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High-performance liquid chromatographic resolution of amino acid enantiomers derivatized with fluorescent chiral Edman reagents

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Abstract

The fluorescent chiral Edman reagents, 4-(3-isothiocyanatopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole [*R*(−)- and *S*(+)-NBD-PyNCS] and 4-(3-isothiocyanatopyrrolidin-1-yl)-7-(N,N-dimethylaminosulfonyl)-2,1,3-benzoxadiazole [*R*(−)- and *S*(+)-DBD-PyNCS], have been utilized for the resolution of amino acid enantiomers as diastereomeric derivatives. These reagents react with amino acid enantiomers in the presence of base catalyst (triethylamine, 1-azabicyclo[2.2.2]octane and 1,8-diazabicyclo[5.4.0]-7-undecene) to produce the corresponding thiocarbamoyl-amino acid diastereomers under the mild reaction conditions of 55°C for 10 min. The resulting derivatives are relatively stable, not only in alkaline reaction solution but also in acidic medium, with no measurable conversion to the thiohydantoin derivatives. The reactivities of both enantiomers of the derivatization reagents with the amino acid enantiomers (L- and D-isoleucine) are comparable. The fluorescence properties (maximal wavelengths and intensities) of the thiocarbamoyl-amino acid derivatives are dependent upon the solvents in the medium. Some amino acids labeled with the proposed reagents are efficiently resolved by an ODS column with water–acetonitrile containing 0.05% trifluoroacetic acid. The R_s values are in the ranges 3.57–0.55 (13 amino acids) for the diastereomers obtained with NBD-PyNCS, and 2.57–0.68 (14 amino acids) for those with DBD-PyNCS. The R_s values obtained from neutral and/or aromatic amino acids are larger than those of basic and acidic amino acids.

1. Introduction

The resolution of amino acid enantiomers has been widely investigated because of their commercial significance and ease of availability. Amino acids are used primarily by the pharmaceutical and food industries. In many cases, the L-isomers of the corresponding amino acids are required by these industries because only L-isomers are used as natural nutrients for the human body. Therefore, the determination of optical purity is important for the manufacture of amino acids. Optically pure amino acids are also required for the syntheses of several chiral reagents, catalysts and physiologically active substances including bioactive peptides and antibiotics. Although peptides and proteins in mammals are composed of L-amino acids, racemiza-

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tion may alter modify the biological activity. The racemization reactions of amino acids have also been used to estimate the age of fossil bones and teeth. Consequently, highly effective resolution of amino acid enantiomers is an important subject.

Chromatographic techniques such as gas chromatography (GC) [1,2] and high-performance liquid chromatography (HPLC) [3,4] can be employed for the resolution of racemates. A great number of methods, which involve both the direct method with a chiral stationary phase (CSP) column and an indirect method requiring derivatization with chiral reagents, have been developed for resolving amino acids racemates [5]. HPLC has emerged as an important tool for this purpose because of its good reproducibility and efficiency for a wide range of compounds. Although chiral separations of amino acids by the direct methods with CSP columns are quite popular, the indirect methods which involve derivatization steps with chiral reagents are also receiving considerable attention. The introduction of chromophores may enhance both the detection and the resolution. Some derivatization reagents such as 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl (GITC) [6] and *R*-α-methylbenzyl isothiocyanate (*R*-AMBI) [7] have been developed for chiral separation of amino acids. Adequate separations of these labels were obtained by reversed-phase chromatography, but the sensitivities seem to be insufficient to trace level detection in real samples. A major advantage of the indirect HPLC method with fluorescence detection is the possibility of excellent sensitivity due to the properties of the derivatization reagent. The combinations of *o*-phthalaldehyde (OPA) and various chiral thiols (e.g. N-acetyl-L-cysteine, Boc-L-cysteine, etc.) are frequently used for derivatization and separation of amino acid enantiomers as fluorescence compounds [8–10]. However, for some applications the isoindole derivatives produced from OPA/thiols do not have adequate stability [11,12].

In a previous paper [13], we reported the syntheses of the novel fluorescence Edman-type reagents, i.e. *S*(+)- and *R*(−)-enantiomers of 4-(3-isothiocyanatopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole (NBD-PyNCS) and 4-(3-isothiocyanatopyrrolidin-1-yl)-7-(N,N-dimethylaminosulfonyl)-2,1,3-benzoxadiazole (DBD-PyNCS). These reagents have been demonstrated as useful for the resolution of various amines including β-blocking drugs. This paper deals with the investigations of the derivatization conditions for amino acids, the fluorescence properties of the resulting thiocarbamoyl-amino acid, and the separation by reversed-phase chromatography of the diastereomers derived from the reactions of some amino acids with the chiral Edman reagents.

