CHROMSYMP. 615

ELECTROKINETIC CHROMATOGRAPHY WITH 2-O-CARBOXYMETHYL-β-CYCLODEXTRIN AS A MOVING "STATIONARY" PHASE

SHIGERU TERABE*, HIROTO OZAKI, KOJI OTSUKA and TEIICHI ANDO

Department of Industrial Chemistry, Faculty of Engineering, Kyoto University, Sakyo-ku, Kyoto 606 (Japan)

(Received March 28th, 1985)

SUMMARY

Preliminary results are presented on a new type of liquid chromatography, based on inclusion complex formation in a solution of a β-cyclodextrin derivative having an ionized group, 2-O-carboxymethyl-β-cyclodextrin (β-CMCD), combined with electrokinetic migrations. Only the host–guest interaction between β-CMCD and the solute operates as the distribution process, and electroosmosis and electrophoresis in an open-tubular capillary filled with a β-CMCD solution permit differential migrations between the host and guest molecules. Separations of some aromatic isomers are shown, and retention parameters and the distribution coefficient are discussed.

INTRODUCTION

One of the two phases constituting a chromatographic system between which a solute is distributed is usually fixed rigid in a column and is therefore called the stationary phase. It is, however, not always necessary for the stationary phase to be held immovable in the column; a relative displacement of the two phases is sufficient for chromatographic separations. Micellar solubilization chromatography[1–3], or more generally electrokinetic chromatography[3], is an example where the two chromatographic phases move in the column with different velocities.

In electrokinetic chromatography where a solution of an ionic surfactant is filled in an open-tubular glass capillary and is subjected to free zone electrophoresis, the whole solution is transported by the electroosmotic flow with an approximately flat velocity profile in a direction determined by the sign of the zeta potential at the solution–glass interface. On the other hand, the ionic micelle is concurrently moved by the electrophoretic attraction generally in the direction opposite to the electroosmotic flow. As the electroosmotic flow is usually much stronger than the electrophoretic migration of the micelle[2,3], the micelle is also transported in the same direction as the whole solution but with a slower velocity than the latter. A mixture of solutes added to the micellar solution at one end of the tube can be separated while it moves towards the other end according to the extent to which each component is incorporated into the micelle by the micellar solubilization phenomenon.

This concept of chromatography can be readily extended to the utilization of some specific interactions, e.g., complexation or clathration. This paper presents preliminary results on electrokinetic chromatography with an ionic cyclodextrin derivative that can be expected to form inclusion complexes with a variety of organic molecules; 2-O-carboxymethyl-β-cyclodextrin (β-CMCD) was employed as a substitute for the chromatographic phase corresponding to the micelle in the above-mentioned chromatographic system.

Recently, cyclodextrins have attracted wide attention in high-performance liquid chromatography (HPLC), e.g., cyclodextrins chemically bonded to silica gel as stationary phases have revealed interesting features for the separation of aromatic isomers[4,5] and optical isomers[6]. In these chemically bonded cyclodextrin phases, adsorption by spacer groups employed to link the cyclodextrin to the silica gel could be involved in the distribution process in addition to the formation of inclusion complexes of cyclodextrins with the solutes[4,5]. In electrokinetic chromatography, however, the cyclodextrin derivative is free in the solution and, therefore, only inclusion complex formation or host–guest interaction should be operating as the distribution process, although inclusion by ionic cyclodextrins may be slightly different from that by electrically neutral cyclodextrins.

EXPERIMENTAL

Reagents and materials

β-Cyclodextrin was purchased from Nakarai Chemical (Kyoto, Japan). All compounds employed as samples and reagents used for the preparation of the cyclodextrin derivative and for the preparation of the chromatographic solution were of analytical-reagent grade and used without further purification.

