Direct chiral resolution of aliphatic α-hydroxy acids using 2-hydroxypropyl-β-cyclodextrin in capillary electrophoresis

The Analyst

Shuji Kodama,* Atsushi Yamamoto and Akinobu Matsunaga

Toyama Institute of Health, 17-1 Nakataikoyama, Kosugi-machi, Toyama 939-0363, Japan.
E-mail: lee07664@niftyserve.or.jp

Received 21st September 1998, Accepted 17th November 1998

Chiral resolution of α-hydroxy acids (lactic acid, 2-hydroxybutyric acid, 2-hydroxy-3-methylbutyric acid, 2-hydroxyisocaproic acid) without derivatization was performed by capillary electrophoresis using 2-hydroxypropyl-β-cyclodextrin (2HP-β-CD). An increase in the bulkiness of the alkyl group in these acids enhanced the resolution. The association constants for complexes of 2HP-β-CD with the α-hydroxy acids were determined by spectrophotometric and electrophoretic methods. Good agreement was found between the two methods. An increase in the bulkiness of the alkyl group in these acids brought about an increase in the association constants. When cyclohexanol that was included in 2HP-β-CD was added to the background electrolyte, chiral separation of these compounds was completely prevented. These results suggest that α-hydroxy acids having a short alkyl chain, and also chiral compounds having aromatic rings, could be included in 2HP-β-CD to be chiroptically separated.

Introduction

According to recent reviews,[1–7] cyclodextrins (CDs) and their derivatives have been widely applied in capillary electrophoresis (CE) for the separation of enantiomers of many compounds. It is well known that at least three-point simultaneous interactions are generally needed between a chiral selector and an enantiomer to cause physical separation, because optical isomers have a three-dimensional spatial character. In the inclusion complexation mechanism, the hydrophobic interaction between the cavity of the native or derivatized CDs and the hydrophobic part of the compounds, such as an aromatic ring, plays an important role in the stereoselective interaction. Inclusion complex formation has been studied by several methods, such as proton nuclear magnetic resonance spectroscopy, ultraviolet and visible spectroscopy, circular dichroism spectroscopy and thermoanalytical methods, and the equilibrium constants for the inclusion–complexation have been calculated.[8–10] The migration time of analytes in CE was well characterized by Guttman *et al*.[11] Wren and Rowe[12] developed a theoretical model relating the mobility to the concentration of a CD selector. An extended model for the separation of enantiomers of weak electrolyte solutes considering both pH and concentration of chiral selector has been proposed by Rawjee *et al*.[13,14] Recently, CE has been successfully used for the calculation of association constants using electrophoretic measurements.[15–19] Rundlett and Armstrong[20] showed the relationship between several standard linear plotting methods that could be used to calculate binding constants with CE and compared these constants calculated by CE with those determined by various methods, including spectroscopy, calorimetry and chromatography.

Most compounds described in these reviews have aromatic rings. It has been reported that monoterpenes such as pinenes, camphene and limonene, which have no aromatic rings and are hydrophobic, are chiroptically separated by CE[21] and also by gas chromatography[22–24] and high-performance liquid chromatography[25] using a cyclodextrin bonded stationary phase. Recently, we reported the direct chiral resolution of the hydrophilic pantothenic acid having no aromatic rings by CE using 2-hydroxypropyl-β-cyclodextrin (2HP-β-CD).[26] However, it remains unclear whether pantothenic acid is included in the cavity of 2HP-β-CD or not. An interesting chiral recognition mechanism between β-CD and dinitrophenyl (DNP)-amino acids (valine and leucine) has been reported.[8] According to this report, the DNP group forms stable inclusion complexes with the β-CD cavity, and the alkyl groups form a secondary inclusion complex with another β-CD cavity (in the case of DNP-L-amino acids) or are sterically repulsed by the hydroxyl groups at the edge of the cavity (in the case of DNP-D-amino acids). This may indicate that an alkyl group in the aliphatic acid is also included in the cavity of the CD. This raised the possibility that the direct chiral resolution of various organic acids having no aromatic rings could be accomplished with CE using CDs.

