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Preparation and enantiomer separation behavior of selectively methylated γ-cyclodextrin-bonded stationary phases for high-performance liquid chromatography

Takashi Araki, Yudai Kashiwamoto, Shinji Tsunoi, Minoru Tanaka*

Research Center for Environmental Preservation, Osaka University, Yamada-oka, Suita, Osaka 565-0871, Japan

Abstract

Native and three selectively methylated γ-cyclodextrin (γ-CD)-bonded stationary phases without an unreacted spacer arm for HPLC were prepared, where octakis(2-*O*-methyl)-γ-CD, octakis(3-*O*-methyl)-γ-CD and octakis(2,3-di-*O*-methyl)-γ-CD were used as the methylated γ-CDs. The enantiomer separation abilities of the resulting γ-CD stationary phases for 11 pairs of dansylamino acid enantiomers as model solutes were investigated. The effects of pH and methanol content of the mobile phase on the retention and resolution were examined to optimize the mobile phase conditions. The optimum resolution was achieved using a mobile phase consisting of 1.0% triethylammonium acetate buffer (pH 5.0)–methanol (8:2, v/v). γ-CD-bonded stationary phase and octakis(3-*O*-methyl)-γ-CD-bonded stationary phase exhibited good enantiomer separation ability for the dansylamino acids among the four γ-CD stationary phases examined in this study. In particular, octakis(3-*O*-methyl)-γ-CD-bonded stationary phase showed the better enantiomer separation than γ-CD-bonded stationary phase except for dansyl-DL-leucine and -glutamic acid. Octakis(2-*O*-methyl)- and octakis(2,3-di-*O*-methyl)-γ-CD-bonded stationary phases had no enantiomer separation abilities for those solutes. The results suggest the important role of the hydroxyl groups at the 2-positions on the rim of the γ-CD cavities in the enantiomer separation for the dansylamino acids.

Keywords: Stationary phases, LC; Cyclodextrin-based stationary phases; Enantiomer separation; Amino acids

1. Introduction

Cyclodextrins (CDs) can encapsulate a variety of guest molecules into their hydrophobic cavities to form inclusion complexes. The formation of the inclusion complex may be influenced by the shape and size of the guest molecule, hydrogen bonding, hydrophobic interactions and so on. Native CDs and their chemically modified derivatives are widely used in analytical chemistry, especially for the purposes of chiral discrimination [1,2]. Both primary and secondary hydroxyl groups located on the external rims of the CDs are considered to play an important role in the formation and stabilization of their inclusion complexes, in essence, the secondary hydroxyl groups at the 2- and 3-positions located inside and outside on the wider rim of the CD cavity, respectively. It is known that the chemical modifications of CDs produce changes in the shape and size of their cavities, in their hydrogen-bonding ability and in other physical properties.

During 1983–1984, three CD-bonded silica stationary phases for high-performance liquid chromatography (HPLC) were successively reported [3–5]. To date, native and chemically-modified CD stationary phases have been marketed. In previous

*Corresponding author.

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work, we have found that the selective methylation of the hydroxyl groups of β-CD and γ-CD at their 2-, 3-, 6-, 2,3-, 2,6- 3,6- or 2,3,6-positions produces remarkable enantioselectivity changes during the capillary zone electrophoretic separations of dansylamino acid [6,7] and naphthalenesulfonylamino acid [8] enantiomers. To our knowledge, there are few reports on chiral stationary phases bearing such selectively methylated CD derivatives, and they are restricted to selectively methylated β-CD-bonded stationary phases [9,10]. These stationary phases have plural spacer arms connecting between the CD units and silica or many unreacted spacer arms. The existence of the plural spacer arms [11] or the unreacted ones [12] remaining on the silica surface affected the selectivity of the CD stationary phases, and the removal of such unreacted spacer arms enhanced the selectivity derived from the CD unit [12]. Consequently, it is of great interest to couple the selectively methylated cyclodextrin derivatives to silica with one spacer arm and without unreacted ones and to preliminarily investigate their enantiomer separation behavior.

