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The facile HPLC enantioresolution of amino acids, peptides on naphthylethylcarbamate-β-cyclodextrin bonded phases using the acetonitrile-based mobile phase after their pre-column derivatization with phenyl isothiocyanate: factors that affect the resolution

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Summary. A variety of α-amino acids are enantioresolved for the first time on naphthylethylcarbamate-β-cyclodextrin bonded phases (i.e., SN- and RN-β-CD) using the acetonitrile-based mobile phase after their pre-column derivatization with phenyl isothiocyanate in alkaline medium. The resolution is better obtained on RN-β-CD phase and fails to reproduce if the amino acid is *N*-benzoylated or *N*-carbobenzyloxylated under the same chromatographic conditions. The enhanced resolution is believed to be due to the re-location of the hydrogen receptor site from sulfur to nitrogen on the isothiocyanyl fragment of derivatizing reagent, which in turn changes the enantioselectivity. Also, the sulfur atom is larger in size and subject to steric hindrance more significantly in comparison with oxygen. The carboxyl group of amino acid is essential toward a satisfactory resolution. The position of the amino group on the backbone affects the resolution as well. Finally, the resolution is either not observed or unsatisfactory in the reversed- or normal phase mode for most of the amino acids examined in this study.

Keywords: Phenyl isothiocyanate – Amino acids – Peptides – Enantioresolution

Introduction

During the past decade, the enantioresolution of optically active molecules has interested many analytical chemists, leading to the development and commercialization of several chiral stationary phases (CSPs) (Armstrong et al., 1985; Oi et al., 1986; Shinbo et al., 1987; Okamoto et al., 1988; Jadaud et al., 1989; Miwa et al., 1990; Pirkle et al., 1991; Armstrong et al., 1994). However, resolving chiral compounds is still challenging and many important enantiomers remain unresolved (e.g., 4-(2-bromoacetamide)-TEMPO, an ESR probe). In general, a pair of enantiomers can be HPLC resolved without derivatization on a deliberately chosen chiral selector, which could be immobilized on silica gel or introduced to the mobile phase as an additive (Armstrong et al., 1993a). The resolution can be readily improved by changing the composition of the mobile phase (Menges et al., 1990; Chen, 2002). For those difficult to be resolved in the native form, chemical derivatization with an electrophilic tagging reagent prior to chromatography is usually an alternative (Zukowski et al., 1992; Pawlowski et al., 1993; Chen et al., 1994). Basically, these approaches can be employed to obtain or improve enantioresolution independently or complementally to one another. For example, poor resolution for enantiomers on a specific chiral column usually can be improved by changing the structure of enantiomers through chemical derivatization or by modifying the composition of mobile phase before switching to another chiral column for different enantioselectivity. A typical application involving this technique is the separation and determination of the optical purity of amino acids derivatized with a highly fluorescent tag at trace levels (Zukowski et al., 1992; Pawlowski et al., 1993). Altering the enantioselectivity through modifying the chiral selector is considered to be tedious, costly and impractical.

Since their successful commercialization in 1983, cyclodextrin-bonded CSPs have proven to be useful for separating enantiomers. Most applications were initially carried out either in the normal phase or reversed-phase mode (Menges et al., 1990). Under these modes, the size and solubility of the analyte in the mobile phase usually controlled

