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The HPLC resolution of *N*-2,4-dinitrophenylated amino acids and peptides stereoisomers on naphthylethylcarbamate-β-cyclodextrin bonded phase using the acetonitrile-based mobile phase: evidence for the chiral recognition pattern

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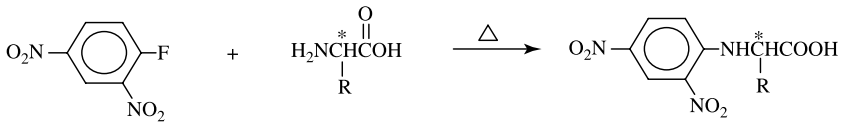
Summary. A facile method of enantioresolving a variety of α-amino acids and peptides on naphthylethylcarbamate-β-cyclodextrin bonded phases (i.e., SN- and RN-β-CDs) under the elution of acetonitrile-based mobile phase makes use of 2,4-dinitrofluorobenzene (DNFB) as the tagging reagent, which undergoes nucleophilic substitution by the free amino group in alkaline medium to give a *N*-2,4-dinitrophenyl (DNP) derivative. The resolution is better obtained on RN-β-CD phase and fails to reproduce on the intact β-cyclodextrin bonded phase under the same chromatographic conditions, which strongly suggests that the observed resolution should be due to the interaction of analyte with naphthylethylcarbamate moiety, not with the residual secondary hydroxyl groups on the β-cyclodextrin.

Keywords: 2,4-Dinitrofluorobenzene – Amino acid – Peptide – Enantioresolution – Chiral recognition pattern

Introduction

Initially, the intact cyclodextrin-bonded CSPs were developed to carry out resolution in the reversed-phase mode and have proven to be useful for separating enantiomers (Menges et al., 1990). In this mode, however, the size of the analyte usually controlled the separation as an inclusion complexation responsible for the resolution is formed. For example, analytes to be successfully resolved should have moieties about the sizes of benzene, naphthalene, and anthracene for tight inclusion complexation with α-, β-, γ-cyclodextrins (α-, β-, γ-CDs), respectively. Also the stereogenic center should be surrounded by functional groups nearing the mouth of CD cavity for hydrogen bonding. With the successful development of derivatized version of β-CD-based CSPs, application is expected to carry out in the normal phase mode for enantioresolving molecules large in size through more profound $\pi-\pi$ interactions on multimodal *R*- or *S*-naphthylethyl carbamated β-CD CSP (RN- or SN-β-CD) (Chang et al., 1986; Armstrong et al., 1990, 1991). For a CSP to be considered ''multimodal'', it needs to separate different classes of compounds in each mode. For those compounds with outstretched stereogenic centers upon forming inclusion complexation with the intact CD, successful enantioresolution can be achieved through hydrogen bonding with prolonging secondary hydroxyl groups on *R*,*S*-2-hydroxy propyl derivatized β-CD CSP (RSP-β-CD) (Chang et al., 1986). Note that a new stereogenic center is introduced to the chiral selector after derivatization and thus expected to form more profound interaction patterns.

The development of acetonitrile-based mobile phase also dramatically increases the number of enantiomers that can be resolved on intact or derivatized cyclodextrin-bonded CSPs (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 enantioresolution mechanism is believed to result from the external association (i.e., non-inclusion complexation) of analyte and cyclodextrin (i.e., intact or derivatized) and thus is not confined to the analyte's size and is quite different from that dominating in the water-based mobile phase (Armstrong et al., 1992). Typical examples include the resolution of 6-aminoquino-



Dissolved in acetonitrile before derivatizing reaction

Dissolved in alkaline medium (e.g., Na_2CO_3 or sat'd Borax solution)

N-2,4-Dinitrophenylated amino acid

Fig. 1. The 2,4-dinitrofluorobenzene derivatization chemistry

lyl-*N*-hydroxysuccinimidyl carbamate (AQC), 9-fluorenylmethyl chloroformate (FMOC) (Zukowski et al., 1992a, b), derivatized amino acids, peptides, dipeptides and tripeptides with structures that are highly ordered (Pawlowski et al., 1993). In general, these analytes are poorly resolved on cyclodextrin-bonded CSPs in the normal or reversed-phase mode.

Enantiomers of native amino acids are water-soluble and consequently must be resolved in a water-based mobile phase (Armstrong et al., 1993a, b). However, these analytes become more hydrophobic after reaction with a highly electrophilic compound as mentioned above. 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, 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 to alter the enantioselectivity.