In this paper, we coupled the selectively methylated γ-CD derivatives (2- and 3-monomethylated and 2,3-dimethylated) as well as native γ-CD on the silica surface without an unreacted spacer arm and examined the enantiomer separation abilities of the resulting CD stationary phases for dansylamino acids as model solutes.

2. Experimental

2.1. HPLC apparatus and conditions

The HPLC system comprised a Toso CCPS pump, a Rheodyne Model-7725 sample injector, a Toso UV-8020 detector operating at 220 nm and a Toso Chromatocorder 21 integrator. Each CD stationary phase was packed into a stainless steel column (15 cm×4 mm I.D.) by a slurry packing method using a Chemco CPP-085 packing apparatus at ca. 550 kg/cm^2.

The buffers were prepared by dissolving the required amount of triethylamine in water, and their pH values were adjusted by adding acetic acid. The mobile phases used were mixtures of the buffer–methanol (8:2–7:3, v/v) and were filtered through a membrane filter before use. All HPLC measurements were run at room temperature at a flow-rate of 1.0 ml/min. The concentration of the sample solutes was 0.2 m*M*, and a volume of less than 5 μl was injected.

2.2. Reagents

Silica gel (Super micro bead B-5) and thin-layer chromatography (TLC) plastic sheets (silica gel $60F_{254}$) were supplied from Fuji Silysia (Aichi, Japan) and Merck (Darmstadt, Germany), respectively. Dansylamino acids were obtained from Sigma (St. Louis, MO, USA) and others from Wako (Osaka, Japan) or Tokyo Kasei (Tokyo, Japan). γ-CD was purchased from Ensuiko Seito (Yokohama, Japan). Water was processed with a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.3. Synthesis of selectively methylated γ-CD derivatives

Octakis(2-*O*-methyl)-γ-CD and octakis(2,3-di-*O*-methyl)-γ-CD were synthesized by the previously reported methods [13,14]. Octakis(3-*O*-methyl)-γ-CD was synthesized as follows. First, the hydroxyl groups of γ-CD at the 6-positions were protected with *tert*.-butyldimethylsilyl chloride in pyridine. Secondly, the 2-hydroxyl groups of the γ-CD derivative were benzylated with benzyl bromide in the presence of barium oxide and barium hydroxide octahydrate in dimethylformamide (DMF). Finally, the remaining 3-hydroxyl groups were methylated as usual. After removing the protecting groups, the desired 3-methylated γ-CD derivative was obtained.

Crude methylated γ-CD derivatives (ca. 10 g) were purified by silica gel (100–200 mesh) column chromatography (length ca. 45 cm, diameter 7.5 cm) using dichloromethane–methanol as eluents. The elution of the products was monitored by TLC [dichloromethane–methanol (2:1, v/v), R_F 0.3 for dimethylated γ-CD and dichloromethane–methanol (1:1, v/v), R_F 0.3 for monomethylated γ-CDs]. The methylated γ-CD derivatives thus obtained were characterized by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy and fast atom bombardment mass spectrometry (FAB-MS). The mono- and dimethylated γ-CDs are denoted by prefixing native

γ-CD with MM- and DM-, respectively. Each fractionated γ-CD derivatives was a mixture of octakis(methyl)-γ-CD and its under- and/or over-methylated products. The composition of each methylated γ-CD derivatives was estimated from the relative intensity of the $(M+Na)^+$ ion in FAB-MS spectra (NaI was adulterated with the sample): 83% for 2MM-γ-CD derivative, 81% for 3MM-γ-CD derivative and 71% for 2,3DM-γ-CD derivative.

