dissolved in water at concentrations 0.5–2 mg/ml to give suitable peak heights. Buffer solutions used for EKC were prepared by mixing a 0.02 *M* sodium dihydrogenphosphate solution or a 0.02 *M* ammonium acetate solution with a 0.02 *M* sodium tetraborate solution in appropriate proportions to give suitable pH values (6–9). The buffer solution was filtered through a 0.45-μm membrane filter prior to use.

Procedure

A capillary tube was filled with a buffer solution by use of a microsyringe and about 10 ml each of the same buffer solution were introduced into the two beakers placed at the same level. To introduce a sample solution into the tube, the positive end of the tube was moved rapidly to a vessel containing a sample solution and the level of the sample solution was raised about 5 cm higher than the level of the buffer solution to allow the sample solution to flow downward into the capillary tube. After 5–10 s, the end of the tube was returned to the previous beaker of the buffer solution, then an high voltage was applied. The injection volume in this system was of the order of a nanolitre. All experiments were performed in the laboratory air-conditioned at *ca.* 27°C.

RESULTS AND DISCUSSION

Electrokinetic separation

A schematic illustration of micellar EKC is shown in ref. 11. An high d.c. voltage up to +25 kV in the present system is applied between both ends of the capillary tube containing an aqueous buffer solution of an anionic surfactant. The whole solution is transported toward the negative end by the electroosmotic flow, while the anionic micelle migrates toward the positive end with a different velocity because of an additional electrophoretic migration. However, since the electrophoretic velocity of the micelle is lower than that of the electroosmotic flow under the usual experimental conditions, the micelle also migrates toward the negative end.

When non-ionic solutes are introduced to the positive end of the tube they are separated by the difference in distribution between the micellar phase and the aqueous phase, and detected at the negative end of the tube. In the case of an ionic solute, the retention time is determined by its own electrophoresis in addition to micellar solubilization and ionic interaction.

So we can summarize the parameters that affect the separation of the solute as follows: (1) applied voltage (current)[2,15,16]; (2) composition, concentration and pH of the buffer[17]; (3) structure and concentration of the surfactant[16]; (4) additives (for example, organic solvent)[18]; (5) length, diameter and characteristics of the inner surface of the capillary[19,20]; (6) composition and concentration of the sample solution[15,21].

Concerning parameter (1), a linear relationship was observed between the current and migration velocity of the solute[2,15]. We examined the migration velocity of some solutes under various applied voltages with a 0.02 *M* phosphate–borate buffer (pH 9.0) without a surfactant. The result is shown in Fig. 2. The same result as previously reported[16] was obtained, and the observed plate height, *H*, values of those solutes under various applied voltages are shown in Fig. 3. It was also reported that

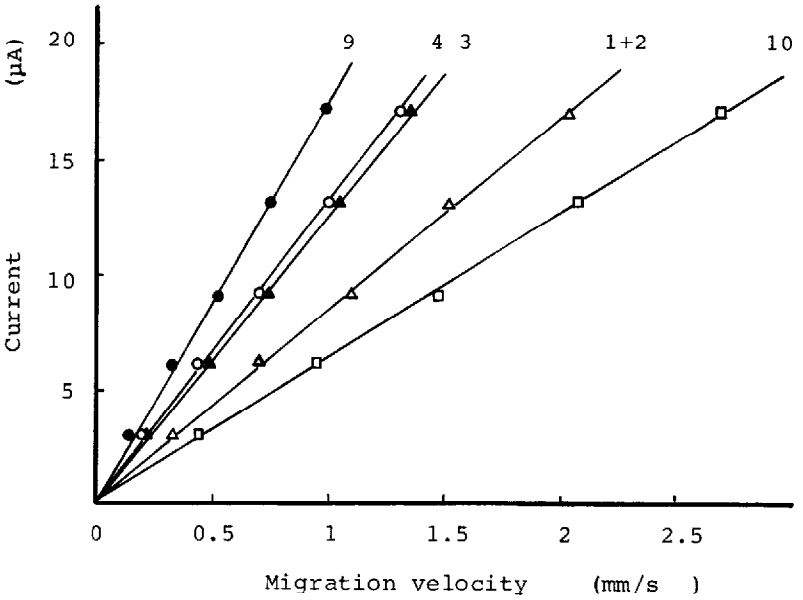


Fig. 2. Velocities of solutes as a function of the current. Solutes are indicated by the numbers given in Fig. 1. Conditions: buffer, 0.02 *M* phosphate–borate (pH 9.0) without a surfactant; separation tube, 650 mm × 0.05 mm I.D.; length of the tube used for separation, 500 mm; temperature, ambient; detection wavelength, 210 nm; attenuation, 0.08 a.u.f.s.

