

Preparation and evaluation of new Pirkle type chiral stationary phases with long alkyl chains for the separation of amino acid enantiomers derivatized with NBD-F

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Received 10th August 1998, Accepted 1st October 1998

In order to improve the high-performance liquid chromatographic separation of α -amino acids derivatized with the fluorogenic reagent 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) on commercially available chiral stationary phases (CSPs) such as SUMICHIRAL OA-2500(S) (CSP 1) and OA-4700 (CSP 3), the preparation of two new CSPs (CSP 2 and CSP 4) having 11-aminoundecanoic acid between the aminopropyl silica gel support and the chiral moiety in CSP 1 and CSP 3 is described. CSP 2 and CSP 4 improved both the mutual and enantiomeric separation of NBD-amino acids compared with CSP 1 and CSP 3. Thus, 17 pairs of NBD-amino acid enantiomers and NBD-glycine were separated on CSP 2 except for six NBD-amino acids (D-Asn, D-Ser, D-Gln, L-Pro, L-Ser and Gly). CSP 2 and CSP 4 also showed better enantiomeric separation of NBD-amino acid esters and amides than CSP 1 and CSP 3. It was considered that the achiral long alkyl chains in the CSPs might form a hydrophobic space which assisted the stereoselective interaction of analytes with the chiral moiety by changing the environment around the chiral moiety. On CSP 1 and CSP 2, NBD- β -amino acid was also enantiomerically separated.

Introduction

Enantiomeric separation methods for protein amino acids by high-performance liquid chromatography (HPLC) have been improved extensively in the last decade,¹⁻³ which stimulated the recent progress in the biochemistry of D-amino acids.⁴ Among these methods, the diastereomer formation of each amino acid enantiomer by precolumn derivatization with chiral reagents followed by their separation on non-chiral stationary phases is now widely used, especially with *o*-phthalaldehyde in the presence of chiral thiols.⁵⁻¹⁰ However, *o*-phthalaldehyde reacts only with primary amino groups and therefore proline (Pro) cannot be determined. Other chiral reagents, such as (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC),^{11,12} 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (FDAA),^{13,14} 4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N,N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole (DBD-PyNCS),¹⁵ 4-(2-chloroformylpyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole (NBD-Pro-COCl)¹⁶ and 4-(2-chloroformylpyrrolidin-1-yl)-7-(*N,N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole (DBD-Pro-COCl)¹⁷ have also been used for diastereomer formation. However, the separation factors (α values) for some amino acid diastereomers were small, such as hydrophilic amino acids, D,L-aspartic acid (Asp) (α = 1.02–1.07) and –serine (Ser) (α = 1.01–1.07). Furthermore, the optical purity of the chiral reagents or their possible racemization during the experiments needs to be taken into consideration. To overcome these problems, we tried to develop a separation method for amino acid enantiomers using chiral stationary phases (CSPs).

We have already achieved the enantiomeric separation of 17 amino acid enantiomers, except for cysteine (Cys) and tryptophan (Trp), derivatized with the fluorogenic reagent 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F),¹⁸ using a commercially

available Pirkle type chiral stationary phase [SUMICHIRAL OA-2500(S) (CSP 1), which has a chiral moiety A, as shown in Fig. 1]^{19,20} with a mobile phase of 5.0 mM citric acid in methanol.²¹⁻²⁴ The reason why we selected this CSP was that it exhibited the superior enantiomeric separation of NBD-amino acids among commercially available CSPs (Pirkle type CSPs,²³ cyclodextrin-bonded stationary phases and polysaccharide carbamate phases). The application of this technique to biological samples²⁴⁻²⁷ led to the discovery of a large amount of D-Asp in rat pineal gland.²⁵ However, some amino acids, especially neutral amino acids such as asparagine (Asn), glutamine (Gln) and Ser enantiomer, could not be determined as they co-eluted. The mutual separation of NBD-amino acids seemed to be mainly affected by the aminopropyl group inserted between the silica gel support and the chiral moiety A (Fig. 1). In order to improve the mutual separation of each amino acid, we utilized CSPs having long alkyl chains between the aminopropyl group and the chiral moiety as tethers which are expected to retain the analytes by non-stereoselective hydrophobic interactions.

In this paper, we describe the preparation and evaluation of these CSPs for the separation of NBD-labelled amino acids and other related chiral compounds.

