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Enantiomer separation of hydrophobic amino compounds by high-performance liquid chromatography using crown ether dynamically coated chiral stationary phase

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Abstract

CROWNPAK CR(+) column, which is powerful for the separation of amino acid enantiomers, must be used at a column temperature below 50°C and a mobile phase containing less than 15% methanol, because the chiral crown ether moiety of the stationary phase is dynamically coated on an ODS matrix. The second peak of the enantiomers of alanine-β-naphthylamide (Ala-β-NA) appeared at 204 min (k_2=148) by using ordinary mobile phase, that is, a mixture of 10 m*M* perchloric acid and 15% methanol. In this study, enantiomer separations of Ala-β-NA and 1-(1-naphthyl)ethylamine (1-NEA), both of which are hydrophobic amino compounds, were investigated through the modification of the mobile phase. Addition of crown ether, cyclodextrins (CDs), cations, etc., affected the stability of the complex between an analyte and the chiral moiety, leading to fast separation. The second peak of the enantiomers of Ala-β-NA appeared at 68 min (k_2=49) through the addition of 10 m*M* β-CD, or at 61 min (k_2=44) using potassium dihydrophosphate as a buffer component. This method was applied for the optical purity testing of L-Ala-β-NA, which is used as one of the chiral derivatization reagents for carboxylic compounds. Validations such as reproducibility and linearity were also demonstrated and this method was found to be sufficient as a quality control method for the optical purity testing of L-Ala-β-NA. As little as 0.05% D-form in L-Ala-β-NA could be determined. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Enantiomer separation; Chiral stationary phase, LC; Crown ethers; Cyclodextrins; Alanine-β-naphthylamide; 1-(1-Naphthyl)ethylamide; Amino compounds, hydrophobic

1. Introduction

Crown ethers, first introduced by Pedersen in 1967 [1,2] are synthetic macrocyclic polyethers that can form selective complexes with cations. Crown ether derivatives have been widely used as chiral selectors for primary amino compounds in analytical chemistry, particularly, high-performance liquid chromatography (HPLC) [3–16]. In the late 1970s, Cram and his co-workers reported the separation of DL-amino acids (DL-AAs) and amino esters by using chiral stationary phases (CSPs) consisting of chiral crown ethers attached to polystyrene [3] or silica gel [4]. Enantiopure crown ethers form complexes enantioselectively with chiral primary amines (in the form of ammonium cations).

In 1987, Shinbo et al. [5,6] reported the separation of underivatized DL-AAs by using a CSP (CROWNPAK CR(+)) in which a hydrophobic chiral crown

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ether is dynamically coated on an ODS column. However, in this type of immobilized stationary phase, there is a disadvantage. That is, only aqueous solutions of inorganic species can be used as the mobile phase. As the crown ether is embedded in the alkyl chain of reversed-phase silica-gel support by the hydrophobic interaction, the passage of methanol-containing solution through the CSP leads to elution of the coated crown ether from the support. Therefore there is a restriction in the mobile phase that can be usable for the CROWNPAK CR(+) column. That is, a column temperature below 50°C and a mobile phase containing less than 15% methanol, according to the brochure [14].

To overcome this problem and to widen the applicability of the crown ether type CSPs for enantiomer separation (elution) of hydrophobic amino compounds, there are two approaches. One is to employ a CSP that can be usable under 100% organic solvents, that is, CSP where chiral crown ether is covalently bonded on the silica-gel support. The other is the modification of the mobile phase used in CROWNPAK CR(+). Addition of 18-crown-6 (18C6), cyclodextrins (CDs) and cations, etc., that is, modification of the mobile phase affects the stability of complex between amino compounds and the chiral crown ether moiety in CSP and will promote the fast elution of analytes.

In the previous works [15,16], we reported synthesis and evaluation of a novel CSP (CSP-18C6I) chemically immobilized (+)-18-crown-6 tetracarboxylic acid, and applied for enantiomer separations of hydrophobic amino compounds. In the present paper, enantiomer separations of alanine-β-naphthylamide (Ala-β-NA) and 1-(1-naphthyl)ethylamine (1-NEA) by CROWNPAK CR(+), which is only one commercially available crown ether type CSP, were investigated through the modification of the mobile phase. Ala-β-NA and 1-NEA were used as examples of hydrophobic amino compounds, although enantiomer separations of these two compounds have been already reported by using other CSPs [15,17,18]. Our aim is to widen the applicability of the CROWNPAK CR(+). The effects of the addition of 18C6 and CDs, both of which can form complexes with analytes, to the mobile phase on the retention time and enantioselectivity were studied (Fig. 1a). Further, the addition of metal cations and counter anions, which may affect the complex formation between the analyte and the chiral crown ether in CROWNPAK CR(+) were also investigated (Fig. 1b). Finally, this method was applied for the optical purity testing of L-alanine-β-naphthylamide (L-Ala-β-NA).

