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Separation of enantiomers and isomers of amino compounds by capillary electrophoresis and high-performance liquid chromatography utilizing crown ethers

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Abstract

Separation of enantiomers and isomers of a wide variety of primary amines by capillary electrophoresis (CE) was investigated employing charged and uncharged crown ethers. Enantiomer separation of primary amines by CE was successful through the addition of a chiral crown ether, 18-crown-6 tetracarboxylic acid ($18C6H_4$). Thirteen out of 17 enantiomers tested were separated by CE with $18C6H_4$. Low pH values (pH 1.9–2.1) and relatively high Tris concentration (20 m*M*) were effective for the fast enantiomer separation. Enantiomer separation of the same primary amines was also investigated by high-performance liquid chromatography (HPLC) with a chiral Crownpak CR(+) column, although different chiral crown ether moieties were employed in CE and HPLC. Other than enantiomers, separation of positional isomers of aromatic amino compounds such as aminophenols, aminocresols was investigated by both CE with three kinds of 18-membered crown ethers and HPLC with a Crownpak CR(+) column. The use of crown ethers for the separation of amino compounds is discussed in viewpoint of practical applications. The optical purity testing of amino compounds is also demonstrated.

Keywords: Enantiomer separation; Crown ethers; Amines; Aminophenols; Aminocresols

1. Introduction

Crown ethers, first introduced by Pedersen in 1967 [1,2] are synthetic macrocyclic polyethers that can form selective complexes with cations. Eighteen-membered crown ethers can make relatively stable complexes with an ammonium ion and protonated primary amino compounds in a tripod arrangement, and have been used successfully in the separation of cations and amino compounds including enantiomers, especially in high-performance liquid chromatography (HPLC) as immobilized stationary phases [3–7] and mobile phase additives [8–10]. An immobilized crown ether stationary phase is commercially available as a Crownpak CR(+) column. This column, first synthesised by Shinbo et al. [7], is effective for enantiomer separation of amino compounds because a chiral 18-membered crown ether is introduced. Selectivity of amino compounds in the reversed-phase HPLC is much improved through the addition of 18-membered crown ethers to the mobile phase.

Capillary electrophoresis (CE) has attracted attention as a promising technique in the separation of pharmaceuticals. Separation is achieved with one capillary tube (without a special column and an

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organic solvent), high resolution and relatively fast migration. Selectivity is manipulated through the modification of a running buffer. Enantiomer separation is one of the successful applications of CE and can be achieved by employing chiral additives in the running buffer. Several chiral selectors have been utilized in CE enantiomer separation [11–13]. Crown ether was first employed as a chiral selector for the CE enantiomer separation by Kuhn et al. [14–18], who reported CE enantiomer separation using the chiral crown ether, 18-crown-6 tetracarboxylic acid ($18C6H_4$). $18C6H_4$ is only one commercially available chiral crown ether. Enantiomers of amino acids [14–17], peptides [15,18], aminoalcohols [14–17], aminotetralins [19,20], etc. have been separated by the method.

In this paper, we mainly investigate the enantiomer separation of pharmaceuticals and hydrophobic compounds by CE utilizing $18C6H_4$. The same samples are analysed by HPLC with a Crownpak CR(+) column, whose chiral crown ether moiety is similar to the 18-membered ring. Then we compare the two methods (CE and HPLC) using crown ethers for the enantiomer separation of primary amines. The optical purity testing by CE with $18C6H_4$ is also demonstrated. Furthermore three kinds of 18-membered crown ethers are employed for the separation of positional isomers of aromatic amino compounds such as aminophenols and aminocresols. The use of these crown ethers in CE is discussed in practical applications.

2. Experimental

2.1. CE instrument

CE experiments were performed on a Beckman P/ACE system 5510 equipped with a photodiode array detector (Beckman Instruments, Fullerton, CA, USA). An uncoated capillary tube (75 μm I.D., effective length 37 cm) was used as the separation tube. The temperature of the capillary tube was maintained at 15 or 20°C with a liquid coolant. The applied voltage was held at constant 15 or 20 kV. The detection wavelength was set at 210, 220 or 235 nm. The tested samples were dissolved in methanol (if necessary, 1 *M* HCl was added) at a concentration of ca. 1 mg/ml and stored in tight, opaque containers at a temperature ca. 5°C when not in use. These sample solutions were diluted with water or methanol to ca. 0.1 mg/ml and introduced from the positive end by the pressure mode (0.5 psi, 2–9 s). Instrument control and data collection were performed with a personal computer (COMPAQ Pro-Linea 4/33). The running buffer solution was prepared by mixing 10–20 m*M* Tris or NaH_2PO_4 with 0.1% H_3PO_4 (v/v) to give pH 1.9–2.2. When a charged crown ether was used, the pH was adjusted after the addition of the crown ether. The running buffer solutions were passed through a membrane filter (0.45 μm) (Gelman Science, Tokyo, Japan) and degassed by sonication prior to use.