2.4. Preparation of selectively methylated γ-CD-bonded stationary phases

2.4.1. Monoallyloxyethylation of γ-CD derivatives

Monoallyloxyethylation of native (γ-CD) and three methylated γ-CDs (2MM-γ-CD, 3MM-γ-CD and 2,3DM-γ-CD) was carried out. 3MM-γ-CD, for instance, was treated as follows. A solution of 2-allyloxyethyl iodide (2.4 g) in 20 ml of dimethyl sulfoxide (DMSO) was added to a solution of 3MM-γ-CD (10 g) and NaH (0.6 g) in 400 ml of DMSO. After stirring at room temperature for 70 h, the mixture was evaporated under reduced pressure. The residue was purified by silica gel column chromatography using dichloromethane–methanol as eluents. The monoallyloxyethylated 3MM-γ-CD (AOE-3MM-γ-CD) thus obtained was characterized by FAB-MS. The purity of AOE-3MM-γ-CD was 76%.

2.4.2. Hydrosilylation of monoallyloxyethylated γ-CD derivatives and their immobilization on silica gel

Under a nitrogen atmosphere, AOE-3MM-γ-CD (3.0 g) dissolved in DMF (50 ml) was added to methyldimethoxysilane (0.4 g). The mixture was stirred at 100°C for 36 h after adding small amounts of hydrogen hexachloroplatinate (IV) hexahydrate. The platinate catalyst was filtered off, and the filtrate was concentrated in vacuum (ca. 10 ml). The residue was added to silica gel (4 g) suspended in DMF (30 ml). The mixture was heated without stirring at 100°C for 50 h. The 3MM-γ-CD-bonded stationary phase (denoted by 3MM-γ-CD–silica) was filtered off, washed successively with DMF, DMF–water, methanol, acetone and diethyl ether, and then dried under vacuum at 40°C for 48 h. Similarly, the other allyloxyethylated γ-CD derivatives (AOE-γ-CD, AOE-2MM-γ-CD and AOE-2,3DM-γ-CD) were also immobilized on silica gel. The resulting γ-CD-bonded stationary phases are denoted by γ-CD–silica, 2MM-γ-CD–silica and 2,3DM-γ-CD–silica, respectively. Fig. 1 shows the preparation scheme for the γ-CD stationary phases used in this study.

The amounts of γ-CD derivatives immobilized were readily evaluated by elemental analysis: 69 μmol/g for γ-CD–silica, 110 μmol/g for 2MM-γ-CD–silica, 76 μmol/g for 3MM-γ-CD–silica and 78 μmol/g for 2,3DM-γ-CD–silica.

2.5. Evaluation of enantiomer separation

The extent of separation of the two peaks of a racemate is usually represented by the factor of the resolution (R_S). However, this R_S does not efficiently represent the extent of the separation for the poorly resolved peaks, because their width cannot be precisely measured. Therefore the extent of resolution was evaluated by R' which is expressed using the following equation

$$R' = \frac{(h_1 + h_2)/2 - h'}{(h_1 + h_2)/2} \cdot 100 \quad (1)$$

Here, h_1 and h_2 are the height of the first and

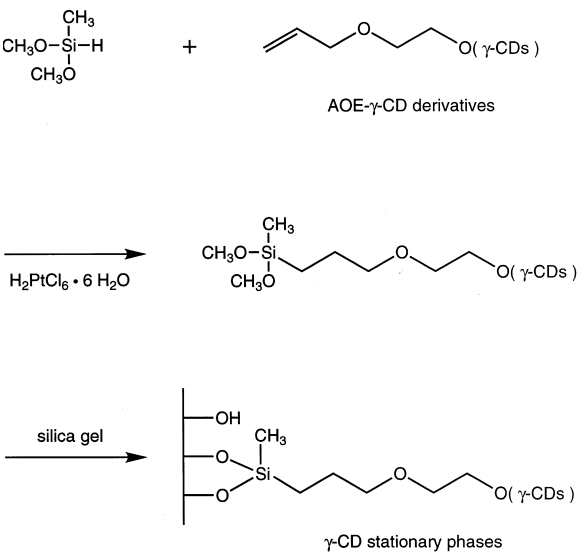


Fig. 1. Preparation scheme for γ-CD stationary phases.

second peaks of the enantiomers, respectively, and h' is the height of the valley between the two peaks. In this definition, the greater the R' value, the better the resolution, and $R' = 100$ represents the baseline separation of the two peaks.

