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Systematic study on the resolution of derivatized amino acids enantiomers on different cyclodextrin-bonded stationary phases

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ABSTRACT

The chiral separation of amino acids by liquid chromatography using cyclodextrin-bonded stationary phases was studied systematically. Six types of native and chemically modified β-cyclodextrin-bonded stationary phases have been used to separate enantiomers of some derivatized amino acids in the reversed-phase high-performance liquid chromatography mode. Chiral separations with (*R*)- and (*S*)-naphthyl-ethylcarbamate-β-cyclodextrin (NEC-β-CD) bonded phases were compared with similar separations with the native β-CD stationary phase. Racemic dansyl amino acids were separated best on β-CD column while 3,5-dinitro-2-pyridyl-, dabsyl-, and 3,5-dinitrobenzoyl-amino acids were resolved best on the (*R*)- or (*S*)NEC-β-CD column. The role of the mobile phase was studied. Effects of organic modifiers, ionic strength, and pH of the mobile phase on retention and enantioselectivity of the analytes were investigated on three stationary phases. The enantiomer elution order of all dansyl amino acids on native and NEC-β-CD bonded stationary phases was L before D enantiomer, while a reverse elution order for dabsyl amino acids was observed. Hence, elution order can be controlled by choosing the appropriate functional group. This provides an alternative to the current method of changing retention order by changing the stationary phase (*i.e.*, obtaining a different column). It appears that the NEC-β-CD bonded phases are highly effective multimodal chiral stationary phases. The chiral recognition model involving inclusion complexation and π–π interactions is discussed.

INTRODUCTION

Two general approaches for the direct liquid chromatographic separation of enantiomers have been used. One involves use of chiral stationary phases (CSPs) and the other makes use of chiral mobile phase additives (CMAs). A number of new and improved CSPs and CMAs have been introduced to solve problems involving enantiomeric separations. A series of the recently developed bonded stationary phases derive from cyclodextrins (CDs), which are cyclic oligosaccharides containing D(+)-glucopyranose units. There are three naturally occurring CDs, α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin commercially available. Since all of the primary and secondary hydroxyl groups of CDs are on the outside of this toroidal shaped molecule, the cavity is relatively non-polar, thus allowing CDs to form inclusion complexes with a variety of polar or non-polar molecules. It is apparent that the size and geometry of a guest molecule in relation to that of the cyclodextrin cavity is an important factor in inclusion complex formation. Inclusion complex formation is determined by the several different factors which include hydrophobic effect, hydrogen bonding, Van der Waals interactions, release of high-energy water from the CD cavity, and a change in ring strain upon complexation [1,2]. CD was attached to silica gel via a linkage chain to form an effective high-performance CSP [3–7]. The β-CD

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bonded phases has been the most widely used of these columns. Enantiomeric resolution of a series of amino acids, barbiturate dioxolane, phenyl acetic acid derivatives, nicotine analogues, and many other compounds were successfully obtained with β-CD CSPs under reversed phase conditions.

Recently, derivatized cyclodextrin bonded phases were introduced for enantiomeric high-performance liquid chromatographic (HPLC) separations in the normal-phase mode [8,9]. They were able to separate a variety of racemates with either hexane–isopropanol or mobile phases of pure alcohol or acetonitrile. These CSPs seems to resemble more closely the cellulosic stationary phases than the native cyclodextrin bonded phase. In contast to the native β-CD stationary phase, the enantiomeric separation mechanism for the derivatized phases was not thought to be dependent on inclusion complexation. It was reported that derivatized CD stationary phases and native CD phases, operated in different modes, resolved different types of racemates [8–11]. The derivatized cyclodextrin bonded phases were utilized in reversed-phase separations due to their stability. It was found that completely different types of enantiomers were resolved by these columns in the reversed-phase mode [10].

Amino acids are found in all known living organisms. Proteins are made by association of twenty primary amino acids which are always L-amino acids. It may be important to be able to detect the presence of D-amino acids in biological systems [12]. The enantiomer separation of some aromatic amino acids was carried out on a α-CD-CSP [11]. Racemic dansyl-amino acids (5-dimethylamino-1-napthalene sulfonyl or Dns) and 2,4-dinitrobenzoyl (DNP)-amino acids were resolved on β-CD bonded CSPs [13–15].

The goal of this paper is to obtain a better knowledge of the chiral recognition mechanism of amino acids by β-CD bonded CSPs. The stationary phase, the mobile phase and the solute itself are all three strongly involved in the chiral recognition mechanism. In the first part of this work, the effect of the stationary phase is studied using six different β-CD and derivatized β-CD CSPs. The second part examines the effect of solute derivatization. Amino acids were derivatized with five different reagents and analyzed on the three best CSPs. The final aspect of this study deals with the mobile phase. The effects of the organic modifier nature (methanol, acetonitrile or tetrahydrofuran) and concentration were studied. The pH and buffer concentration effects also were investigated.