2.2. HPLC instrument

HPLC experiments were performed on an LC-10A liquid chromatograph (Shimadzu, Kyoto, Japan) consisting of a LC-10AD pump, a SPD-10 A UV detector, a CTO-10A column oven and a SIL-10A auto sample injector. Instrument control was performed with a SIL-10A system controller and data processing was carried out with a Shimadzu Chromatopac C-R5A. The crown ether immobilized column used was Crownpak CR(+) (15 cm, 4 mm I.D.) (Daicel, Tokyo, Japan). A hydrophobic chiral crown ether (see Fig. 1) is coated on 5 μm ODS-silica via physical adsorption [7]. The mobile phase

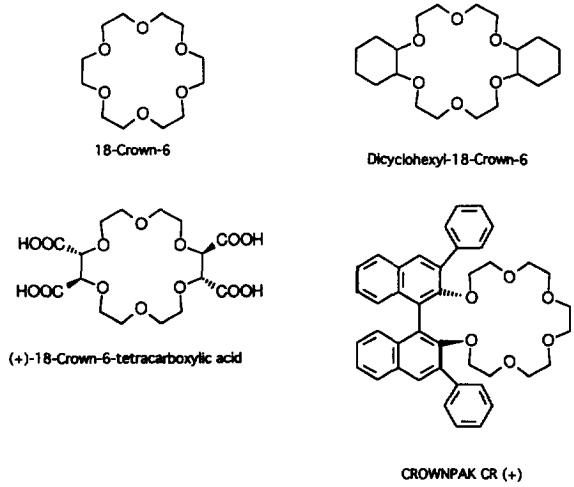

Fig. 1. Structures of crown ethers employed.

was prepared by mixing 0.1% $HClO_4$ (v/v) (pH 1.9) with methanol. These mobile phases were filtered with a type FR-40 membrane (0.45 μm) (Fuji Photo Film, Tokyo, Japan). The flow-rate and detection wavelength were set at 1.0 ml/min and 210 nm, respectively. The column temperature, except 40°C, (in the range 10–30°C) was maintained with a RM-6 type thermostat (Lauda, Germany). The samples were dissolved in the mobile phase at a concentration ca. 0.1 mg/ml and maintained at 15°C with a Shimadzu sample cooler.

2.3. Reagents and materials

Three types of crown ethers used were as follows: 18-crown-6 tetracarboxylic acid ($18C6H_4$), 18-crown-6 (18C6) and dicyclohexyl-18-crown-6 (DC18C6). These structures are shown in Fig. 1 with the chiral moeity of a Crownpak CR(+) column. $18C6H_4$ was purchased from Merck (Darmstadt, Germany), but may also be available from Aldrich-Sigma (WI, USA). Other crown ethers were purchased from Nihon Soda Chemicals (Tokyo, Japan). Methanol of HPLC grade was purchased from Katayama Kagaku Kogyo (Osaka, Japan). Tris of biochemical grade, H_3PO_4 (85%), $HClO_4$ (70%) of analytical reagent grade were purchased from Katayama Kagaku. Water was purified by a Milli-RO 60 water system (Millipore, Tokyo, Japan).

Enantiomers of drugs or compounds tested were as follows: tyrosine (Tyr), DOPA, noradrenaline, norephedrine, ephedrine, phenylglycine (PheG), octopamine, baclofen, mexiletine, kynurenine, aminoglutethimide, primaquine, epinastine, phenylethylamine (PEA), 1-naphthylethylamine (NEA), alanine-β-naphthylamide (Ala-β-NA), and 1,1′-binaphthyl-2,2′-diamine (BNA). The structures are shown in Fig. 2. Most of them are racemic drugs. Some single enantiomers were purchased from commercial sources to determine the migration order. Mexiletine and epinastine were extracted from commercial preparations and others were purchased from Katayama Kagaku, Wako Pure Chemicals (Tokyo, Japan), Nacalai Tesque (Kyoto, Japan), Tokyo Kasei Kogyo (Tokyo, Japan) and Aldrich Chemicals. The aromatic amino compounds tested were as follows: *o*-aminophenol, *m*-aminophenol, *p*-aminophenol, *o*-aminobeozoic acid, *m*-aminobenzoic acid, *p*-amino-

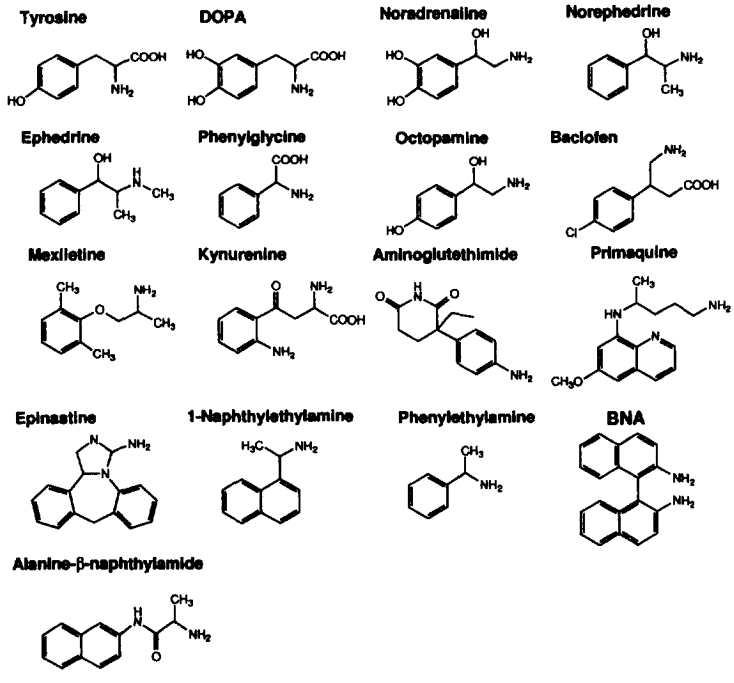

Fig. 2. Structures of enantiomers investigated.

benzoic acid, 2-amino-*m*-cresol, 4-amino-*m*-cresol, 6-amino-*m*-cresol, 2-amino-*p*-cresol, 3-amino-*o*-cresol and 5-amino-*o*-cresol. These were purchased from Wako Pure Chemicals and Nacalai Tesque.