2. Experimental

2.1. Materials and reagents

S(+)- and *R*(−)-NBD-PyNCS and *S*(+)- and *R*(−)-DBD-PyNCS were synthesized as described previously [13]. Both enantiomers of amino acids were obtained from Sigma (St. Louis, MO, USA). 1-Azabicyclo[2.2.2]octane (quinuclidine) and 1,8-diazabicyclo[5.4.0]-7-undecene (DBU) were purchased from Nacalai Tesque (Kyoto, Japan). Trifluoroacetic acid (TFA), acetonitrile, and water were of HPLC grade (Wako). All other chemicals were of analytical-reagent grade and were used without further purification.

2.2. HPLC

The HPLC system consisted of two LC-9A pumps (Shimadzu) and an SCL-6B system controller (Shimadzu). Sample solutions were injected with a SIL-6B auto injector (Shimadzu). The analytical column was an Inertsil ODS-80A (150 × 4.6 mm I.D., 5 μm) for reversed-phase chromatography (GL Sciences, Tokyo, Japan). The columns were maintained at 40°C with a CTO-6A column oven (Shimadzu). A Shimadzu RF-550 fluorescence monitor equipped with a 12-μl flow cell was employed for the detection. The excitation and emission wavelengths were fixed at 490 and 530 nm for the derivatives of

amino acids with NBD-PyNCS, and at 460 and 540 nm for those with DBD-PyNCS. The peak areas obtained from the fluorescence monitor were calculated with a C-R4A Chromatopac (Shimadzu). All mobile phases were de-gassed with an on-line degasser (DEGAS; Shodex, Tokyo, Japan). The flow-rate of the eluent was 1.0 ml/min.

2.3. Derivatization procedure for amino acids

A 10-μl volume of the reagent [5 mM $R(-)$- or $S(+)$-enantiomer] in acetonitrile and 10 μl of amino acid enantiomers (1 mM of each enantiomer) in acetonitrile–water (1:1) containing 2% triethylamine (TEA) were mixed in a 1.5-ml mini-vial (GL Sciences). The vials were tightly capped and heated at 55°C with a dry heat block for 10 min. Then 480 μl of 1 M acetic acid (AcOH) in acetonitrile–water (1:1) were added to the reaction mixture to stop the derivatization reaction. The acidic solution was diluted ten times with acetonitrile and then 5 μl of the solution was injected into the column. The reagent blanks without amino acids were also treated in the same manner.

The capacity factor (k'), separation factor (α) and the resolution value (R_s) were calculated from the equations $k' = (t_R - t_0)/t_0$, $\alpha = k'_2/k'_1$ and $R_s = 2(t_{R2} - t_{R1})/(w_1 + w_2)$, where t_R, t_{R1} and t_{R2} are the peak retention times, t_0 is the retention time of an unretained compound (void volume of the column) ($t_0 = 1.27$ min) and w_1 and w_2 are the widths of the bases formed by triangulation of the peaks.

2.4. Fluorescence properties of the reagents and their derivatives

For the fluorescent spectra measurements, 20 μl of the acid solutions of the D-tyrosine derivatives, obtained from recommended procedures, were injected onto the column and the peak corresponding to the thiocarbamoyl-amino acid derivative was collected downstream from the detector (ca. 2-ml portion). The eluent collected in the tube was evaporated under reduced pressure, and then a small quantity of acetonitrile was added to dissolve the residue. Equal volumes of the solution was added to portions of 2 ml of various solvents, and maximal wavelengths (excitation and emission) and relative fluorescence intensities (RFIs) were measured with a fluorescence spectrophotometer. Fluorescence properties of the chiral reagents themselves were also measured at 2 μM concentration without chromatographic separation.

3. Results and discussion

Fig. 1 shows the derivatization reaction of amino acids with the chiral fluorescent Edman reagents, NBD-PyNCS and DBD-PyNCS. The reaction proceeds in alkaline medium to produce corresponding thiocarbamoyl-amino acids.