EXPERIMENTAL

Chemicals and methods

Amino acids and other compounds were obtained from Sigma (St. Louis, MO, USA) or Aldrich, (Milwaukee, WI, USA). HPLC-grade methanol (MeOH), tetrahydrofuran (THF) and acetonitrile (ACN) were purchased from Fisher Scientific (St. Louis, MO, USA). Water was deionized by passing distilled water through a Barnstead water purification system. Free amino acids were dried and dabsylated according to the procedure described [16]. A 1-ml dabsyl (4-dimethylaminoazobenzene-4′-sulfonyl) chloride solution (0.65 mg/ml in ACN) was mixed with 1 ml of 0.1 *M* sodium carbonate solution containing excess free amino acid. The reaction mixture was shaken in water bath at 70°C for 30 min. The resulting solution was directly introduced to the injector of the liquid chromatograph. In the 3,5-dinitrobenzoyl (DNB) derivatization, approximately 2–5 mg of amino acid were dissolved in 2 ml acetone. Next, 2–3 mg of 3,5-dinitrobenzoyl chloride derivatizing agent were added. The reacting solution was heated at 60°C for 10 min under gentle stirring. The resulting solution was cooled and injected directly into the liquid chromatograph.

Apparatus

A liquid chromatographic system containing a Shimadzu LC-6A chromatograph (Columbia, MD, USA) was used in this study. A variable-wavelength detector, Model SPD-6AV, was also used and interfaced with a CR601 Chromatopac data system. Dabsyl-amino acids were detected at 436 nm. All other compounds were detected at 254 nm. Rheodyne's Model 7125 sample injection valve with a 20-μl loop was used.

The six different 25-cm columns (4.6 mm I.D., 5 μm particle diameter) are listed in Table I. The first column was a Cyclobond I column (BC) with β-CD molecules chemically bonded to silica gel with a five-atom, non-nitrogen-containing spacer. The second column was a Cyclobond I Acetylated (AC)

TABLE I

COLUMNS USED

All columns: 25 cm × 4.6 mm I.D., 5 μm particle diameter.

Designation	Bonded moiety	Number of substitutes per β-CD ring
RN	(*R*)NEC-β-CD	6.7
SN	(*S*)NEC-β-CD	3.5
HSN	(*S*)NEC-β-CD	6.6
TC	Toluoyl-β-CD	*ca.* 13
BC	Native β-CD	0
AC	Acetyl-β-CD	*ca.* 16

column which is the peracetylated form of the bond β-CD column. Both columns were obtained from Astec (Whippanny, NJ, USA). Four more 25-cm columns were packed with CSPs prepared in the laboratory. Two (*S*)-1(1-naphthyl)-ethyl carbamate [(*S*)NEC]-derivatized β-CD bonded phase columns (SN and HSN), one (*R*)-1(1-naphthyl)-ethyl carbamate (*R*)NEC-derivatized β-CD bonded phase column (RN), and one toluoyl-derivatized β-CD column (TC) were prepared according to the procedure described in previous papers [8,9].

RESULTS AND DISCUSSION

Resolution of racemic derivatized amino acids on five CD stationary phases

Dansyl-amino acids and 3,5-dinitro-2-pyridyl (DNPy)-amino acids (DNPy) were analyzed on the six stationary phases listed in Table I. Two mobile phases were used (A and B, Table II). Table III lists the results, retention, expressed with the capacity factor, k', and enantioselectivity, expressed with the ratio of the k' values of the two enantiomers, α.

TABLE II

MOBILE PHASES USED

ACN = Acetonitrile; TEAA = triethylammonium acetate.

Designation	Composition (v/v)	pH
A	ACN–1% TEAA buffer (65:35)	4.5
B	ACN–1% TEAA buffer (50:50)	4.5
C	ACN–0.5% TEAA buffer (50:50)	4.1
D	ACN–0.5% TEAA buffer (21:79)	4.1

TABLE III

CAPACITY FACTOR (k') AND SELECTIVITY α OF SOME DERIVATIZED AMINO ACID ENANTIOMERS ON SEVERAL CHIRAL STATIONARY PHASES

Capacity factor (k') given for the first eluting enantiomer. PhG = Phenylglycine.

Compound	Column	k'	α	Mobile phase
Dansyl-amino acids				
Dns-Glu	RN	4.77	1.05	B
	SN	1.94	1.05	A
	HSN	5.77	1.04	A
	TC	3.42	1.03	A
	BC	2.26	1.11	A
	AC	1.15	1.04	A
Dns-Leu	RN	5.38	1.00	B
	SN	1.66	1.00	A
	HSN	6.55	1.00	B
	TC	4.60	1.00	B
	BC	0.91	1.19	B
	AC	0.70	1.00	B
Dns-Met	RN	4.69	1.00	B
	SN	1.46	1.00	A
	HSN	6.13	1.00	B
	TC	3.98	1.00	B
	BC	0.73	1.10	B
	AC	0.61	1.00	B
Dns-Nle	RN	5.46	1.00	B
	SN	1.58	1.00	A
	HSN	3.38	1.00	A
	TC	4.87	1.00	B
	BC	0.76	1.11	A
	AC	0.66	1.00	B
Dns-Nva	RN	4.51	1.00	B
	SN	1.46	1.00	A
	HSN	6.97	1.00	B
	TC	4.07	1.00	B
	BC	0.78	1.10	B
	AC	0.66	1.00	B
Dns-Phe	RN	7.01	1.09	B
	SN	1.94	1.08	A
	HSN	4.43	1.11	A
	TC	5.65	1.05	B
	BC	0.99	1.13	B
	AC	0.76	1.00	B
Dns-Ser	RN	2.48	1.04	B
	SN	1.01	1.06	A
	HSN	1.86	1.00	A
	TC	2.03	1.00	B
	BC	0.79	1.11	A
	AC	0.44	1.00	A
Dns-Thr	RN	2.96	1.06	B
	SN	1.09	1.08	A