3. Results and discussion

3.1. Enantiomer separation of amino compounds by CE

The crown-ether $18C6H_4$ has been used for the CE enantiomer separation of amino acids, aromatic amino compounds and catecholamines by Kuhn et al. [14–18]. In this paper, we mainly investigated the enantiomer separation of synthetic drugs and hydrophobic compounds having a primary amino group. The tested amines are 13 drugs and 4 aromatic amino compounds (Fig. 2). Kuhn et al. used 10–30 m*M* $18C6H_4$ in 10 m*M* Tris–citrate buffer (pH 2.2–6.5) as the running buffer solution. There are four carboxylic acid groups on $18C6H_4$ and their p*K* values are 2.13, 2.84, 4.29 and 4.88 [21]. Accordingly the buffer pH strongly affects the complex formation between the analyte and $18C6H_4$. Above pH 2.2, $18C6H_4$ has negative charge and it tends to migrate toward the positive end. Electrostatic interaction between the analyte and anionic $18C6H_4$ must be also strong if the analyte has a cationic nature. It requires a long analysis time for the migration of the compound. Below pH 2.1, the degree of dissociation of $18C6H_4$ is small and $18C6H_4$ will migrate toward the negative end with the slow electroosmotic flow. However, there is no suitable tracer of $18C6H_4$. Buffer composition also has a marked influence on the migration of the analytes [16]. At preliminary testing, high concentration (20 m*M*) of Tris buffer gave smaller migration times compared with those in 10 m*M* Tris buffer. This result is in contrast to results by Kuhn et al. [16]. Tris is a primary amine and it must compete with the analytes for the crown ether sites, and higher concentration of Tris leads to smaller migration of the analytes. The column temperature is another important factor affecting enantioseparation [14,15]. Thus in this investigation, 10 m*M* $18C6H_4$ in 20 m*M* Tris–H_3PO_4 buffer (pH 1.9–2.1) was used as the running buffer solution and the column temperature was set at 15°C expecting fast enantiomer separation.

The results of enantiomer separation at pH 2.06 are summarized in Table 1 with the results obtained from the same running buffer containing no $18C6H_4$. These amino compounds migrated fast at around 6 min except PheG in the absence of crown ethers. With the addition of crown ethers, migration times increased and the selectivity, including enantiomer separation, was improved, indicating the effective complexation of the analytes with the crown ether. Resolution (R_s) was calculated as $2(t_2-t_1)/(w_1+w_2)$, where t_1, t_2 ($t_2>t_1$) and w_1, w_2 are migration times and peak width of the enantiomers, respectively. The separation factor, α, was calculated as t_2/t_1 by the equation used by Kuhn [17]. As expected, relatively fast enantiomer separation (within 15 min) was performed without loss of enantiorecognition, compared with the Kuhn's paper. Migration order of L/D or *R*/*S* of some amino compounds were also investigated. For amino acid analogues (Tyr, DOPA, PheG, Ala-β-NA), L-forms migrated faster than corresponding D-forms.

According to the reported papers [15,16], primary amine functionality of the analyte is essential. Bulky

Table 1
Enantiomer separation of amino compounds by CE with $18C6H_4$

Compound	0 m*M*	10 m*M* $18C6H_4$			
	t (min)	t_1 (min)	t_2 (min)	α	R_s
Ala-β-NA	6.45	9.91(L)	12.75(D)	1.29	9.00
Aminoglutethimide	5.00	12.47	12.69	1.02	0.70
Baclofen	6.30	11.81	12.32	1.04	2.48
BNA	6.82	7.48(*R*)	9.94(*S*)	1.33	9.00
DOPA	6.93	10.94(L)	11.28(D)	1.03	1.60
Epinastine	5.70	6.01	–	–	–
Ephedrine	5.69	5.45	–	–	–
Kynurenine	6.74	13.96	14.78	1.06	2.42
Mexiletine	5.84	6.96	7.46	1.07	2.00
NEA	5.94	10.43(*S*)	12.23(*R*)	1.17	6.28
Noradrenaline	5.93	12.86	13.17	1.02	0.89
Norephedrine	5.72	7.54	7.77	1.03	1.21
Octopamine	5.63	11.45	11.94	1.04	1.12
PEA	5.94	8.87	–	–	–
PheG	10.57	16.74(L)	21.98(D)	1.31	13.70
Primaquine	4.58	9.60	9.67	1.01	1.18
Tyr	8.89	14.51(L)	14.96(D)	1.03	1.51