A very successful method of identifying the N-terminal residue, introduced in 1945 by Frederick Sanger, makes use of 2,4-dinitrofluorobenzene (DNFB), which undergoes nucleophilic substitution by the free amino group to give a *N*-2,4-dinitrophenyl (DNP) derivative as described in Fig. 1. The derivatized peptide is hydrolyzed to the component amino acids, and the N-terminal residue, labeled by the 2,4-dinitrophenyl group in the heated acidic solution (not shown), is separated and identified. In the past, there has been several reports concerning with the use of DNP as the tagging reagent in resolving enantiomers with a variety of chiral selectors (Edwards et al., 1996; Ryu et al., 1998; Lämmerhofer et al., 1999; Mandl et al., 1999; Lämmerhofer et al., 2000; Franco et al., 2000). Note that amino acid is characterized by a dipolar ion structure and considered to be hydrophilic. However, the hydrophobicity increases after derivatization and the detection limit also is expected to be lowered due to the introduced chromophores. The other advantage for carrying out resolution with organic solvent (e.g., acetonitrile, methanol) as the mobile phase is the life span of column can be extended.

In this report, a variety of amino acids, peptides are chemically derivatized with 2,4-dinitrofluorobenzene 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 carried out on the intact β-cyclodextrin bonded CSP for comparison to rationalize the chiral recognition pattern involving in the resolution. The factors that affect the resolution will be discussed as well.

Experimental

Apparatus

The RN-, SN-β-CD and the intact β-CD CSPs (250×4.6 mm i.d., 5 μm particle diameter) used for all the separations carried out at ambient temperature ($\sim$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. The wavelength was set at 270 nm for all the separations.

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). The water treated with a Millipore water purifying system was used in all cases. The ACN, MeOH, HOAC, TEA and EE are abbreviations for acetonitrile, methanol, acetic acid, triethylamine and ethyl ether, respectively.

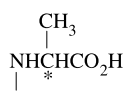
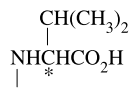
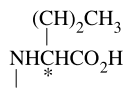
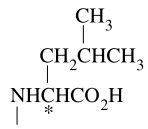
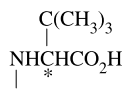
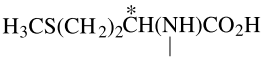
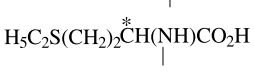
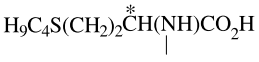
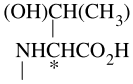
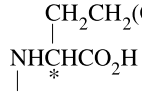
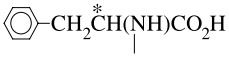
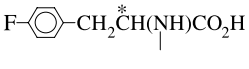
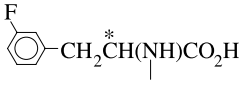
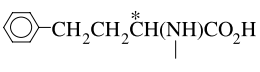
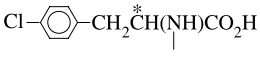
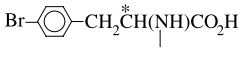
Methods

The purchased amino acids and peptides were dissolved in alkaline medium (e.g., sodium carbonate solution at 0.50 M) for chemical derivatization with derivatizing reagent in acetonitrile according to the procedure described previously (Chen, 1996). The resulting solution was then injected for HPLC analysis without further purification. The amount of *N*-2,4-dinitrophenylated analyte injected was estimated to be about 5×10^{-2} mg.

Results and discussion

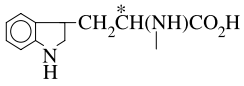
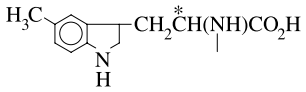
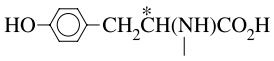
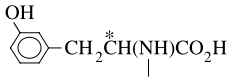
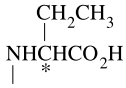
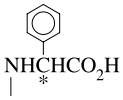
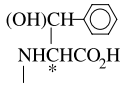
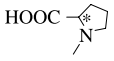
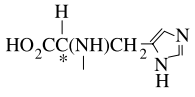
The chromatographic data for the enantiomeric resolution of *N*-2,4-dinitrophenylated (DNP) amino acids on RN- and SN-β-CD bonded phases using the acetonitrile-based mobile phase are listed in Table 1. As can be seen, most