Experimental

Materials

D- or L-Amino acids were purchased from Kyowa Hakko Kogyo (Tokyo, Japan), citric acid monohydrate and tetrahydrofuran (THF) from Kanto Kagaku (Tokyo, Japan) and

prilocaine was from Sigma (St. Louis, MO, USA). Aminopropylsilica for APS, APS-6 and APS-11 was APS-SP-120-HP (5 μ m) obtained from Daiso (Osaka, Japan). For the four CSPs, LiChrosorb NH₂ (5 μ m)₂ Merck, Darmstadt, Germany) was used. Pirkle type CSPs used were SUMICHIRAL OA-2500(S) (CSP 1) and SUMICHIRAL OA-4700 (CSP 3) (250 \times 4.6 mm id, 5 μ m) (Sumika Chemical Analysis Service, Osaka, Japan). CSP 1 and CSP 3 were composed of 3,5-dinitrophenylamide-L-1-naphthylglycine and *N*-(*R*)-1-(α -naphthyl)ethylaminocarbonyl]-*L*-*tert*-leucine modified silica gel, respectively. NBD-F, 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl) and *N,N*-dimethylaminopyridine (DMAP) were obtained from Tokyo Kasei (Tokyo, Japan) and *N,N'*-dicyclohexylcarbodiimide (DCC) from Wako (Osaka, Japan). Methanol, trifluoroacetic acid (TFA) and acetonitrile for the mobile phase were of HPLC grade (Kanto Chemicals, Tokyo, Japan) and all other chemicals were of analytical reagent grade. The water used was purified on a Milli RO-Milli Q system (Nippon Millipore, Tokyo, Japan).

Apparatus

Proton nuclear magnetic resonance (¹H NMR) spectra were recorded on a GSX-400 instrument (JEOL, Tokyo, Japan) using tetramethylsilane as an internal standard. Mass spectra were measured on an M-1200H mass spectrometer [atmospheric chemical ionization (APCI) system] (Hitachi, Tokyo, Japan).

HPLC conditions

The HPLC system consisted of a Model 655 liquid chromatograph (Hitachi), a Rheodyne (Cotati, CA, USA) Model 7125 injector with a 20 μ l injection loop, an F1000 spectrofluorimeter (Hitachi) and a Model 655-60 processor (Hitachi). The column temperature was ambient. A C₁₈ guard column (Tosoh, Tokyo, Japan) was fitted between the injector and the analytical column. The flow rate of the mobile phase was 1.0 ml min⁻¹. The fluorescence of each NBD derivative was detected at 530 nm with excitation at 470 nm.

Derivatization of standard amino acids with NBD-F

A 10 μ l volume of 100 μ M D- or L-amino acid in 0.2 M borate buffer (pH 8.0) containing 4.0 mM Na₂EDTA and 30 μ l of 50 mM NBD-F in acetonitrile were mixed and heated at 60 °C for 5 min. After the addition of 960 μ l of 1% acetic acid in

methanol, a 10 μ l aliquot of the resultant mixture was injected into the HPLC system.

Synthesis of NBD-Ala methyl ester

A 160 mg amount of NBD-Cl in 20 ml of acetonitrile was added dropwise to 60 mg of Ala in H₂O in the presence of 2.5 g of NaHCO₃ at room temperature. The reaction mixture was stirred at 60 °C for 1 h and then acetonitrile was removed by evaporation. The residue was extracted twice with 50 ml of ethyl acetate to remove the excess NBD-Cl. The aqueous phase was acidified with HCl and extracted three times with 50 ml of ethyl acetate. The organic phase was dried over anhydrous Na₂SO₄. The solution was filtered and the filtrate was condensed *in vacuo* to give orange crystals (NBD-Ala), 50 mg of which were dissolved in ethyl acetate (30 ml) at 0 °C with 100 mg of DCC and 50 mg of DMAP. A 100 μ l volume of dried methanol was added to the mixture and stirred at 50 °C for 1 h. The reaction mixture was evaporated to dryness. After silica gel chromatography [ethyl acetate–hexane (1 + 1 v/v)], 10 mg (20%) of orange crystals (NBD-Ala methyl ester) were obtained. APCI-MS, *m/z* 267 ([M + H]⁺); ¹H NMR (CDCl₃), δ ppm 1.2 (3H, s), 1.6 (3H, d), 4.4 (1H, m), 6.1 (1H, d, *J* = 8.4 Hz), 8.4 (1H, d, *J* = 8.4 Hz).