In this work, the direct chiral resolution of the aliphatic α-hydroxy acids using 2HP-β-CD in CE was studied. The association constants between 2HP-β-CD and α-hydroxy acids by using spectrophotometric and electrophoretic measurements were also studied.

Experimental

Reagents

Heptakis(2,6-di-*O*-methyl)-β-cyclodextrin, 2-hydroxypropyl-β-cyclodextrin (average degree of substitution 7) and D-lactic acid lithium salt were obtained from Sigma (St. Louis, MO, USA). 2-Hydroxypropyl-α-cyclodextrin, 2-hydroxypropyl-γ-cyclodextrin and racemic 2-hydroxy-3-methylbutyric acid were obtained from Aldrich (Milwaukee, WI, USA). 2,3,6-Tri-*O*-methyl-β-cyclodextrin, DL-lactic acid lithium salt, DL-2-hydroxybutyric acid lithium salt, 0.05 mol l^{-1} iodine solution, cyclohexanol and other chemicals (guaranteed grade) were

purchased from Wako (Osaka, Japan). D- and DL-2-Hydroxyisocaproic acid were purchased from Tokyo Kasei (Tokyo, Japan).

Apparatus

Electrophoretic experiments were carried out using an HP^{3D} capillary electrophoresis system (Hewlett-Packard, Palo Alto, CA, USA). Samples were injected by applying a pressure of 5 kPa for 4 s. The separations were performed in a poly(vinyl alcohol) (PVA) coated bubble cell capillary of 50 cm × 50 μm id (Hewlett Packard). The capillary was kept at 15 °C. The analytes were detected at 200 nm. The power supply was operated in the constant-voltage mode, at −30 kV, and the substances migrated towards the positive pole.

Spectrophotometric examinations were carried out using a Hitachi (Tokyo, Japan) U-2000 spectrophotometer. The sample compartment contained a 1 cm thick quartz cell that was kept at 25 °C.

Buffer and sample preparation for CE

The background electrolyte (BGE) in the electrophoretic experiments, unless stated otherwise, was 60 mM phosphate buffer (pH 6.0) containing 0–220 mM 2HP-β-CD and was filtered with a 0.22 μm filter before use. De-ionized water was prepared using a Yamato (Tokyo, Japan) WA-52G auto still.

Stock standard solutions of 100 mM DL-lactic acids, DL-2-hydroxybutyric acid, racemic 2-hydroxy-3-methylbutyric acid and DL-2-hydroxyisocaproic acid were separately prepared in de-ionized water, stored at 4 °C and diluted to 2 mM before use.

Calculation of resolution

Each electropherogram that was obtained was subtracted from a blank run and the resolution (R_s) of the enantiomer was calculated by using the following equation:

$$R_s = 2[(t_2 - t_1)/(w_2 + w_1)]$$

where t is the migration time and w is the width of the peak at the baseline.

Determination of association constants by UV/VIS spectrophotometry

The association constant (K_a) for a 2HP-β-CD–α-hydroxy acid system was determined by spectrophotometric examination of the inhibitory effect of the α-hydroxy acid on the association of the CD with a light-absorbing substance as reported by Matsui and Mochida,[9] except that iodine was used as the light-absorbing substance. A 2 ml volume of 30 mM phosphate buffer (pH 7.0) containing various concentrations of α-hydroxy acid was added to 2 ml of 30 mM phosphate buffer (pH 7.0) containing 0.08 mM iodine and 2 mM 2HP-β-CD and their absorption spectra (250–450 nm) were measured. The value was calculated according to the following equations:

$$[I] = (\Delta I_\infty - \Delta I)/\Delta\varepsilon$$

$$[C] = K_d([I]_0 - [I])/[I]$$

$$K_a = \{[C]_0 - [C] - ([I]_0 - [I])\}/ [C]\{[H]_0 - [C]_0 + [C] + ([I]_0 - [I])\}$$

where ΔI_∞ is the difference in absorbance between free and complexed iodine, ΔI is the change in absorbance of an iodine solution with the addition of 2HP-β-CD and α-hydroxy acid, $\Delta\varepsilon$ is the difference in the molar absorptivities for free and complexed iodine, K_d is the dissociation constant for the 2HP-β-CD–iodine complex as reported by Li and Purdy,[8] $[C]_0$, $[I]_0$ and $[H]_0$ are the total concentrations of 2HP-β-CD, iodine and α-hydroxy acid, respectively, and [C] and [I] are the equilibrium concentrations for 2HP-β-CD and iodine, respectively. The absorption spectra were measured at five concentrations of α-hydroxy acid.

