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Direct separation of enantiomers using multiple-interaction chiral stationary phases based on the modified β-cyclodextrin-bonded stationary phase

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ABSTRACT

Several multiple-interaction chiral stationary phases have been developed. These stationary phases contain a hydrophobic cavity capable of inclusion complexation, aromatic groups capable of π–π interaction, polar hydroxyl groups capable of hydrogen-bonding with the polar functional groups of the solute, and bulky non-polar groups providing steric repulsion. The characteristics and properties of these stationary phases are described. The direct separation of enantiomers of a wide variety of chiral compounds are reported. The effect of mobile phase composition on the retention and resolution is discussed.

INTRODUCTION

In recent years, direct separation of enantiomers using high-performance liquid chromatographic (HPLC) chiral stationary phases has been a very active and fast-moving field due to its importance in optical purity determination, monitoring asymmetric synthesis, pharmacokinetics studies, metabolism studies, and dating of archaelogical materials. Today, more than 50 different chiral stationary phases are commercially available.

Cyclodextrin-bonded stationary phase, especially β-cyclodextrin stationary phase, developed by Armstrong [1], is one of the most widely used chiral stationary phases. β-Cyclodextrin stationary phase offers the following advantages: (i) it is chemically and physically more robust and (ii) it can be used in a reversed-phase mode with solvents containing water and organic modifiers. However, β-cyclodextrin stationary phase has a limited range of applications. Chiral recognition is significant only for those molecules which have a large hydrophobic group in the molecule. This limitation is mainly related to the nature of β-cyclodextrin itself and the chiral recognition mechanism.

As was known, the structure of β-cyclodextrin has the shape of a toroid or hollow truncated cone. The side of the torus with the larger circumference contains the secondary hydroxyl groups (on carbons 2 and 3 of the glucose units) while the primary hydroxyl groups (on carbon 6 of the glucose units) are on the small side. The interior of the cavity contains two rings of C–H groups with a ring of glycosidic oxygens in between. As a result, the cavity is relatively hydrophobic while the external faces are hydrophillic [2]. Chiral recognition mechanism studies [3,4] show that in reversed-phase applications, chiral recognition is mainly caused by hydrophobic interaction between the cavity of cyclodextrin and the hydrophobic moiety of the solute. The chief problem is that because the interior of the cavity, with glycosidic oxygens, is not quite non-polar and both ends of the cavity are open to solvent, only those guest molecules which have large hydrophobic groups and appropriate shape can form

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strong inclusion complexes with it. Therefore, chiral recognition is significant only for these larger molecules. To overcome this problem, much work has been done to increase the binding forces by modifying the cyclodextrins through the reaction of the hydroxy groups with a variety of modifiers [5,6].

In this work, a regiospecific modification was carried out by attaching groups on the primary hydroxyl side of the β-cyclodextrin. This modified β-cyclodextrin stationary phase contains: (i) a hydrophobic cavity, capable of inclusion complexation; (ii) aromatic groups, capable of π–π interaction; (iii) polar hydrogen-bonding and/or dipole stacking sites; and (iv) bulky non-polar groups, providing steric repulsion, Van der Waals interaction, and/or confirmational control. Therefore, it can be said that it is a multiple-interaction type of chiral stationary phase. The enantiomeric separations of various compounds including amino acid derivatives, phenothiazine and related drugs, and other pharmaceuticals have been achieved on these multiple-interaction chiral stationary phases and are presented in this paper. The characteristics and chromatographic properties are discussed.

EXPERIMENTAL

Chemicals

β-Cyclodextrin was from Chemical Dynamics (South Plainfield, NJ, USA). HPLC-grade methanol and triethylamine were from Fisher Scientific (Montreal, Canada). N-(2-Aminoethyl-3-aminopropyl)trimethoxysilane was from Huls America (Bristol, PA, USA). Amino acids and their derivatives, and the phenothiazines were from Sigma (St. Louis, MO, USA). All other chemicals were from Aldrich (Milwaukee, WI, USA).