2. Experimental

2.1. Apparatus

The HPLC system (Shimadzu, Kyoto, Japan) consisted of an LC-10AD high-pressure pump, an SPD-10A variable-wavelength UV detector and a CTO-10AC column oven. Samples were applied to the column with a Rheodyne Model 7725i injector equipped with a 50-μl sample loop. Peak integration was carried out with a Shimadzu Chromatopac C-R7A plus data processor. The column (15×0.4 cm I.D.) used was a CROWNPAK CR(+) (Daicel Chemicals, Tokyo, Japan).

2.2. Materials

Methanol of HPLC grade, salts used as buffer, hydrochloric acid and perchloric acid (70%) of analytical reagent grade were purchased from Katayama Kagaku Kogyo (Osaka, Japan). 18C6 was purchased from Nacalai Tesque (Kyoto, Japan). α-CD, β-CD and γ-CD were purchased from Katayama Kagaku Kogyo. DL-Ala-β-NA was purchased from Aldrich Chemicals (Milwaukee, WI, USA). L-Ala-β-NA was purchased from USB (Cleveland, OH, USA). Epinastine was extracted from commercial preparations and the other amino compounds used in Table 5 were purchased from Aldrich Chemicals, Katayama Kagaku Kogyo, Tokyo Kasei Kogyo (Tokyo, Japan) and Wako Pure Chemicals (Tokyo, Japan).

2.3. Chromatographic conditions

Chromatographic runs were performed at a constant flow-rate of 0.8 ml/min and a constant tem-