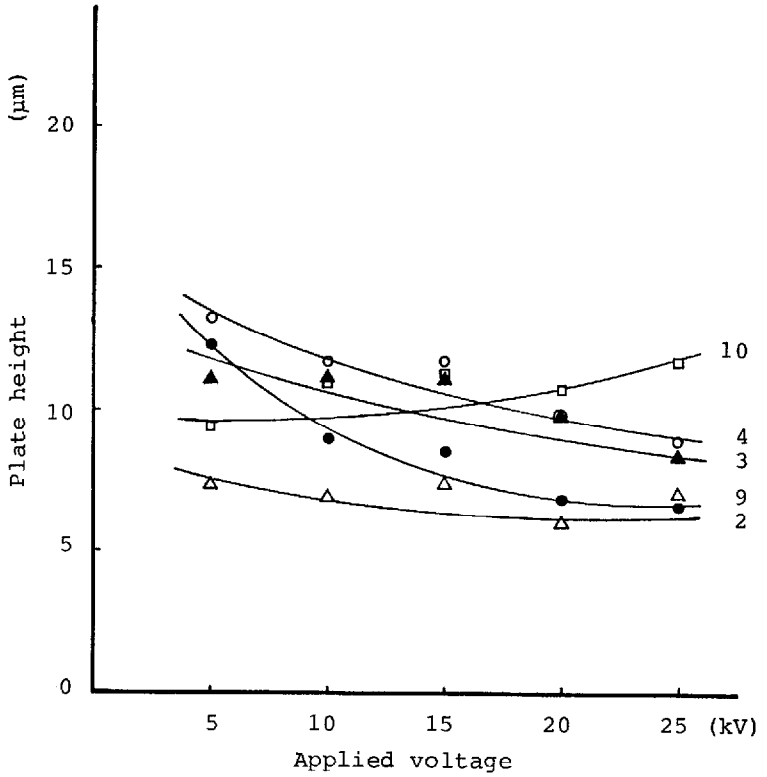


Fig. 3. Dependence of *H* values of solutes on applied voltage. Solutes and conditions as in Fig. 2.

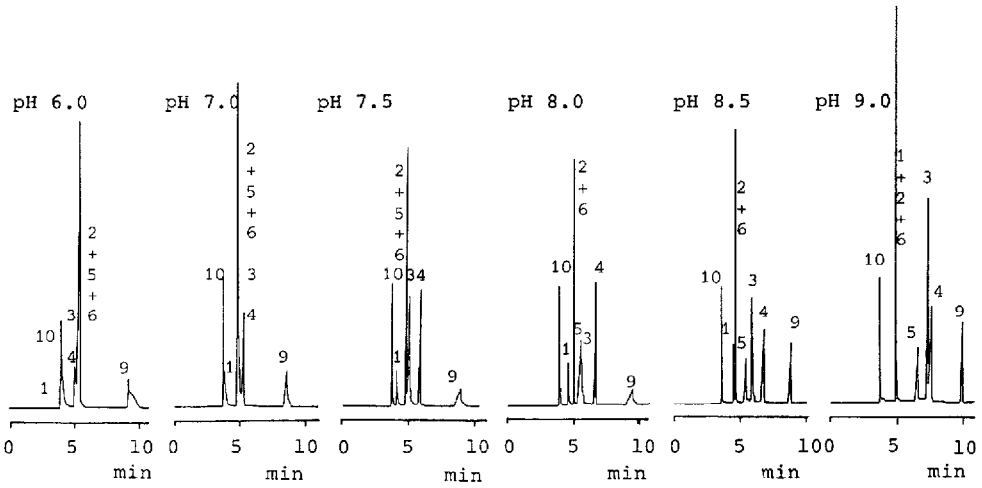


Fig. 4. Effect of pH on retention times and peak shapes of eight water-soluble vitamins. Conditions: applied voltage, 20 kV. Other conditions as in Fig. 2 except for the pH.

the higher the applied voltage is the lower is the H value[19]. We decided to operate the power supply at 20 or 25 kV in the following experiments. Concerning parameter (5), we selected 0.05 mm I.D. fused-silica capillary tube of length about 65 cm.