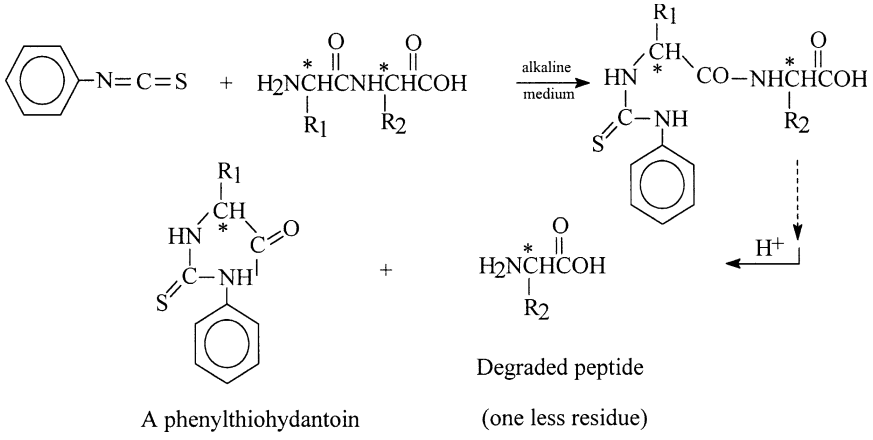


Fig. 1. The phenyl isothiocyanate derivatization chemistry

the separation. With the development of acetonitrile-based mobile phases, the number of enantiomers that can be resolved on cyclodextrin-based CSPs has been dramatically increased (Armstrong et al., 1992; Chang et al., 1993). In this mobile phase, it was found that analytes separated on cyclodextrin bonded CSPs were relatively large in size with the stereogenic centers surrounded by functional groups. The chiral recognition mechanism is believed to result from the external association of analyte and cyclodextrin (i.e., noninclusion complexation) and is different from that dominating in the water-based mobile phase (i.e., inclusion complexation) (Armstrong et al., 1992). A typical example is the resolution of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) derivatized amino acids, peptides, dipeptides and tripeptides with structures that are highly ordered (Pawlowski et al., 1993). In general, enantiomers of these analytes are poorly resolved on cyclodextrin-bonded CSPs in the normal or reversed-phase mode.

Enantiomers of native amino acids and amino alcohols are water-soluble and consequently must be resolved in a water-based mobile phase (Armstrong et al., 1993b; 1993c). However, these analytes become more hydrophobic after reacting with a highly electrophilic compound such as dansyl chloride. The resulting increase of solubility in organic solvent allows separation on cyclodextrin bonded CSPs using acetonitrile-based, but not water-based mobile phase (Lee et al., 1992). Previous studies have indicated that enantioselectivity of analyte is affected to some extent by the moiety pre-column tagged, which in turn influences the resolution (Chen et al., 1994; Chen, 1996). In light of these findings, separation for enantiomers that are difficult to resolve in native form can be improved by pre-column derivatization with a proper tagging moiety.

In its various modifications, however, the most widely used method of *N*-terminal residue analysis seems to be one introduced in 1950 by Pehr Edman (Edman, 1967; Stark, 1971). This is based on the reaction between an amine group and phenyl isothiocyanate (PHES) to form a substituted thiourea. Mild hydrolysis with hydrochloric acid selectively removes *N*-terminal residue as the phenyl thiohydantoin as shown in Fig. 1. The great advantage of this method is that it leaves the rest of the peptide chain intact, so that the analysis can be repeated to sequence the peptide chain. Besides the hydrophobicity, the detection limit is expected to be lowered due to the introduced chromophores. The other advantage for carrying out resolution with organic solvent (e.g., acetonitrile) as the mobile phase is the life span of column can be extended.

In this report, a variety of α-amino acids are chemically derivatized with phenyl isothiocyanate, an electrophilic tagging reagent used in protein sequencing, in alkaline medium before being enantioresolved on naphthylethylcarbamate-β-cyclodextrin bonded CSPs for the first time using the acetonitrile-based mobile phase. Under the same chromatographic conditions, the resolution is compared to that for *N*-benzoylated or *N*-carbobenzyloxylated amino acids to rationalize the mechanism involving in the enhanced enantioresolution for phenyl isothiocyanated amino acids. The factors that affect the resolution will be discussed as well.

Experimental

Apparatus

The RN- and SN-β-CD CSPs (250 × 4.6 mm i.d., 5 μm particle diameter) used for all the separations carried out at ambient temperature (~28°C) and at a flow rate of 1.0 mL/min were obtained from Advance Separation Technologies (Whippany, NJ, USA). The HPLC system used in this study is a Hitachi model L-7100 linked to a D-2500 Chromatopac data station and a variable wavelength UV detector.

