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ELECTROKINETIC CHROMATOGRAPHY WITH MICELLAR SOLUTIONS

SEPARATION OF PHENYLTHIOHYDANTOIN-AMINO ACIDS

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SUMMARY

A mixture of 22 phenylthiohydantoin-amino acids was separated by electrokinetic chromatography with a sodium dodecyl sulphate (SDS) solution and a 650 × 0.05 mm I.D. fused-silica tube. This chromatography is based on micellar solubilization and electrokinetic migration in an open-tubular capillary. The retention times of the solutes could be controlled by changing the SDS concentration, the applied voltage or the length of the separation tube. Dodecyltrimethylammonium bromide (DTAB) solutions were also employed. The separation characteristics with SDS solutions were completely different from those with DTAB solutions. The resolutions were better with the former than with the latter solution, but for some solutes the latter gave better results.

INTRODUCTION

Micellar solubilization chromatography[1–3], or more generally electrokinetic chromatography[3], is a new type of chromatography based on micellar solubilization and electrokinetic migration. In this method, micellar solubilization operates as the partition mechanism, that is, a solute is distributed between the micelle and the aqueous phase, and the electroosmotic flow of a solution filled in an open-tubular capillary and the electrophoretic migration of the ionic micelle make possible differential displacement between the micelle and the aqueous phase. Although micelles are understood to be dynamic structures and are called a pseudo-phase, this technique can be regarded as belonging to a branch of liquid–liquid partition chromatography having no solid support. We have previously described[3] the fundamental characteristics of this method from the viewpoints of electrokinetic migration, chromatographic parameters and the micellar solubilization phenomenon.

In previous work[2,3], we employed solutes that were electrically neutral under the experimental conditions used. If a solute can be ionized, the chromatographic behaviour of the ionized solute should be different from that of the neutral solute, because the ionized solute is subjected to electrophoretic attraction in addition to the

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electroosmotic migration of the whole solution when it exists in the aqueous phase. The electrophoretic attraction for the ionized solute should also be effective when the solute is incorporated into the micelle as well as that for the ionic micelle. This kind of electrophoretic effect on the ionized solute should be taken into account in order to achieve a good separation of a complex mixture containing ionized species.

Phenylthiohydantoin (PTH)-amino acids, which are amino acid derivatives resulting from the Edman degradation of peptides and proteins, are important materials for determining amino acid sequences. Since Zimmerman *et al.*[4] first introduced high-performance liquid chromatography (HPLC) for separating PTH-amino acids, several studies have been published in which various kinds of techniques were used for HPLC. Isocratic[5–10] and binary gradient[11–16] elution were performed with acidic eluents, in some instances at high temperatures. In any event, however, some disadvantages were noted, such as long analysis times, complexity of the operation or failure of complete separation. Recently, it has been shown that the use of micro HPLC under the conditions of isocratic elution and room temperature operation can shorten the analysis time[17], but satisfactory separation was not obtained.

In this paper, the separation of PTH-amino acids is described to demonstrate the applicability of electrokinetic chromatography with micellar solutions. Some strategies for improving the resolution with this technique are also considered.

EXPERIMENTAL

The same apparatus as described previously[2,3] was used. As a chromatographic column, a 650 × 0.05 mm I.D. or an 1150 × 0.05 mm I.D. fused-silica tube was employed. The temperature of the thermostated oven was maintained at 35°C.

All the reagents and samples were used as received. Sodium dodecyl sulphate (SDS) solutions were prepared by dissolving SDS in 0.05 *M* sodium dihydrogen phosphate–0.0125 *M* sodium tetraborate buffer solution (pH 7.0). Dodecyltrimethylammonium bromide (DTAB), supplied by Tokyo Kasei Kogyo (Tokyo, Japan), was dissolved in 0.1 *M* tris(hydroxymethyl)aminomethane–0.1 *M* hydrogen chloride buffer (Tris–HCl buffer) (pH 7.0). PTH-amino acids, obtained from Wako (Osaka, Japan), were dissolved in acetonitrile–water (1:1) or in acetonitrile and stored at −20°C. Sudan III or Yellow OB (Tokyo Kasei Kogyo) was used after dissolution in surfactant solution or methanol.