CZE separation

We investigated the separation of eight water-soluble vitamins with a buffer solution containing no SDS. In CZE, one of the effective parameters in separation is the pH value of the buffer. The pH dependence of the retention time and the peak shape in the range pH 6–9 are shown in Fig. 4. At low pH, the retention times of some solutes were very close to each other and some peaks were very broad. However, the peak shape and separation were gradually improved and retention times increased with increasing pH value, although the electroosmotic flow whose velocity was evaluated from the retention time of methanol or that of neutral compounds such as nicotinamide or vitamin B_{12} in this case was not greatly changed in this pH range. The increase in the retention times of some solutes at high pH is due to the increase in ionization ratios of the solutes at the specified pH. That is, negatively charged solutes were more strongly retained by the electrophoretic effect. In general, the elution order of a solute in CZE can be explained by its ionization characters. In the case of cationic solutes, they are eluted first because the electroosmotic flow and its electrophoretic migration are in the same direction to the negative end of the tube in this system. Electrically neutral solutes migrate with the same velocity as the electroosmotic flow. The pH dependence of the retention times shown in Fig. 4 is in good agreement with the above mentioned behaviours: cationic vitamin B_1 which has a positively ionized nitrogen atom was eluted first and niacin which has a carboxyl group was eluted last.

In CZE, the selection of the buffer composition is also important in order to obtain symmetrical peaks. Instead of the phosphate–borate buffer, we also used an acetate–borate buffer in the above experiments, and chromatograms with the two

buffer solutions are compared in Fig. 5. The higher column efficiency was achieved by use of the phosphate–borate buffer. From these results, we employed this buffer solution at pH 9.0 in subsequent experiments, except when different pH conditions were investigated.

Micellar EKC separation

The effect of SDS concentrations on the retention time is shown in Fig. 6. Vitamin B_{12}, nicotinamide and pyridoxamine were eluted at the same position as methanol which was a tracer of the electroosmotic flow when the SDS concentration is zero, moreover the peak shape of pyridoxal 5′-phosphate was seriously distorted. However, the addition of SDS to the buffer, by which micellar EKC was effected, improved the separation of solutes. A typical chromatogram of the separation of eleven water-soluble vitamins by micellar EKC with a 0.05 *M* SDS solution at pH 9.0 is shown in Fig. 7. The number of theoretical plates are in the range of 100 000 (pyridoxal 5′-phosphate)–350 000 (pyridoxamine 5′-phosphate) calculated from the equation $N = 2\pi(t_R h/A)^2$, where t_R, h and A are the retention time, peak height and peak area obtained by using a Shimadzu C-R2AX.

The retention times of nicotinamide and pyridoxamine became slightly longer than that of methanol (electroosmotic flow) as the SDS concentration increased. This was due to the gradual increase in solubilization by the micelle with increasing SDS concentrations. On the other hand, large variations of the retention time were observed for vitamins B_1, B_{12} and B_2. The increase of the retention time of vitamin B_2 is explained in terms of its high lipophilicity. For vitamins B_1 and B_{12}, which are cationic solutes having N^+ or Co^{2+}, an ion pair might be formed between the cation-