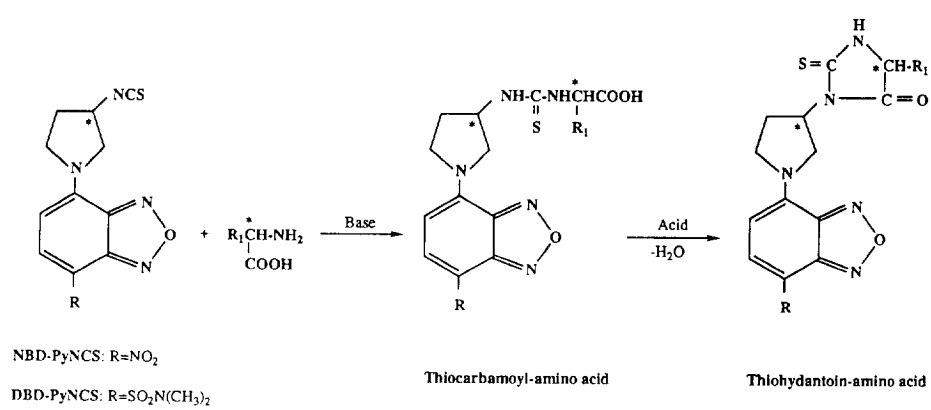

Fig. 1. Reaction of amino acids with optically active fluorescent Edman reagents.

Thiocarbamoyl-amino acids, produced with Edman reagents like phenylisothiocyanate (PITC), are converted to corresponding thiohydantoin derivatives in strong acid solution [14,15]. In the case of the thiocarbamoyl-amino acids derived from NBD-PyNCS and DBD-PyNCS, however, no such conversion was found with 1 *M* acetic acid which was added to the reaction mixture to stop the derivatization reaction. When hydrochloric acid at the same concentration level was used for the termination of the derivatization reaction, no substantial differences were observed on the chromatograms. Furthermore, no degradation of the derivatives is observed as compared with both chromatograms before and after storage for more than 24 h at 5°C. The fluorescent derivatization reagents in neutral acetonitrile solution are stable for at least 3 weeks at 5°C and 1 week at room temperature. It is noteworthy that the reagents are sufficiently stable to retain the –NCS functional group in the acidic solution, even after heating at 50°C for 3 h. In contrast, the –NCS functional group of previously reported Edman reagents decompose to produce the corresponding $-NH_2$ compounds in strong acidic medium [16]. The results suggest that the reagents and the derivatives are relatively stable in the acidic medium. Good stability of the reagents and the derivatives is an important property for a useful reagent for trace quantities of amino acids.

Some parameters affecting the derivatization reaction were studied to select optimal conditions. Alkaline solution is essential for the derivatization with Edman-type reagents; therefore, the effect of base catalyst on the derivatization was examined with tryptophan and isoleucine. These amino acids were selected as the representatives of aromatic and aliphatic amino acids, respectively. As shown in Fig. 2, three organic bases —TEA, DBU and quinuclidine— gave similar yields of the derivatives. The low yields with $NaHCO_3$ may be due to the poor nucleophilic properties of the inorganic salt. Although TEA, DBU and quinuclidine are equally effective as catalysts for the derivatization, TEA was selected for the derivatization

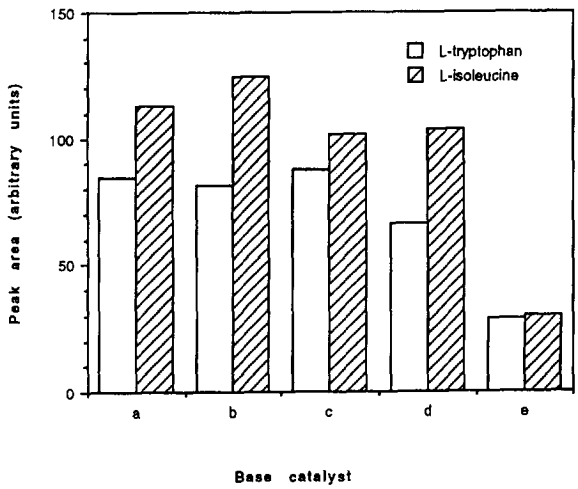

Fig. 2. Comparison of base catalysts for the derivatization of amino acids with *R*(−)-NBD-PyNCS. a = 0.5% TEA; b = 0.5% quinuclidine; c = 0.5% DBU; d = 0.05 *M* borate buffer (pH 10); e = 0.5 *M* $NaHCO_3$ buffer (pH 9.5).

because of its ready availability. With respect to the concentration of TEA, similar results were observed in the range from 0.5 to 3% (Fig. 3); however, concentrations higher than 3% reduced the production of the thiocarbamoyl-amino acid derivatives. Therefore, 1% TEA was selected in the following experiments. Fig. 4 shows the influence of the reagent concentration on the derivatization. The peak areas were almost constant at reagent-to-analyte ratios in excess of 5.