The experimental procedure was the same as that described previously[2].

RESULTS AND DISCUSSION

Fig. 1 shows an electrokinetic chromatogram of a mixture of 22 PTH-amino acids with 0.05 *M* SDS solution. In this chromatogram, the peaks of all the solutes in the mixture were separately recognized. However, four pairs of peaks were poorly resolved: Thr (T)–Ser (S), Val (V)–Met (M), Leu (L)–Trp (W) and Phe (F)–Nle (n-L). Here, three- or one-letter abbreviations for the amino acid are used to indicate the corresponding PTH-amino acid. As noted previously[2], the capacity factor, $\tilde{k}'$, of an electrically neutral solute in this chromatography is given by

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

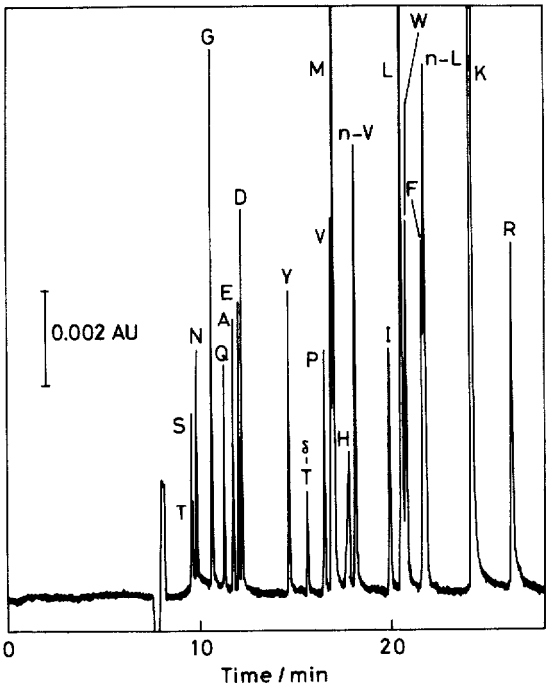

Fig. 1. Electrokinetic chromatogram of a mixture of 22 PTH-amino acids. Micellar solution, 0.05 *M* SDS in phosphate–borate buffer (pH 7.0); separation tube, 650 × 0.05 mm I.D.; length of the tube used for separation, 500 mm; total applied voltage, *ca.* 10 kV; current, 14 μA; detection wavelength, 260 nm; temperature of oven, 35°C. The peaks are labelled with one-letter abbreviations for the amino acid.

where t_R, t_0 and t_{mc} are the elution times of the solute, the insolubilized solute and the micelle, respectively. Here, the symbol, $\tilde{k}'$ is used instead of k', which is widely accepted in conventional HPLC[3]. In the experiments, t_0 was regarded as the retention time of methanol and t_{mc} that of Sudan III or Yellow OB[3]. The resolution, R_s, of two adjacent peaks whose capacity factors are $\tilde{k}'_1$ and $\tilde{k}'_2$ is expressed as follows[3]:

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{\tilde{k}'}{1 + \tilde{k}'}\right)\left[\frac{1 - t_0/t_{mc}}{1 + (t_0/t_{mc})\tilde{k}'}\right] \quad (2)$$

where N is the plate number, α is the separation factor and $\tilde{k}'$ is approximately given by $\tilde{k}' = \tilde{k}'_1 = \tilde{k}'_2$. The term t_0/t_{mc} is a specific parameter for electrokinetic chromatography which shows the width of the total range of elution[3], and in Fig. 1 its value was about 0.25: $t_0 = 470$ sec and $t_{mc} = 1860$ sec. We have already shown[3] that R_s should be maximal at $\tilde{k}' = 2.0$ for the value of $t_0/t_{mc} = 0.25$. In the chromatogram shown in Fig. 1, the retention time corresponding to $\tilde{k}' = 2.0$ is about 16 min. The value of $\tilde{k}'$ of a solute changes linearity with the concentration of the surfactant and with the applied voltage[3]: an increase in the concentration of surfactant or a decrease in the applied voltage causes an increase in the value of $\tilde{k}'$. Therefore, for pairs of peaks that are eluted at a time far from the optimum, *e.g.*, pairs