(Continued on p. 86)

TABLE III *(continued)*

Compound	Column	k'	α	Mobile phase
	HSN	2.04	1.10	A
	TC	2.45	1.00	B
	BC	0.92	1.19	B
	AC	0.62	1.00	B
Dns-Trp	RN	6.71	1.05	B
	SN	1.91	1.00	A
	HSN	9.42	1.09	B
	TC	5.98	1.00	B
	BC	0.95	1.00	B
	AC	0.44	1.00	A
Dns-Val	RN	4.50	1.06	B
	SN	1.48	1.09	A
	HSN	2.93	1.05	A
	TC	2.24	1.04	A
	BC	0.66	1.18	A
	AC	0.71	1.00	B
3,5-Dinitro-2-pyridyl-amino acids				
DNPy-Ala	RN	3.17	1.06	B
	SN	1.26	1.00	A
	HSN	4.69	1.00	B
	TC	2.99	1.00	B
	BC	0.51	1.00	B
	AC	0.49	1.00	B
DNPy-Leu	RN	4.88	1.10	B
	SN	1.55	1.00	A
	HSN	7.31	1.00	B
	TC	4.73	1.00	B
	BC	0.62	1.13	B
	AC	0.59	1.00	B
DNPy-Met	RN	4.49	1.06	B
	SN	1.45	1.00	A
	HSN	7.22	1.08	B
	TC	4.06	1.00	B
	BC	0.63	1.00	B
	AC	0.51	1.00	B
DNPy-Nva	RN	4.30	1.09	B
	SN	1.38	1.00	A
	HNS	6.97	1.00	B
	TC	4.20	1.00	B
	BC	0.57	1.10	A
	AC	0.52	1.00	B
DNPy-Phe	RN	7.46	1.02	B
	SN	2.41	1.04	A
	HSN	4.76	1.08	A
	TC	6.39	1.02	B
	BC	0.89	1.00	B
	AC	0.69	1.00	B
DNPy-Trp	RN	6.57	1.05	B
	SN	1.93	1.00	A
	HSN	10.64	1.00	B
	TC	6.20	1.00	B
	BC	0.52	1.00	B
	AC	0.61	1.00	B
Other derivatized amino acids				
DNB-Leu	RN	4.08	1.06	A
	SN	1.33	1.00	A
	HSN	5.72	1.03	B
	TC	3.27	1.00	B
	BC	0.49	1.00	A
	AC	0.50	1.00	B
DNB-PhG	RN	4.73	1.17	B
	SN	1.36	1.25	A
	HSN	6.78	1.39	B
	TC	3.70	1.00	A
	BC	0.36	1.00	A
	AC	0.37	1.00	A
Benzoyl-Ala	RN	1.37	1.00	B
	SN	0.75	1.00	A
	HSN	1.50	1.00	B
	TC	1.10	1.00	B
	BC	1.04	1.00	A
	AC	0.55	1.00	A
Carbobenzoxy-Phe	RN	4.09	1.06	B
	SN	1.43	1.00	A
	HSN	2.78	1.08	A
	TC	3.19	1.00	B
	BC	0.94	1.00	B
	AC	0.60	1.00	B

Retention. The polarity for the six CSPs used increased from the less polar naphthylethylcarbamate derivatized β-CD CSPs (RN, SN and HSN), the toluoyl-derivatized β-CD CSP (TC), the bare β-CD CSP (BC), to the most polar peracetylated β-CD CSP (AC). The longer retention times were observed on the less polar RN, SN and HSN stationary phases. The retention differences between the SN and the HSN phases were due to the differing bonding density (Table I). The HSN CSP had a higher number of naphthylethyl units per CD ring which made it less polar than the SN CSP. Mobile phase A was richer in organic modifier than mobile phase B. The retention times obtained with A were shorter than the ones obtained with B.

Enantioselectivity. The most polar peracetylated β-CD CSP (AC) did not separate any racemic amino acids, but Dns-glutamic acid with a low 1.04

factor (Table III). The toluoyl-derivatized β-CD CSP (TC) was a little better and could separate three racemic amino acids out of the 20 listed in Table III. The α-values were low. The NEC-derivatized β-CD and the original β-CD CSPs gave the best results with 15 and 11 amino acids-resolved, respectively. For three amino acids, Dns-Leu, Dns-Met and Dns-Nle, the underivatized β-CD CSP was the only one able to separate the enantiomers with selectivity higher than 1.1. Six racemates, Dns-Trp, DNPy-Met, DNPy-Phe, DNPy-Trp, DNB-PhG and carbobenzoxy-Phe, were separated only by the NEC-derivatized β-CD CSPs. When a racemic amino acid pair was separated on both NEC-derivatized and underivatized β-CD CSP, the selectivity was always higher with the underivatized β-CD phase (Dns-Glu or DNPy-Leu, for example, Table III). Fig. 1 shows that the 5% average gain in selectivity could produce a doubling (100% increase) in resolution of the BC chromatogram (Fig. 1A).