Results and discussion

Chiral separation of α-hydroxy acids

Lactic acid (LA), 2-hydroxybutyric acid (HBA), 2-hydroxy-3-methylbutyric acid (HMBA) and 2-hydroxyisocaproic acid (HICA) were separated by CE using a BGE containing separately 90 or 200 mM α-CD, γ-CD, 2,6-di-*O*-methyl-β-CD, 2,3,6-tri-*O*-methyl-β-CD, 2HP-α-CD, 2HP-β-CD or 2HP-γ-CD (Table 1). A PVA-coated capillary, in which the electroosmotic flow was almost completely suppressed, was used to avoid the longer migration time with a fused-silica capillary. In the case of α-CD, γ-CD and methylated β-CDs, the above analysis was carried out at a concentration of only 90 mM owing to their low solubilities. Of these CDs, 2HP-β-CD was found to be most effective for the resolution of all the α-hydroxy acids. This was in close agreement with the results of other studies in which 2HP-β-CD was suitable for the resolution of the enantiomers of a series of α-hydroxy acids having an aromatic ring, such as mandelic acid,[27,28] and for that of pantothenic acid,[26] having no aromatic rings.

Fig. 1 shows the effect of 2HP-β-CD concentration on the resolution and migration time of these acids. Since the carboxyl group of each α-hydroxy acid is dissociated in the BGE at pH 6 and the electroosmotic flow is almost completely suppressed by using a PVA-coated capillary, the analytes migrate electrophoretically to the anode. Since all of the α-hydroxy acids have the same negative charge, the acids of smaller molecular size migrated faster in the absence of 2HP-β-CD. For both LA and HICA, the L-isomers were found to move faster than the D-isomers. This might indicate that the D-isomers formed a stronger diastereomer complex with 2HP-β-CD than the L-isomers, because neutral 2HP-β-CD hardly migrates at all. For both HBA and HMBA, it is unknown whether the L-isomers moved faster than the D-isomers, because their D- or L-isomers are not commercially available. It was found that the resolution and migration time of all the α-hydroxy acids increased with increasing amount of 2HP-β-CD in the range of the 2HP-β-CD concentrations used in Fig. 1. Wren and Rowe[12] developed a theoretical model relating mobility to the concentration of a CD selector. Also, it has been suggested that maximum resolution can be expected at the optimum CD concentration, $C_{opt} = (K_A K_B)^{-1/2}$, where K_A and K_B are equilibrium constants.

Table 1 Resolution of enantiomers of four α-hydroxy acids using different cyclodextrins as chiral selectors

Cyclodextrin	Concentration/mM	Resolution (R_S)			
		LA	HBA	HMBA	HICA
α-CD	90	NS[a]	0.72	0.68	0.82
γ-CD	90	NS	NS	NS	0.47
2,6-Di-*O*-methyl-β-CD	90	NS	NS	1.03	NS
2,3,6-Tri-*O*-methyl-β-CD	90	NS	NS	NS	0.57
2HP-α-CD	90	NS	NS	0.67	0.68
	200	0.79	1.02	1.40	1.26
2HP-β-CD	90	NS	1.14	1.62	1.96
	200	1.02	1.91	2.00	3.00
2HP-γ-CD	90	NS	NS	NS	1.17
	200	NS	NS	1.45	3.62

[a] NS, not separated.