Chemicals

All chemicals used in this study were purchased from Sigma (St. Louis, MO, USA) and Aldrich (Milwaukee, WI, USA). All HPLC grade solvents (acetonitrile, methanol, triethylamine, glacial acetic acid, etc.) were obtained

from Fisher Scientific (Pittsburgh, PA, USA) and Merck Taiwan Ltd. (Taipei, Taiwan, ROC). Double filtered and distilled water was used in all cases.

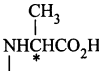
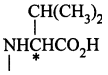
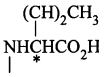
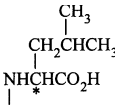
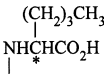
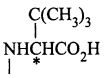
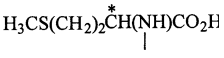
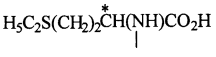
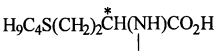
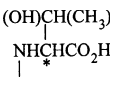
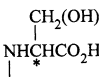
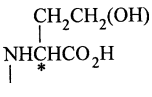
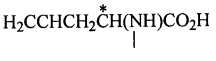
Methods

The purchased enantiomers were dissolved in proper solvents for chemical derivatization according to the procedure described in Fig. 1 before being injected for HPLC separation. The resulting solution contained native enantiomers due to the incomplete derivazation and was purified through ethyl ether extraction. The ethyl ether layer was collected and further concentrated under reduced pressure before being injected for HPLC analysis.

Results and discussion

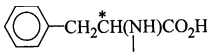
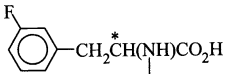
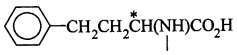
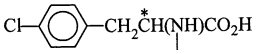
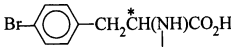
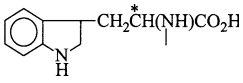
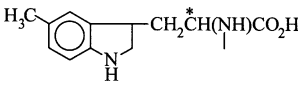
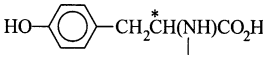
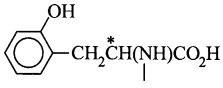
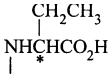
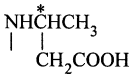
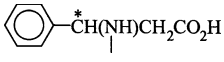
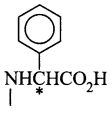
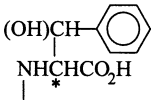
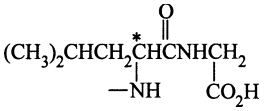
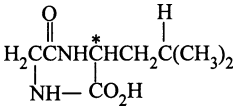
The chromatographic data for the enantiomeric resolution of phenyl isothiocyanated α-amino acids on RN- and SN-

Table 1. The chromatographic data for the resolution of phenyl isothiocyanated amino acids on naphthylethylcarbamate-β-cyclodextrin chiral stationary phases using the acetonitrile-based mobile phase

Compound	Structure	CSP[a]	k′[b]	α[b]	R_s[b]	Mobile phase[c]
Alanine		RN	3.30	1.52	4.00	A
		SN	2.88	1.07	1.02	A
			1.53	1.09	0.96	B
Valine		RN	2.46	1.15	1.32	A
		SN	1.19	1.05	0.65	B
Norvaline		RN	2.62	1.43	3.58	A
		SN	2.31	1.14	1.31	A
			1.21	1.13	1.24	B
Leucine		RN	2.78	1.74	5.31	A
		SN	1.38	1.60	4.77	B
Norleucine		RN	2.66	1.53	3.85	A
		SN	1.24	1.33	1.74	B
tert-Leucine		RN	2.61	1.28	2.45	A
		SN	2.54	1.19	1.80	A
			1.41	1.15	1.23	B
Methionine		RN	2.97	1.40	3.46	A
		SN	2.41	1.19	1.79	A
			1.27	1.19	1.64	B
Ethionine		RN	3.14	1.43	2.94	A
		SN	2.52	1.18	1.80	A
			1.37	1.20	1.75	B
Buthionine		RN	3.49	1.32	3.33	A
		SN	2.60	1.13	1.20	A
			1.40	1.13	1.22	B
Threonine		RN	2.47	1.15	1.28	A
		SN	1.99	1.12	1.22	A
			1.07	1.13	1.01	B
Serine		RN	4.25	1.11	1.10	A
Homoserine		RN	4.69	1.04	0.71	A
2-Amino-4-p-entenoic acid		SN	1.11	1.09	0.61	B