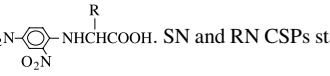
Table 1. The chromatographic data for the enantioresolution of *N*-2,4-dinitrophenylated amino acids on (*R*)-, (*S*)-naphthylethylcarbamate-β-cyclodextrin chiral stationary phases using the acetonitrile-based mobile phase

Compound	Structure[a]	CSP	k[b]	α[b]	R_s[b]	Mobile phase[c]
Alanine[d]		RN	1.83	1.05	0.69	A
Valine[d]		RN	1.54	1.10	1.12	A
Norvaline[d]		RN	1.47	1.07	0.89	A
Leucine[d]		RN	1.53	1.11	1.04	A
tert-Leucine		RN	2.63	1.24	2.07	A
		SN	2.31	1.08	0.95	A
Methionine[d]		RN	1.59	1.05	0.65	A
Ethionine		RN	1.68	1.08	0.89	A
		SN	1.31	1.10	0.97	A
Buthionine		RN	1.69	1.11	1.12	A
Threonine[d]		RN	1.22	1.08	0.82	A
			1.79	1.04	0.65	B
Homoserine[d]		RN	2.04	1.05	0.71	B
Phenylalanine[d]		RN	2.09	1.08	0.90	A
			2.59	1.07	0.95	B
		SN	1.64	1.09	0.90	A
p-Fluorophenylalanine		RN	1.71	1.19	1.79	A
			2.28	1.05	0.65	B
		SN	1.39	1.12	1.17	A
m-Fluorophenylalanine		RN	1.51	1.06	0.70	A
			2.27	1.06	0.88	B
Homophenylalanine		RN	2.97	1.07	0.93	A
			3.10	1.04	0.75	B
		SN	1.91	1.14	0.95	A
p-Chlorophenylalanine		RN	2.01	1.24	2.25	A
		SN	1.57	1.10	0.96	A
p-Bromophenylalanine		RN	2.20	1.28	2.50	A
		SN	1.69	1.12	1.21	A

(*continued*)

Table 1 (*continued*)

Compound	Structure[a]	CSP	k^b	α^b	$R_s{}^b$	Mobile phase[c]
Tryptophan[d]		SN	2.76	1.03	0.61	A
5-Methyltryptophan		RN	3.33	1.05	0.71	A
		SN	2.63	1.06	0.83	A
Tyrosine[d]		RN	1.93	1.09	0.91	A
			3.43	1.06	0.91	B
m-Tyrosine		RN	1.55	1.08	0.95	A
			3.16	1.06	0.94	B
α-Amino-*n*-butyric acid		RN	1.63	1.07	0.80	A
3-Amino-3-phenylpropionic acid		RN	2.45	1.22	2.35	A
2-Phenylglycine		RN	1.12	1.15	1.27	A
3-Phenylserine		RN	1.38	1.09	0.93	A
			1.11	1.05	0.65	A
Proline[d]		RN	2.07	1.31	2.74	A
		SN	1.83	1.19	1.41	A
Histidine[d]		RN	5.18	1.17	1.55	A

[a] The general structure for 2,4-dinitrophenyl tagged amino acid is  . SN and RN CSPs stand for (*S*)-, (*R*)-naphthylethylcarbamate-β-cyclodextrin chiral stationary phases, respectively

[b] The selectivity factor, α, is equal to 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 are for the *N*-benzoylated phenylalanine

[c] Mobile phase is a solvent mixture of A: 495 ACN/5 MeOH/1 HOAC/1 TEA, B: 95 MeOH/5 EE/0.4 HOAC/0.2 TEA by volume, (v/v). The ACN, MeOH, HOAC, TEA and EE are abbreviations for acetonitrile, methanol, acetic acid, triethylamine and ethyl ether, respectively

[d] The optical pure enantiomers of these amino acids are commercially available. The D-enantiomer of amino acids examined is eluted first except for phenylalanine

amino acids in the derivatized form are resolved using a single acetonitrile-based mobile phase on RN-β-CD phase. The D-enantiomer of the amino acids examined in this study is eluted first except for phenylalanine.