Therefore, it could be suggested that the 2HP-β-CD concentrations used were still below the optimum concentrations. Also, it was considered that the increased migration times resulted both from complexation and from the increased viscosity of the buffer due to the high cyclodextrin concentration. It was also found that an increase in the bulkiness of the alkyl group in these acids lowered the amount of 2HP-β-CD required for chiral separation.

The effect of capillary temperature on the resolution and the migration time of α-hydroxy acids was studied. It was found that a lower capillary temperature caused increases in both the resolution and the migration time of all the α-hydroxy acids. This result was the same as those observed with other chiral compounds.[6,26,29–31] According to Heuermann and Blaschke,[31] the increase in the R_s value with a decrease in temperature might be explained by a decrease in rotational and/or vibrational energy, increasing the fixing of the enantiomers inside or at the rim of CD and thus increasing the enantioselectivity.

Spectrophotometric determination of association constants

In order to establish whether these racemic α-hydroxy acids were included in the cavity of 2HP-β-CD or not, the association constants K_a for the inclusion–complexation were measured by spectrophotometry as reported by Matsui and Mochida,[9] except that iodine was used as a light-absorbing substance. In the presence of 2HP-β-CD, the absorption maxima of iodine at 287 and 351 nm were shifted to longer wavelengths (290 and 356 nm, respectively), and the absorption intensity was significantly enhanced. As a result, the dissociation constant K_d for the 2HP-β-CD–iodine complex calculated from the absorbance at 290 nm was 1.13 mM and was identical with that calculated from the absorbance at 356 nm. The K_a value was determined by using the absorbance at 356 nm, because the absorbance of the complex at 356 nm was not affected by an increase of the ionic strength to 0.6, but the absorbance at 290 nm was slightly increased. Fig. 2 shows that the addition of DL-HICA to the 2HP-β-CD–iodine system resulted in a decrease in absorbance, indicating that part of the added DL-HICA is included by 2HP-β-CD to expel the complexed iodine to the bulk solution. The calculated K_a value of cyclohexanol used as a control was 0.198 ± 0.009 l mmol^{-1}, which was lower than the value (0.501 l mmol^{-1}) reported for the association of cyclohexanol with β-CD.[9] This difference may result from the effect of the substituted group of β-CD and also the fact that the association constant for propranolol binding to β-CD[17] is twice that for binding to methyl-β-CD.[32] The K_a value of DL-mandelic acid, used as another control having an aromatic ring, was 0.0088 ± 0.0004 l mmol^{-1}, which was higher than the values (0.0028 l mmol^{-1} for the D-isomer and 0.0024 l mmol^{-1} for the L-isomer) reported for the association of mandelic acid with γ-CD.[16] It could be suggested that this difference in the values resulted from the difference of the cavity size in these CDs to include the acid.

The calculated K_a values of DL-LA, DL-HBA, racemic HMBA and DL-HICA with 2HP-β-CD were 0.0004 ± 0.0001, 0.0044 ± 0.0002, 0.0095 ± 0.0005 and 0.0144 ± 0.0007 l mmol^{-1}, respectively. Although these values are 10–100 times lower than those of hydrophobic compounds having aromatic rings with cyclodextrins as reported previously,[15,17–20] it could be suggested that these α-hydroxy acids were apparently included in the cavity of 2HP-β-CD. The CD cavity possesses a hydrophobic character and exhibits an affinity for the hydrophobic group of the guest compound. Hence it was found that an increase in the bulkiness of the alkyl group in these acids enhanced the association constants.

Calculation of association constants using CE

Fig. 1 shows that an increase in the amount of 2HP-β-CD increased the resolution and the migration time of the α-hydroxy acids. The resolution increases as the difference in migration time between two enantiomers increases and as the width of peak at the baseline decreases. However, the width of the peak is more difficult to describe mathematically, because it is affected by band broadening due to diffusion and other factors.[12] We propose defining the differential time selectivity coefficient, S_{DT}, as the ratio of the average migration time of a pair of enantiomers to the difference in migration time:

$$S_{DT} = (T_B - T_A)/[(T_A + T_B)/2] \quad (1)$$

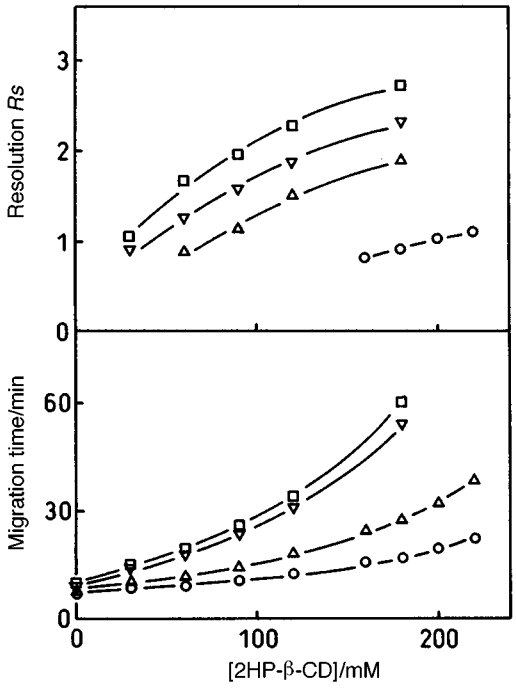

Fig. 1 Effect of 2HP-β-CD concentration on the enantiomeric resolution and migration time of α-hydroxy acids. Racemic α-hydroxy acids (2 mM) were separated by CE. The BGE was composed of various concentrations of 2HP-β-CD containing 60 mM phosphate buffer (pH 6.0). ○, LA; △, HBA; ▽, HMBA; and □, HICA.

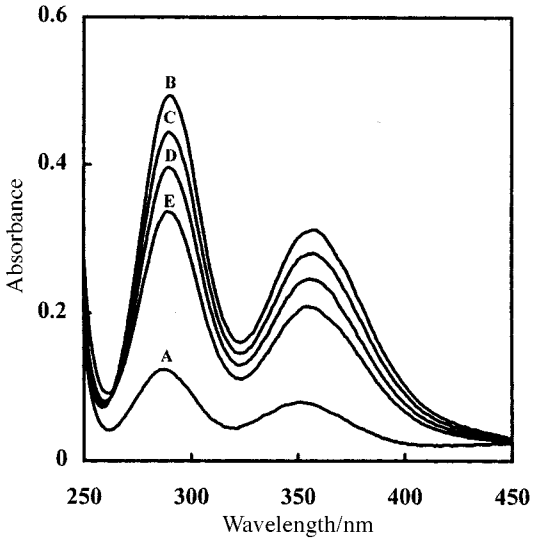

Fig. 2 Effect of DL-HICA addition on the absorption spectra of the 2HP-β-CD–iodine complex. A, 0.04 mM iodine; B, A + 1 mM 2HP-β-CD; C, B + 30 mM DL-HICA; D, B + 75 mM DL-HICA; and E, B + 150 mM DL-HICA.

where T_A and T_B are migration times of enantiomers A and B. The buffer viscosity will affect the mobility of all the ionic species and hence the resultant current. It has been reported that the relative viscosity determined from measuring the current agreed well with that determined by direct measurement.[12] According to that report, it was found that the current values for the buffer without 2HP-β-CD and with 200 mM 2HP-β-CD were 45.2 and 19.3 μA to give a ratio of 2.34. Goodall and co-workers[17, 33] reported that observed mobilities should be converted into corrected mobilities before calculation of association constants. However, an advantage using S_{DT} is to be able to cancel out the factors such as viscosity and diffusion.

Expressing the migration time (T_A) in terms of effective mobility of enantiomer (μ_A) , the operating voltage (V), the total capillary length (L) and the capillary length to the detector (l), yields

$$T_A = lL/\mu_A V \quad (2)$$

As a PVA-coated capillary was used, we assumed that electroosmotic flow was negligible. Wren and Rowe[12] described a separation model that incorporated equations for mobility and differential mobility. Mobility was defined as

$$\mu_A = (\mu_F + \mu_C K_A[C])/(1 + K_A[C]) \quad (3)$$

where μ_F is the electrophoretic mobility of both enantiomers A and B in the free state, μ_C is the electrophoretic mobility of both enantiomers A and B in the complexed state, [C] is the concentration of 2HP-β-CD and K_A and K_B are formation constants for inclusion complexes.