Preparation of 2-O-carboxymethyl-β-cyclodextrin (β-CMCD)

β-CMCD was synthesized by the reaction of sodium iodoacetate with an excess of β-cyclodextrin anion in dimethyl sulphoxide (DMSO) according to the method described by Kitaura and Bender[7] for the preparation of carboxymethyl-α-cyclodextrin (α-CMCD), and isolated by the method described by Gruhn and Bender[8] with some modifications, viz., DEAE Toyopearl 650M (Toyo Soda, Tokyo, Japan) was employed instead of DEAE-Sephadex A-25 and 0.02 M phosphate buffer solution (pH 4.6) was delivered into the column as an isocratic eluent. Each eluate fraction was subjected to HPLC analysis with a DEAE-5500PW column (15 cm × 6 mm I.D.) (Toyo Soda) and a 0.02 M phosphate buffer solution (pH 3.85). Fractions containing β-CMCD were collected, concentrated by evaporation and desalted with a Sephadex G-10 (Pharmacia, Uppsala, Sweden) column to give an aqueous solution of the sodium salt of β-CMCD. β-CMCD was isolated by lyophilization and its molecular structure was confirmed by NMR spectroscopy as described below.

About 40 mg of the sodium salt of β-CMCD were dissolved in ca. 0.5 ml of ^2H_2O and proton NMR spectra at 90 MHz and carbon-13 NMR spectra at 22.49 MHz were measured at room temperature by using tetramethylsilane as an external standard. The proton NMR spectrum of β-CMCD was similar to that reported for α-CMCD[8]. Two peaks at δ 5.34 (1H, doublet, $J = 4$ Hz) and δ 4.21 (2H, singlet) were apparently observed, distinct from the spectrum of β-cyclodexrin recorded

under the same conditions. These peaks can be assigned to the C_1 anomeric proton on the carboxymethylated glucose unit and methylene protons of the carboxymethyl group in the order cited above.

Only two weak peaks were recognized in the carbon-13 NMR spectrum of β-CMCD other than those observed for β-cyclodextrin[9], i.e., δ 100.0 and δ 70.4, which may be assigned to C_1 and C_3, respectively, of the glucose unit having the substituent. Major peaks of C_1 and C_3 were observed at δ 101.9 and δ 72.2, respectively. The absorption peak due to the saturated carbon of the carboxymethyl group seemed to overlap with the peak due to C_5 (δ 71.9), because only the C_5 peak was stronger than the others. Whereas the major peak of C_2 appeared at δ 73.1, the absorption by the C_2 of the glucose unit having the substituent could not be detected. Probably it accidentally coincides with that due to C_4 (δ 81.1).

Apparatus

Electrokinetic chromatography was performed at room temperature with an apparatus similar to that previously described[2]. The current supply of a Shimadzu IP-2A isotachophoretic analyser was used, which could deliver a constant current up to 500 μA at below 25 kV. The on-column detection technique was employed with a Jasco Uvidec-100-V spectrophotometric detector with a modified slit, as described elsewhere[2]. Proton NMR spectra were recorded on a Varian EM-390 and carbon-13 NMR spectra on a Jeolco FX-90Q NMR spectrometer.

Procedure

The chromatographic solution was prepared by dissolving β-CMCD in 0.1 M phosphate buffer solution (pH 7.0) consisting of sodium salts alone. The two solutions at the two electrodes were interchanged at an appropriate interval to avoid changes in pH owing to the electrolysis of water. The apparatus was operated at the constant current of 50 μA. All the solutes were injected as methanol solutions. Other chromatographic procedures were the same as those described elsewhere[2].

RESULTS AND DISCUSSION

Retention data obtained in this work are listed in Table I. The retention time of unretained solutes, t_0, was regarded as being equal to that of methanol used as the solvent for the solutes. On the assumption that methanol cannot be included at all by β-CMCD, the velocity of methanol band should be identical with that of the aqueous phase that is moved by electroosmosis[3]. The observed variations in t_0 shown in Table I for identical solutions are considered to be due to temperature fluctuations and/or slight changes in the pH of the solution as a result of the electrolysis of water. As the tube is filled with β-CMCD solution of a constant concentration, it is necessary for the measurement of the velocity of β-CMCD to label β-CMCD with a UV-absorbing molecule. Iodine was employed as a candidate for the tracer of β-CMCD, but the iodine peak could not be recognized when it was injected in the same way as the other samples. The chromatogram for the separation of cresol isomers is shown in Fig. 1 and that for the separation of xylidine isomers in Fig. 2.

The solutes listed in Table I can be assumed to be electrically neutral under the experimental conditions used, except for nitrophenols. These neutral solutes were

TABLE I

RETENTION TIMES, CAPACITY FACTORS* AND DISTRIBUTION COEFFICIENTS** OF SUB-
STITUTED BENZENES

Conditions as in Fig. 1. Three to six solutes listed under the same run number were injected as a mixture.