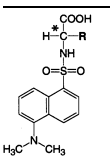
3. Results and discussion

3.1. Enantiomer separation of dansylamino acids on γ-CD-bonded stationary phases

At first, the resolution of the eleven pairs of dansylamino acid enantiomers on the four γ-CD-bonded stationary phases were compared under the same eluent conditions. The mobile phase was composed of 1.0% triethylammonium acetate buffer (pH 5.0)–methanol (7:3, v/v).

Table 1 shows the capacity factors (k') and the extent of resolution (R' values) for the dansylamino acids on γ-CD–silica, 2MM-γ-CD–silica, 3MM-γ-CD–silica and 2,3DM-γ-CD–silica. The retention on 2MM-γ-CD–silica is shown to be stronger than that on the other γ-CD stationary phases. This stronger retention might be ascribed to the larger amount of 2MM-γ-CD immobilized on silica (110 μmol), compared to those of the other γ-CDs (γ-CD–silica; 69 μmol, 3MM-γ-CD–silica; 76 μmol and 2,3DM-γ-CD–silica; 78 μmol). On the contrary, the retention on 2,3DM-γ-CD–silica is the weakest among the γ-CD stationary phases [7]. These results suggest that the important participation of the secondary hydroxyl groups on the wider rim of the γ-CD cavities in the retention of the solutes.

Table 1
Enantiomer separation of dansylamino acids on γ-CD stationary phases [mobile phase: 1.0% triethylammonium acetate (TEAA) buffer (pH 5.0)–methanol (7:3, v/v)]

Dansylamino acid		γ-CD–silica		2MM-γ-CD–silica		3MM-γ-CD–silica		2,3DM-γ-CD–silica	
	R	k'[a]	R'	k'	R'	k'[a]	R'	k'	R'
Alanine	CH_3	1.96	0	5.03	0	2.42	74	1.53	0
α-Aminobutyric acid	CH_2CH_3	2.53	65	6.78	0	2.78	100	1.69	0
Norvaline	$CH_2CH_2CH_3$	3.07	83	9.93	0	3.16	77	1.94	0
Norleucine[b]	$CH_2CH_2CH_2CH_3$	3.52	71	15.92	0	4.41	88	2.53	0
Valine	$CH(CH_3)_2$	2.61	58	8.58	0	3.11	86	1.79	0
Leucine	$CH_2CH(CH_3)_2$	2.75	100	11.71	0	3.15	49	2.21	0
Serine	CH_2OH	1.18	0	2.64	0	1.50	0	1.93	0
Threonine	$CH(CH_3)OH$	1.47	48	3.20	0	1.58	96	1.05	0
Phenylalanine	$CH_2C_6H_5$	9.20	100	24.38	0	8.58	86	2.77	0
Aspartic acid	CH_2COOH	1.45	0	2.32	0	1.63	19	1.16	0
Glutamic acid	CH_2CH_2COOH	1.37	Shoulder	2.30	0	1.67	0	1.11	0

[a] Capacity factor of the first eluted L-enantiomer.
[b] Neither enantiomer was obtained.

As can be seen from the R' values, γ-CD–silica and 3MM-γ-CD–silica exhibited high enantiomer separation abilities, particularly higher in the case of 3MM-γ-CD–silica except for dansyl-DL-norvaline, -leucine and -phenylalanine. The L-enantiomers were eluted faster than the corresponding D-enantiomers, indicating the stronger interaction of the D-enantiomers with the γ-CD cavities. On the other hand, 2MM-γ-CD–silica and 2,3DM-γ-CD–silica could not resolve the solutes at all. These results indicate that the secondary hydroxyl groups at the 2-positions are essential for the chiral recognition of the dansylamino acids. Also, the methyl groups at the 3-positions considerably enhance the ability. Fig. 2 shows the typical liquid chromatograms for dansyl DL-α-aminobutyric acid on γ-CD–silica, 2MM-γ-CD–silica, 3MM-γ-CD–silica and 2,3DM-γ-CD–silica. As mentioned above, methylation of the 3-hydroxyl groups (3-monomethylation) in native γ-CD enhanced the enantiomer separation ability, but 2-monomethylation drastically lost its high enantiomer separation ability.