The RN and SN columns are both NEC-derivatized β-CD CSPs. The RN column was obtained from the (*R*)NEC enantiomer derivative. The SN column was obtained from the (*S*)NEC derivative (Table I). The RN and SN columns behaved different. Four racemates were separated on the *R* column and not on the *S* version (Fig. 2). All four compounds are DNPy derivatives (DNPy-Ala, DNPy-Leu, DNPy-Nva and DNPy-Trp, Table III). Any racemate that was separated on *S* columns was

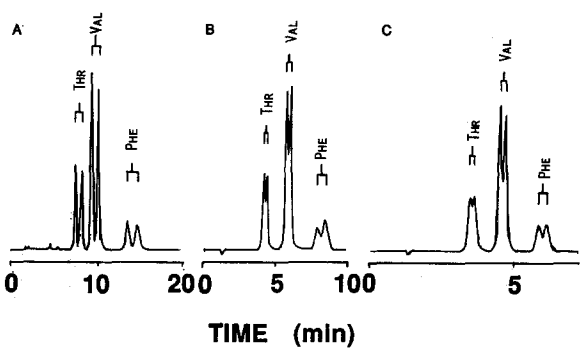

Fig. 1. Chromatograms of the separation of racemic dansyl-amino acids on three different chiral stationary phases. (A) BC column; mobile phase, ACN–0.5% TEAA buffer, pH 4.48 (25:75), 2 ml/min. (B) RN column; mobile phase, ACN–0.7% TEAA buffer, pH 4.48 (50:50), 2 ml/min. (C) SN column; mobile phase, ACN–0.7% TEAA buffer, pH 4.48 (50:50), 2 ml/min. Detection, UV at 254 nm.

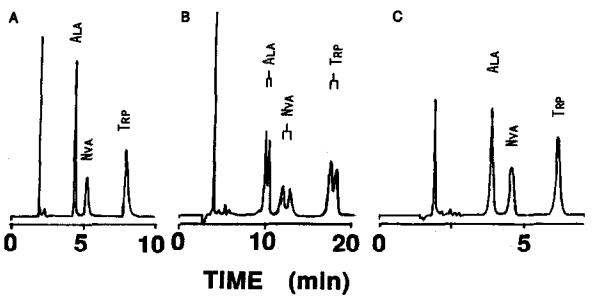

Fig. 2. Chromatograms of the separation of racemic DNPy-amino acids on three different chiral stationary phases. All experimental conditions as in Fig. 1.

also separated on the *R* column. However, the Dns-serine racemate was separated on the SN column and not on the HSN. Conversely, Dns-Trp, DNPy-Met, DNB-Leu and carbobenzoxy-Phe racemates were separated on the HSN column and not on the SN one.

These results show that the chiral mechanism of CDs is intricate. Inclusion complexation and chiral interaction with secondary alcohols located at the mouth of the CD cavity are parts of the mechanism. When the chiral recognition by β-CD disappears upon NEC derivatization (Dns-Leu, Dns-Nle and Dns-Met, Table III), a predominance of the inclusion complex formation may be suspected. Conversely, when the NEC-derivatized β-CD phases can separate racemates not separated by the native β-CD phase, a reversed-phase Pirkle-type recognition mechanism may be part of the chiral resolution process.

The peracetylated β-CD column (AC) and the toluoyl β-CD column (TC) were abandoned due to the poor enantioselectivity obtained in derivatized amino acid resolution. The highly substituted (*S*) NEC column (HSN) was also discarded due to long retention times compared to the similar SN column. Only three columns, the RN, SN and β-CD columns (Table I) were used to investigate solute derivatization effects (next part) and mobile phase effects (last part).

Amino acid derivatization effects on chiral recognition by β-CD columns

The derivatives. The amino acids were derivatized using four different reagents: dansyl chloride (Dns) DNB, DNPy and dabsyl chloride. All derivatiza-

TABLE IV

CAPACITY FACTOR (k') AND SELECTIVITY (α) OF 3,5-DINITROBENZOYL AMINO ACID ENANTIOMERS ON SOME CHIRAL STATIONARY PHASES

Np = Naphthyl; F = Fluoro; Hphe = homophenylalanine; PhG = phenylglycine.

DNB-Amino acid	Column	k'	α	Mobile phase
Ala	RN	2.90	1.03	C
	SN	1.88	1.06	C
3-(1-Np)-Ala	RN	11.3	1.09	C
	SN	5.42	1.11	C
	BC	5.44	1.00	D
3-(2-Np)-Ala	RN	9.80	1.05	C
	SN	5.49	1.13	C
Asn	RN	2.26	1.03	C
	SN	1.52	1.07	C
	BC	1.22	1.00	D
Asp	RN	6.91	1.00	C
	SN	3.71	1.07	C
	BC	2.52	1.00	D
Glu	RN	6.39	1.00	C
	SN	3.18	1.09	C
	BC	2.29	1.00	D
Leu	RN	5.34	1.05	C
	SN	3.01	1.03	C
	BC	2.67	1.00	D
Met	RN	4.60	1.08	C
	SN	2.66	1.06	C
	BC	1.98	1.00	D
Nle	RN	5.30	1.09	C
	SN	2.81	1.06	C
	BC	2.61	1.00	D
Phe	RN	7.89	1.11	C
	SN	4.06	1.07	C
	BC	3.40	1.06	D
Hphe	RN	13.58	1.31	C
	SN	5.03	1.00	C
	BC	6.42	1.06	D
o-F-Phe	RN	7.00	1.07	C
	SN	3.53	1.07	C
	BC	3.28	1.06	D
PhG	RN	4.73	1.17	B
	SN	1.36	1.25	A
	BC	0.36	1.00	A
Pro	RN	2.92	1.00	C
	SN	1.84	1.00	C
	BC	2.15	1.00	D
Ser	RN	2.35	1.00	C
	SN	1.65	1.08	C
	BC	1.22	1.00	D
Thr	RN	3.08	–	C
	SN	1.73	1.07	C
	BC	1.28	1.00	D
Trp	RN	7.58	1.09	C
	SN	4.08	1.12	C
	BC	3.34	1.03	D