Silica gel

Silica gel purchased from Chromatographic Separation (Montreal, Canada) as Spherisorb was used as the support material. This gel consists of spherical particles with a pore diameter of 8 nm and a mean particle size of 5 μm. The surface area of the silica gel, according to the manufacturer, is 220 m^2/g. Before carrying out reactions, the silica support was acid-hydrolysed in 0.2 *M* hydrochloric acid at 90°C for 24 h, so that its surface had a maximum number of Si–OH groups per unit of surface area. After cooling and filtration, the silica was washed with distilled water until neutral, and then dried at 170°C *in vacuo* for 12 h.

Apparatus

All chromatographic experiments were performed on a liquid chromatographic system which consisted of a Model 590 pump (Waters Assoc., Milford, MA, USA), a Model 440 254-nm ultraviolet detector (Water Assoc.) and a Model 7125 injector containing a 10-μl loop (Rheodyne, Cotati, CA, USA). Injections were made on column using precision sampling syringes. All column evaluations were carried out at ambient temperature (*ca.* 20°C).

The column was 25 cm × 0.46 cm I.D. 0.655 cm O.D. stainless-steel tubing (Chromatographic Separation) with a mirror-finish. Columns were prepared by a high-pressure balanced-density slurry packing technique using a Shandon HPLC packing pump (Chromatographic Separation).

Preparation of the multiple-interaction stationary phases

The preparation procedure used in this investigation is shown in Fig. 1. It involves four steps: (i) bonding the N-(2-aminoethyl-3-aminopropyl)trimethoxysilane to the silica gel; N-(2-aminoethyl-3-aminopropyl)trimethoxysilane was used as a linkage material to join the β-cyclodextrin to the silica; (ii) regiospecific sulfonation of the primary hydroxyl groups of the β-cyclodextrin with *p*-toluenesulfonyl chloride [7]; (iii) reaction of 2-aminoethyl-3-aminopropyl-bonded silica gel (I) with toluenesulfonyl-β-cyclodextrin (II); the reaction between the amino groups of the N-(2-aminoethyl-3-aminopro-

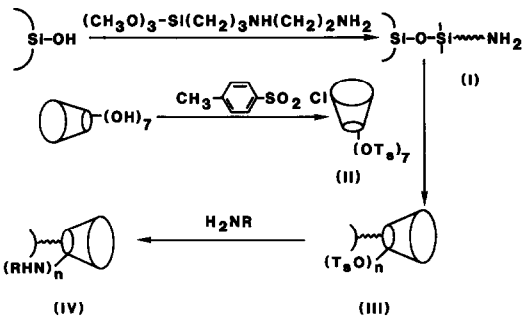


Fig. 1. General procedure for the preparation of the modified β-cyclodextrin stationary phases.

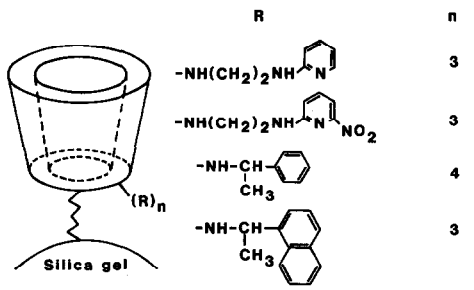


Fig. 2. Structures of modified β-cyclodextrin stationary phases.

pyl)-bonded silica and the toluenesulfonyl groups of the cyclodextrin links the cyclodextrin to the silica gel [8]; and (iv) modification of the bonded cyclodextrin by further reaction of the unreacted toluenesulfonyl groups of the cyclodextrin (III) with various modifiers.

Fig. 2 shows the structural diagrams of these modified β-cyclodextrin stationary phases.

Analyses of the surface species

Identification of the surface species is made by means of infrared spectroscopy using transmission and the attenuated total reflection techniques. In this investigation, the amounts of cyclodextrin bonded to the silica gel were determined by means of a colorimetric method based on the reaction of D-glucose with tetrazolium blue [8,9]. The stationary phase (0.5 g) was hydrolysed in 30 ml of 0.5 *M* sulfuric acid at 100°C for 5 h. The hydrolysate was neutralized with sodium hydroxide and made up to 50 ml with water. In a reaction vessel with a glass stopper, 1 ml of this solution was made to react at 50°C for 10 min with 1 ml of a solution of 0.5% tetrazolium blue in ethanol–0.2 *M* aqueous sodium hydroxide (80:20, v/v). After the vessel had cooled, 3 ml methanol were added, and sodium sulfate was removed by centrifugation. The absorbance of the clear solution was measured at 525 nm.