Buffer: 10 m*M* $18C6H_4$ in 20 m*M* Tris–H_3PO_4 (pH 2.06); temperature: 15°C; applied voltage: 20 kV; detection: 210 nm or 235 nm.

substituents of the chiral carbon give better enantiorecognition. That is, norephedrine was successfully separated while ephedrine was not separated. Excellent resolution of NEA was achieved but PEA was not resolved. Compounds with a large large substituent naphthalene ring, NEA, Ala-β-NA and BNA, gave large R_s values, compared with the others. For example, the R_s and migration times of Ala-β-NA, which has been used as a chiral derivatization reagent for carboxylic acids such as naproxen [22] and imidapril hydrochloride [23], was 9.0 and ca. 10 min, respectively. For NEA, R_s 6.28 was obtained with a migration time of 12 min in our investigation, while Kuhn et al. reported R_s and migration time of 1.79 and ca. 23 min. These good values in our investigation must be ascribed to pH and Tris concentration. As mentioned above, pH value and the concentration of the buffer solution influences the migration times of the analytes. The migration times of Ala-β-NA was twice as great at pH 2.24. Separations of five drugs and three aromatic amino compounds at pH 1.90 are shown in Fig. 3A and 3B. Each analyte migrated faster compared with Table 1 (pH 2.06). However, enantiomer separation

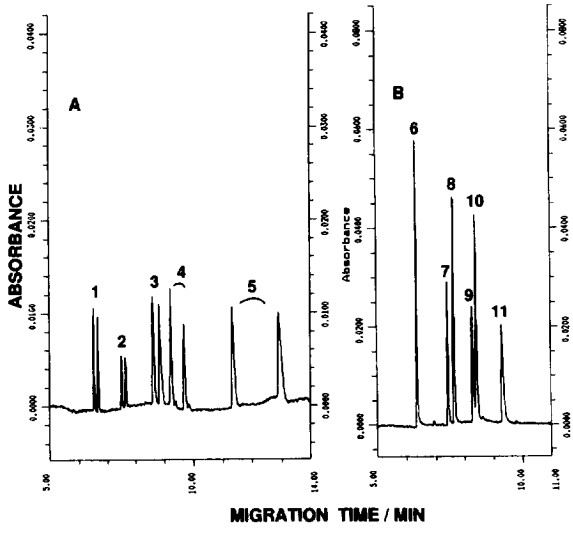

Fig. 3. Enantiomer separation of (A) five drugs and (B) three aromatic amino compounds by CE with 18C6H$_4$. Buffer: 20 m*M* Tris–H_3PO_4 (pH 1.90) containing 10 m*M* 18C6H$_4$; column temperature: 15°C; applied voltage: 20 kV; detection: 210 nm. Samples: (A) 1=mexiletine; 2=DOPA; 3=octopamine; 4=baclofen; 5=PheG; (B) 6=*R*-(+)-BNA; 7=*S*-(−)-BNA; 8=*S*-(−)-NEA; 9=L-Ala-β-NA; 10=*R*-(+)-NEA; 11=D-Ala-β-NA.

of norephedrine was unsuccessful at pH 1.90. The pH difference of 0.1 at around the p*K* value of 18C6H$_4$ was very critical for the enantiorecognition and the migration. The enantiomer separation of PheG was also successfully achieved under our conditions. Kuhn [15] reported a migration time of PheG of 59 min and were unsuccessful in the enantiomer separation of PheG. Only L-PheG was detected under the condition of Ref. [15], probably because of the long migration time of D-PheG. Fast enantiomer separation was achieved with the running buffer solution of pH 1.9–2.1 and 20 m*M* Tris.

For the amino drugs, enantiomer separations of primaquine and aminoglutethimide were successful, although the distance of amine functional group and the stereogenic centre for both drugs is long. In general, this long distance is unfavourable for the enantiomer separation, and optimum separation is obtained if the stereogenic centre is in close proximity to the amine group. DOPA, which was not enantioseparated by cyclodextrin modified capillary zone electrophoresis (CZE) [24], was also successfully separated as shown in Fig. 3A. The wide enantioselectivity obtained by 18C6H$_4$ indicates that an ionic crown ether having chirality and an 18-membered ring is effective for the enantioseparation of primary amines.

Since we were also interested the optical purity testing of drugs, the CE method with 18C6H$_4$ was evaluated for use as a purity testing method. The desirable detection limit is usually 0.1%. There is only one report for optical purity testing by CE with 18C6H$_4$ [20]. The detection limit reported for dihydroxy-2-aminotetralins was 0.3% (*S*/*N*=2). By using the same running buffer solution as in Table 1 (pH 2.06), optical purity determination of *S*-(−)-BNA, which was purchased from the commercial source, was performed. An example of *S*-(−)-BNA spiked ca. 0.2% *R*-(+)-BNA is shown in Fig. 4. Less than 0.1% of the minor enantiomer can be detectable by the method. This low detection level can be ascribed to the high R_s value and small migration times in the enantiomer separation of BNA, compared with those of aminotetralins. The minor enantiomer, *R*-(+)-BNA was not detected in the sample, i.e. the optical purity of *R*-(+)-BNA is more than 99.9%. This means that the optical purity of 18C6H$_4$ is also more than 99.9%, and there is no