tions make the amino acids highly UV detectable. The Dns derivatization adds a somewhat π-basic group on one branch of the amino acid's stereogenic center. The DNB derivatization adds a π-acid group to the amino group of amino acids. The DNPy group has an intermediate π-acidity with an electron rich (π-basic) pyridine ring and two electron attracting nitro groups (π-acid). The dabsyl derivative is rather π-basic, it was chosen for its long and rigid azobenzene backbone which may favor CD inclusion. Tables III and IV and V list the capacity factors and enantioselectivity factors of the four derivatized amino acids on the selected columns.

Retention. As a general rule, the retention order for any derivative was RN > SN > BC. It corresponds to the CSP polarity order. The underivatized β-CD column has the highest polarity. The retention times of the solutes were so short that it was necessary to use a water-rich polar mobile phase (solution D, 79%, v/v, water, Table II) to increase them. As far as polarity is concerned, the RN and SN columns have the same type of substituent. However, they do have differing polarities because the NEC bonding density is different (Table I). For a given amino acid, the retention times were similar for the DNB, DNPy and Dns derivatives. The dabsyl derivatives always had significantly higher retention times.

Enantioselectivity. The highest enantioselectivity coefficients were obtained with dabsyl derivatives. For example, α values of 1.56, 1.29, 1.23 or 1.22 were obtained for dabsyl-Leu, dabsyl-Glu, dabsyl-NvA or dabsyl-Thr, respectively, on the (*R*)NEC or (*S*)NEC columns (Table V). The data of Tables

TABLE V

CAPACITY FACTOR (k') AND SELECTIVITY (α) OF DABSYL-AMINO ACID ENANTIOMERS ON SOME CHIRAL STATIONARY PHASES

Dabsyl-amino acid	Column	k'	α	Mobile phase
Ala	RN	4.08	1.13	A
	SN	2.27	1.04	A
	BC	2.01	1.00	A
3-(1-Np)-Ala	RN	5.65	1.11	A
	SN	2.60	1.08	A
	BC	1.29	1.07	B
3-(2-Np)-Ala	RN	7.97	1.13	A
	SN	3.16	1.00	A
	BC	1.46	1.00	B
Glu	RN	10.57	1.29	A
	SN	4.39	1.09	A
	BC	5.07	1.00	A
His	RN	1.46	1.16	A
	SN	1.01	1.15	A
	BC	1.24	1.09	B
Leu	RN	–	–	A
	SN	8.12	1.56	A
	BC	–	–	B
Nva	RN	–	–	A
	SN	2.61	1.23	A
Phe	RN	10.99	1.00	A
	SN	4.25	1.07	A
	BC	2.01	1.00	B
o-F-Phe	RN	6.93	1.12	A
	SN	3.19	1.04	A
	BC	1.65	1.00	A
m-F-Phe	RN	8.46	1.00	A
	SN	3.34	1.07	A
Pro	RN	5.10	1.10	A
	SN	2.52	1.00	A
	BC	2.93	1.00	B
Ser	RN	3.33	1.17	A
	SN	1.77	1.06	A
	BC	2.21	1.00	B
Thr	RN	4.22	1.22	A
	SN	2.19	1.07	A
	BC	2.50	1.00	B
Trp	RN	5.59	1.12	A
	SN	2.68	1.06	A
	BC	1.61	1.10	B
Tyr	SN	3.14	1.15	A
Val	RN	–	–	A
	SN	5.04	1.18	A
	BC	2.49	1.00	B

III–V lead to the following observations. The dansyl-amino acid derivatives were best separated on the underivatized β-CD CSP (Table III). The DNB-amino acid derivatives were best separated on the (*S*)NEC-β-CD column (SN) (Table IV). The dabsyl and DNPy derivatives were best separated on the (*R*)NEC-β-CD column (RN) (Tables III and V).

The dansyl derivatives, with a naphthyl group, seem to fit well in the β-CD cavity. It is difficult to access this cavity when bulky NEC groups are grafted on its mouth. π–π interactions (Pirkle-type) and steric hindrance can be involved to explain the chiral recognition of DNB derivatives on the SN column. Most compounds also were separated, with a somewhat lower enantioselectivity, by the corresponding *R* column (RN). Circular dichroism studies showed opposite elution orders between the D and L forms of some DNB derivatives eluted on the RN and SN columns. The D forms eluted first on the RN column and the L forms on the SN column. The chiral mechanism for DNB derivatives recognition seems to be dominated by π–π interaction of the Pirkle-type. Fig. 3 is an example of DNB derivative separation on the BC and RN columns. The L form of all dansyl derivatives eluted first on the three CSPs used. Conversely, the D form of all dabsyl derivatives eluted first on the three CSPs (RN, SN and BC). Inclusion complexation seems to predominate in the chiral recognition mechanism of these derivatives because there was no significant difference between the RN and SN columns. Dabsyl and DNPy derivatives have somewhat intermediate π-acidities. The RN column separated these analytes better than the SN column did. This shows that steric hindrance and π-π interactions due to the NEC moieties cannot be neglected: the (*R*)NEC density was higher than the (*S*)NEC density (Table I). This point is illustrated by Fig. 4 which shows a dabsyl derivative separation on RN and SN columns. One exception: dabsyl-phenylalanine was better separated by the SN column. It should be noted that the observed efficiency for all dabsyl-amino acid peaks was about 50% lower than the efficiency observed for the other derivatives. The dabsyl peaks appeared broader (Figs. 3 and 4). This was due to a slow mass transfer. It is a possible sign of CD inclusion complexation. All this shows that the chiral recognition mechanism for β-CD-CSPs and specially for NEC-derivatized β-CD CSPs cannot be simply reduced to one kind of behavior, rath-