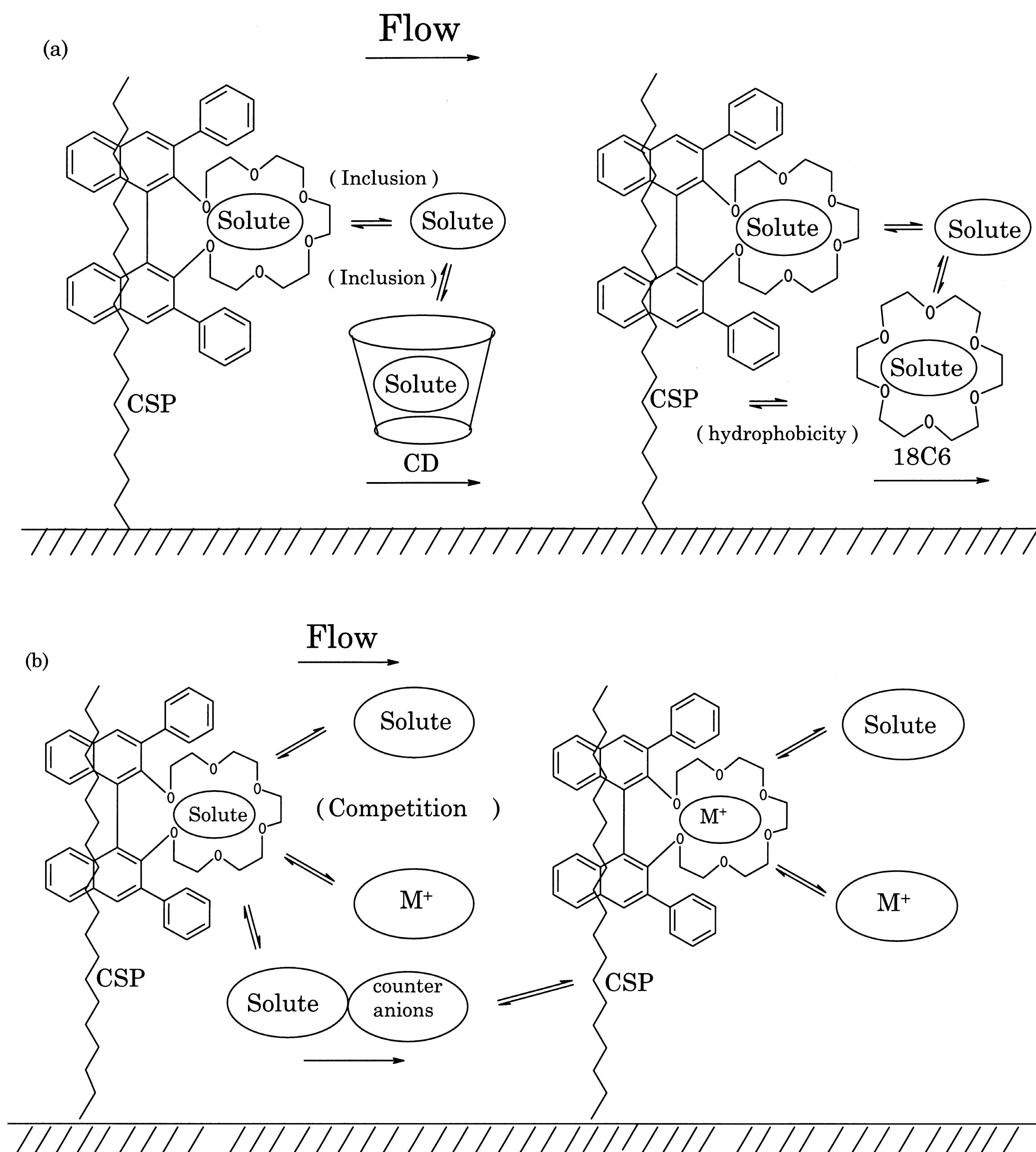

Fig. 1. Schematic illustrations of enantiomer separation of hydrophobic amino compounds. (a) Addition of 18-crown-6 or cyclodextrin; (b) addition of metal cations or counter anions.

perature of 40°C. The pH of buffer was adjusted by hydrochloric acid excepted for buffers using perchloric acid. The solute was detected at 254 nm. Typically, 2 μl of a 1% solution of racemate dissolved in mobile phases or 0.1 *N* hydrochloric acid was injected.

3. Results and discussion

3.1. Enantiomer separation by addition of crown ether or cyclodextrin

The CROWNPAK CR(+) column was first designed and used for the separation of DL-AAs. The hydrophobic chiral crown ether is dynamically coated on an ODS matrix in this column [5,6]. Thus, retention manipulation through the addition of an organic solvent is limited to below 15% methanol [14]. Column temperature is also effective for fast elution, although enantioselectivity decreases with an increase of temperature [5]. Accordingly, the harshest operation conditions permitted for this column is as follows: 50°C column temperature and a mobile phase containing 15% methanol [14]. First, enantiomer separations of hydrophobic amino compounds such as Ala-β-NA and 1-NEA with a CROWNPAK CR(+) column were investigated through the addition of 18C6 or CDs to the mobile phase.

Capacity factors, k_1 and k_2 ($k_1 < k_2$), separation factor ($\alpha = k_2/k_1$) and resolution (R_s) obtained for Ala-β-NA and 1-NEA are summarized in Table 1. Mobile phase consisting of 10 m*M* perchloric acid and 15% methanol (entry 1) was used for the separation as the standard (ordinary mobile phase). Enantiomers of Ala-β-NA were resolved with $\alpha =$ 4.10, $R_s = 10.6$ and a long retention time (204 min) of the second peak ($k_2 = 148$) by employing an ordinary mobile phase as shown in Fig. 2b. The effects of 18C6, α-, β- or γ-CD addition on the enantioselectivity and the retention were investigated by adding each one to the ordinary mobile phase, i.e., a mixture of 10 m*M* perchloric acid and 15% methanol. Addition of 18C6 (2 or 10 m*M*) gave larger capacity factors, and smaller α and R_s values for Ala-β-NA and 1-NEA (entries 2 and 3), compared with those in the ordinary mobile phase (entry 1). Under the acidic mobile phase, the protonated primary amino group in an amino compound associated with 18C6, which resulted in the enhanced hydrophobicity of the analyte. Furthermore, hydrophobicity of 18C6 itself also contributed to the increase of the retention of an analyte, because 18C6 probably adsorbed physically on the stationary phase, resulting in the increased crown ether moiety in the CSP. It is also reported that addition of 18C6 to the mobile phase in the reversed-phase HPLC caused the selective enhancement of retention of amino compounds, and the degree of its enhancement depended on both of the stability of the complexes and the hydrophobicity of the crown ethers employed [19,20].