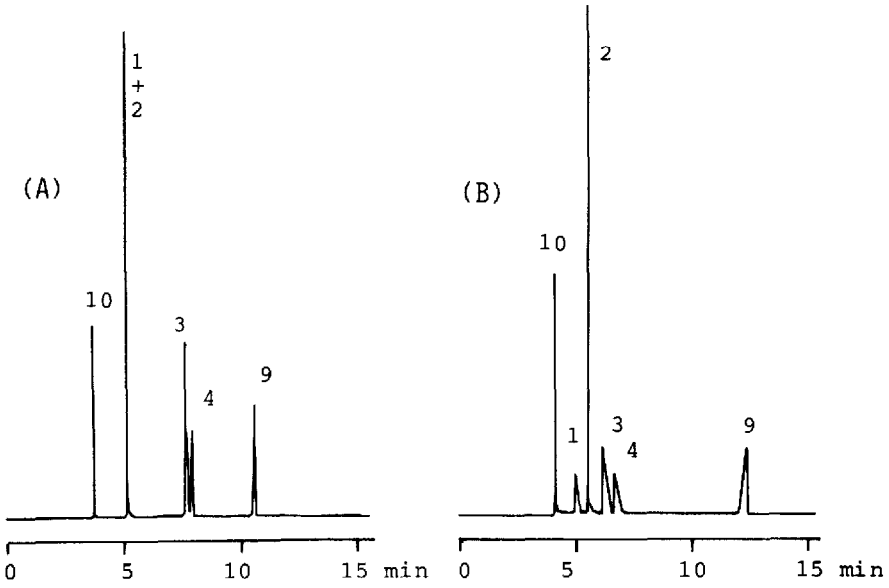


Fig. 5. Effect of the buffer composition on peak shapes: (A) 0.02 *M* phosphate–borate buffer (pH 9.0); (B) 0.02 *M* acetate–borate buffer (pH 9.0). Solutes are indicated by the numbers given in Fig. 1.

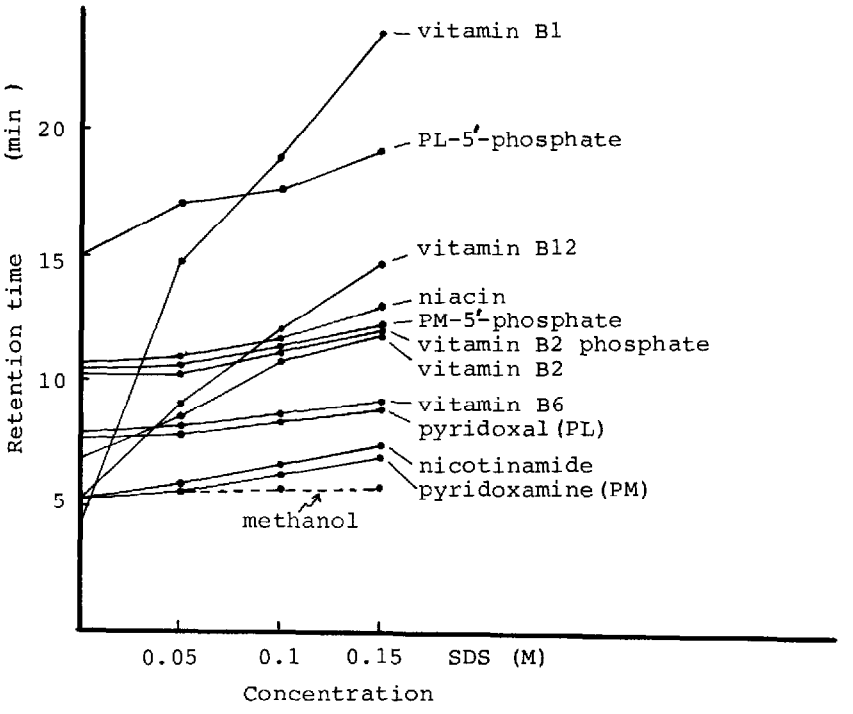


Fig. 6. Effect of SDS concentrations on the retention time of eleven water-soluble vitamins. Applied voltage: 20 kV. Other conditions as in Fig. 2 except for the addition of SDS.

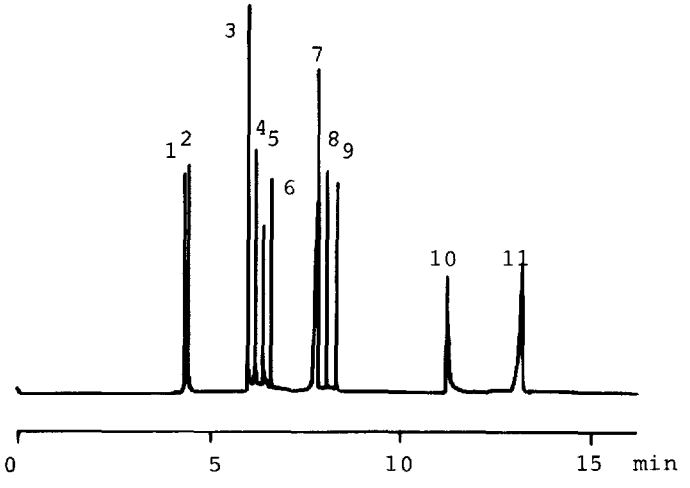


Fig. 7. Micellar EKC of 11 water-soluble vitamins. Conditions: applied voltage, 25 kV; SDS concentration 0.05 *M*. Solutes are indicated by the numbers given in Fig. 1. Other conditions as in Fig. 2.