No.	Solute	t_0 (min)	t_R (min)	$\bar{k}'$	K
1	Acetophenone	15.6	18.5	1.00	49
	Anisole		18.8	1.19	58
	Methyl benzoate		19.6	1.88	92
	Ethyl benzoate		19.9	2.23	109
	Propyl benzoate		20.4	3.04	149
	Butyl benzoate		20.7	3.71	182
2	*o*-Cresol	15.6	18.1	0.78	38
	m-Cresol		18.6	1.04	51
	p-Cresol		19.3	1.55	76
3	*m*-Nitroaniline	16.1	18.6	0.75	37
	o-Nitroaniline		18.9	0.89	44
	p-Nitroaniline		20.8	2.54	124
4	*o*-Chloroaniline	15.8	18.7	0.96	47
	m-Chloroaniline		18.9	1.08	53
	p-Chloroaniline		19.7	1.68	82
5	*m*-Dinitrobenzene	14.5	15.5	0.29	14
	p-Dinitrobenzene		15.7	0.36	18
	o-Dinitrobenzene		16.6	0.78	38
6	*m*-Nitrophenol	15.3	18.9	—***	—
	o-Nitrophenol		20.3	—***	—
	p-Nitrophenol		22.5	—***	—
7§	*m*-Nitrophenol	9.8	10.4	—	—
	p-Nitrophenol		12.3	—	—
	o-Nitrophenol		14.2	—	—
8§§	*m*-Nitrophenol	8.0	8.6	—	—
	o-Nitrophenol		12.2	—	—
	p-Nitrophenol		13.0	—	—
9	3,5-Xylidine	15.6	16.4	0.18	9
	2,6-Xylidine		17.0	0.35	17
	2,3-Xylidine		17.4	0.48	24
	2,5-Xylidine		18.1	0.77	38
	2,4-Xylidine		18.3	0.86	42
	3,4-Xylidine		18.6	1.02	50
10	3,5-Xylenol	15.4	17.6	0.68	33
	2,6-Xylenol		17.6	0.68	33
	2,5-Xylenol		18.5	1.18	58
	2,4-Xylenol		18.8	1.41	69
	2,3-Xylenol		19.1	1.67	82
	3,4-Xylenol		19.4	2.00	98

* An electrophoretic mobility of $9.03 \cdot 10^{-3}$ mm^2 sec^{-1} V^{-1} for β-CMCD was adopted in cal-
culating t_{CD}.
** As specific volume of 0.671 ml g^{-1} for β-CMCD was assumed in calculating V_{CD}.
*** See text.
§ α-Cyclodextrin (0.032 M) was employed instead of β-CMCD.
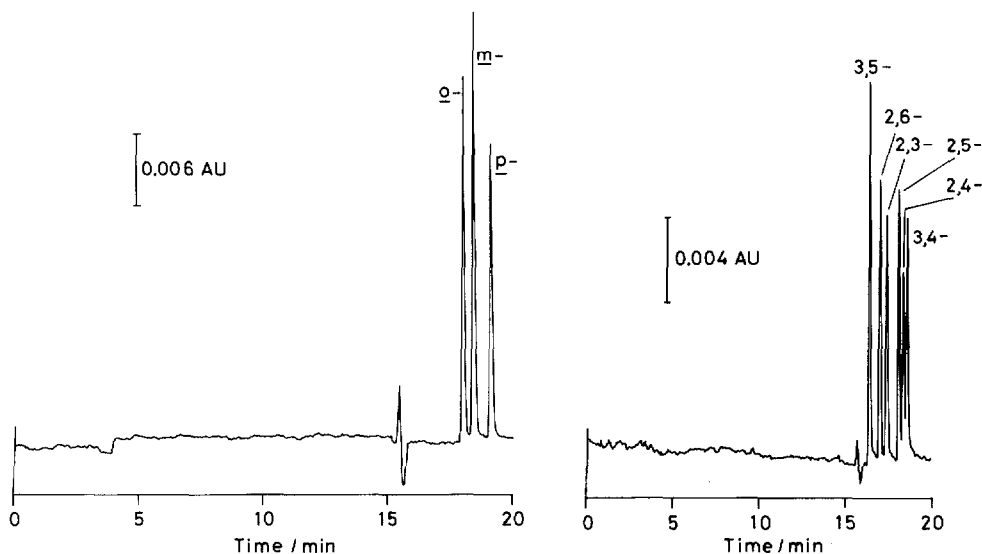§§ Phosphate buffer (0.1 M, pH 7.0) containing no cyclodextrin was used.