Substitution of eqns. (2) and (3) into eqn. (1) and rearrangement yield

$$S_{DT} = 2(\mu_F - \mu_C)(K_B - K_A)[C]/\{2\mu_F + (\mu_F + \mu_C)(K_A + K_B)[C] + 2\mu_C K_A K_B [C]^2\} \quad (4)$$

The double reciprocal expression of eqn. (4) yields eqn. (5), which shows the relationship between the $1/S_{DT}$ and 1/[C]:

$$1/S_{DT} = \mu_F/(\mu_F - \mu_C)(K_B - K_A)[C] + (\mu_F + \mu_C)(K_A + K_B)/2(\mu_F - \mu_C)(K_B - K_A) + \mu_C K_A K_B [C]/(\mu_F - \mu_C)(K_B - K_A) \quad (5)$$

The molecular masses of the complexes are 12–18 times higher than those of the free acids, whereas both the free acids and the complexes have the same charge. Therefore, it is thought that the effective mobility of free α-hydroxy acids is much higher than that of complexes. When $\mu_F >> \mu_C$ is postulated for the sake of simplicity, we obtain

$$1/S_{DT} = 1/(K_B - K_A)[C] + (K_A + K_B)/2(K_B - K_A) \quad (6)$$

That is, when $1/S_{DT}$ is plotted against 1/[C], the slope is $1/(K_B - K_A)$ and the intercept on the ordinate is $(K_A + K_B)/2(K_B - K_A)$. As shown in Fig. 3, a straight line ($r > 0.99$) was obtained by plotting $1/S_{DT}$ *versus* 1/[C]. It was found that the differences between the K_B and K_A values of these four α-hydroxy acids were very small and that K_B and K_A values calculated by the CE method were similar to the K_a values determined by spectrophotometry (Table 2). Therefore, it could be suggested that this calculation method, using the CE system with a PVA-coated capillary, was useful for the determination of the association constant.

Inhibition of chiral resolution of DL-α-hydroxy acids by cyclohexanol

Since cyclohexanol has a high association constant with 2HP-β-CD, as mentioned above, it was hoped that cyclohexanol would act as a competitor between the guest and host molecules. It has been reported that when cyclohexanol was added to the BGE, a higher concentration of β-CD was required for maximum resolution of tioconazole enantiomers.[17] In order to confirm the inclusion of the α-hydroxy acids in the cavity of 2HP-β-CD, the effect of cyclohexanol on the resolution was studied by CE (Fig. 4). It was found that the addition of cyclohexanol to the BGE completely prevented the resolution of the α-hydroxy acids and resulted in a decrease in their migration times. It could be suggested that cyclohexanol inhibited the formation of the inclusion complex of the α-hydroxy acids with 2HP-β-CD and that the free enantiomers of each analyte migrated with the same mobility. Therefore, this supported the result obtained by using spectrophotometry that the α-hydroxy acids could be included in the cavity of 2HP-β-CD.

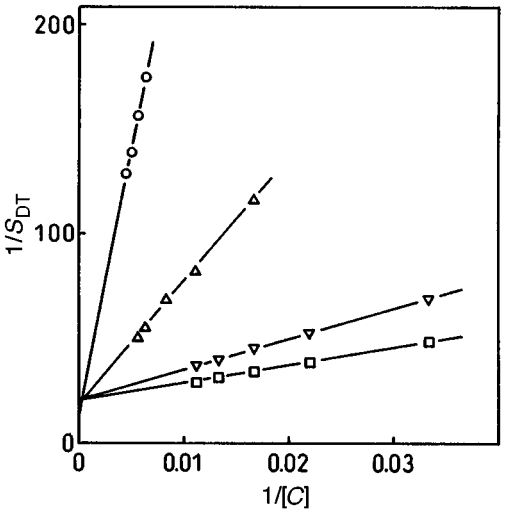

Fig. 3 Relationship between $1/S_{DT}$ and 1/[C]. ○, LA; △, HBA; ▽ HMBA; and □, HICA.