Immediate reaction is generally more likely to

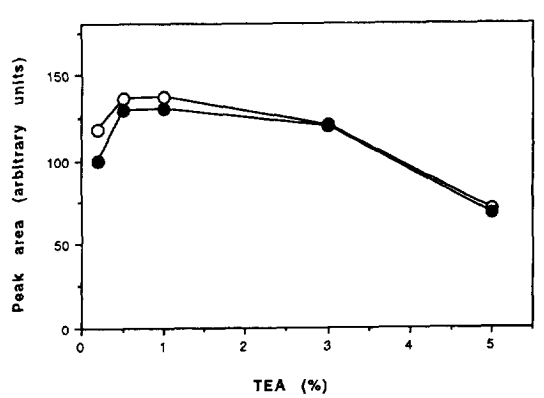

Fig. 3. Effect of TEA concentration on the derivatization with *R*(−)-NBD-PyNCS. ○ = L-Tryptophan; ● = L-isoleucine.

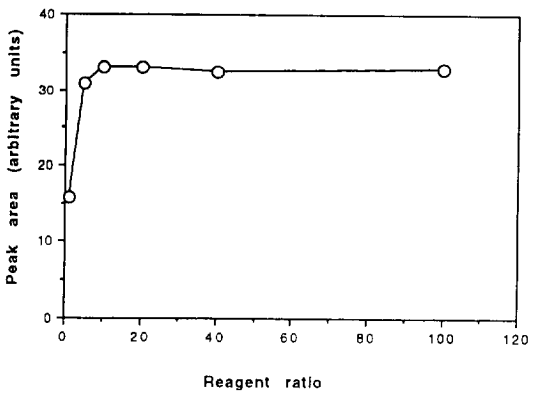

Fig. 4. Effect of $R(-)$-NBD-PyNCS concentration on the derivatization of L-isoleucine.

yield preferable highly reproducible results than a very slow reaction. In addition, the chiral derivatization reagents must react at similar rates with both enantiomers. Big differences in reaction rate will yield uncertain results, especially in the detection of trace quantities. The reactivity of the reagent was evaluated with both enantiomers of isoleucine. Fig. 5 shows the time courses of the derivatization reaction with $R(-)$-NBD-PyNCS. The reactions were essentially complete after 10 min at 55°C. Judging from both curves, the reaction rates seem to be comparable for both enantiomers of NBD-PyNCS. The reaction of amino acids with DBD-PyNCS is considered to be essentially the same as those with NBD-PyNCS, since the effect of

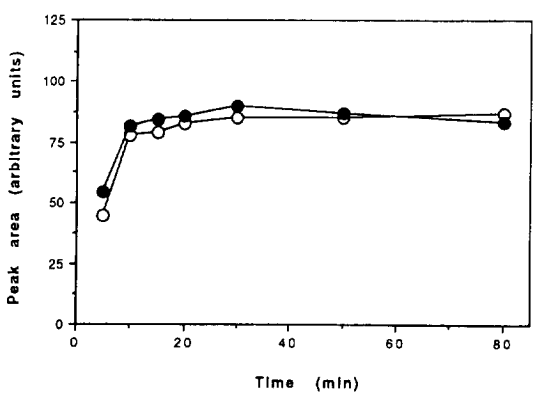

Fig. 5. Time courses of the derivatization reaction with $R(-)$-NBD-PyNCS at 55°C in the presence of 1% TEA. ○ = L-Isoleucine; ● = D-isoleucine.

the functional groups ($-NO_2$ and SO_2NMe_2) at 4-position in the benzofurazan structure would be expected to be negligible. Because the derivatization reaction occurs at a site well removed from the 4-position [17,18]. Based upon the above observations, derivatization at 55°C for 10 min in water–acetonitrile (1:1) in the presence of 1% TEA is recommended for the preparation of diastereomers of the amino acid enantiomers.