Preparation of APS-6 and APS-11

6-Aminohexanoic acid (10 mmol) was dissolved in a mixture of dioxane (20 ml), H₂O (10 ml) and 1 M NaOH (10 ml) and chilled in an ice-bath. After adding di-*tert*-butyl dicarbonate (11 mmol), the mixture was stirred for 1.5 h at room temperature. The resultant mixture was acidified to pH 3–4 with 10% citric acid, and extracted twice with 20 ml of ethyl acetate. The combined extracts were condensed to dryness *in vacuo* to give oily Boc-6-aminohexanoic acid, which was used without further purification for the preparation of APS-6. Boc-11-aminoundecanoic acid was prepared in the same way as Boc-6-aminohexanoic acid, and gave colorless crystals from 80% ethanol, mp 67–71 °C. Elemental analysis: calculated for C₁₆H₃₁O₄N, C 63.75, H 10.37, N 4.65; found, C 63.63, H 10.24, N 4.69%.

Boc-6-aminohexanoic acid or Boc-11-aminoundecanoic acid condensed with aminopropylsilica gel in dioxane–dichloromethane (1 + 1, v/v) by using dicyclohexylcarbodiimide and triethylamine as condensing agents at 2–3 °C for 2 h and then

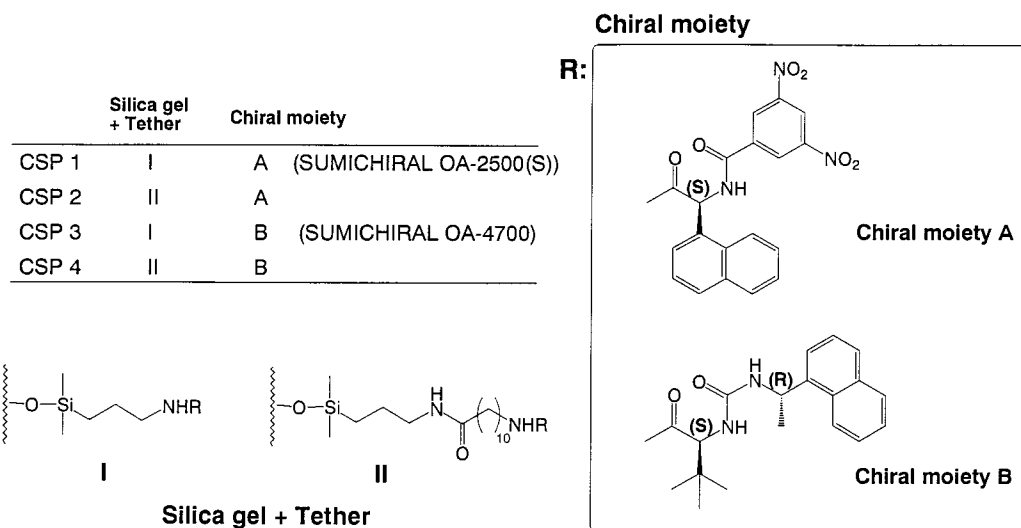


Fig. 1 Structures of chiral stationary phases.

left to stand overnight at room temperature. The modified silica gels (5 g) were hydrolyzed in a TFA–dichloromethane solution (5 ml of TFA + 25 ml of dichloromethane) for 1 h, collected by filtration, transferred into a flask and then suspended in 25 ml of dichloromethane. A 25 ml volume of 20% *N*-ethyl-diisopropylamine in dichloromethane was added to the suspension with stirring. The gels were collected by filtration, transferred into a new flask, stirred in 50 ml of dichloromethane and recollected. They were washed repeatedly with methanol and dried *in vacuo*. Elemental analysis for APS-6 and APS-11: C 8.48, H 1.72, N 1.94% (APS-6); C 8.36, H 1.68, N 1.68% (APS-11). The modified silicas were slurry packed into stainless-steel columns (250 × 4.6 mm id) as described previously.²⁸