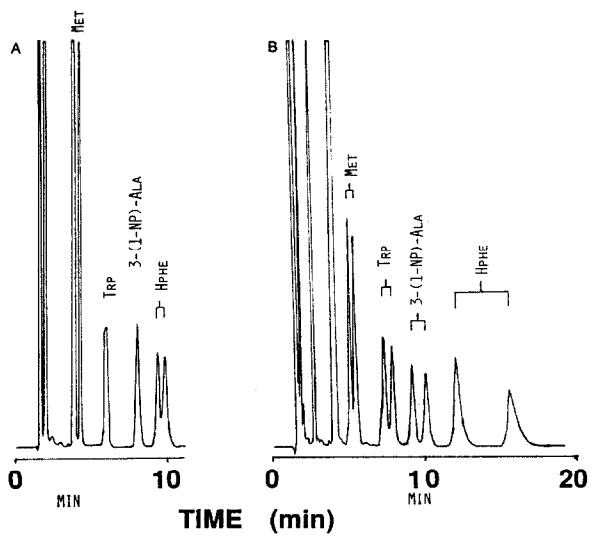

Fig. 3. Chromatograms of the separation of DNB-amino acids on the BC (A) and RN (B) chiral stationary phases. All experimental conditions as in Fig. 1.

er it involves several types. From these results, it appears that NEC-β-CD CSP columns are particularly effective and versatile.

Mobile phase and enantioselectivity

The mobile phase solvates the stationary phase and the solutes. It is most often a mixture of solvents, which means that the solvatation may be different when the local polarity is changing. Despite its importance, the mobile phase is probably the least known and studied parameter in chiral recognition mechanism studies. In this work, three mobile phase parameters were studied (i) the organic modifier nature, (ii) the pH effect, (iii) the ionic strength effect through buffer concentration.

Nature of the organic modifier. Table VI lists the

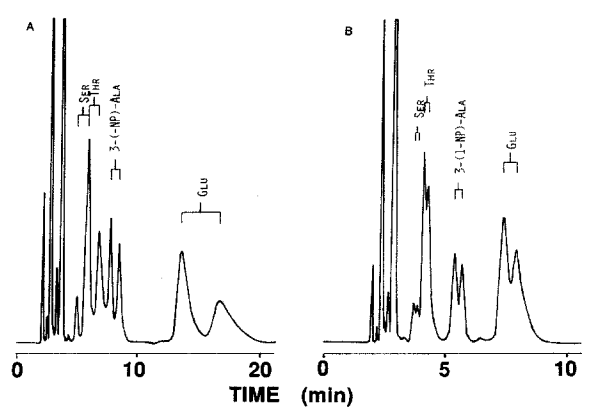

Fig. 4. Chromatograms of the separation of racemic dabsyl-amino acids on the RN and SN derivatized β-CD stationary phases. The mobile phase is ACN–0.7% TEAA buffer, pH 4.48 (65:35), 2 ml/min. (A) RN column. (B) SN column.

TABLE VI

ENANTIOMERIC SEPARATION DATA FOR DERIVATIZED AMINO ACIDS ON THE DIFFERENT ORGANIC MODIFIERS

Column RN and 1% TEAA buffer (pH 4.1) solutions are used.

Compound	50% ACN		50% MeOH		40% THF	
	k'	α	k'	α	k'	α
Dns-Glu	2.42	1.03	4.84	1.00	2.41	1.00
Dns-Leu	2.73	1.00	5.25	1.00	2.75	1.00
Dns-Met	2.75	1.00	5.81	1.00	3.06	1.00
Dns-Nle	2.86	1.00	–	–	3.10	1.00
Dns-Nva	2.52	1.00	4.82	1.00	2.75	1.00
Dns-Phe	3.71	1.07	9.32	1.00	3.92	1.00
Dns-Ser	1.76	1.00	3.65	1.00	1.93	1.00
Dns-Thr	1.90	1.06	3.77	1.00	2.24	1.00
Dns-Trp	3.38	1.04	9.00	1.00	3.63	1.00
Dns-Val	2.47	1.04	4.55	1.00	2.59	1.00
DNB-PhG	3.05	1.19	8.72	1.25	5.28	1.16
Benzoyl-Ala	1.10	1.00	1.54	1.00	1.02	1.00
Carbobenzoxy-Phe	2.43	1.05	4.82	1.04	2.53	1.00
DNPy-Ala	2.36	1.05	5.47	1.06	3.88	1.00
DNPy-Met	3.03	1.05	8.33	1.07	5.33	1.00
DNPy-Nva	2.81	1.08	6.68	1.09	4.87	1.00
DNPy-Phe	4.48	1.00	13.47	1.00	6.24	1.00
DNPy-Trp	4.03	1.04	14.36	1.07	6.34	1.00
Dabsyl-Ala	4.81	1.10	7.72[a]	1.28	3.22	1.00
Dabsyl-3(1-Np)-Ala	7.65	1.08	10.84[a]	1.13	5.77	1.00
Dabsyl-3(2-Np)-Ala	10.75	1.12	16.88[a]	1.17	6.34	1.00
Dabsyl-Glu	7.25	1.42	16.13[a]	1.00	3.69	1.00
Dabsyl-His	1.19	1.14	2.21[a]	1.13	0.56	1.00
Dabsyl-Nva	7.67	1.00	8.95[a]	1.00	–	–
Dabsyl-Phe	7.43	1.00	–	–	5.32	1.00
Dabsyl-*o*-F-Phe	9.41	1.10	–	–	5.10	1.00
Dabsyl-*m*-F-Phe	7.71	1.09	17.73[a]	1.00	–	–
Dabsyl-Pro	6.00	1.09	11.56[a]	1.07	3.19	1.00
Dabsyl-Ser	3.94	1.16	7.90[a]	1.13	2.71	1.00
Dabsyl-Thr	4.70	1.18	8.87[a]	1.21	2.83	1.00
Dabsyl-Trp	6.92	1.10	11.56[a]	1.14	–	1.00
Dabsyl-Tyr	10.01	1.00	10.54[a]	1.00	3.82	1.00