α-CD (2 m*M*, entry 4) and γ-CD (2 m*M*, entry 7) gave the same retention times and α values for these analytes as in the ordinary mobile phase (entry 1). These results indicate that the analytes would not fit tightly into the cavity of α-CD and γ-CD. However, in the case of β-CD (2 m*M*, entry 5), as we had expected, a smaller capacity factor ($k_2 = 117$) was obtained without loss of α and R_s values, compared with those in the ordinary mobile phase (entry 1). Further, the capacity factor much decreased with an increase of β-CD concentration. β-CD (10 m*M*) gave about one-fourth capacity factor without change

Table 1
Enantiomer separations of hydrophobic amino compounds by CROWNPAK CR(+) through the modification of the mobile phase

Entry	Buffer	Ala-β-NA				1-NEA			
		k_1	k_2	α	R_s	k_1	k_2	α	R_s
1	10 m*M* $HClO_4$	36.1	148.2	4.10	10.6	25.4	30.6	1.21	1.04
2	10 m*M* $HClO_4$+2 m*M* 18-crown-6	52.8	164.1	3.11	9.89	35.8	40.7	1.14	0.92
3	10 m*M* $HClO_4$+10 m*M* 18-crown-6	114.6	233.7	2.04	6.63	79.7	84.9	1.06	0.56
4	10 m*M* $HClO_4$+2 m*M* α-CD	41.1	177.1	4.31	12.0	26.1	31.4	1.21	1.20
5	10 m*M* $HClO_4$+2 m*M* β-CD	25.3	117.3	4.63	11.3	26.1	31.5	1.20	1.23
6	10 m*M* $HClO_4$+10 m*M* β-CD	9.89	48.6	4.91	9.56	21.5	26.2	1.22	1.21
7	10 m*M* $HClO_4$+2 m*M* γ-CD	34.2	141.5	4.13	10.3	24.8	29.8	1.20	1.15
8	500 m*M* KCl, adjusted at pH 2.0	20.7	52.5	2.53	11.0	7.04	7.66	1.09	0.89
9	500 m*M* KH_2PO_4, adjusted at pH 2.0	17.7	43.7	2.47	10.0	6.67	7.30	1.09	0.90

Column temperature, 40°C; flow rate, 0.8 ml/min; mobile phase, containing 15% methanol.

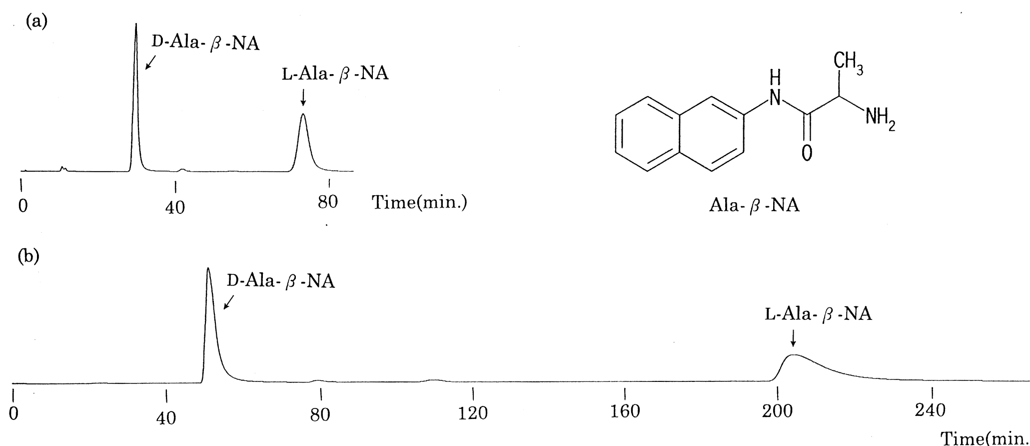

Fig. 2. Enantiomer separation of Ala-β-NA by the mobile phase containing KCl. (a) Mobile phase: methanol/500 m*M* potassium chloride (15:85); column temperature, 40°C; detection, 254 nm. (b) Mobile phase: methanol/10 m*M* perchloric acid (15:85); column temperature, 40°C; detection, 254 nm.

of α and R_s values for Ala-β-NA (entry 6). β-CD included the analytes in the cavity, and the complex eluted without interaction with the CSP because of the hydrophilic nature of the outside of the CD. This will contribute to the fast elution of the hydrophobic amino compounds. More strongly included analyte, in other words, more stable complex must elute faster. A schematic illustration of the separation mechanism through the addition of 18C6 or CDs is shown in Fig. 1a.