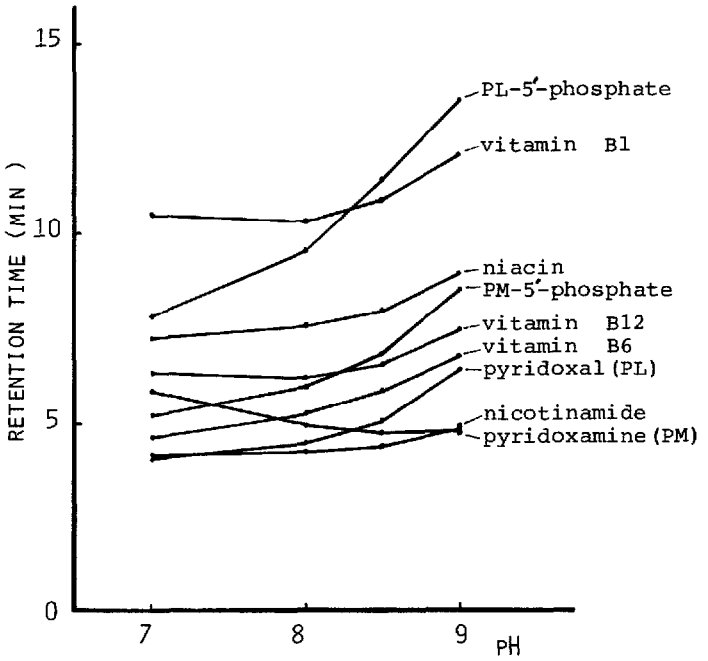


Fig. 8. Effect of pH on the retention time of nine water-soluble vitamins in micellar EKC. Conditions: applied voltage, 25 kV; SDS concentration, 0.05 *M*. Other conditions as in Fig. 2.

ic group of the solutes and the polar group of anionic surfactants (SO_4^- in the case of SDS) and its formation contributes to the large variation of the retention time. This mechanism will be discussed in the following sections. The effect of the pH on the retention time is shown in Fig. 8, which was obtained by keeping the SDS concentration constant at 0.05 *M*. The retention times of pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate increased gradually with increasing pH; on the contrary, that of pyridoxamine decreased and the retention behaviour observed might be explained in terms of the repulsive ionic interaction between solutes and anionic surfactants.

Instead of SDS, LMT was also employed as an anionic surfactant and the effect of its concentration on the retention times of seven solutes was examined. The results are shown in Figs. 9 and 10. When the effects of the two surfactants were compared a difference was observed in the retention behaviours of vitamins B_1 and B_{12}. Vitamin B_1 was eluted between vitamin B_6 and niacin in a 0.1 *M* LMT solution although its retention time was the longest in a 0.1 *M* SDS solution. This marked difference can be attributed to the difference in physico-chemical properties of the two surfactants. Some physical parameters and the structures of the two surfactants are summarized in Table I, where it is seen that the physical parameters are almost the same. The separation of seven parabens as a neutral sample with the two surfactants at 0.05 *M* is shown in Fig. 11. For these neutral solutes, the difference was not so distinct and almost the same chromatograms were obtained. However, in the case of ionic samples, especially cationic samples, in anionic surfactant solutions just like the present experimental conditions, the difference was obvious as mentioned above. The reason for this difference is probably the difference in the polar group of the surfactant, *i.e.*,

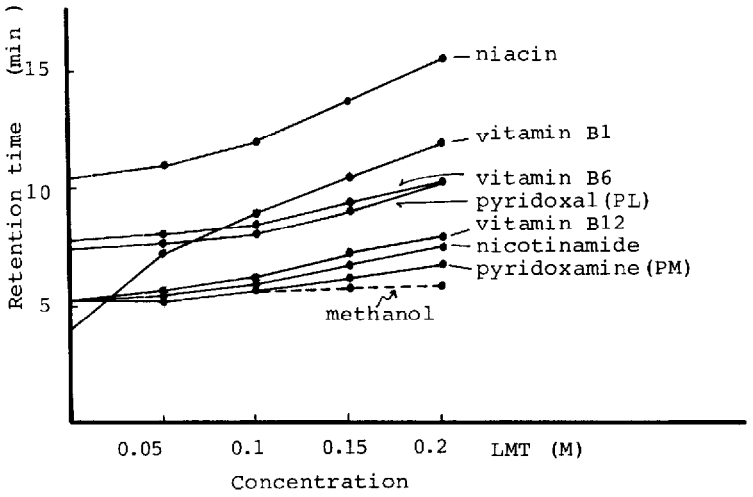


Fig. 9. Effect of LMT concentration on the retention time of seven water-soluble vitamins. Applied voltage, 20 kV. Other conditions as in Fig. 2 except for the addition of LMT.