Preparation of long alkyl chain CSPs

3,5-Dinitrophenylamide-*L*-1-naphthylglycine and *N*-[(*R*)-1-(α -naphthyl)ethylaminocarbonyl]-*L*-*tert*-leucine were synthesized by the procedure described previously¹⁹ and each compound was coupled with APS-11 by swirling gently in dry THF in the presence of triethylamine at room temperature for 5 h and then heating at 50 °C for 3 h. After cooling, the modified silica was collected by filtration and washed exhaustively with THF, methanol and diethyl ether and then, dried under vacuum. Elemental analysis for CSP 2 and CSP 4: C 14.61, N 2.54% (CSP 2); C 17.5, N 2.58% (CSP 4). The modified silicas were slurry packed into stainless-steel columns (250 × 4.6 mm id) as described previously.²⁸

Results and discussion

Mutual separation on aminoalkylsilica stationary phases

We first prepared two non-chiral stationary phases (APS-6 and APS-11, Fig. 2), which have aminoalkyl groups of different chain lengths (C₅ and C₁₀) attached to the aminopropylsilica support. The effects of alkyl chain lengths on the mutual separation of NBD-amino acids were investigated by comparison with APS (Fig. 2) using 5.0 mM citric acid in methanol as the mobile phase as in previous studies.^{21,22} As shown in Fig. 2, the length of the alkyl chains in the stationary phase apparently affected the retention and the mutual separation of NBD-amino acids. APS-11 gave the largest capacity factors for NBD-amino acids among these three stationary phases, indicating that alkyl chains used as a tether formed a hydrophobic space which interacted with NBD-amino acids. The elution order of NBD-amino acids was slightly different between these columns: threonine (Thr) eluted after phenylalanine (Phe) on both APS and APS-6, whereas it eluted before Phe on APS-11. This might be due to different interactions between the alkyl chains and NBD-amino acids.

The numbers of peaks detected after injection of 17 NBD-amino acids were 14, 15 and 16 for APS, APS-6 and APS-11, respectively. In the case of APS, the pairs arginine (Arg)–histidine (His), glycine (Gly)–Thr, and proline (Pro)–lysine (Lys) co-eluted, whereas for APS-6, Gly–Gln and Ser–Asn–Lys co-eluted. In contrast, only Gln and Pro co-eluted in the case of APS-11. Even if the concentration of citric acid in the mobile phase used was decreased, the mutual separation of NBD-amino acids was not improved on APS and APS-6, even though the retention times were longer. These results show that amino alkyl groups attached to the aminopropyl silica have affinities to the side chain of NBD-amino acid and improve the mutual separation of NBD-amino acids. Since APS-11 was the most appropriate stationary phase for the mutual separation of NBD-amino acids, we selected it for the further experiments.

Separation of NBD-amino acids on CSP 2 and CSP 4

CSP 1 and CSP 3 having the chiral moieties (3,5-dinitrophenylamide)-*L*-1-naphthylglycine (chiral moiety A, Fig. 1) and *N*-[(*R*)-1-(α -naphthyl)ethylaminocarbonyl]-*L*-*tert*-leucine (chiral moiety B, Fig. 1), respectively gave good enantiomeric separations of NBD-amino acids.²³ We therefore attached these chiral moieties to the end of the APS-11 to afford new CSPs (CSP 2 and CSP 4, Fig. 1). When 5.0 mM citric acid in methanol was used as the mobile phase, these NBD-amino acids were retained as a result of ionic interactions between the carboxyl group of the NBD-amino acids and the amino group remaining in the CSPs. Table 1 gives the capacity factors and α values for NBD-amino acid enantiomers obtained on CSP 2 and CSP 4, together with the data on CSP 1 and CSP 3, and Fig. 3 illustrates typical chromatograms for the enantiomeric separation of NBD-amino acids. The capacity factors on CSP 2 and CSP 4 were larger than those on CSP 1 and CSP 3, except for Pro on CSP 4, indicating that the extended hydrophobic space formed by the tethers of long alkyl chains was not disturbed after attachment of the chiral moieties. In terms of chiral recognition, the α values for many NBD-amino acid enantiomers on CSP 2 were slightly smaller than those on CSP 1. However, the α values for two hydrophobic amino acids [leucine (Leu) and Phe] and Lys (α - and ϵ -amino groups were derivatized with NBD-F) increased on CSP 2 compared with those on CSP 1. The α values for 10 out of 15 amino acids on CSP 4 were larger than those on CSP 3, indicating that the enantiomeric separation of hydrophobic amino acids was more improved on CSP 4. These increments in α values for the hydrophobic amino acids on the CSP 2 and CSP 4 indicated that the extended hydrophobic space