(*continued*)

Table 1 (*continued*)

Compound	Structure	CSP[a]	k′[b]	α[b]	R_s[b]	Mobile phase[c]
Phenylalanine		RN	2.79	1.80	5.86	A
			*4.75	1.08	0.95	A
		SN	1.45	1.55	3.75	B
m-Fluorophenylalanine		RN	2.24	1.19	2.00	A
Homophenylalanine		RN	5.13	1.76	5.00	A
		SN	3.92	1.58	4.67	A
p-Chlorophenylalanine		SN	2.69	1.09	0.97	A
			1.47	1.05	0.66	B
p-Bromophenylalanine		SN	3.05	1.12	1.37	A
			1.58	1.14	0.92	B
Tryptophan		RN	5.93	1.18	1.82	A
		SN	4.86	1.07	0.95	A
			2.39	1.08	0.95	B
5-Methyltryptophan		RN	5.45	1.21	2.14	A
		SN	4.60	1.09	1.10	A
			2.25	1.10	1.07	B
Tyrosine		RN	6.61	1.08	0.90	A
m-Tyrosine		RN	5.56	1.17	1.75	A
		SN	4.73	1.08	1.03	A
			2.15	1.09	0.95	B
α-Amino-n-butyric acid		RN	2.87	1.27	2.55	A
		SN	2.53	1.05	0.71	A
β-Amino-n-butyric acid		RN	3.47	1.05	0.75	A
3-Amino-3-phenylpropionic acid		RN	3.05	1.19	1.17	A
		SN	1.47	1.05	0.65	B
Phenylglycine		RN	2.34	1.56	4.67	A
		SN	1.07	1.20	1.30	B
3-Phenylserine		RN	1.65	1.47	2.90	A
		SN	0.73	1.53	3.53	B
Leucylglycine		RN	4.17	1.23	2.12	B
		SN	3.70	1.09	1.12	B
Glycylleucine		RN	11.19	1.35	3.10	A
			5.11	1.38	3.30	B
		SN	4.77	1.22	2.60	B

(*continued*)

Table 1 (*continued*)

Compound	Structure	CSP[a]	k′[b]	α[b]	R_s[b]	Mobile phase[c]
Leucylglycyl glycine	$(CH_3)_2CHCH_2\overset{*}{C}HCNHCH_2CONHCH_2CO_2H$ (O; —NH)	RN	7.49	1.27	2.30	B
		SN	5.86	1.19	1.60	B
Alanylglycine	$CH_3\overset{*}{C}HCNHCH_2CO_2H$ (O; —NH)	RN	4.83	1.06	0.84	B
Alanylglycyl glycine	$CH_3\overset{*}{C}HCNHCH_2CONHCH_2CO_2H$ (O; —NH)	RN	7.65	1.27	2.19	B
		SN	7.67	1.13	1.59	B

[a] SN and RN CSPs stand for (*S*)-, (*R*)-naphthylethylcarbamate-β-cyclodextrin chiral stationary phases, respectively
[b] The selectivity factor, α, is equal to $\frac{k_2'}{k_1'}$ and resolution factor, R_s, is equal to $2(tr_2 - tr_1)/(W_2 + W_1)$ and capacity factor, k′, is equal to $(t_r - t_0)/t_0$. The chromatographic data marked with the asterisk are for the *N*-benzoylated phenylalanine
[c] Mobile phase is a solvent mixture of A: 495 ACN/5 MeOH/1 HOAC/1 TEA, B: 480 ACN/20 MeOH/1 HOAC/2 TEA by volume, (v/v). The ACN, MeOH, HOAC and TEA are abbreviations for acetonitrile, methanol, acetic acid and triethylamine, respectively