Table 2 Association constants between α-hydroxy acids and 2HP-β-CD

α-Hydroxy acid	K_A[a] l mmol^{-1}	K_B[a] l mmol^{-1}	K_a[b] l mmol^{-1}
LA	0.00038	0.00042	0.0004
HBA	0.00288	0.00304	0.0044
HMBA	0.0137	0.0144	0.0095
HICA	0.0225	0.0238	0.0144

[a] K_A and K_B calculated by electrophoretic experiments are association constants of each enantiomer of the α-hydroxy acids for inclusion complexes with 2HP-β-CD. [b] K_a measured by spectrophotometry is the association constant between racemic α-hydroxy acids and 2HP-β-CD.

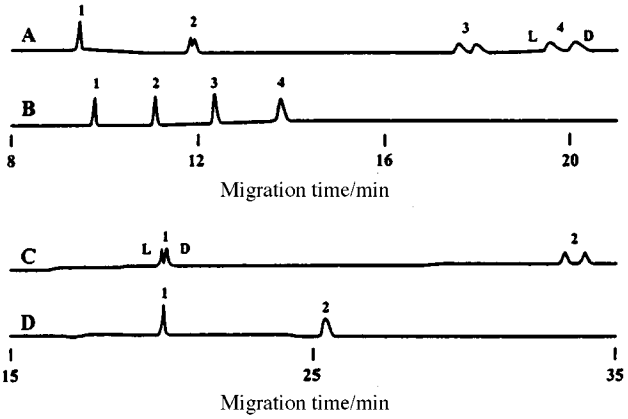

Fig. 4 Inhibition effect of cyclohexanol on the enantiomeric resolution of α-hydroxy acids. Racemic α-hydroxy acids (2 mM) were separated by CE using the following BGEs. A, 60 mM 2HP-β-CD containing 60 mM phosphate buffer (pH 6.0); B, A + 180 mM cyclohexanol; C, 200 mM 2HP-β-CD containing 60 mM phosphate buffer (pH 6.0); D, C + 200 mM cyclohexanol. Peaks: 1 = LA; 2 = HBA; 3 = HMBA; and 4 = HICA . L and D are indicated L- and D-isomers, respectively.

Conclusion

Direct chiral resolution of aliphatic α-hydroxy acids was performed by CE using 2HP-β-CD. Using two methods, spectrophotometry and CE, we showed that these α-hydroxy acids could be included in the cavity of 2HP-β-CD. It is well known that at least three-point simultaneous interactions are generally needed between a chiral selector and an enantiomer to cause physical separation. It can be assumed that one of the three-point interactions is the inclusion of an alkyl group in the α-hydroxy acid with 2HP-β-CD. As the hydrophobic interaction with the cavity alone is not sufficient to permit the separation of enantiomers,[5] it is suggested that two other interactions between the hydroxyl and carboxyl groups on the asymmetric center of α-hydroxy acids and the secondary and/or primary hydroxypropyl groups of the CD are responsible for chiral recognition.

We propose defining the differential time selectivity coefficient, S_{DT}, as the ratio of the average migration time of a pair of enantiomers to the difference in migration time. By calculating S_{DT} as a function of 2HP-β-CD concentration, the association constants of the α-hydroxy acids with 2HP-β-CD can be obtained, and they show satisfactory agreement with those obtained by spectrophotometry.

There have been many reports on the direct chiral resolution of various compounds with one or more aromatic rings by CE using CDs and their derivatives. However, using the present CE method, we were able to achieve the chiral separation of α-hydroxy acids having no aromatic rings, and without having to derivatize the acids. Therefore, the chiral resolution of various organic compounds having no aromatic rings may become possible with CE using native and derivatized CDs.