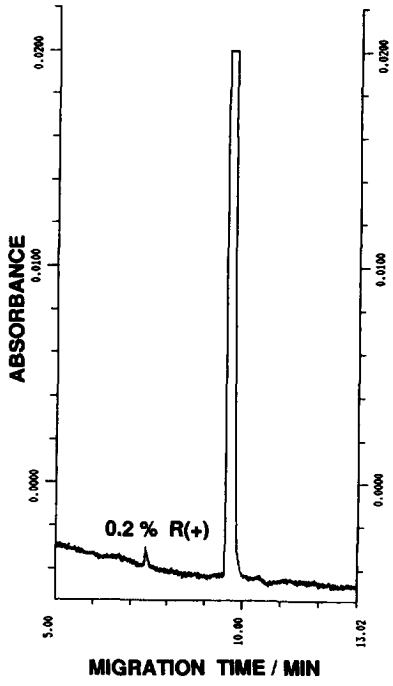

Fig. 4. Optical purity testing of *S*-(−)-BNA by CE with $18C6H_4$. Sample: *S*-(−)-BNA spiked ca. 0.2% *R*-(+)-BNA. Conditions are the same as in Table 1.

problem about the optical purity of $18C6H_4$ as a chiral selector. It is important to assure the optical purity of a chiral selector which is being used for optical purity testing. Similarly optical purity of NEA was checked, and the minor enantiomer was not found in the commercial samples. These results show that BNA and NEA can be used as chiral derivatization reagents because of their high optical purity (>99.9%) and reactive amino group. Actually these amines have been employed for HPLC enantiomer separation [25].

3.2. Enantiomer separation of primary amines by HPLC

Enantiomer separation of the same primary amines was investigated by HPLC with a Crownpak CR(+) column. This column was first used for the separation of DL-amino acids. The hydrophobic chiral crown ether (see Fig. 1) is dynamically coated to an ODS-silica in this column [7]. Thus, retention manipulation through the addition of an organic solvent is limited to below 15% methanol [26]. Column temperature is also effective for the fast elution, although enantioselectivity decreases with increased temperature [7]. Accordingly, the harshest operation condition permitted for this column is 50°C column temperature and the mobile phase containing 15% methanol.

The results of enantiomer separation under three conditions are summarized in Table 2. Separation of five drugs and three aromatic amino compounds are shown in Fig. 5A and B. The separation factor, α, was calculated by the ratio of the capacity factors of the enantiomers, k_2/k_1. BNA, Ala-β-NA and epinastine did not elute within 60 min under 40°C and 15% methanol mobile phase. These are very hydrophobic and more than 15% methanol will be necessary for their elution. However, in this type of immobilized stationary phase, there is a restriction for organic solvents. A covalently bonded column will be favourable for the enantiomer separation of these hydrophobic amino compounds. Other samples eluted under the conditions, all except mexiletine and ephedrine were successfully enantioseparated by a Crownpak CR(+) column. Partial separation of aminoglutethimide and baseline separation of primaquine were achieved by this column. These results corresponded to those obtained by CE with $18C6H_4$. PEA, which was not enantioseparated by CE with $18C6H_4$, was enantioseparated by this column. Other than DL-amino acids, this column was found to be useful for the enantiomer separation of amino compounds. Although the structures of the two chiral crown ethers employed in CE and HPLC (Fig. 1) are quite different, these 18-membered chiral crown ethers showed marked enantioselectivity for a wide variety of primary amino compounds.

The effect of methanol concentration on the elution and enantioselectivity in a Crownpak CR(+) column was investigated for five analytes. The results are shown in Table 3. By increasing methanol content, elution of analytes became more rapid as in the reverse-phase HPLC, and the enantioselectivity (resolution) was decreased. The effect of column temperature on the elution and enantioselectivity was also investigated for four analytes. The results are shown in Table 4. By decreasing column temperature elution of the analytes was delayed and enantioselectivity was improved.

Table 2
Enantiomer separation of primary amines by HPLC with a Crownpak CR(+) column

Compound	t_1 (min)	t_2 (min)	α	R_s	Condition[a]
Ala-β-NA	>60	–	–	–	A
Aminoglutethimide	10.08	11.05	1.11	0.72	B
Baclofen	9.32	14.23	1.59	4.22	A
BNA	>60	–	–	–	A
DOPA	1.48	1.70	1.46	1.21	A
Epinastine	>60	–	–	–	A
Kynurenine	2.95	4.53	1.81	3.25	A
Mexiletine	17.37	–	–	–	A
Naphthylethylamine	32.77	38.57	1.18	1.59	A
Noradrenaline	3.88	4.18	1.10	0.71	C
Norephedrine	2.95	3.38	1.22	1.34	A
Octopamine	4.64	4.95	1.09	0.65	C
PEA	3.34	3.89	1.24	1.47	A
PheG	1.68	3.09	3.07	4.96	A
Primaquine	35.17	39.67	1.13	1.32	A
Tyr	1.69	1.96	1.39	1.36	A

Column: Crownpak CR(+) (150×4 mm I.D.); mobile phase: 0.1% $HClO_4$ (v/v) (pH 1.9) with methanol; flow-rate: 1.0 ml/min; detection: 210 nm.
[a] A: 40°C, 15% MeOH; B: 25°C, 15% MeOH; C: 25°C, 0% MeOH.