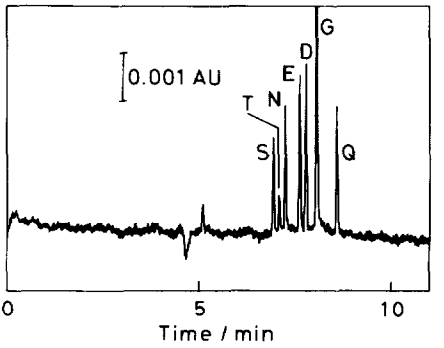

Fig. 2. Electrokinetic chromatogram of a mixture of some PTH-amino acids, showing shorter retention times in Fig. 1. Micellar solution, 0.10 *1M* SDS; total applied voltage, *ca.* 15 kV; current, 31 μA. Other conditions as in Fig. 1.

such as Ser–Thr, Leu–Trp or Phe–Nle, we can adjust the $\tilde{k}'$ to more appropriate values by changing the SDS concentration and/or the applied voltage, and expect to obtain better resolutions than those given under the conditions used in Fig. 1.

For the pair Ser–Thr, whose $\tilde{k}'$ value is small (*ca.* 0.33) in Fig. 1, an increase in the SDS concentration gave a better separation, as shown in Fig. 2, where the Ser–Thr pair is completely resolved. It should be noted in Fig. 2 that Glu (E) and Asp (D) were eluted faster than Gly (G) and Gln (Q), in contrast to the elution order in Fig. 1. The result can be interpreted by considering two effects on the retention of PTH-amino acids in this chromatography: one is the micellar solubilization phenomenon in combination with electrophoresis of micelles and the other is electrophoresis of an ionized solute. Both PTH-Glu and -Asp have a free carboxyl group but PTH-Gly and -Gln do not. That is, PTH-Gly and -Gln are transported in the tube with a velocity $v_s(n)$, which is given by[3]

$$v_s(n) = \frac{1 + (t_0/t_{mc})\tilde{k}'_n}{1 + \tilde{k}'_n} \cdot v_{eo} \tag{3}$$

where $\tilde{k}'_n$ is the capacity factor for the electrically neutral solute and v_{eo} is the electroosmotic velocity, which is equal to the migration velocity of the unretained solute. On the other hand, PTH-Glu and -Asp can be considered to be ionized under the conditions given in Fig. 2, so that these solutes are subjected to the electrophoretic attraction to the positive electrode in addition to the electrokinetic chromatographic migration expressed by eqn. 3. By assuming that the electrophoretic velocity of the micelle, $v_{ep}(mc)$, remains constant, even when the micelle solubilizes the ionized solute, the migration velocity of the ionized solute, $v_s(i)$, is represented by

$$v_s(i) = v_{eo} + \frac{1}{1 + \tilde{k}'_i} \cdot v_{ep}(i) + \frac{\tilde{k}'_i}{1 + \tilde{k}'_i} \cdot v_{ep}(mc) \tag{4}$$

where $\tilde{k}'_i$ is the capacity factor for the ionized solute and $v_{ep}(i)$ is the electrophoretic velocity of the ionized solute itself when it exists in the aqueous phase. Here, it should

be noted that the sign of the migrating direction is taken into account. As the migration velocity of the micelle, v_{mc}, is given by[3]

$$v_{mc} = v_{eo} + v_{ep}(mc) \tag{5}$$

eqn. 4 can easily be rewritten as

$$v_s(i) = \frac{1 + (t_0/t_{mc})\bar{k}'_i}{1 + \bar{k}'_i} \cdot v_{eo} + \frac{1}{1 + \bar{k}'_i} \cdot v_{ep}(i) \tag{6}$$

where the direction of $v_{ep}(i)$ is the reverse of that of v_{eo} or $v_s(i)$. Comparison of eqn. 3 with eqn. 6 reveals that an increase in capacity factor, which is caused by an increase in the SDS concentration, should decrease the velocity of the non-ionized solute, $v_s(n)$, to a greater extent than that of the ionized solute, $v_s(i)$, because the second term in eqn. 6 becomes smaller in absolute value with an increase in $\bar{k}'_i$. As had been expected, the retention times of PTH-Gly and -Gln could be made longer than those of PTH-Glu and -Asp by increasing the SDS concentration, as shown in Figs. 1 and 2.