Table 1 shows the maximum fluorescent wavelengths (excitation and emission) and the RFIs of the tyrosine derivatives. The fluorescence properties of the derivatives are dependent upon the solvent in the medium. The maximal excitation and emission wavelengths appear to be dependent on the strength of hydrophobicity of the solvents as suggested by the shift towards blue with increasing hydrophobicity of the medium. On the other hand, the fluorescence intensity is not so easily explained and differs in each solvent. The RFIs of the derivatives with NBD-PyNCS and DBD-PyNCS are higher in aprotic organic solvents (AcOEt and CH_2Cl_2, etc.) than in protic solvents such as methanol and ethanol. The fluorescence maxima (excitation and emission wavelengths) and the fluorescence intensities were almost the same as those with and without 0.1% TFA in water–acetonitrile. Essentially the same fluorescence characteristics were also obtained with the reagent themselves (data not shown); therefore, the fluorescence seems to be due to the benzofurazan structure of the reagents.

The separation of the enantiomers of 18 amino acids, which are usually found in acid hydrolysates of peptides, enzymes and proteins, was tried by reversed-phase HPLC after derivatization with the chiral fluorescent reagents. In the present experiments, column temperature at 40°C was selected to obtain reproducible results of retention time of the derivatives. The capacity factor (k'), separation factor (α) and resolution value (R_s) for each pair of the thiocarbamoyl-amino acids derived from $R(-)$-NBD-PyNCS and $R(-)$-DBD-PyNCS are listed in Tables 2 and 3. The diastereomers derived from neutral and/or aromatic amino acids were well resolved

Table 1
Fluorescence properties of tyrosine derivatives with the reagents in various solvents

Solvent	With DBD-PyNCS			With NBD-PyNCS		
	Excitation (nm)	Emission (nm)	RFI	Excitation (nm)	Emission (nm)	RFI
CH_3CN–water (1:1)	458	540	13.5	488	528	36
CH_3CN	458	541	100	480	525	100
MeOH	459	536	33	475	525	56
EtOH	460	537	40	475	525	43
DMF	464	541	99	485	530	75
Acetone	458	536	132	475	521	100
AcOEt	456	532	120	470	520	200
n-Hexane	436	515	131	450	508	172
CH_2Cl_2	456	528	127	470	512	194

MeOH = Methanol; EtOH = ethanol; DMF = dimethylformamide; AcOEt = ethyl acetate; RFI = relative fluorescence intensity.

by reversed-phase HPLC with water–acetonitrile containing TFA as the eluent. However, the resolution of the basic amino acid derivatives was inadequate with this solvent mixture. Attempts to resolve acidic amino acids such as asparagic acid and glutamic acid were also unsuccessful. The extent of separation varies with different amino acids and the derivatization reagents, NBD-PyNCS and DBD-PyNCS. The difference of R_s values with DBD-PyNCS is smaller than that with NBD-PyNCS (ca. 2.57–0.68 versus ca. 3.57–0.55). Therefore, DBD-PyNCS appears to be a superior reagent than NBD-PyNCS for the resolution of amino acids. When *R*(−)-isomers of the reagents are used as the derivatization reagents, the diastereomers of D-amino acids are eluted faster than those of L-amino acids. Opposite elution order is ob-

Table 2
Enantiomeric separation of amino acids after derivatization with *R*(−)-NBD-PyNCS

Amino acid	k'		α	R_s	Eluent
	D-Isomer	L-Isomer			
Alanine	10.71	11.19	1.05	0.55	a
Cystine	3.34	4.03	1.21	1.13	e
Isoleucine	10.94	12.30	1.12	1.78	c
Leucine	5.61	6.29	1.12	1.04	d
Lysine	7.91	9.13	1.15	1.65	d
Methionine	6.37	6.92	1.09	0.97	c
Norleucine	12.17	13.75	1.13	1.85	c
Proline	4.87	6.06	1.20	1.50	b
Phenylalanine	12.52	14.90	1.19	3.57	c
Threonine	7.77	8.25	1.06	0.71	a
Tryptophan	5.95	7.05	1.18	1.54	d
Tyrosine	9.20	10.00	1.09	1.20	b
Valine	6.11	6.81	1.12	1.62	c

Eluents: CH_3CN–water mixtures containing 0.05% TFA. CH_3CN contents: a = 25%; b = 30%; c = 35%; d = 40%; e = 45% (v/v).