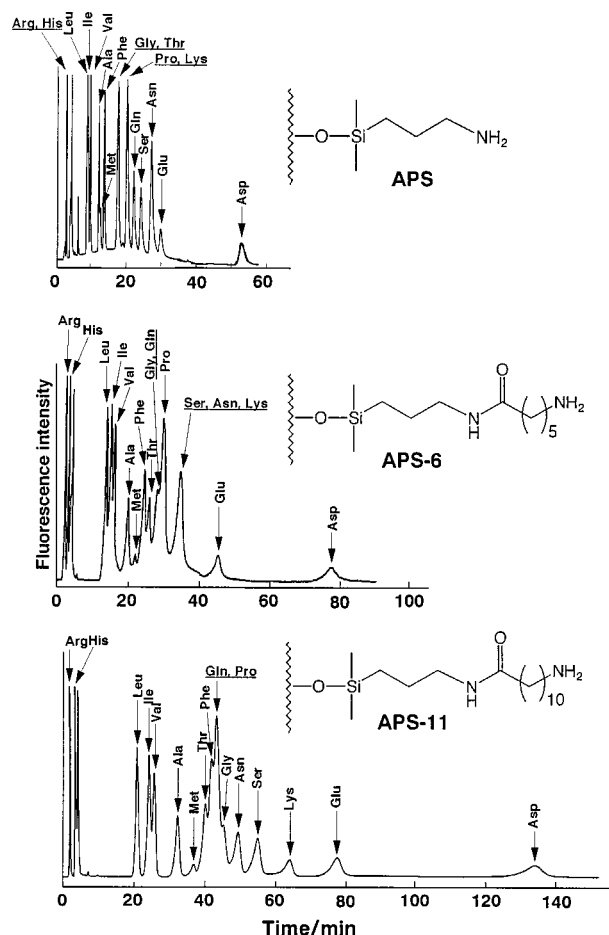


Fig. 2 Structures of non-chiral stationary phases and chromatograms for 16 NBD-*L*-amino acids and NBD-Gly. Column, (a) APS, (b) APS-6, and (c) APS-11; mobile phase, 5.0 mM citric acid in methanol; temperature, ambient; flow rate, 1.0 ml min⁻¹; Detection, λ_{ex} 470 nm, λ_{em} 530 nm.

assisted the stereoselective interaction of the NBD-amino acid with the chiral moiety.

The elution intervals of each peak were wider on CSP 2 and CSP 4 than those on CSP 1 and CSP 3, respectively, without any difference in peak widths. Therefore, a sharper separation of NBD-amino acid enantiomers was achieved on CSP 2 and CSP 4, indicating their superior separation of NBD-amino acid enantiomers compared with other commercially available CSPs. Finally, the use of CSP 2 gave the best separation of NBD-amino acid enantiomers among these CSPs: the separation of a mixture of 17 pairs of NBD-amino acid enantiomers and NBD-Gly was achieved on CSP 2, except for the three pairs D-Asn–L-Gln, Gly–D-Ser and L-Ser–L-Pro) (data not shown). If a CSP having an even longer alkyl chain such as an octadecyl group as a tether is available, we could expect a better mutual separation of NBD-amino acids on the CSP. However, such aminoalkylcarboxylic acids are not yet available commercially.

Separation of NBD-amino acids ester and amide

The good enantiomeric separations of hydrophobic amino acids on CSP 2 and CSP 4 prompted us to use these CSPs for the enantiomeric separation of hydrophobic NBD derivatives. Since these compounds were assumed to penetrate more easily than NBD-amino acids into the extended hydrophobic space to

form the stereoselective complex, better enantiomeric separations would be achieved on both CSP 2 and CSP 4.

We then investigated the separation of methyl esters of NBD-Phe and NBD-Ala and NBD-Phe amide as representatives of hydrophobic compounds on these CSPs. The mobile phase of methanol containing citric acid was suitable for enantiomeric separation of amino acids,^{21,22} but it resulted in these ester and amide compounds eluting near the void volume. Therefore, mixtures of water, acetonitrile and TFA or water, methanol and citric acid were used in further experiments.