For other poorly resolved pairs in Fig. 1, *i.e.*, Leu–Trp and Phe–Nle, which were eluted with larger $\bar{k}'$ (4.9 and 5.8) than the optimum values, a decrease in SDS concentration was required in order to attain better separations. The chromatogram in Fig. 3 shows that a decrease in SDS concentration from 0.05 to 0.02 *M* brought about better resolutions of both pairs than those observed in Fig. 1.

The pair Val–Met was not completely separated in Fig. 1, although it was eluted with the nearly optimum value of $\bar{k}' = 2.6$. We tried to improve the resolution of this pair by doubling the length of the initially used column to 100 cm. Under these conditions, the number of theoretical plates should be twice and the resolution about 1.4 times as large as those in Fig. 1. The two peaks were successfully separated, as shown in Fig. 4.

Dodecyltrimethylammonium bromide (DTAB), which is a cationic surfactant, was also used to examine the effect of surfactant molecules on the separation of

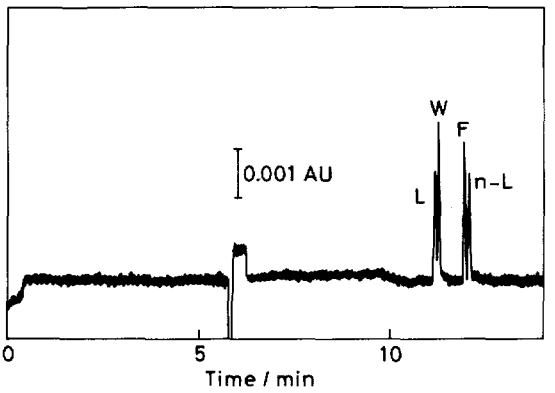

Fig. 3. Electrokinetic chromatogram of a mixture of PTH-Leu, Trp, -Phe and -Nle. Micellar solution, 0.02 *M* SDS; total applied voltage, *ca.* 12.5 kV; current, 13 μA. Other conditions as in Fig. 1.

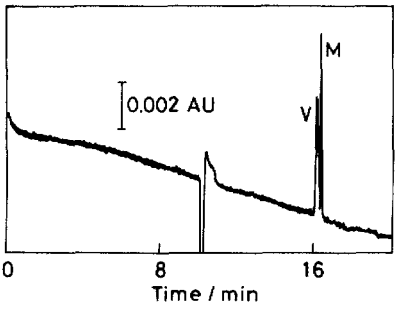

Fig. 4. Electrokinetic chromatogram of a mixture of PTH-Val and -Met. Separation tube, 1150 × 0.05 mm I.D.; length of the tube used for separation, 1000 mm; total applied voltage, *ca.* 25 kV; current, 17 μA. Other conditions as in Fig. 3.

PTH-amino acids. The directions of both electroosmotic flow and electrophoretic migration of the DTAB micelle were the opposite of those in SDS solutions: the direction of the electroosmotic flow was from the negative to the positive electrode and that of the electrophoretic migration of the DTAB micelle was from positive to negative. These observations can be explained in terms of the reversal of the signs of the zeta potentials at the fused-silica wall and the micelle when DTAB is substituted for SDS. In fact, the zeta potential of the quartz/DTAB solution has been reported[18] to be positive if the concentration of DTAB is higher than 10^{-4} *M*, and the micelle of DTAB apparently has positive charges. The chromatogram shown in Fig. 5 is an example of the separation of PTH-amino acids with 0.05 *M* DTAB solution. It is