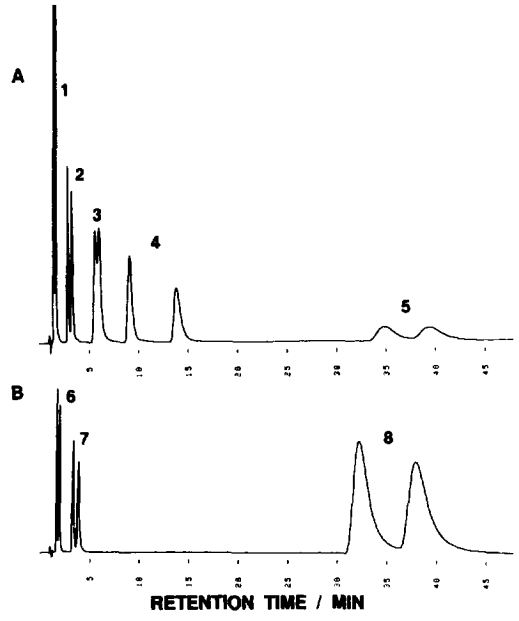

Fig. 5. Enantiomer separation of (A) five drugs and (B) three aromatic amino compounds by HPLC with a Crownpak CR(+) column. Mobile phase: methanol–0.1% $HClO_4$ (v/v) (pH 1.9)= 15:85; column temperature: 40°C; detection: 210 nm; flow-rate: 1.0 ml/min. Samples: (A) 1=DOPA; 2=norephedrine; 3= aminoglutethimide; 4=baclofen; 5=primaquine; (B) 6=Tyr; 7= PEA; 8=NEA.

3.3. Separation of positional isomers of primary amines by CE

Separation of positional isomers of aromatic amino compounds by CE with crown ethers was investigated. First, CZE separation of a mixture of three kinds of isomers, aminophenols, aminocarboxylic acids and aminoresols, was performed with crown ether-free running buffer of pH 2.1. Electropherogram is shown in Fig. 6A. Except *m*-aminobeozoic acid, the same type of positional isomers migrated with the same electrophoretic mobility under these conditions. Typical CZE is not suitable for the separation of analytes having the same charge and molecular weight (i.e. isomers). Successful separation of each positional isomer was obtained by adding 10 m*M* $18C6H_4$ to the running buffer solution as shown in Fig. 6B and 6C, except, *m*-aminophenol and *p*-aminophenol. Crown ether addition was found to be effective in CE for the separation of positional isomers of primary amines as well as the separation of enantiomers. The migration order of positional isomers of aminophenols and aminobeozoic acids was as follows: *ortho*-isomer>*meta*-isomer>*para*-isomer. This order will be interpreted by the steric hindrance in the complexation formation. That is, complexation of *ortho*-isomer, which

Table 3
Effect of methanol addition on the retention times and the enantioseparation

Compound	Methanol concentration							
	0%		5%		10%		15%	
	t (min)	R_s	t (min)	R_s	t (min)	R_s	t (min)	R_s
DOPA	3.14	2.86	2.77	2.97	2.26	2.72	1.84	2.11
	4.17		3.82		3.11		2.41	
Noradrenaline	3.88	0.71	3.53	0.52	3.09	–	2.53	–
	4.18		3.73					
Norephedrine	8.52	2.50	6.76	2.50	5.12	2.39	4.08	2.24
	10.52		8.62		6.63		5.32	
Octopamine	4.64	0.65	4.21	0.42	3.61	–	3.09	–
	4.95		4.39		–		–	
Tyr	4.16	2.91	3.57	2.95	2.80	2.85	2.18	2.29
	5.50		4.91		3.88		2.89	

Mobile phase: 0.1% $HClO_4$ (v/v) (pH 1.9) with methanol; column temperature: 25°C; flow-rate: 1.0 ml/min; detection: 210 nm.

has a substituent next to the amino group, was sterically hindered and consequently the delay of the migration time was least among the three isomers. The migration order of three aminocresols also will be interpreted by the steric hindrance, that is, substitution pattern near the amino group.

Next, separation of the same analytes was investigated with 18C6. The buffer used was 20 m*M* NaH_2PO_4–H_3PO_4. An electropherogram of a mixture of three kinds of isomers at 100 m*M* 18C6 is shown in Fig. 7. In case of 18C6, concentration higher than 50 m*M* was required for the successful separation, although 10 m*M* was effective in $18C6H_4$. This must be ascribed to the effect of the substituent of crown ethers. In $18C6H_4$, four carboxyl groups must contribute to the stable complexation of the analyte with the crown ether, leading to the improvement of the selectivity at low concentrations. DC18C6 was also employed for the separation of positional isomers. Because of its poor solubility in water, preparation of the running buffer solution containing 20 m*M* DC18C6 required 20% methanol. Complete separation of three aminobenzoic acids and partial separation of three aminocresols and three aminophenols were obtained using this running buffer solution. However, the successful separation

Table 4
Effect of column temperature on the retention times and the enantioseparation

Compound	Temperature							
	10°C		20°C		30°C		40°C	
	t (min)	R_s	t (min)	R_s	t (min)	R_s	t (min)	R_s
DOPA	4.68	3.94	3.45	3.17	2.72	2.37	2.24	1.64
	7.51		4.81		3.42		2.61	
Noradrenaline	7.90	0.88	4.72	0.79	3.17	0.65	2.43	–
	8.80		5.15		3.37			
Octopamine	9.42	0.80	5.62	0.70	3.75	0.54	2.79	–
	10.34		6.05		3.95		–	
Tyr	6.38	3.92	4.60	3.18	3.53	2.49	2.82	1.82
	9.94		6.35		4.44		3.31	

Mobile phase: 0.1% $HClO_4$ (v/v) (pH 1.9); flow-rate: 1.0 ml/min; detection; 210 nm.