[a] The mobile phase of methanol–buffer (75:25) is used for all the dabsyl-amino acid separations.

retention and enantioselectivity obtained for the derivatized amino acids on the RN column. Three mobile phases were prepared with the same buffer solution (water + 1%, v/v, triethylamine (TEA) adjusted to pH 4.10 adding acetic acid) and ACN, MeOH or THF. The results are straightforward: as far as retention is concerned, THF was the strongest eluting solvent and MeOH was the weakest. However, THF should not be used because it did not give any enantioselectivity. We think the low polarity ether ring of THF may enter the β-CD cavity and/or associate with π-systems hindering both the inclusion complex formation and π–π interactions. Clearly, ACN gave the best enantioselectivity. It was able to resolve some Dns derivatives that were not resolved with MeOH or THF modifiers. It resolved most of the DNPy and dabsyl-amino acid derivatives, somewhat better than MeOH and with shorter retention times.

TABLE VII

pH EFFECT FOR ENANTIOMERIC SEPARATION OF DERIVATIZED AMINO ACIDS

The mobile phase contains ACN–1% TEAA buffer (65:35) unless indicated otherwise; column RN.

Compound	pH 4.50		pH 6.10		pH 7.00	
	k'	α	k'	α	k'	α
Dns-Glu	4.77[a]	1.05	1.12	1.07	0.54	1.00
Dns-Phe	7.01[a]	1.09	1.23	1.11	0.95	1.11
Dns-Ser	2.48[a]	1.04	0.60	1.00	0.42	1.00
Dns-Thr	2.96[a]	1.06	0.65	1.09	0.49	1.09
Dns-Trp	6.71[a]	1.05	1.18	1.07	0.93	1.07
Dns-Val	4.50[a]	1.06	0.94	1.10	0.74	1.24
DNPy-Ala	3.17[a]	1.06	0.77	1.00	0.49	1.00
DNPy-Leu	4.88[a]	1.10	0.99	1.10	0.66	1.12
DNPy-Met	4.49[a]	1.06	0.90	1.05	0.59	1.09
DNPy-Nva	4.30[a]	1.09	0.86	1.09	0.57	1.09
DNPy-Phe	7.46[a]	1.02	1.40	1.00	0.93	1.00
DNPy-Trp	6.57[a]	1.05	1.21	1.05	0.82	1.05
Carbobenzoxy-Phe	4.09[a]	1.06	0.98	1.00	0.64	1.00
DNB-Leu	3.33	1.17	1.43	1.19	0.95	1.20
DNB-PhG	4.22[a]	1.22	1.86	1.24	1.20	1.06
Dabsyl-Ala	5.10	1.10	2.25	1.10	1.47	1.09
Dabsyl-3-(1-Np)-Ala	5.65	1.11	2.36	1.14	1.57	1.11
Dabsyl-3-(2-Np)-Ala	7.97	1.13	3.47	1.14	2.21	1.13
Dabsyl-Glu	4.73[a]	1.17	0.92	1.16	0.63	1.18
Dabsyl-*o*-F-Phe	6.93	1.12	3.16	1.15	2.01	1.15
Dabsyl-Pro	4.08[a]	1.06	0.90	1.08	0.61	1.10
Dabsyl-Ser	10.57	1.29	3.35	1.16	1.58	1.13
Dabsyl-Thr	4.08	1.13	1.82	1.14	1.17	1.14
Dabsyl-Trp	5.59	1.12	2.60	1.13	1.69	1.13

[a] ACN–1% TEAA buffer (50:50), (mobile phase B in Table II).

pH effects. It has been reported that the pK_a value of the carboxylic acid group of amino acids varies from 1.7 to 2.7 [15,17]. Substitution of the amino acid's amino group with electron acceptor moieties (Dns, DNPy, DNB or dabsyl) should increase the acid dissociation constant. All derivatized amino acids exist in the anionic form over the pH range 4.1 to 7.0. Table VII shows rapid amino acid retention decreases with pH increases. In the reversed-phase mode, such a decrease means either the solute becomes more polar or the mobile phase strength increases or the stationary phase becomes more polar. Since the solute ionization and the mobile phase becomes more polar. Since the solute ionization and the mobile phase composition are not supposed to change much, pH changes are suspected to induce stationary phase changes. The silica network underneath the β-CD layer is pH sensitive. Upon pH increase, residual silanols may ionize, rendering the stationary phase somewhat negatively charged. Triethylammonium ions (TEA^+) should cancel negative charges. They may not cancel all negative charges. The good point is that, for these particular analytes, enantioselectivity is little affected by pH changes (Table VII). The chiral recognition is due to solute-β-CD interactions that take place far enough from the silica surface. Elevated mobile phase pH values may make the chiral analysis faster. However, it should not be forgotten that silica solubilization occurs from pH 7. To extend column life, it is safer to work below pH 6.