3.2. Enantiomer separation by addition of cations and/or anions

The effects of addition of cations and/or anions (potassium chloride, potassium dihydrogenphosphate) on the enantioselectivity and the retention were investigated with buffers containing 15% methanol. The concentration of salts and pH of the buffer were adjusted at 500 m*M* and 2.0, respectively. Both potassium chloride (Fig. 2a, entry 8) and potassium dihydrogenphosphate (entry 9) gave smaller capacity factors and α values without loss of R_s values for Ala-β-NA and 1-NEA, compared with the ordinary mobile phase (entry 1). It seems that the competition between the analyte and metal cation in the eluent, or ion-pair formation between the analyte and anion in the eluent, contribute to the fast elution of the hydrophobic amino compounds. Further, salt addition was effective for the peak shape (theoretical plate number), judging from the same R_s values and smaller α values (entries 8, 9).

Next, the effects of cation and anion species on the enantioselectivity and the retention were investigated in detail with buffers containing 15% methanol. Cations employed were potassium, ammonium, sodium and lithium (as chloride form). The concentration of salts and pH of the buffer were fixed at 500 m*M* and 2.0, respectively. The results obtained are summarized in Table 2. The results indicate that the capacity factors of amino compounds decreased with the addition of cations in the order: Li^+, Na^+, NH_4^+, K^+.

K^+ was found to be most effective and gave about one-fourth capacity factor for 1-NEA, about half capacity factor for Ala-β-NA with slight loss of α and R_s values (entry 10), compared with those in the ordinary mobile phase (entry 1). Decrease of R_s and α values may be interpreted by the decreased k' values themselves. Li^+ addition did not affect the retention (entry 13), although R_s values increased.

Metal cations are incorporated into the ring cavity of the crown ether based on ion–dipole interaction. These cations inhibited an interaction between the analyte and the crown ether moiety of the stationary phase, and contributed to the decrease of stability of the complex between the two. The stability of the complex between crown ether and cations signifi-

Table 2
Effect of cations on the retention and enantioselectivity of hydrophobic amino compounds

Entry	Buffer	Ala-β-NA				1-NEA			
		k_1	k_2	α	R_s	k_1	k_2	α	R_s
10	500 m*M* KCl	20.7	52.5	2.53	11.0	7.04	7.66	1.09	0.89
11	500 m*M* NH_4Cl	25.4	79.0	3.11	13.0	11.1	12.5	1.12	1.28
12	500 m*M* NaCl	27.4	86.6	3.16	13.3	11.7	13.1	1.12	1.25
13	500 m*M* LiCl	37.5	146.8	3.92	14.9	21.6	25.2	1.17	1.62

Column temperature, 40°C; flow rate, 0.8 ml/min; mobile phase, containing 15% methanol.

cantly depends on the fitness of the size of the cavity relative to that of the cation (Li^+, 1.36 Å; Na^+, 1.94 Å; K^+, 2.66 Å; NH_4^+, 2.84 Å). The 18C6 ring (the diameter of the cavity is estimated as 2.6 Å from CPK molecular model [21]) forms the most stable complex with K^+, which has an ionic diameter that best fits the cavity size of 18C6. The effect of the addition of cation species on retention of the analytes in CROWNPAK CR(+) can be explained by the complex stability (fitness).

The effects of the anion species on the enantioselectivity and the retention were investigated. The results obtained are summarized in Table 3. The concentration of potassium salts and pH of the buffer were fixed at 500 m*M* and 2.0, respectively. The results indicate that the capacity factor decreases as follows: I^-, NO_3^-, Br^-, Cl^-, $H_2PO_4^-$.

$H_2PO_4^-$ was most effective for the reduction of k' values (entry 14), compared with the other anions. The capacity factor of amino compounds increased by the addition of counteranions that were more polarizable. This observation or order is compatible with the theory of chaotropicity [10,22]. An anion with high chaotropicity is characterized by high polarizability with a consequent low degree of hydration. Chaotropic counteranions, when ion-paired to the analyte, facilitate the approach of the analyte to the stationary phase and thus increase their interaction. This hydrophobic interaction, affected by the chaotropicity of the counteranion, leads to a change in the capacity factor. Almost the same k' values as in the ordinary mobile phase were observed for Ala-β-NA when I^- was employed, a K^+ cation even existed (entry 18). A schematic illustration of the separation mechanism through the addition of cations and/or anions is shown in Fig. 1b.