RESULTS AND DISCUSSION

Characteristics of the packings

Surface coverage. The most valuable parameter for characterization of bonded-phase packings is the surface concentration of bonded functional groups. However, most of the commercial chiral stationary phases are only characterized by their carbon content, which gives poor and incomplete information. In general, it is very difficult to precisely and/or accurately evaluate the content of the cyclodextrins bonded to the surface of silica gel by the method based on differences in the gel weights before and after the bonding reaction or by elemental analysis. In this work, the amount of cyclodextrin bonded to the silica gel was colorimetrically determined by a method based on the reaction of D-glucose produced by hydrolysing cyclodextrins with 3,3′-[3,3′-dimethoxy-(1,1′-biphenyl-4,4′-diyl)]bis-(2,5-diphenyl-2H-tetrazolium)dichloride, called tetrazolium blue. The maximum coverage for these stationary phases was determined to be 50.6 μmol/g.

To determine the degree of the substitution on the β-cyclodextrin of the modified β-cyclodextrin stationary phases, an HPLC method has been used. The degree of substitution for each of the stationary phases was calculated and is reported in Fig. 2.

Stability. The chemical stability of the stationary phase is primarily determined by that of silica and the bonded cyclodextrins. Consequently, most solvents can be used if they are within the pH range of 3.0–7.0. Although cyclodextrin molecules and their derivatives are fairly stable in alkaline solution, they are quite suspectible to hydrolysis in strongly acidic solution. For example, the rate constants of hydrolysis of β-cyclodextrin at pH 0.13, 40°C and 100°C, are $1.0 \cdot 10^{-5}$ and $4.8 \cdot 10^{-2}$ min^{-1}, respectively, corresponding to half-lives of 48 days and 14 min [4]. The stability in aqueous solutions of pH > 7 is influenced by the presence of organohydroxylsilyl groups resulting from the hydrolysis of Si–$(OCH_3)_3$ or Si–$(OC_2H_5)_3$ groups of the linkage materials. These stationary phases can be used at temperature up to 65°C.

Chromatographic properties

Column efficiency. Each of the slurry-packed modified β-cyclodextrin columns was conditioned with a solvent series of increasing polarity. This series consisted of isopropanol, methanol and methanol–water (50:50). After conditioning, all columns were evaluated using a test mixture of *o*-chlorophenol, *p*-chlorophenol, 3-nitroaniline, and 2-biphenylol and a mobile phase of methanol–water (50:50). The HETP values obtained on two of these columns

TABLE I

COLUMN EFFICIENCY (HETP VALUES)

Columns	HETP (cm)	
	0.5 ml/min	1.0 ml/min
β-CD	0.0014	0.0015
β-CD–NHCH(CH$_3$)–	0.0035	0.0040
β-CD–NHCH(CH$_3$)–	0.0039	0.0046

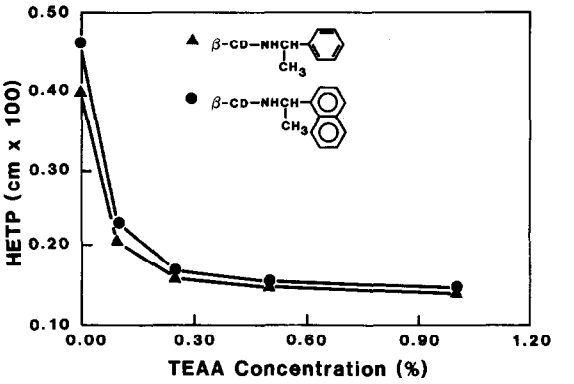


Fig. 3. Effect of TEAA on the column efficiency. Mobile phase: methanol–TEAA buffer (50:50) (pH 5.5).

at flow-rates of 0.5 and 1.0 ml/min are listed in Table I. As can be seen, the efficiences of these modified columns are lower than that of the unmodified β-cyclodextrin column.