References

1 J. Snopek, I. Jelinek, and E. Smolkova-Keulemansova, *J. Chromatogr.*, 1992, **609,** 1.
2 S. Terabe, K. Otsuka and H. Nishi, *J. Chromatogr.*, 1994, **666**, 295.
3 M. Novotny, H. Soini and M. Stefansson, *Anal. Chem.*, 1994, **66**, 646A.
4 H. Nishi and S. Terabe, *J. Chromatogr. A*, 1995, **694**, 245.
5 S. Fanali, *J. Chromatogr. A*, 1996, **735**, 77.
6 S. Fanali, *J. Chromatogr. A*, 1997, **792**, 227.
7 I. S. Lurie, *J. Chromatogr. A*, 1997, **792**, 297.
8 S. Li and W. C. Purdy, *Anal. Chem.*, 1992, **64**, 1405.
9 Y. Matsui and K. Mochida, *Bull. Chem. Soc. Jpn.*, 1979, **52**, 2808.
10 E. A. Lewis and L. D. Hansen, *J. Chem. Soc., Perkin Trans. 2*, 1973, 2081.
11 A. Guttman, A. Paulus, A. S. Cohen, N. Brinberg and B. L. Karger, *J. Chromatogr.*, 1988, **448,** 41.
12 S. A. C. Wren and R. C. Rowe, *J. Chromatogr.*, 1992, **603**, 235.
13 Y. Y. Rawjee, D. U. Staerk and G. Vigh, *J. Chromatogr. A.*, 1993, **635**, 291.
14 Y. Y. Rawjee and G. Vigh, *Anal. Chem.*, 1994, **66**, 619.
15 S. A. C. Wren, *J. Chromatogr.*, 1993, **636**, 57.
16 I. E. Valko, H. A. H. Billiet, J. Frank and K. Ch. A. M. Luyben, *Chromatographia*, 1994, **38**, 730.
17 S. G. Penn, G. Liu, E. T. Bergstrom, D. M. Goodall and J. S. Loran, *J. Chromatogr., A*, 1994, **680**, 147.
18 K.-H. Gahm and A. M. Stalcup, *Anal. Chem.*, 1995, **67**, 19.
19 S. Piperaki, S. G. Penn and D. M. Goodall, *J. Chromatogr. A*, 1995, **700**, 59.
20 K. L. Rundlett and D. W. Armstrong, *J. Chromatogr. A*, 1996, **721**, 173.
21 K.-H. Gahm, L. W. Chang and D. W. Armstrong, *J. Chromatogr. A*, 1997, **759**, 149.
22 A. Steinborn, R. Reinhardt, W. Engewald, K. Wyssuwa and K. Schulze, *J. Chromatogr. A*, 1995, **697**, 485.
23 K. L. Rundlett and D. W. Armstrong, *J. Chromatogr. A*, 1996, **721**, 173.
24 B. E. Kim, K. P. Lee, K. S. Park, S. H. Lee and J. H. Park, *Chromatographia*, 1997, **46**, 145.
25 D. W. Armstrong and J. Zukowski, *J. Chromatogr. A.*, 1994, **666**, 445.
26 S. Kodama, A. Yamamoto and A. Matsunaga, *J. Chromatogr. A*, 1998, **811**, 269.
27 A. Nardi, A. Eliseev, P. Bocek and S. Fanali, *J. Chromatogr.*, 1993, **638**, 247.
28 I. E. Valko, H. A. H. Billiet, J. Frank, and K. Ch. A. M. Luyben, *J. Chromatogr. A*, 1994, **678**, 139.
29 A. Guttman and N. Cooke, *J. Chromatogr. A*, 1994, **680**, 157.
30 K. D. Altria, D. M. Goodall and M. M. Rogan, *Chromatographia*, 1992, **34**, 19.
31 M. Heuermann and G. Blaschke, *J. Chromatogr.*, 1993, **678**, 267.
32 S. A. C. Wren, *Electrophoresis*, 1995, **16**, 2127.
33 L. Knights, G. Liu, A. Ruddick and D. M. Goodall, *J. Phys. Chem.*, 1995, **99**, 3875.

Paper 8/07351A