In general, the resolution is better obtained on RN-β-CD phase as compared to that on SN-β-CD phase and fails to reproduce on the intact β-CD phase under the same chromatographic conditions for all analytes examined in this study. Typical chromatograms for the resolutions of DNP-Pro and DNP-His under the same chromatographic conditions are shown in Fig. 2. It is thought that the strong π–π interaction between the 2,4-dinitrophenyl tagging moiety and the naphthylethylcarbamate group on the chiral selector is responsible for the resolution observed on the RN-β-CD phase according to the recognition mechanism proposed by Pirkle (Pirkle et al., 1991). The nearby polar nitrogen atoms, carbonyl groups on both the analyte and the naphthylethylcarbamate moiety of chiral

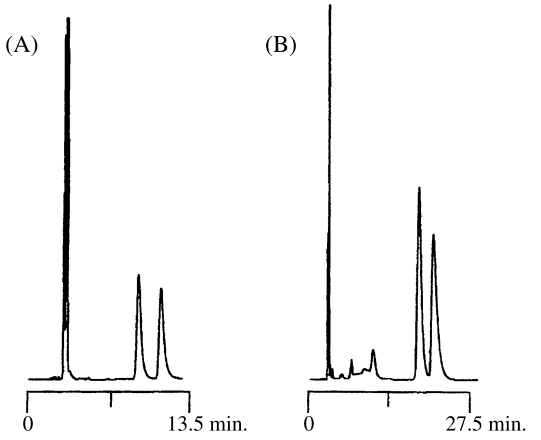


Fig. 2. Chromatograms showing the enantioresolution of 2,4-dinitrophenylated (**A**) proline and (**B**) histidine on RN-β-CD bonded phase using the acetonitrile-based mobile phase of 480 ACN/20 MeOH/1 HOAC/2 TEA by volume, (v/v)

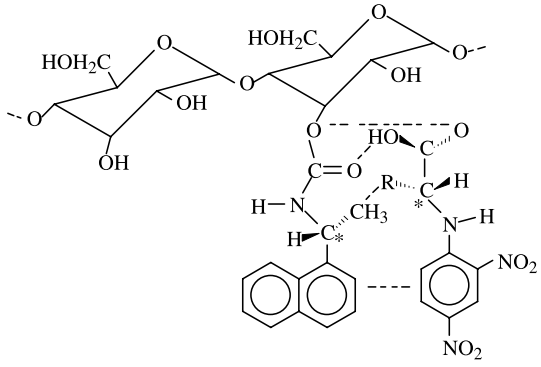


Fig. 3. The oversimplified interaction model between the analyte and the naphthylethylcarbamate group on the CD chiral selector. Note that the structure of chiral selector is only partially shown and the interaction model is not drawn to the scale

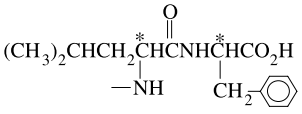
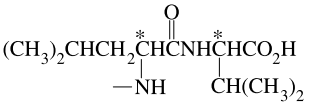
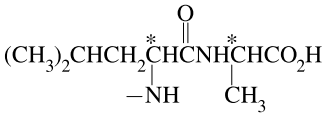
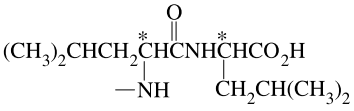
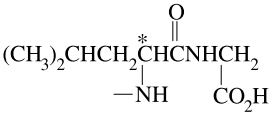
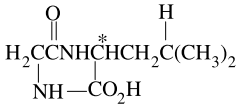
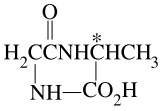
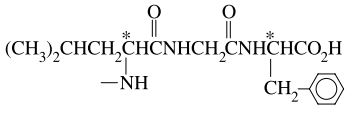
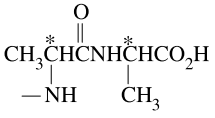
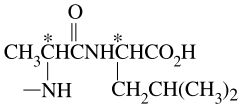
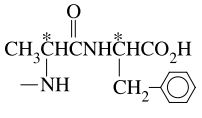
selector also can form additional hydrogen bonding, dipole–dipole interactions leading to the chiral resolution. The simplified interaction model between the analyte and the chiral selector is described in Fig. 3. As can be seen, the analyte is not long enough to reach the residual secondary hydroxyl groups on RN-β-CD phase for interactions. Otherwise, the resolution should have been observed on the intact β-CD phase under the same chromatographic conditions. As for on the SN-β-CD phase, the resolution is not satisfactory since RN- and SN-β-CD phases have identical chemical constitution, but differ as regards the arrangement of the atoms or groups in space leading to the diverse, however, almost complementary enantioselectivity.