the sulphate group for SDS and the sulphonate group for LMT. In addition to the difference in charge-bearing groups, the neighbouring groups are different: LMT has $-N(CH_3)-C(=O)-$ near the ionic group, in comparison with the linear hydrocarbon chain structure of SDS. Therefore, ion-pair formation between solutes and the ionic group of LMT might be partially blocked by steric hindrance.

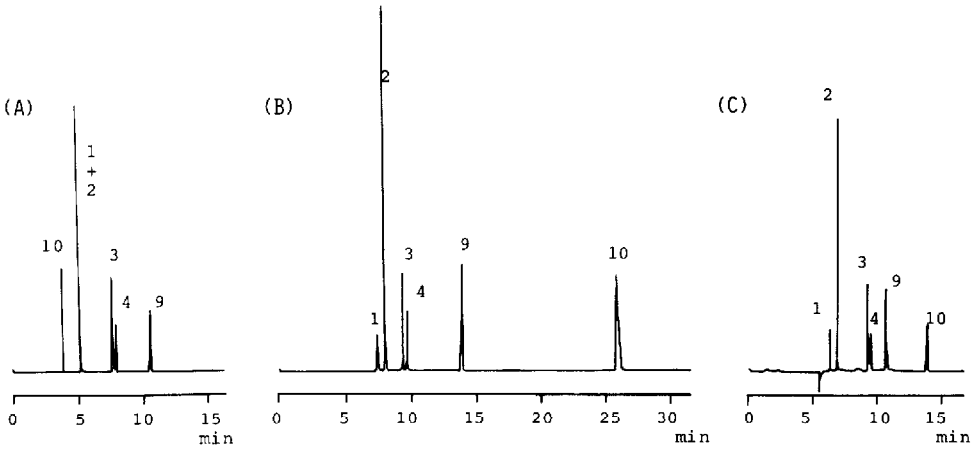
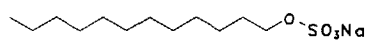


Fig. 10. Comparison between CZE separation and micellar EKC separations. Applied voltage; 20 kV. (A) CZE separation, 0.02 *M* phosphate–borate buffer (pH 9.0), 210 nm; (B) micellar EKC separation, 0.15 *M* SDS in the above buffer, 210 nm; (C) micellar EKC separation, 0.15 *M* LMT in the above buffer, 220 nm.

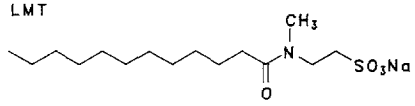
TABLE I

PHYSICOCHEMICAL PROPERTIES OF SDS AND LMT

SDS



LMT



Surfactant	*Mol.wt.*	*Kraft point (°C)*	*CMC (mmol/l)*
SDS	288.38	*ca.* 8–9	8.1
LMT	343.46	<0	8.7

To clarify the effect of the micelle, we investigated the effect of an ion-pairing reagent that is often used in reversed-phase high-performance liquid chromatography. As an ion-pairing reagent, sodium pentanesulphonate was employed at 0.1 *M*. The result is shown in Fig. 12. The retention times of all the solutes were increased in the presence of the ion-pairing reagent because of the decrease in electroosmotic flow. However, the elution orders observed in 0.1 *M* SDS were not altered in this system. In conclusion, the drastic change in the elution orders of vitamins B_1 and B_{12} in micellar EKC mentioned above was due to the effect of the micelle because sodium pentanesulphonate does not form the micelle structure. The marked increase in retention times was probably the result of ion-pair formation between the Stern layer of the micelle and the solute.