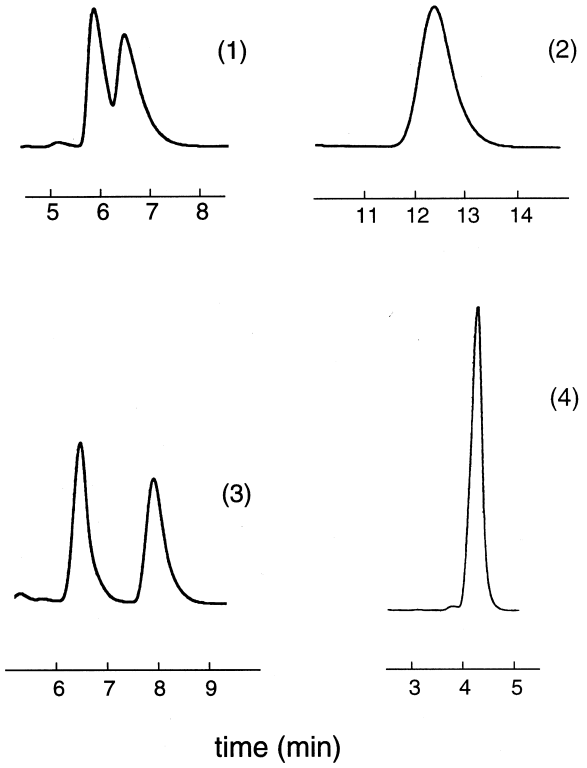


Fig. 2. Enantiomer separation of dansyl-DL-α-aminobutyric acid on (1) γ-CD–silica, (2) 2MM-γ-CD–silica, (3) 3MM-γ-CD–silica and (4) 2,3DM-γ-CD–silica.

3.2. Effect of pH and methanol content on the retention and resolution

The effect of pH of the mobile phase, 1.0% triethylammonium acetate buffer–methanol (7:3, v/v), on the retention and resolution was investigated by changing the pH of the buffer from 5.0 to 7.0. Table 2 lists both k' and R' values for the dansylamino acids γ-CD–silica and 3MM-γ-CD–silica. On the whole, the retention and resolution of the dansylamino acids decreased with increasing pH of the buffer. Similar retention behavior was observed on 2MM-γ-CD–silica and 2,3DM-γ-CD–silica, and the resolution was zero in all cases.

Next we examined the effect of methanol content on the retention and resolution. Table 3 shows the results of both k' and R' values together with the R_S values for the dansylamino acids on γ-CD–silica and 3MM-γ-CD–silica in the mobile phases of 1.0% triethylammonium acetate buffer (pH 5.0)–methanol (7:3 and 8:2, v/v). In the case of 1.0% triethylammonium acetate buffer (pH 5.0)–methanol (9:1, v/v), the elution of the solutes was too slow. The capacity factors and resolution decreased as the methanol content increased. This observation is in agreement with that reported by Fujimura et al. [15]. On increasing the methanol content, the resolution may probably decrease due to the competition of methanol with the solutes in the inclusion complex formation process. Similarly, the effect of acetonitrile content was investigated, but further results were not recognized. Considering the retention and enantiomer separations of the dansylamino acids, the optimum mobile phase was the 1.0% triethylammonium acetate buffer (pH 5.0)–methanol (8:2, v/v). Under this condition, 3MM-γ-CD–silica could resolve all of the dansylamino acids. Similar trend for the mobile phase are also true for γ-CD–silica. Typical chromatograms for dansyl-DL-valine and -glutamic acid on γ-CD–silica and 3MM-γ-CD–silica are shown in Fig. 3.