Like the conventional β-cyclodextrin columns, the efficiency of these modified β-cyclodextrin columns is dramatically improved by adding TEAA (triethylammonium acetate) buffer in the mobile phase. As shown in Fig. 3, the presence of 0.3% TEAA in the mobile phase can produce a three-fold increase in the column efficiency.

Retention behaviour. For most solutes, the retention time on these multiple-interaction stationary phases is much longer than the retention time on the β-cyclodextrin-bonded stationary phase. For instance, the capacity factors of *o*- *m*-, and *p*-chlorophenols are more than two times larger than those on the β-cyclodextrin stationary phase (see Table II). The longer retention time means that the modification indeed increases the binding forces of inclusion complex formation between the guest and the cyclodextrin. However, the retention time for dansylamino acids is shorter than that obtained on the β-cyclodextrin column. The reason for this behaviour is that the modification may make the cyclodextrin cavity too shallow, so that the large naphthylamine group cannot completely fit into the cavity. The strength of the inclusion complex is, therefore, decreased.

TABLE II

CAPACITY FACTORS OBTAINED ON β-CYCLODEXTRIN AND MODIFIED β-CYCLODEXTRIN COLUMNS

Solute	Capacity factors		
	β-CD	β-CD–NHCH(CH$_3$)–	β-CD–NHCH(CH$_3$)–
o-Chlorophenol	0.87	2.56	2.32
m-Chlorophenol	1.00	2.35	2.15
p-Chlorophenol	1.10	2.16	2.01
COOH CHNHSO$_2$– CH$_2$OH –N(CH$_3$)$_2$	2.06	1.71	1.83

Enantioselectivities

It has been found that these multiple-interaction stationary phases exhibited higher enantioselectivity toward a wider variety of chiral compounds. The improved enantioselectivity may be rationalized in terms of the following two factors.

(1). The attached groups on the primary hydroxyl side of β-cyclodextrin cavity increase the capability of inclusion complexation with a wide variety of compounds.

It is believed that to achieve chiral separation on a β-cyclodextrin stationary phase, the guest molecule must form an enantioselective complex with β-cyclodextrin. Typically, cyclodextrin inclusion complex formation is associated with a favourable enthalpy and an unfavourable entropy change [4,10]. Although the exact nature of the bonding

forces involved in the complex formation still remains controversial, several proposals have been made to interpret the binding forces between the guest and the cyclodextrin molecule in solution [10–13]: (i) Van der Waals–Landon dispersion forces (the so-called "hydrophobic effect"); (ii) hydrogen bonding between the polar group of the guest molecule and the secondary hydroxyl groups of the cyclodextrin; (iii) release of high-energy water molecules in complex formation; and (iv) release of the strain energy in the ring frame system of the cyclodextrin. In most cases, a combination of these factors seems to be operative, with the first dominant. However, because the interior of the cavity, with a ring of ether oxygens, is not quite non-polar and both ends of the cavity are open to solvent, only those guest molecules which have large hydrophobic groups and appropriate shape can form strong inclusion complexes with it.

For these modified β-cyclodextrin stationary phases, the attached groups on the primary hydroxyl side can cluster to form a flexible cap on the smaller side of β-cyclodextrin cavity [14]. This will increase the hydrophobicity of the cavity, thus increasing the capability of the inclusion complex formation with the smaller molecules. It was found that the solutes which have only one benzyl ring in the molecule were indeed bound strongly on these modified β-cyclodextrin stationary phases as shown by the increased retention time on these columns.