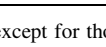
In the case of peptides (i.e., dipeptides and tripeptides), the resolution is generally improved since the analyte is long enough to reach these residual secondary hydroxyl groups on the β-cyclodextrin for additional interactions and found relatively insensitive to the size effect as in the resolution of phenyl isothiocyanated amino acids reported previously (Chen, 2003). The chromatographic data are summarized in Table 2 and Fig. 4 shows a typical chromatogram for the resolution of DNP-Gly-Leu. Ignoring the extension of stereogenic center away from the 2,4-dinitrophenyl group by two carbon atoms due to the glycine, the resolution is still significantly enhanced as compared to that for DNP-Leu under the optimized conditions. The same argument also holds in the case of DNP-Gly-Ala and DNP-Ala. Similarly, the resolution of dipeptides, and tripeptides with two stereogenic centers was observed as well. Typical chromatograms showing the resolutions of DNP-Ala-Leu and DNP-Ala-Phe on RN-β-CD phase are in Fig. 5. Figures 6 and 7 show the resolutions of DNP-Ala-Phe and DNP-Leu-Ala on SN-β-CD phase under the same chromatographic conditions. The corresponding data are also summarized in Table 2 for comparison. As can be seen, the resolution is better obtained on RN-β-CD phase under the same chromatographic condition in both cases and can not be reproduced on the intact β-CD phase. These results all 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 π–π interaction appears to be extremely important and mainly responsible for the resolution observed, which accounts for why the resolution is not reproducible on the intact β-CD phase under the same chromatographic conditions.

When the side-chain group becomes bulky as in the cases of methionine/ethionine/buthionine, leucine/*tert*-leucine and norvaline/valine, the resolution deteriorates with the enantioselectivity, α, remains almost unchanged suggesting the kinetic part of (i.e., the adsorption/desorption rates) resolution is influenced, not the thermodynamics. However, the opposite is observed in the cases of phenylalanine/p-bromophenylalanine. It is thought that the size of the side-chain group is bulky enough to signify the hindrance, which may result in the additional attractive interactions with chiral selector leading to the increasing retention factor and the enantioselectivity. In this case, the attractive interaction is just as important as the repulsive interaction (Pirkle et al., 1991). Note that the aromatic side-chain group of the amino acids may also enhance the chiral recognition resulting in the change of both retention and selectivity factors in a way of forming the π–π interaction with the chiral selector.

It has been indicated that the role of carboxyl group on the analyte is essential toward a successful resolution on teicoplanin phase in the reversed-phase mode (Armstrong

Table 2. The chromatographic data for the resolution of *N*-2,4-dinitrophenylated di- and tripeptides stereoisomers on (*R*)-, (*S*)-naphthylethylcarbamate-β-cyclodextrin bonded chiral stationary phases using the polar organic mobile phase

Compound[a]	Structure ($R_1=$)[b]	k[c]				CSP	Mobile phase[d]
Leu-Phe		1.68 1.35	2.14 1.46	 1.51		RN SN	A A
Leu-Val		1.19 1.08	1.43 1.16	2.66		RN SN	A A
Leu-Ala		1.71 1.36	1.87 1.49	6.80 1.75		RN SN	A A
Leu-Leu		1.20 1.15	1.28 1.21	1.99 1.32	5.07 1.59	RN SN	A A
+Leu-Gly		3.30 2.12	9.11 2.33	 		RN SN	A A
+Gly-Leu		3.11	5.94			RN	A
+Gly-Ala		3.71	5.51			RN	A
Leu-Gly-Phe		4.57 2.11 3.71	5.62 3.41 3.95	5.99 3.58 4.86	9.39 7.29 6.72	RN RN SN	A B A
Ala-Ala		2.27 1.85	2.32	12.11	20.95	RN SN	A A
Ala-Leu		1.79 1.59	2.14 1.82	2.35 2.15	3.91	RN SN	A A
Ala-Phe		2.46 1.79	2.73 1.98	4.17 3.15	5.97	RN SN	A A

[a] *N*-2,4-dinitrophenyl-$R_1=$  . All analytes except for those marked with symbol "+" are of two stereogenic centers

[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, B: 450 ACN/50 MeOH/1 HOAC/4 TEA, by volume (v/v)

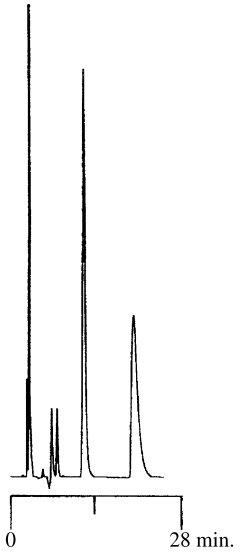


Fig. 4. Chromatogram showing the enantioresolution of DNP-Gly-Leu on the RN-β-CD bonded phase using the acetonitrile-based mobile phase of 480 ACN/20 MeOH/1 HOAC/2 TEA by volume, (v/v). Due to the glycine, the retention scale is usually larger than that for DNP-Leu. Also, the resolution is better under the same chromatographic conditions