Table 2
Effect of the methanol content on the retention and resolution on γ-CD–silica and 3MM-γ-CD–silica [mobile phase: 1.0% triethylammonium acetate (TEAA) buffer (pH 5.0)–methanol (7:3, v/v)]

Dansylamino acid	γ-CD–silica						3MM-γ-CD–silica					
	pH 5.0		pH 6.0		pH 7.0		pH 5.0		pH 6.0		pH 7.0	
	k'^a	R'	k'^a	R'	k'^a	R'	k'^a	R'	k'^a	R'	k'^a	R'
Alanine	1.96	0	1.15	0	0.65	0	2.42	74	1.53	56	0.99	25
α-Aminobutyric acid	2.53	65	1.42	52	0.79	18	2.78	100	1.78	100	1.11	97
Norvaline	3.07	83	1.74	74	0.90	43	3.16	77	2.09	67	1.32	40
Norleucine[b]	3.52	71	2.08	64	1.10	35	4.41	88	3.02	80	1.91	69
Valine	2.61	58	1.61	48	0.78	16	3.11	86	2.08	77	1.22	56
Leucine	2.75	100	1.71	100	0.83	91	3.15	49	2.14	25	1.33	Shoulder
Serine	1.18	0	0.77	0	0.33	0	1.50	0	1.01	0	0.56	0
Threonine	1.47	48	0.91	18	0.45	0	1.58	96	1.14	91	0.54	61
Phenylalanine	9.20	100	5.90	100	2.89	97	8.58	86	6.11	75	3.57	55
Aspartic acid	1.45	0	0.94	0	0.25	0	1.63	19	0.88	0	0.28	0
Glutamic acid	1.37	Shoulder	0.85	0	0.22	0	1.67	0	0.86	0	0.32	0

[a] Capacity factor of the first eluted L-enantiomer.
[b] Neither enantiomer was obtained.

4. Conclusions

We have developed for the first time selectively methylated γ-CD-bonded stationary phases for HPLC and evaluated their enantiomer recognition abilities. By controlling the mobile phase composition, the γ-CD-bonded stationary phases can provide efficient and selective separation of dansylamino acid enantiomers. In particular, the methanol content in the mobile phase is an important factor. As the methanol content decreased, the capacity factors and resolution clearly increased. γ-CD–silica and 3MM-

Table 3
Effect of methanol content on the retention and resolution on γ-CD–silica and 3MM-γ-CD–silica [mobile phase: 1.0% triethylammonium acetate (TEAA) buffer (pH 5.0)–methanol]

Dansylamino acid	γ-CD–silica						3MM-γ-CD–silica					
	TEAA–MeOH (7:3, v/v)			TEAA–MeOH (8:2, v/v)			TEAA–MeOH (7:3, v/v)			TEAA–MeOH (8:2, v/v)		
	k'^a	R'	R_S	k'^a	R'	R_S	k'^a	R'	R_S	k'^a	R'	R_S
Alanine	1.96	0	0	3.69	0	0	2.42	74	0.91	5.78	98	1.58
α-Aminobutyric acid	2.53	65	0.74	4.96	91	0.87	2.78	100	2.21	7.09	100	3.28
Norvaline	3.07	83	1.15	6.39	100	1.94	3.16	77	1.05	8.53	100	1.64
Norleucine[b]	3.52	71	0.85	7.96	97	1.25	4.41	88	1.18	13.92	100	1.97
Valine	2.61	58	0.67	5.89	85	0.99	3.11	86	1.13	8.14	100	1.76
Leucine	2.75	100	1.81	6.83	100	2.41	3.15	49	0.70	8.70	94	1.15
Serine	1.18	0	0	2.23	0	0	1.50	0	0	3.09	33	0.46
Threonine	1.47	48	0.59	2.89	88	1.07	1.58	96	1.54	3.29	100	2.07
Phenylalanine	9.20	100	1.53	23.66	100	2.30	8.58	86	1.24	27.17	100	1.56
Aspartic acid	1.45	0	0	2.75	0	0	1.63	19	0.38	3.18	85	1.05
Glutamic acid	1.37	Shoulder	0	2.40	57	0.65	1.67	0	0	3.17	38	0.58

[a] Capacity factor of the first eluted L-enantiomer.
[b] Neither enantiomer was obtained.