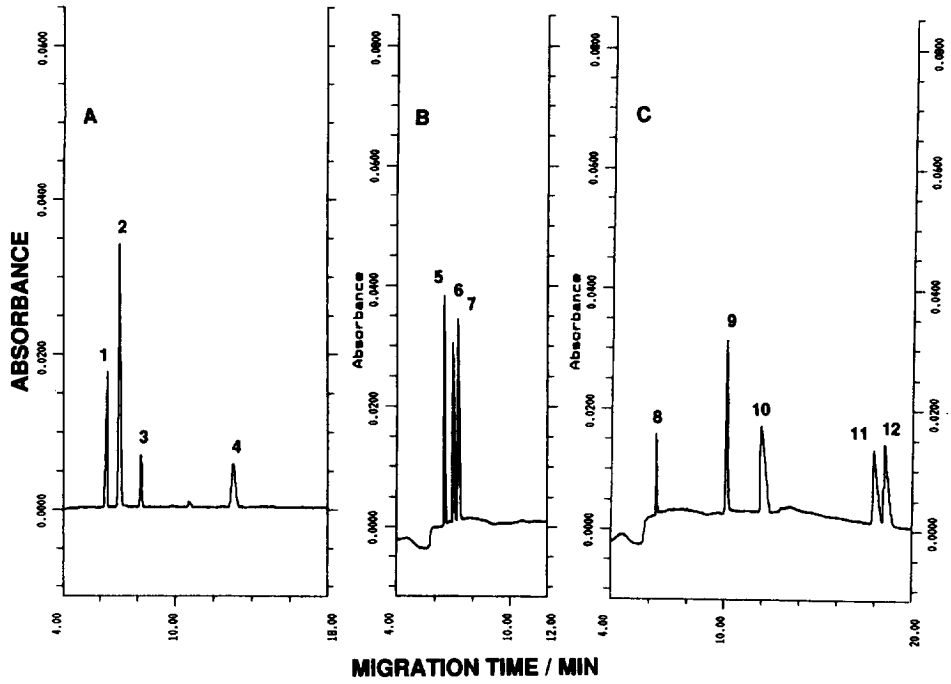

Fig. 6. Separation of positional isomers by CE with $18C6H_4$. Buffer: (A) no crown ethers, (B) and (C) 10 m*M* $18C6H_4$ in 20 m*M* Tris–H_3PO_4 buffer (pH 2.1). Column temperature: 20°C; applied voltage: 15 kV; detection: 220 nm. Samples: (A) 1=*o*-aminophenol+*m*-aminophenol+*p*-aminophenol; 2=4-amino-*m*-cresol+6-amino-*m*-cresol+2-amino-*p*-cresol; 3=*m*-aminobenzoic acid; 4=*o*-aminobenzoic acid+*p*-aminobenzoic acid; (B) 5=4-amino-*m*-cresol; 6=6-amino-*m*-cresol; 7=2-amino-*p*-cresol; (C) 8=*o*-aminophenol; 9=*o*-aminobenzoic acid; 10=*m*-aminophenol+*p*-aminophenol; 11=*m*-aminobenzoic acid; 12=*p*-aminobenzoic acid.

of three aminobenzoic acids was due to the addition of methanol rather than DC18C6. At a concentration of 20 m*M*, DC18C6 was not effective for the successful separation of each isomer. Higher concentrations of DC18C6 will be necessary for the perfect separation of the isomers as in 18C6.

The migration order of isomers in CE with three types of crown ethers is summarized in Table 5. The migration order in $18C6H_4$ was interpreted successfully by the steric hindrance as mentioned above. However, in 18C6 and DC18C6, the degree of the delay of the migration time was smaller than that in $18C6H_4$ and the migration order for aminobenzoic acids was not interpreted by the steric hindrance alone. Selectivity for aminocresols was interestingly quite different among three types of crown ethers. This may be ascribed to the number of the substituent of aminocresols: amino, methyl and hydroxy groups. The complexation of aminocresols is not simple compared with two substituted compounds such as aminophenols and aminobenzoic acids. Therefore, the difference of the substituent of three crown ethers will give the different migration order for aminocresols. *m*-Aminophenol and *p*-aminophenol were not separated at all under the given conditions. In practical applications of crown ethers, especially in CE, solubility of crown ether is important because organic solvents are not used in most CE analysis. Among three crown ethers, $18C6H_4$ gave the best separation, probably because of good solubility and effective substituents. An ionic 18-membered crown ether will be powerful for the separation of primary amines in CE, and if it has chirality, it will have also splendid enantioselectivity.