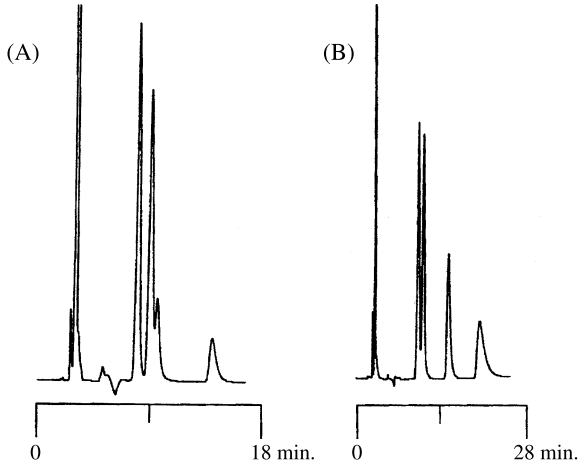


Fig. 5. Chromatograms showing the enantioresolution of (**A**) DNP-Ala-Leu and (**B**) DNP-Ala-Phe on RN-β-CD bonded phase using the acetonitrile-based mobile phase of 480 ACN/20 MeOH/1 HOAC/2 TEA by volume, (v/v). Based on the results of altering the detection wavelength, the first two peaks appear to be a pair of enantiomers

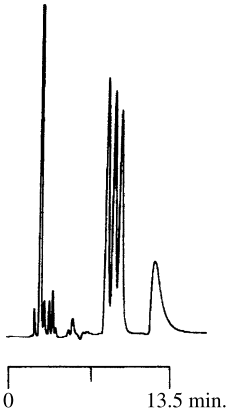


Fig. 6. Chromatogram showing the enantioresolution of DNP-Ala-Phe on SN-β-CD bonded phase using the acetonitrile-based mobile phase of 480 ACN/20 MeOH/1 HOAC/2 TEA by volume, (v/v). Note that the retention scale is smaller and the resolution is worse as compared to that obtained on RN-β-CD phase under the same chromatographic conditions

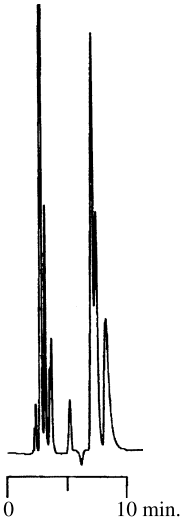


Fig. 7. Chromatogram showing the enantioresolution of DNP-Leu-Ala on SN-β-CD bonded phase using the acetonitrile-based mobile phase of 480 ACN/20 MeOH/1 HOAC/2 TEA by volume, (v/v). Note that the retention scale is relatively small. The middle two peaks appear to be a pair of enantiomers based on the results of altering the detection wavelength

et al., 1996). The failure in attempting to resolve amino alcohols in derivatized form, whose structure is highly similar to that of the resolved amino acids, also shows the importance of carbonyl group in obtaining a resolution on RN-β-CD phase. Typical examples include the DNP-2-amino-3-methyl-1-butanol/DNP-Val and DNP-2-amino-1-propanol/DNP-Ala. 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 DNP-alanine (α-amino acid). However, the resolution disappeared in DNP-3-aminoisobutyric acid (β-amino 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 DNP-α-amino-n-butyric acid to the DNP-β-amino-n-butyric acid. Under the same chromatographic conditions, only the DNP-α-amino-n-butyric acid was resolved.

Conclusion

The resolution of a variety of α-amino acids, peptides on naphthylethylcarbamate-β-cyclodextrin bonded chiral phases using the acetonitrile-based mobile phase after their pre-column derivatization with 2,4-dinitrofluorobenzene in alkaline medium has been demonstrated. The resolution is considered to be much better on RN-β-CD phase as compared to that on SN-β-CD phase for a given amino acid under the same chromatographic conditions and can not be reproduced on intact β-CD phase. The resolution is due to the interaction of the analyte with naphthylethylcarbamate moiety, not with the residual secondary hydroxyl groups on the β-cyclodextrin. The importance of carbonyl group on the analyte essential toward a successful resolution has been demonstrated. Also, the position of amino group on the analyte's skeleton affects the enantioresolution. 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.

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