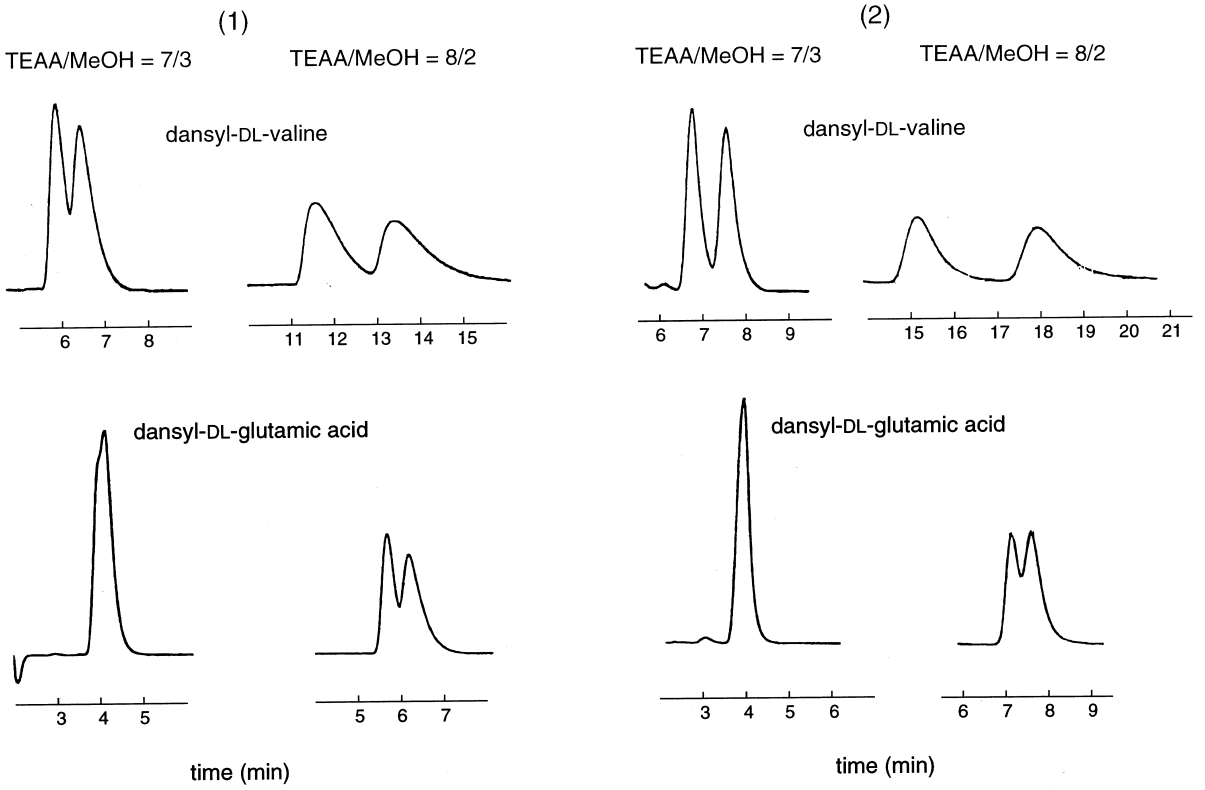


Fig. 3. Enantiomer separation of dansyl-DL-valine and -glutamic acid on (1) γ-CD–silica and (2) 3MM-γ-CD–silica.

γ-CD–silica exhibited high enantioselectivities for the dansylamino acids, while 2MM-γ-CD–silica and 2,3DM-γ-CD–silica showed no enantioselectivities. These results show that the secondary hydroxyl groups of γ-CD at the 2-positions are essential for the enantiomer recognition process for the dansylamino acids.

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