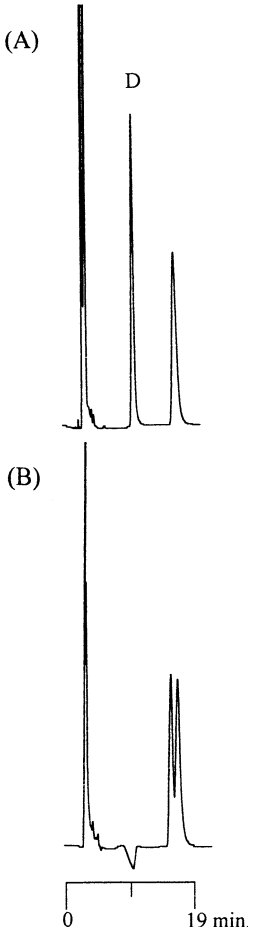


Fig. 2. Chromatograms showing the enantioresolution of (**A**) phenyl isothiocyanated phenylalanine and (**B**) *N*-benzoylated phenylalanine on RN-β-CD bonded phase using the acetonitrile-based mobile phase of 495 ACN/5 MeOH/1 HOAC/1 TEA by volume, (v/v). As can be seen, phenylalanine is much better resolved in phenyl isothiocyanate form under the same chromatographic conditions

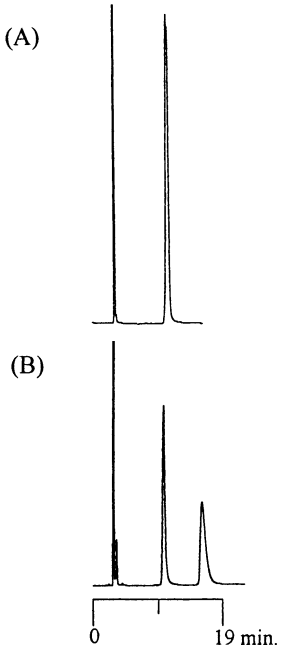


Fig. 3. Chromatograms showing the enantioresolution of phenyl isothiocyanated leucine (**A**) on SN-β-CD and (**B**) on RN-β-CD bonded phases bonded phases using the acetonitrile-based mobile phase of 495 ACN/5 MeOH/1 HOAC/1 TEA by volume, (v/v). As can be seen, phenyl isothiocyanated leucine is much better resolved on RN-β-CD under the same chromatographic conditions

β-CD bonded phases using the acetonitrile-based mobile phase are listed in Table 1. As can be seen, most amino acids are much better than baseline resolved in the derivatized form using a single acetonitrile-based mobile phase. In general, the resolution is better obtained on RN-β-CD phase and fails to reproduce if the amino

acid is *N*-benzoylated or *N*-carbobenzyloxylated under the same chromatographic conditions. A typical chromatogram for the resolution of PHES-Phe is shown in Fig. 2(A). Under the same chromatographic conditions, *N*-benzoylated phenylalanine is poorly resolved as shown in Fig. 2(B). Note that these tagging reagents are highly similar in structure. It is believed that the enhanced resolution is due to the re-location of hydrogen receptor site from sulfur (which is the oxygen atom in the case of *N*-benzoyl and *N*-carbobenzyloxyl reagents) to the nitrogen atom on the isothiocyanyl fragment of the derivatizing reagent. Note that sulfur is also larger in size and subject to steric hindrance more significantly in comparison with oxygen. Figure 3 shows the resolution of PHES-Leu on SN-β-CD (A) and on RN-β-CD (B) phases under the same chromatographic conditions, which clearly indicates that the resolution obtained on RN-β-CD phase is much better. This conclusion also holds for other amino acids phenyl isothiocyanated and examined in this study.