3.3. The effects of the concentration of potassium chloride and pH of buffer

The effects of the concentration of potassium chloride (range, 50–2000 m*M*) on the enantioselectivity and the retention were investigated with buffers of pH 2.0. The results are shown in Fig. 3. An increase of the potassium chloride concentration resulted in an increase of the capacity factor (k_1) of D-Ala-β-NA, whereas the capacity factor (k_2) observed for L-Ala-β-NA decreased in the range from 50 to 500 m*M*. Then it started to increase. In case of 1-NEA, an increase of the potassium chloride concentration in the eluent causes a decrease (from 50 to 500 m*M*) and an increase (above 500 m*M*) of the

Table 3
Effect of anions on the retention and enantioselectivity of hydrophobic amino compounds

Entry	Buffer	Ala-β-NA				1-NEA			
		k_1	k_2	α	R_s	k_1	k_2	α	R_s
14	500 m*M* KH_2PO_4	17.7	43.7	2.47	10.0	6.67	7.30	1.09	0.90
15	500 m*M* KCl	20.7	52.5	2.53	11.0	7.04	7.66	1.09	0.89
16	500 m*M* KBr	25.9	71.1	2.75	12.6	8.15	8.85	1.09	0.95
17	500 m*M* KNO_3	28.4	70.4	2.48	11.4	9.05	9.76	1.08	0.90
18	500 m*M* KI	42.7	140.5	3.29	14.8	13.0	14.1	1.09	1.00

Column temperature, 40°C; flow rate, 0.8 ml/min; mobile phase, containing 15% methanol.

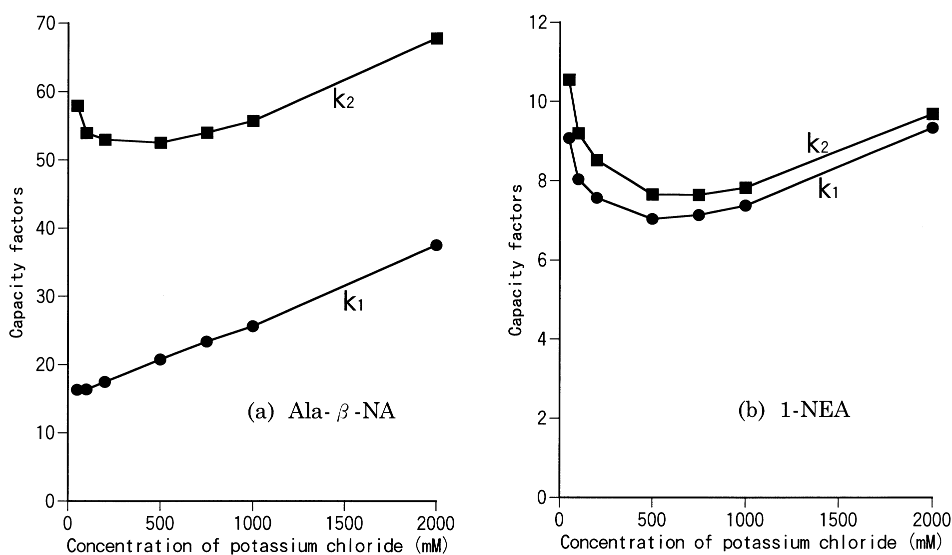

Fig. 3. The effect of KCl concentration on the capacity factor: (a) Ala-β-NA; (b) 1-NEA.

capacity factors for both of the enantiomers, as in the L-Ala-β-NA. The observed changes in trends may be ascribed to the sum of the two mechanisms, that is, the competition between the analyte and potassium cation, and the ion-pair formation between the analyte and chloride anion (counteranion). In the range from 50 to 500 m*M*, the competition is dominant. Then, above 500 m*M*, the ion-pair formation (increase of hydrophobicity of the analyte) becomes a major factor for the retention.

The effects of the pH (range, 2.0–5.8) of buffer on the enantioselectivity and the retention were investigated. The results are summarized in Table 4. The pH in the range 2.0–4.0 did not significantly affect the k' and α values. The capacity factors slightly increased at pH 5.8 for Ala-β-NA and 1-NEA, degree of ionization in a primary amino group of the analyte decreased and peak tailing of the analyte became remarkable.