Fig. 1. Electrokinetic chromatogram of cresol isomers. Chromatographic solution, 0.025 M β-CMCD in 0.1 M phosphate buffer (pH 7.0); separation tube, 650 × 0.05 mm I.D.; length of the tube used for separation, 500 mm; current, 50 μA; total applied voltage, $ca.$ 12 kV; detection wavelength, 210 nm.

Fig. 2. Electrokinetic chromatogram of xylidine isomers. Conditions as in Fig. 1.

not separated by electrophoresis alone when 0.032 M α-cyclodextrin was used in place of β-CMCD. The lower solubility of β-cyclodextrin than α-cyclodextrin in water is the reason why β-cyclodextrin was not used in the blank experiment. The fact that the separation of these isomers was effected when β-CMCD was present in the solution clearly implies that the inclusion of solutes by β-CMCD takes part in the distribution process. The order of elution of isomers of cresol, nitroaniline and dinitrobenzene is the same as that reported[4,5] in HPLC with β-cyclodextrin-bonded stationary phases. It should be noted that isomers of xylidine or xylenol were successfully separated even if 3,5- and 2,6-xylenols were not resolved. These results suggest that this method is promising for separating isomers of substituted benzenes.

The isomers of nitrophenol were separated in every instance shown in Table I (Nos. 6, 7 and 8), and these results can be explained as follows. In the absence of cyclodextrins (Table I, No. 8), the isomers were separated only by electrophoresis of free isomers in the phosphate buffer, that is, the isomers were eluted in decreasing order of their pK_a values. When α-cyclodextrin was added to the buffer solution (Table I, No. 7), the p-isomer was included by α-cyclodextrin to a much greater extent than the other isomers and the electrophoretic mobility was reduced, causing reversal of the order of elution between the o- and p-isomers. Comparison of the results with α-cyclodextrin (Table I, No. 7) and β-CMCD (Table I, No. 6) reveals that the p-isomer was also included by β-CMCD more strongly than the o-isomer.

The capacity factor, $\bar{k}'$, which is defined as the ratio of the total number of moles of the solute included by β-CMCD, n_{CD}, to those in the aqueous phase, n_{aq}, i.e., $\bar{k}' = n_{CD}/n_{aq}$, is related to the retention time, t_R, for electrically neutral solutes

by the following equation, which is similar to that derived for electrokinetic chromatography with micellar solutions[3]:

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

where t_{CD} is the elution time of β-CMCD and indicates a longer limit of retention times for electrically neutral solutes. As shown in eqn. 1, the values of both t_{CD} and t_0 are required for calculation of the capacity factor. This means that the possible range of the retention time should be known in order to determine the capacity factor in this method.

In order to measure the elution time of β-CMCD, an experimental approach was used in addition to the tracer method mentioned above. A *ca.* 0.015 *M* aqueous solution of β-CMCD was injected and detected by UV absorption at 200 nm under the same conditions as those given in Table I, except that 0.1 *M* phosphate buffer solution (pH 7.0) containing no β-CMCD was used instead of that containing β-CMCD. The current was kept at 50 μA, but the voltage increased to 15 kV from the 12 kV observed with the 0.025 *M* β-CMCD solution. The electroosmotic velocity was measured with methanol added to the aqueous β-CMCD solution. The electrophoretic mobility of β-CMCD in the 0.1 *M* phosphate buffer solution (pH 7.0) was $9.03 \cdot 10^{-3}$ mm^2 sec^{-1} V^{-1}. The estimated value of t_{CD}/t_0 from the above value of the electrophoretic mobility was about 1.4 under the conditions given in Table I.