It appears that the resolution of phenyl isothiocyanated amino acids on RN- and SN-β-CD bonded phases is relatively insensitive to the size effect as compared to that on teicoplanin phase (Chen S, 2003). This can be seen in the resolution of PHES-Leu, PHES-Gly-Leu and PHES-Gly-Gly-Leu under the same chromatographic conditions. Due to the glycyl moiety, the stereogenic center of PHES-Gly-Leu and PHES-Gly-Gly-Leu is extended away from the phenyl isothiocyanate by two or four carbon atoms. However, the resolution is still obtained for these two analytes. As expected, the resolution of dipeptides, and tripeptides with two stereogenic centers and with high similarity in structure was observed as well. Typical chromatograms showing the resolution of PHES-Leu-Val and PHES-Leu-Phe on RN- and SN-β-CD phases are in Figs. 4 and 5, respectively. The corresponding data are summarized in Table 2. As can be seen, the resolution is better obtained on RN-β-CD phase under the same chromatographic condition in both cases. All these results suggest that chiral recognition for the resolution on RN- and SN-β-CD selectors under the elution of acetonitrile-based mobile phase is through external association. The $\pi-\pi$ interaction seems to be important since no resolution is obtained on native β-CD phase under the same chromatographic conditions.

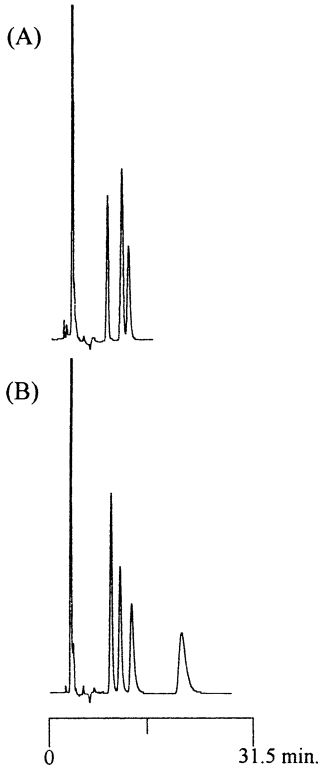


Fig. 4. Chromatograms showing the enantioresolution of phenyl isothiocyanated Leu-Val (**A**) on SN-β-CD and (**B**) on RN-β-CD bonded phases bonded phases using the acetonitrile-based mobile phase of 480 ACN/20 MeOH/1 HOAC/2 TEA by volume, (v/v). As can be seen, four enatiomers of phenyl isothiocyanated Leu-Val are fully resolved on RN-β-CD under the same chromatographic conditions

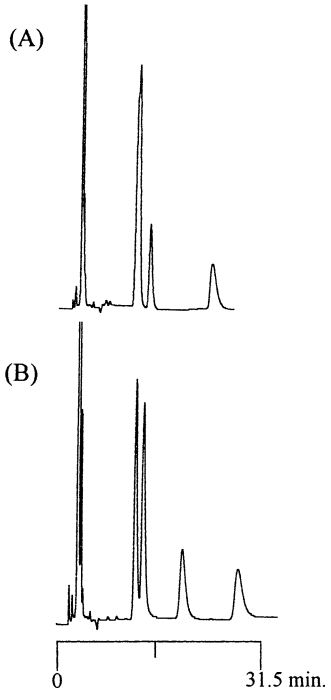
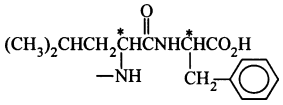
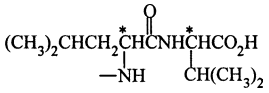
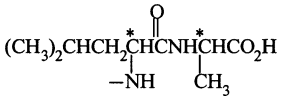
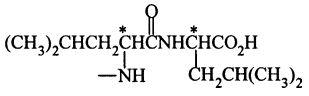
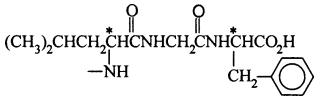
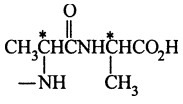
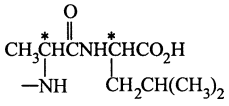
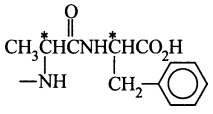


Fig. 5. Chromatograms showing the enantioresolution of phenyl isothiocyanated Leu-Phe (**A**) on SN-β-CD and (**B**) on RN-β-CD bonded phases bonded phases using the acetonitrile-based mobile phase of 480 ACN/20 MeOH/1 HOAC/2 TEA by volume, (v/v). As can be seen, four enatiomers of phenyl isothiocyanated Leu-Phe are better resolved on RN-β-CD under the same chromatographic conditions