Buffer concentration effect. Table VIII lists the effects of buffer concentration on retention and resolution of amino acid derivatives on the RN column. A dramatic retention decrease was observed when the TEAA concentration increased. An efficiency increase was also observed at high TEAA concentration, that is why resolution factors were listed in Table VIII instead of selectivity factors. This indicates that the solute, the mobile phase and the stationary phase were modified by TEAA. Hydrophilic $TEAA^+$-derivatized $[amino\ acid]^-$ ion pairs may be formed and eluted faster at high TEAA concen-

TABLE VIII

EFFECT OF TEAA BUFFER CONCENTRATION IN THE MOBILE PHASE ON THE RETENTION AND RESOLUTION

Mobile phase: ACN–buffer, pH 4.10 (50:50); column RN.

Compound	0.2% TEAA		0.5% TEAA		1.0% TEAA	
	k'	R_s	k'	R_s	k'	R_s
Dns-Glu	16.74	0.7	5.13	0.5	2.42	0.5
Dns-Leu	10.49	0.5	4.70	0.0	2.73	0.0
Dns-Nva	9.71	0.0	4.40	0.0	2.52	0.0
Dns-Phe	15.18	1.0	6.67	0.8	3.71	0.9
Dns-Ser	6.97	0.6	3.09	0.3	1.76	0.0
Dns-Thr	7.51	0.9	3.34	0.7	1.90	0.6
Dns-Trp	13.32	0.9	5.98	0.6	3.38	0.5
Dns-Val	9.25	0.8	4.25	0.6	2.47	0.6
DNPy-Ala	9.22	1.0	4.24	0.7	2.36	0.8
DNPy-Leu	12.34	1.5	5.63	1.2	–	–
DNPy-Met	12.36	1.0	5.53	0.8	3.03	0.7
DNPy-Nva	11.44	1.5	5.16	1.1	2.81	1.0
DNPy-Phe	19.28	0.3	8.52	0.0	4.48	0.0
DNPy-Trp	17.20	1.0	5.65	1.2	4.03	0.8
DNB-Leu	9.53	0.9	4.50	0.5	2.60	0.3
DNB-PhG	12.77	3.5	5.69	3.0	3.05	3.0
Dabsyl-Ala	19.87	2.0	8.86	1.2	4.81	1.1
Dabsyl-3(1-Np)-Ala	34.10	2.0	14.86	1.5	7.65	1.0
Dabsyl-Glu	–	–	17.02	2.5	7.25	1.5
Dabsyl-*o*-F-Phe	–	–	17.41	0.5	9.41	0.4
Dabsyl-Pro	23.75	1.0	10.97	0.8	6.00	0.9
Dabsyl-Ser	16.47	4.0	7.24	2.0	3.94	1.7
Dabsyl-Thr	19.55	2.0	8.51	1.2	4.70	1.3
Dabsyl-Trp	29.66	2.0	13.06	1.0	6.92	1.0

trations. Mobile phases were prepared by putting the quoted amount (v/v) of liquid triethylamine in water and adding acetic acid to the desired pH. The 1% (v/v) TEAA water solution at pH 4.1 contained about 3% (v/v) of organic modifiers (TEA and acetic acid). Mobile phase ionic stength changed much upon TEAA concentration changes. The TEAA ion pair may enter the β-CD cavity thus weakening the strength of derivatized amino acid's inclusion complex. 1% (v/v) TEAA concentrations produced a decrease in enantioselectivity that was not compensated by the efficiency increase, *i.e.* the overall resolution decreased (Table VIII).

CONCLUSIONS

The classical native β-CD CSP column (BC) is well-suited for dansyl-derivatized amino acid analysis. However, the NEC-β-CD CSP columns (RN, SN and HSN), and especially the (*R*)NEC derivatized β-CD CSP column (RN) gave excellent results with π-acid derivatized amino acids (DNB-amino acids). The RN column was also able to resolve dabsyl-amino acids and DNPy-amino acids. The linear azobenzene group of the dabsyl derivative makes dabsyl-amino acids easy to resolve by the RN column. ACN is better than MeOH as an organic mobile phase modifier. TEAA buffer concentrations of 0.5% (v/v) and a pH around 5 are the best mobile phase conditions to resolve racemic derivatized amino acids. Clearly the (*R*)NEC-β-CD column (RN) was the most widely useful derivatized cyclodextrin based CSP. The chiral recognition mechanism of this CSP involves (i) inclusion complexation with the β-CD cavity, (ii) π–π interaction (Pirkle type) with the naphthylethyl moiety, (iii) interactions with remaining chiral secondary alcohols at the CD-mouth and (iv) steric hindrance.

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