Typical chromatograms for NBD-Phe methyl ester and amide on CSP 1 and CSP 2 are shown in Fig. 4. CSP 2 showed a better enantiomeric separation of each enantiomer than CSP 1 when each peak was adjusted to give similar retention times by changing the acetonitrile and TFA concentrations in the mobile phase. Separation parameters for each NBD-amino acid derivative on four CSPs are summarized in Table 2. Although the enantiomers of NBD-Phe methyl ester were not completely separated on CSP 1 ($\alpha = 1.03$), they were well separated on CSP 2 ($\alpha = 1.06$). The elution order of amide derivatives on CSP 4 was reversed compared with that of CSP 3, the reason of which is unclear. The improvement of the enantiomeric separation of the esters on CSP 2 and CSP 4 and of the amide in the case of CSP 2 supports our hypothesis that the extended hydrophobic space assisted the stereoselective interaction of NBD-amino acid derivatives with the chiral moiety by changing the environment around the chiral moiety.

Table 1 Capacity factors (k') and α values for NBD-amino acids on CSP 1–4. Mobile phase, 5.0 mM citric acid in methanol; temperature, ambient; flow rate, 1.0 ml min⁻¹; detection, λ_{ex} 470 nm, λ_{em} 530 nm

Amino acid	CSP 1			CSP 2			CSP 3			CSP 4		
	k' of D-form	k' of L-form	α	k' of D-form	k' of L-form	α	k' of D-form	k' of L-form	α	k' of D-form	k' of L-form	α
Leu	2.02	2.27	1.12	3.29	3.80	1.16	1.65	2.00	1.21	1.96	2.61	1.33
Ile	2.41	2.73	1.13	4.28	4.83	1.13	2.09	2.35	1.13	2.58	2.98	1.15
Val	2.57	2.89	1.13	4.47	5.01	1.12	2.28	2.53	1.11	2.72	3.01	1.11
Ala	3.29	3.70	1.12	5.86	6.43	1.10	3.18	3.58	1.13	3.77	4.38	1.16
Thr	3.70	4.07	1.10	6.56	7.06	1.08	3.29	3.81	1.16	3.86	4.57	1.18
Gln	5.01	5.38	1.07	7.64	8.04	1.05	3.84	4.32	1.13	3.86	4.49	1.16
Met	4.38	5.08	1.16	7.66	8.47	1.11	3.42	4.10	1.20	4.44	5.62	1.26
Asn	4.84	5.44	1.13	8.08	8.88	1.10	4.06	4.91	1.21	4.43	5.46	1.23
Gly	4.96			8.89			4.99			5.99		
Ser	5.02	5.51	1.10	9.34	9.96	1.07	4.69	5.33	1.14	5.75	6.51	1.13
Pro	6.39	6.10	1.04	9.23	8.91	1.04	5.17	5.47	1.06	4.47	4.59	1.03
Phe	5.03	6.00	1.19	9.02	10.88	1.21	4.02	4.73	1.18	5.34	6.73	1.26
Glu	6.29	6.89	1.10	12.79	13.72	1.07	5.34	6.05	1.13	6.82	7.73	1.13
Asp	8.94	9.81	1.10	21.31	22.79	1.07	8.41	9.40	1.12	11.26	12.39	1.10
Lys	10.68	11.66	1.06	21.27	25.11	1.18	5.91	6.78	1.15	9.32	11.31	1.21
Tyr	14.82	18.31	1.24	22.77	27.30	1.20	8.01	9.32	1.16	11.04	14.61	1.32