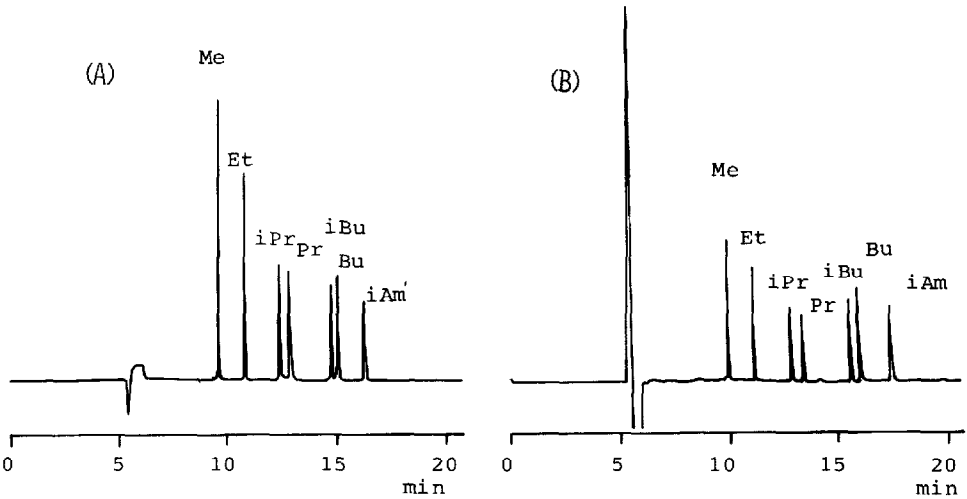


Fig. 11. Micellar EKC separation of seven parabens. Applied voltage: 20 kV. Buffer solution: 0.02 *M* phosphate–borate buffer (pH 9.0) containing 0.05 *M* SDS, 210 nm (A) and 0.05 M LMT, 220 nm (B). Me = Methyl paraben; Et = ethyl paraben; iPr = isopropyl paraben; Pr = propyl paraben; iBu = isobutyl paraben; Bu = butyl paraben; iAm = isoamyl paraben. Other conditions as in Fig. 2.

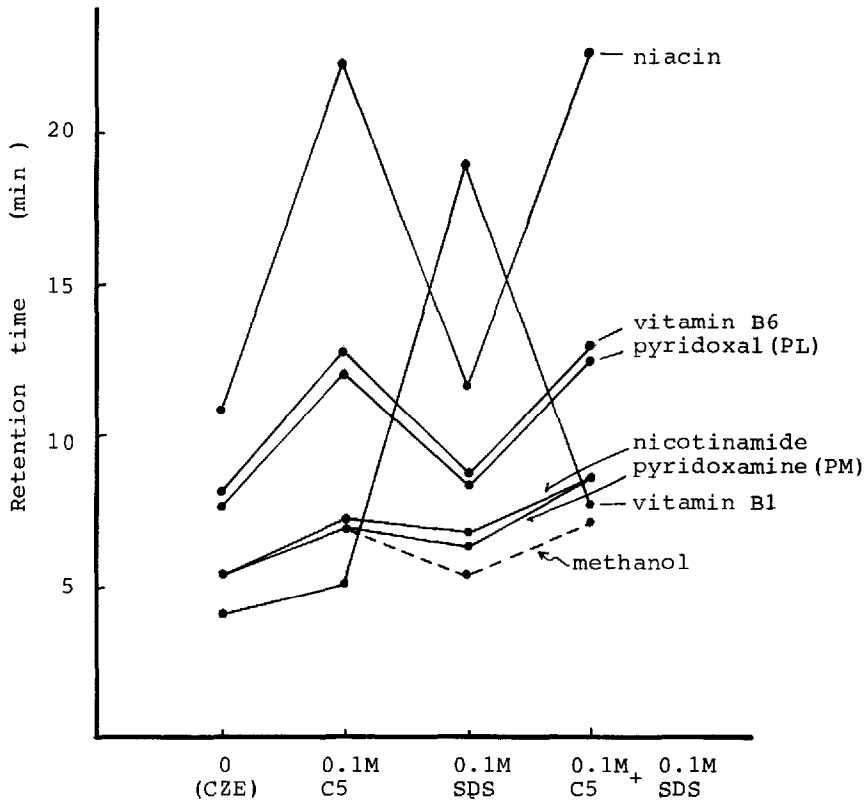


Fig. 12. Effect of the ion-pairing reagent on the separation of six water-soluble vitamins. (0) 0.02 *M* phosphate–borate buffer (pH 9.0) CZE separation; (C_5) sodium pentanesulphonate. Applied voltage; 20 kV. Other conditions as in Fig. 2.

We also expect an improvement in selectivity in separation by mixing an additive with a surfactant to separate a complex mixture if a successful separation is not obtained by changing the surfactant concentrations, although the addition of 0.1 *M* sodium pentanesulphonate to a 0.1 *M* SDS solution was not so effective in case of Fig. 12.

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