Table 2. The chromatographic data for the resolution of phenyl isothiocyanated di- and tripeptides on naphthylethylcarbamate-β-cyclodextrin chiral stationary phases using the acetonitrile-based mobile phase

Compound[a]	Structure ($R_1 =$)[b]	CSP	k′[c]				Mobile phase[d]
Leu-Phe		RN	3.24	3.65	5.77	8.81	A
		SN	3.01	3.13	3.81	7.17	A
Leu-Val		RN	2.28	2.77	3.41	6.09	A
		SN	2.00	2.77	3.15		A
			4.43	6.35	7.62		B
Leu-Ala		RN	2.80	3.03	3.17	4.99	A
		SN	2.36	2.87	3.18		A
Leu-Leu		RN	2.41	2.61	3.13	6.00	A
		SN	2.16	2.81	3.65		A
Leu-Gly-Phe		RN	6.60	6.99	7.65	9.74	A
		SN	5.50	5.98	7.64	10.65	A
Ala–Ala		RN	3.32	3.42	3.56	3.90	A
			7.95	7.91	8.40	9.25	B
		SN	3.01	3.34			A
Ala-Leu		RN	2.85	3.02	3.53	3.83	A
		SN	2.78	3.33	3.47		A
Ala-Phe		RN	4.24	4.70	4.88	11.21	A
		SN	3.88	4.13	4.63	9.28	A

[a] Phenyl isothiocyanate-$R_1 =$  . The RN and SN CSPs stand for the (*R*)-, (*S*)-naphthylethylcarbamate-β-cyclodextrin chiral stationary phases, respectively

[b] Only the structure of peptides is shown

[c] Capacity factor, k′, is equal to $(t_r - t_0)/t_0$

[d] Mobile phase is either a mixture of A: 480 (ACN)/20 (MeOH)/1 (HOAC)/2 (TEA), or B: 495 (ACN)/5 (MeOH)/1 (HOAC)/1 (TEA) by volume (v/v)

The attempt has been made to resolve amino alcohols in derivatized form, whose structure is highly similar to that of the resolved amino acids. A typical example is the PHES-2-amino-3-methyl-1-butanol. As compared to PHES-valine, the only difference in structure is the hydoxyl group, instead of carboxyl group in PHES-valine. The resolution of PHES-2-amino-3-methyl-1-butanol turns out to be a failure indicating the role of carboxyl group on the analyte is essential toward a successful resolution. This holds for other amino alcohols such as 2-amino-1-propanol, which structure is similar to that of alanine as well. The other factor that affects the enantioresolution is the position of amino group on the analyte skeleton. This can be seen in the resolution of PHES-alanine. However, the resolution disappeared in PHES-3-aminoisobutyric acid under the same chromatographic conditions. These two analytes are highly similar in structure except for the position of amino group on the skeleton. The other example is the comparison of PHES-α-amino-n-butyric acid to the PHES-β-amino-n-butyric

acid. Under the same chromatographic conditions, only the PHES-α-amino-n-butyric acid was resolved.

Conclusion

The resolution of a variety of α-amino acids on naphthylethylcarbamate-β-cyclodextrin bonded chiral phases using the acetonitrile-based mobile phase after their pre-column derivatization with phenyl isothiocyanate in alkaline medium has been demonstrated. The resolution is considered to be much better as compared to that in *N*-benzoylated or *N*-carbobenzyloxylated form for a give amino acid under the same chromatographic conditions. The enhanced resolution is believed to be due to the re-location of the hydrogen receptor site from sulfur to nitrogen on the isothiocyanyl fragment of derivatizing reagent, which in turn changes the enantioselectivity. Besides the increase in the sensitivity due to the introduced chromophores, the other advantage for carrying out resolution with polar-organic mobile phase is the life span of column can be extended as there is no hydrolysis in the absence of water.

Acknowledgments

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