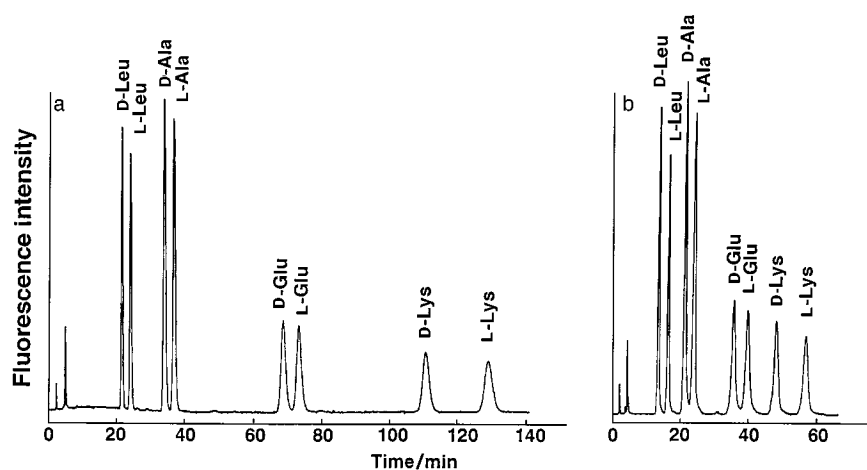


Fig. 3 Chromatograms for NBD-amino acids. Column, (a) CSP 2 and (b) CSP 4; mobile phase, 5.0 mM citric acid in methanol; temperature, ambient; flow rate, 1.0 ml min⁻¹; detection, λ_{ex} 470 nm, λ_{em} 530 nm.

Table 2 Capacity factors and α values for NBD-amino acids methyl esters or amide

Stationary phase	NBD-Phe methyl ester				NBD-Ala methyl ester				NBD-Phe amide			
	k'	α	Elution order	Mobile phase ^a	k'	α	Elution order	Mobile phase ^a	k'	α	Elution order	Mobile phase ^a
CSP 1	2.66, 2.73	1.03	D, L	a	1.22, 1.23	1.01	D, L	a	1.44, 1.59	1.10	D, L	b
CSP 2	6.09, 6.46	1.06	D, L	a	2.45, 2.86	1.03	D, L	a	3.38, 3.83	1.13	D, L	b
CSP 3	2.75	1.00	D, L	c	5.97, 6.11	1.02	D, L	c	9.92, 10.28	1.04	D, L	d
CSP 4	12.14, 13.28	1.09	D, L	c	4.12, 4.42	1.07	D, L	c	23.58, 24.53	1.04	L, D	d

^a Mobile phase: (a) H₂O–acetonitrile–TFA (50 + 50 + 0.015); (b) H₂O–acetonitrile–TFA (50 + 50 + 0.05); (c) H₂O–methanol (2 + 3) containing 5.0 mM citric acid, (d) H₂O–methanol (1 + 1) containing 5.0 mM citric acid.

Many researchers have examined the effect of the alkyl chain present in CSPs on enantiomeric separations in the normal-phase mode using a mixture of *n*-hexane and a polar solvent (chloroform, propan-2-ol or THF) as the mobile phase.^{29,30} Some of them demonstrated that enantiomeric separation was obstructed by non-stereoselective interactions between the alkyl chains and analytes.^{31,32} Others reported an improvement in the enantiomeric separations on the CSPs having long alkyl chains.^{33,34} They suggested that the straightened long alkyl chain blocked the non-stereoselective interaction (hydrogen bonding) between silanol group and analytes in the normal-phase mode. Thus, large α values were obtained with small retention times of the enantiomers.³⁵ Our present data obtained in the reversed-phase mode using methanol containing citric acid or a mixture of an organic solvent, water and acid are consistent with the latter's data except for the retention of the analytes. The α values became large with the longer retention times. It was suggested that stereoselective interactions with a chiral moiety were increased together with an increment in non-stereoselective interactions (hydrophobic interactions) with the achiral long alkyl chains in our experiments. It seemed that the penetration of analytes into the extended hydrophobic space occurred to make non-stereo- and stereoselective interactions, rather than blocking the non-stereoselective interaction with an unmodified support.

Enantiomeric separation of α - and β -amino acid type compounds (prilocaine and β -aminobutyric acid)

We applied these four CSPs to the enantiomeric separation of other related chiral compounds derivatized with NBD-F, and evaluated the usefulness of these CSPs. First we tried to separate

prilocaine (propitocaine, Fig. 5), a local anesthetic frequently used for regional, nerve block and topical anesthesia.³⁶ Both enantiomers have equal biological activity, while the (*R*)-(-)-enantiomer is rapidly hydrolyzed to form toluidine.³⁷ Prilocaine is an amide derivative of an α -amino acid (Ala), and therefore the NBD-labelled prilocaine (NBD-prilocaine) could be separated enantiomerically, especially on CSP 2 with a mobile phase containing water. Fig. 5 illustrates the chromatograms for NBD-prilocaine on CSP 1 and CSP 2, showing that a better enantiomeric separation was achieved on CSP 2 with an α value of 1.09 (*versus* 1.05 on CSP 1). The elution order of NBD-prilocaine enantiomers was not confirmed, since we had only racemic and not optically active prilocaine. CSP 4 also showed better enantiomeric separation than CSP 3 ($\alpha = 1.03$ and 1.00, respectively). The data also indicate that NBD- α -amino acid derivatives are separated enantiomerically on CSP 2 and CSP 4.