3.4. Enantiomer separation of some amino compounds by the modified mobile phase

Separations of hydrophobic amino compounds by the ordinary mobile phase and the modified mobile phase were performed and compared. The results are summarized in Table 5. The simultaneous enantiomer separation of four amino compounds (kynurenine, α-methyltryptophan, 1-NEA and primaquine: entries 26–29) is shown in Fig. 4 (a, modified mobile phase; b, ordinary mobile phase). All enantiomers were separated within 14 min without co-elution by the modified mobile phase (Fig. 4a). In the case of the ordinary mobile phase,

Table 4
Effect of the buffer pH on the retention and enantioselectivity of hydrophobic amino compounds

Entry	Buffer pH	Ala-β-NA				1-NEA			
		k_1	k_2	α	R_s	k_1	k_2	α	R_s
19	2.0	20.7	52.5	2.53	11.0	7.04	7.66	1.09	0.89
20	3.0	20.8	52.7	2.54	10.8	7.06	7.69	1.09	0.86
21	4.0	20.8	53.3	2.57	10.5	7.22	7.87	1.09	0.82
22	5.8	23.1	56.5	2.45	6.09	8.05	8.57	1.06	0.42

Column temperature, 40°C; flow rate, 0.8 ml/min; mobile phase, containing 15% methanol.

Table 5
Enantiomer separations of amino compounds by the modified mobile phase

Entry		Modified mobile phase					Ordinary mobile phase[a]			
		k_1	k_2	α	R_s	M.P.[b]	k_1	k_2	α	R_s
23	2-Amino-9-hydroxyfluorene	13.6	15.3	1.12	1.11	A	16.7	22.5	1.35	2.64
24	Baclofen	5.99	6.91	1.15	1.53	A	6.51	10.3	1.59	4.25
25	Epinastine	33.7	35.7	1.06	0.61	C	47.3	—	1.00	—
26	Kynurenine	0.56	0.91	1.63	1.85	B	1.55	2.77	1.79	3.04
27	α-Methyltryphtophan	2.91	3.31	1.14	1.01	B	7.23	8.80	1.22	1.40
28	1-NEA	8.17	9.35	1.14	1.32	B	25.4	30.6	1.21	1.04
29	Primaquine	6.62	7.41	1.14	0.94	B	30.9	35.1	1.14	0.90

Column temperature, 40°C; flow rate, 0.8 ml/min; detection, 254 nm.
[a] 15% methanol/10 m*M* $HClO_4$.
[b] (A) 5% methanol/500 m*M* KCl, pH 2.0; (B) 15% methanol/100 m*M* KCl, pH 2.0; (C) 15% methanol/10 m*M* $HClO_4$+10 m*M* β-CD.

however, seven peaks for four enantiomeric pairs were found, and the last peak appeared at 46 min (Fig. 4b). 2-Amino-9-hydrofluorene (entry 23) and baclofen (entry 24), which were enantioseparated by the ordinary mobile phase with 32 min (k_2=23), were enantioseparated within 22 min (k_2=15) using a buffer containing 100 m*M* potassium chloride and 5% methanol.

On the other hand, epinastine which was not enantioseparated by CROWNPAK CR(+) with a mixture of 10 m*M* perchloric acid and 15% methanol, was enantioseparated by the addition of 10 m*M* β-CD with α=1.06 (entry 25). This probably can be ascribed to the effect of chirality of β-CD.

3.5. The optical purity testing of L-Ala-β-NA

The optical purity testing of L-Ala-β-NA was performed according to the method shown in Fig. 2a (entry 8). Some validations such as reproducibility and linearity were also demonstrated. The reproducibility was investigated by six repeated injections of L-Ala-β-NA solution spiked with 1.0% of D-Ala-β-NA (Fig. 5a). The range of detected D-form (%)