Table 3
Enantiomeric separation of amino acids after derivatization with $R(-)$-DBD-PyNCS

Amino acid	k'		α	R_s	Eluent
	D-Isomer	L-Isomer			
Alanine	4.61	4.96	1.08	0.85	c
Cystine	4.28	5.39	1.26	1.85	e
Histidine	4.03	4.39	1.09	0.68	a
Isoleucine	7.95	9.40	1.18	2.29	d
Leucine	8.02	9.31	1.16	2.04	d
Lysine	7.11	8.24	1.16	2.06	e
Methionine	4.93	5.51	1.12	1.45	d
Norleucine	5.17	5.98	1.16	1.72	e
Proline	5.61	6.15	1.10	1.48	c
Phenylalanine	13.09	13.95	1.07	2.29	e
Threonine	6.46	7.03	1.09	1.06	b
Tryptophan	6.12	8.42	1.38	2.57	e
Tyrosine	5.96	6.54	1.10	0.93	c
Valine	10.40	10.96	1.05	1.87	c

Eluents: CH_3CN–water mixtures containing 0.05% TFA. CH_3CN contents: a = 25%; b = 30%; c = 35%; d = 40%; e = 45% (v/v).

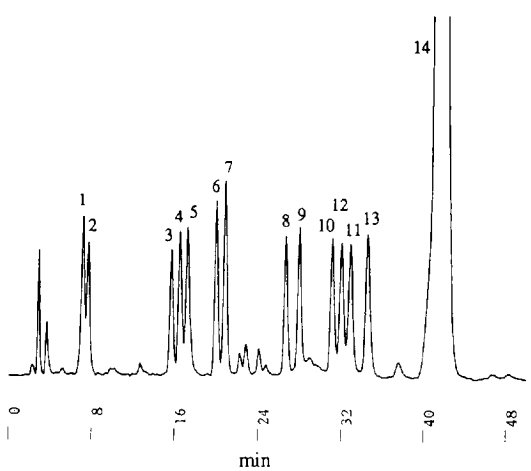

Fig. 6. Chromatograms of thiocarbamoyl-amino acid diastereomers derived from $R(-)$-DBD-PyNCS. Peaks: 1 = D-histidine; 2 = L-histidine; 3 = glycine; 4 = D-threonine; 5 = L-threonine; 6 = D-proline; 7 = L-proline; 8 = D-valine; 9 = L-valine; 10 = D-leucine; 11 = L-leucine; 12 = D-tryptophan; 13 = L-tryptophan; 14 = $R(-)$-DBD-PyNCS. Each peak except the reagent is corresponding to 20 pmol. Eluents: (A) water containing 0.05% TFA; (B) acetonitrile containing 0.05% TFA; isocratic elution of A–B (75:25) for 5 min, linear gradient elution from A–B (75:25) to A–B (60:40) for 20 min, and then isocratic elution of A–B (60:40) for 25 min. Other HPLC conditions are given in the Experimental section.

served with use of $S(+)$-isomers of the reagents. Fig. 6 shows a separation by reversed-phase chromatography of the thiocarbamoyl-amino acids formed from $R(-)$-DBD-PyNCS. A sole peak corresponding to glycine derivative appeared in the chromatogram because there is no asymmetric carbon in the glycine structure. Each pair of the other amino acids tested were clearly separated by the linear gradient elution with water–acetonitrile containing 0.05% TFA. The largest peak (ca. 41 min) observed in the chromatogram is the unreacted reagent; while other small peaks seem to be caused by impurities in the analytes. The total analysis of the enantiomers of 18 amino acids, which are usually produced by acid hydrolysis of polypeptides, are under study in our laboratory.

4. Conclusions

Amino acid enantiomers derivatized under the mild reaction conditions with DBD-PyNCS and NBD-PyNCS enantiomers are separated by the reversed-phase chromatography. The resulting thiocarbamoyl-amino acids exhibit good stability

and strong fluorescence at long wavelengths. The sensitivity of the proposed methods with NBD-PyNCS and DBD-PyNCS is almost comparable with the reported methods using OPA/thiols and Edman-type reagents. However, our methods offer superior resolution of amino acid enantiomers and simplicity of the operation. In addition, the stability of the derivatives is better than that of the derivatives with OPA/chiral thiol. The excitation maxima at around 488 nm for the thiocabamoyl-amino acids with NBD-PyNCS is suitable for the determination with argon-ion laser-induced fluorescence detection [19,20]; whereas those with DBD-PyNCS are detectable at ca. fmol–amol range with peroxyoxalate chemiluminescence detection [21]. Hence, the proposed methods with DBD-PyNCS and NBD-PyNCS may serve for enantiomeric quantification of chiral amino acids in real samples. Chiral sequence analysis of peptides and proteins might be another important application, as described in the previous paper [13]. As well as the determination of antipodo enantiomer in each amino acid, the sequence analysis of polypeptides is currently in progress.

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