Next, we examined the enantiomeric separation of a β -amino acid which has a methylene group between the carboxyl group and the asymmetric carbon, bearing the amino group. We selected β -aminobutyric acid as a representative β -amino acid. These CSPs using a mobile phase of 2.0 mM citric acid in methanol showed better enantiomeric separation of NBD- β -aminobutyric acid [$\alpha = 1.21$ (CSP 1) and 1.10 (CSP 2)], except for CSP 3 and CSP 4 ($\alpha = 1.00$). The data indicate that the enantiomers of not only NBD- α -amino acids but also NBD- β -amino acids could be separated on CSP 1 and CSP 2. A further experiment on the applicability of the CSPs is in progress.

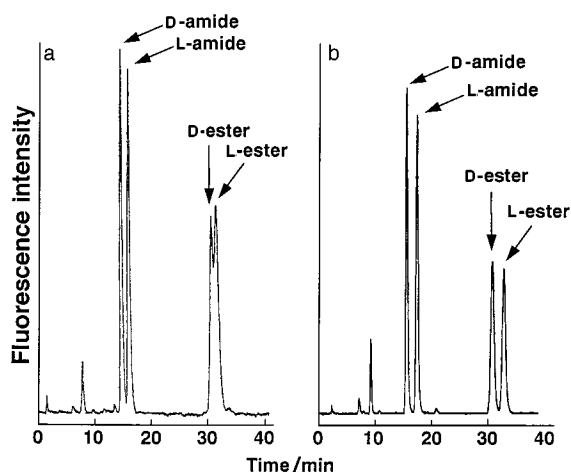


Fig. 4 Chromatograms for NBD-D, L-Phe methyl ester or amide. Column, (a) CSP 1 and (b) CSP 2; mobile phase, H₂O–acetonitrile–TFA, (a) 60 + 40 + 0.05 and (b) 58 + 42 + 0.025; temperature, ambient; flow rate, 1.0 ml min⁻¹; detection, λ_{ex} 470 nm, λ_{em} 530 nm.

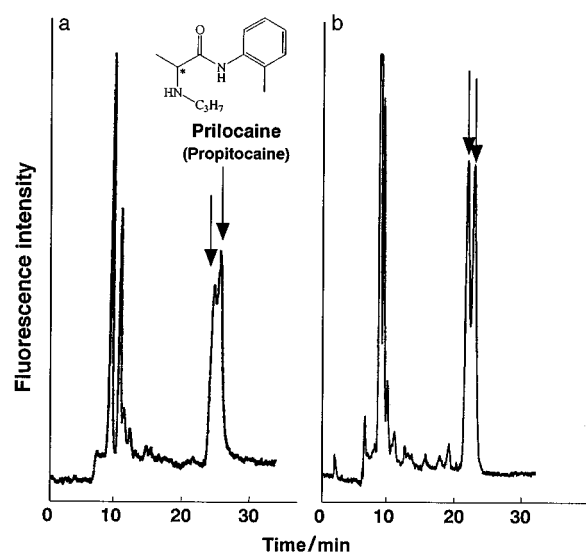


Fig. 5 Chromatograms for NBD-prilocaine. Column, (a) CSP 1 and (b) CSP 2; mobile phase, (a) H₂O–methanol (7 + 13) containing 5.0 mM citric acid and (b) H₂O–methanol (3 + 7) containing 5.0 mM citric acid; temperature, ambient; flow rate, 1.0 ml min⁻¹, detection, λ_{ex} 470 nm, λ_{em} 530 nm.

Acknowledgements

We gratefully acknowledge Drs. C. K. Lim and H. Homma for valuable discussions and comments. Thanks are due to Tosoh for supplying the C₁₈ guard column. M.K. was supported by a research fellowship of the Japan Society for the Promotion of Science for Young Scientists. This work was supported in part from the Ministry of Education, Science and Culture of Japan.

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Paper 8/06304D