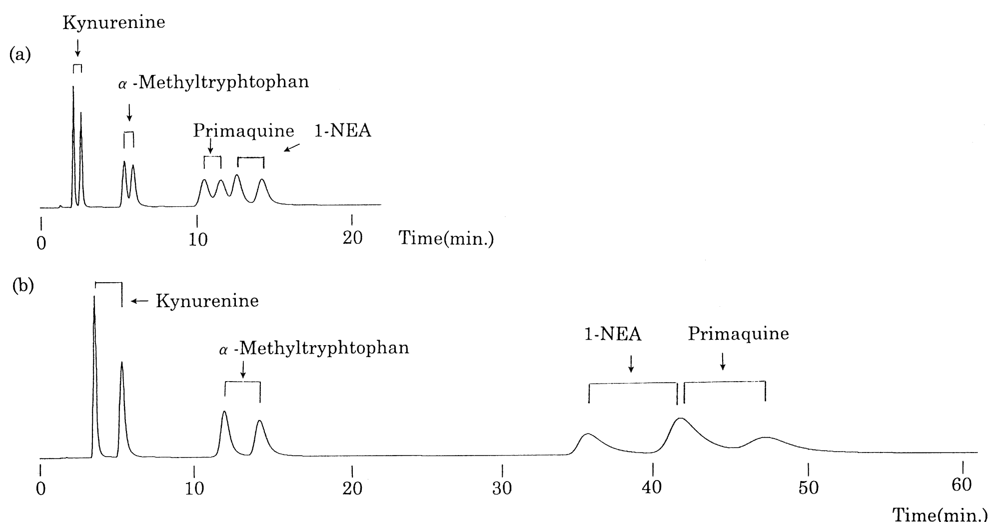

Fig. 4. Enantiomer separation of amino compounds with the modified mobile phase and ordinary mobile phase. (a) Mobile phase: methanol/100 m*M* potassium chloride (15:85); column temperature, 40°; detection, 254 nm. (b) Mobile phase: methanol/10 m*M* perchloric acid (15:85); column temperature, 40°C; detection, 254 nm.

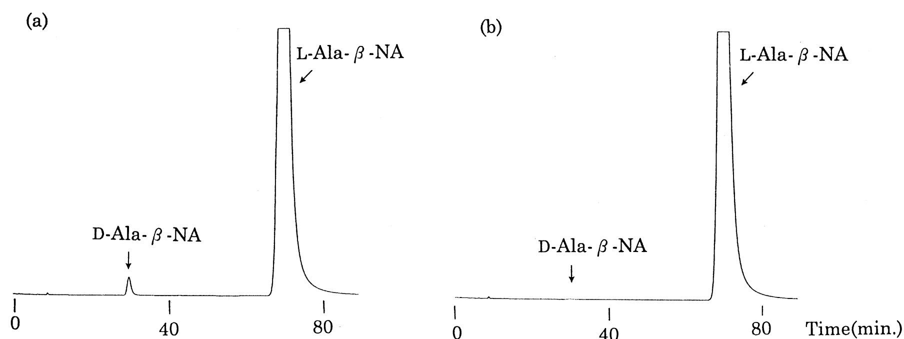

Fig. 5. Optical purity testing of L-Ala-β-NA. Mobile phase: methanol/500 m*M* potassium chloride (15:85); column temperature, 40°C; detection, 254 nm. (a) Chromatogram of L-Ala-β-NA spiked with 1.0% D-Ala-β-NA; (b) Chromatogram of L-Ala-β-NA.

was from 1.000 to 1.006%, and the relative standard deviation (R.S.D.) was 0.25%. The good reproducibility of this method was confirmed. The linearity and accuracy were investigated from the determinations of the content of the D-form in L-form standard spiked with D-form over the range from 0.05 to 5.0%. The relationship between the found values (y) and the theoretical values (x) was found to be a straight line ($y=1.0019x+0.0089$) (correlation coefficient=0.9998); the good linearity and accuracy on this method were confirmed. The detection limit of the D-form with this method was investigated, and as little as 0.05% D-form in L-Ala-β-NA could be determined. Finally, optical purity testing for L-Ala-β-NA (USB), which was purchased from the commercial source, was performed with this method. Chromatograms are shown in Fig. 5b. D-Ala-β-NA was not detected (detection limit, 0.05%), indicating that this reagent can be usable as a chiral derivatizing reagent as it is.

4. Conclusion

The effects of 18C6, CDs (α-CD, β-CD and γ-CD), metal cations or counter anions addition on the retention and enantioselectivity of hydrophobic amino compounds in CROWNPAK CR(+) were investigated. The second peak of the enantiomers of Ala-β-NA appeared at 204 min using the ordinary mobile phase. Among these additives, β-CD effectively contributed to the fast elution of the hydrophobic amino compounds such as Ala-β-NA (68 min) and 1-NEA without loss of α and R_s values, because β-CD included the analyte in the cavity. The addition of cation to the eluent, especially K^+ addition, as we had expected, also contributed to the fast elution of the hydrophobic amino compounds. Because the addition of cations caused a competition in the formation of the complex between the analyte and the chiral crown ether moiety, leading to the fast elution of the analyte. To compare the modified mobile phase and the ordinary mobile phase, fast enantiomer separations were performed using the modified mobile phase. Additionally, epinastine which was not separated with the ordinary mobile phase, was enantioseparated with $\alpha=1.06$ by β-CD addition (10 m*M*). Finally, optical purity testing of L-Ala-β-NA, which is an important chiral derivatizing reagent, was performed. As little as 0.05% D-form in L-Ala-β-NA could be determined in this method. Some validations, such as reproducibility and linearity, were also demonstrated and this method was found to be sufficient as a quality control method.

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