Capacity factors calculated by eqn. 1 from the retention time and t_0 listed in Table I and the values of t_{CD}/t_0 estimated from the electrophoretic mobility of β-CMCD described above are also given in Table I, together with the distribution coefficient, K, which was obtained according to

$$\tilde{k}' = K \cdot \frac{V_{CD}}{V_{aq}} \tag{2}$$

where V_{aq} and V_{CD} are the volumes of the aqueous phase and β-CMCD, respectively, in the tube. The specific volume of β-CMCD required in order to know V_{CD} was assumed to be equal to that of α-cyclodextrin (0.671 ml g^{-1})[10]. It should be stressed that the capacity factors and hence the distribution coefficients listed in Table I are approximate values because the estimated value of t_{CD} is adopted in the calculation.

The resolution equation in electrokinetic chromatography is given by considering eqn. 1[3]:

$$R_s = \frac{\sqrt{N}}{4} \left(\frac{\alpha - 1}{\alpha} \right) \left(\frac{\tilde{k}'}{1 + \tilde{k}'} \right) \left[\frac{1 - t_0/t_{CD}}{1 + (t_0/t_{CD}) \tilde{k}'} \right] \tag{3}$$

where R_s is the resolution, N is the plate number and α is the separation factor. In eqn. 3, capacity factors for two closely eluted peaks are assumed to be approximately equal. The resolution expressed by eqn. 3 is apparently a function not only of N, α and $\tilde{k}'$, but also of t_0/t_{CD}. As discussed in a previous paper[3], the capacity factor that gives the maximum resolution at constant values of the other parameters is dependent

on the ratio t_0/t_{CD}. As the value of t_0/t_{CD} is estimated to be about 0.7, as discussed above, the maximum value at $\bar{k}' = 1.1$ can be predicted to be about 0.07 for the product of the last two terms of the right-hand side of eqn. 3, which depend only on the capacity factor among four terms. This large value of 0.7 for t_0/t_{CD} in this method results in a much poorer resolution in comparison with electrokinetic chromatography with micellar solutions where t_0/t_{mc} lies in the range[3] 0.2–0.3, where t_{mc} is the elution time of the micelle and corresponds to t_{CD}. The low electrophoretic mobility of β-CMCD, which results from the small ratio of the charge number to the molecular weight, must be the reason for the large value of t_0/t_{CD} and it is therefore desirable to introduce more charges into a molecule in order to improve the resolution in this method.

Plate number calculated from peak area, peak height and retention time are 120 000–130 000 in the chromatogram shown in Fig. 1. These numbers correspond to plate heights of about 4 μm. These plate heights are considerably higher than the 2–3 μm observed for chromatograms with micellar solutions[3], the reason for which remains to be clarified.

CONCLUSION

The method described is an example of chromatography with a homogeneous solution and electrokinetic migrations, supplementing that with micellar solutions reported previously[3]. It demonstrates the possibility of widening the scope of electrokinetic chromatography. Only the formation of inclusion complexes or the host–guest interaction between the cyclodextrin derivative and the solute, both of which are free in the solution, operates as the distribution process. This method seems very promising, especially for separating aromatic isomers, although much remains to be done before practical application.

ACKNOWLEDGEMENT

REFERENCES

1 T. Nakagawa, *Newsl., Div. Colloid Surf. Chem., Chem. Soc. Jap.*, 6, No. 3 (1981) 1.
2 S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya and T. Ando, *Anal. Chem.*, 56 (1984) 111.
3 S. Terabe, K. Otsuka and T. Ando, *Anal. Chem.*, 57 (1985) 834.
4 K. Fujimura, T. Ueda and T. Ando, *Anal. Chem.*, 55 (1983) 446.
5 Y. Kawaguchi, M. Tanaka, M. Nakae, K. Funazo and T. Shono, *Anal. Chem.*, 55 (1983) 1852.
6 W. L. Hinze, T. E. Riehl, D. W. Armstrong, W. DeMond, A. Alak and T. Ward, *Anal. Chem.*, 57 (1985) 237.
7 Y. Kitaura and M. L. Bender, *Bioorg. Chem.*, 4 (1975) 237.
8 W. B. Gruhn and M. L. Bender, *Bioorg. Chem.*, 3 (1974) 324.
9 K. Takeo, K. Hirose and T. Kuge, *Chem. Lett.*, (1973) 1233.
10 P. C. Manor and W. Saenger, *J. Amer. Chem. Soc.*, 96 (1974) 3630.