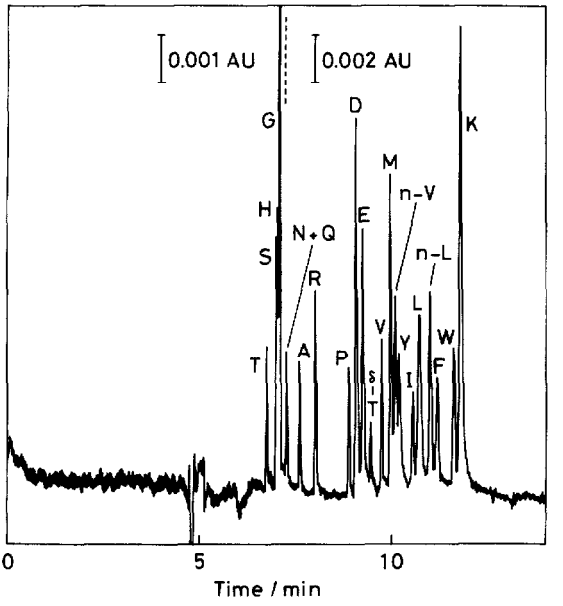

Fig. 5. Electrokinetic chromatogram of a mixture of 22 PTH-amino acids. Micellar solution, 0.05 *M* DTAB in Tris–HCl buffer (pH 7.0); total applied voltage, *ca.* 15 kV; current, 37 μA. Other conditions as in Fig. 1.

TABLE I

COMPARISON OF VARIABLES* OF ELECTROKINETIC MIGRATION BETWEEN SDS AND DTAB SOLUTIONS

Separation tube, 650 × 0.05 mm I.D.; total applied voltage, *ca.* 15 kV; temperature of oven, 35°C.

Surfactant	*Concentration (M)*	*I (μA)*	v_{eo} *(mm sec*$^{-1}$*)*	v_{mc} *(mm sec*$^{-1}$*)*	t_0/t_{mc}
SDS	0.03	19.4	1.76	0.522	0.298
	0.05	22.1	1.71	0.494	0.288
	0.10	30.4	1.68	0.363	0.217
DTAB	0.03	32.2	1.73	0.733	0.425
	0.05	37.4	1.70	0.698	0.412
	0.10	45.5	1.71	0.651	0.380

* I, current; v_{eo}, electroosmotic velocity; v_{mc}, migration velocity of micelles.

worth noting that any pair of solutes that were eluted as partially overlapping peaks under the conditions employed in Fig. 1 was completely resolved in the chromatogram shown in Fig. 5, although some other solutes were not separated in Fig. 5 even with concentrations of DTAB different from 0.05 *M*.

In Table I, some migration variables in elektrokinetic chromatography for the SDS and DTAB solutions are presented . The current in each surfactant solution increased with increase in the concentration of the surfactant[3]. A higher current is observed in the DTAB solution than in the SDS solution at the same concentration of the surfactants. The differences in these values seem to be due to the differences in the buffer solutions used, borate–phosphate and Tris–HCl, rather than to the difference in the surfactants themselves. The electroosmotic velocities, v_{eo}, were almost constant, regardless of the surfactant concentration, but the migration velocities of micelles, v_{mc}, decreased with increase in the micellar concentration in both SDS and DTAB solutions. These facts can be explained in terms of the change in the viscosity of the solutions and the temperature rise due to Joule heat[3]. As a specific parameter in electrokinetic chromatography, we obtained smaller values for t_0/t_{mc} in the SDS solutions than in DTAB solutions at any concentration. From the viewpoint of the maximum resolution, a smaller value of t_0/t_{mc} is advantageous, and this is probably the main reason why the SDS solution gave a better resolution as a whole than the DTAB solution for the 22 PTH-amino acids.

In conclusion, a satisfactory separation of 22 PTH-amino acids was achieved by electrokinetic chromatography with a micellar solution and an open-tubular capillary, and this indicates the high resolving power of this chromatographic technique.

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