3.4. Separation of positional isomers of primary amines by HPLC

Separation of three kinds of positional isomers tested above were investigated by HPLC with a

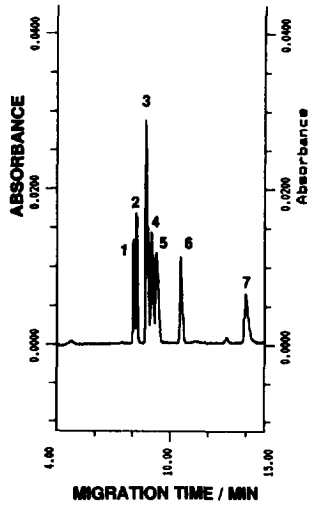

Fig. 7. Separation of positional isomers by CE with 18C6. Buffer: 100 m*M* 18C6 in 20 m*M* NaH_2PO_4–H_3PO_4 buffer (pH 2.1). Samples: 1=4-amino-*m*-cresol; 2=*o*-aminophenol; 3=*m*-aminophenol+*p*-aminophenol+2-amino-*p*-cresol; 4=6-amino-*m*-cresol; 5=*m*-aminobeozoic acid; 6=*o*-aminobenzoic acid. Other conditions are the same as in Fig. 6.

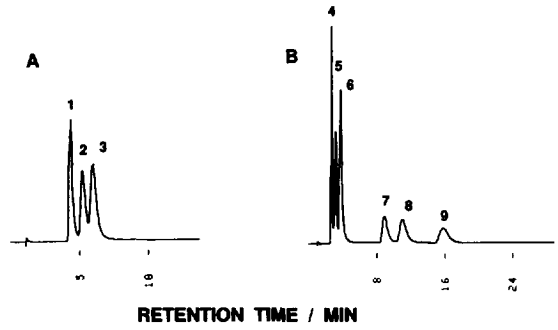

Fig. 8. Separation of positional isomers by HPLC with a Crownpak CR(+) column. Mobile phase: methanol–0.1% $HClO_4$ (v/v) (pH 1.9)=15:85; column temperature: (A) 40°C (B) 25°C, detection: 210 nm; flow-rate: 1.0 ml/min. Samples: (A) 1=*o*-aminobenzoic acid; 2=*p*-aminobenzoic acid; 3=*m*-aminobenzoic acid; (B) 4=2-amino-*m*-cresol; 5=4-amino-*m*-cresol; 6=3-amino-*o*-cresol; 7=2-amino-*p*-cresol; 8=6-amino-*m*-cresol; 9=5-amino-*o*-cresol.

Crownpak CR(+) column. Separations of three aminobenzoic acids and six aminocresols are shown in Fig. 8. Aminophenols were partially separated using 0.1% $HClO_4$ (v/v) (pH 1.9) and 5% methanol mobile phase at 25°C. It was found that a Crownpak CR(+) column is effective for the separation of primary amines as well as the separation of enantiomers. In this column, an analyte which can form stable complex with the crown ether moeity retains strong. That is, elution order will have the same tendency as in CE. For aminophenols and aminobenzoic acids, it was as follows: *ortho*>*para*>*meta*. The *ortho*-isomer eluted first among the three isomers as in CE with $18C6H_4$ due to the steric hindrance. However, the elution order for the three aminocresols investigated in CE was different to that in HPLC.

4. Conclusions

Addition of crown ethers to the running buffer solution of CE was effective for the separation of primary amines, which were not separated in the normal CZE mode. Using the chiral crown ether, $18C6H_4$, many enantiomers having a primary amino group were successfully separation. Epimers, which have ca. 1000 molecular mass and a primary amino group and were not separated by HPLC, were also successfully separated by CE with $18C6H_4$ [24]. Besides $18C6H_4$, which is only one commercially available chiral crown ether, another ionic chiral crown ether will be desired for the separation of enantiomers and isomers. A Crownpak CR(+) col-

Table 5
Migration order of positional isomers in CE

Compound	(No crown ethers)	$18C6H_4$	18C6	DC18C6
Aminocresol[a]	4*m*=2*p*=6*m*	4*m*>6*m*>2*p*	4*m*>2*p*>6*m*	6*m*>2*p*>4*m*
Aminophenol	*o*=*m*=*p*	*o*>*m*=*p*	*o*>*m*=*p*	*o*>*m*=*p*
Aminobenzoic acid	*m*>*o*=*p*	*o*>*m*>*p*	*m*>*p*>*o*	*m*>*p*>*o*

Running buffer: 20 m*M* Tris or NaH_2PO_4–H_3PO_4 buffer (pH 2.1) containing crown ethers; column temperature: 20°C; detection: 220 nm; applied voltage: 15 kV.

[a] 4*m*: 4-amino-*meta*, 2*p*: 2-amino-*para*, 6*m*: 6-amino-*meta*.

umn was found to be effective for the enantiomer separation of many pharmaceuticals having a primary amino group. However, there is a restriction of the usable organic solvent. Covalently bonded types of chiral crown ether columns will be more powerful for the separation of enantiomers and isomers, which have a primary amino group. From comparison between CE and HPLC, CE will be very useful for the method development of an enantiomer separation. CE method development for a compound can be performed within a week.

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