(2). These stationary phases provide multiple-in-

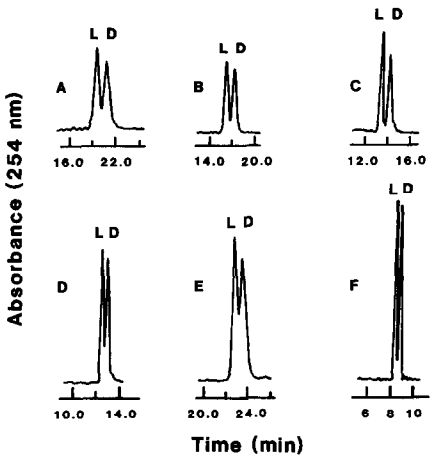


Fig. 4. Chromatograms for the resolution of dansyl-DL-amino acids. (A) dansyl-DL-norleucine; (B) dansyl-DL-valine; (C) dansyl-DL-leucine; (D) dancyl-DL-aspartic acid; (E) dansyl-DL-glutamic acid; (F) dansyl-DL-threonine. Column, (*S*)-(−)-α-methylbenzylamine-modified β-cyclodextrin-bonded column (250 × 4.6 mm I.D.); mobile phase, methanol–TEAA buffer solution (35:65) (0.3% TEAA, pH 5.5).

TABLE III

OPTICAL RESOLUTION OF THE ENANTIOMERS OF DANSYLAMINO ACIDS

Solute	β-CD–NHCH(CH$_3$)–⬡				β-CD
	k'^a	α	R_s	Mobile phase[b]	R_s[c]
Norleucine	5.27	1.10	1.01	30:70	2.30
Aspartic acid	5.00	1.11	0.90	30:70	–
Serine	2.50	1.09	0.80	30:70	0.43
Leucine	4.93	1.14	1.30	30:70	–
Valine	3.13	1.10	1.10	30:70	2.10
Norvaline	3.20	1.08	0.74	30:70	0.83
Glutamic acid	3.60	1.08	0.75	30:70	–
Methionine	2.97	1.07	0.75	30:70	0.70
Threonine	2.16	1.13	1.20	30:70	2.00
Phenylalanine	6.27	1.08	0.80	30:70	1.10

[a] The capacity factor of the first-eluted enantiomer.

[b] The numbers represent the volume ratio of methanol to TEAA buffer (0.3% TEAA, pH 6.2)

[c] Data from ref. 23.

teraction sites which increase the number of specific, discrete, and simultaneous interactions between chiral solute molecules and the stationary phase.

This modified β-cyclodextrin stationary phase has the following functional groups: (i) an hydrophobic cavity, capable of inclusion complexation; (ii) aromatic groups, capable of π–π interaction; (iii) polar hydrogen-bonding sites, capable of hydrogen binding with the polar functional groups on the chiral solutes; and (iv) bulky non-polar groups, providing steric repulsion. It has been believed that the greater the number of specific, discrete, and simultaneous interactions between chiral solutes molecules and a chiral locus on the stationary phase, the greater the likelihood of effective chiral discrimination, and thus of chromatographic resolution of enantiomeric solutes [15].

Enantiomeric separations

These modified stationary phases have demonstrated a broader range of applications. The enantiomeric separations of various compounds, including amino acids and their derivatives, carboxylic acids, phenothiazine and related drugs, and other pharmaceuticals have been achieved on these modified β-cyclodextrin columns.

TABLE IV

OPTICAL RESOLUTION OF THE ENANTIOMERS OF DNP-AMINO ACIDS

Solute[a]	Structure	β-CD–NHCH(CH$_3$)–C$_6$H$_5$				β-CD
		k'[b]	α	R_s	Mobile phase[c]	R_s[d]
DNP-DL-α-amino-*n*-butyrc acid	CH_3CH_2–CHCOOH | NHR	3.20	1.05	0.70	30:70	0.60
DNP-DL-norvaline	$CH_3CH_2CH_2$–CHCOOH | NHR	3.87	1.12	1.20	30:70	0.80
DNP-DL-norleucine	$CH_3(CH_2)_3$–CHCOOH | NHR	6.35	1.13	1,83	30:70	2.45
DNP-DL-α-amino-*n*-caprylic acid	$CH_3(CH_2)_5$–CHCOOH | NHR	11.01	1.21	1.62	30:70	3.40
DNP-DL-methionine sulfone	$CH_3SO_2CH_2CH_2$–CHCOOH | NHR	1.67	1.08	0.90	30:70	0.90
DNP-DL-methionine	$CH_3SCH_2CH_2$–CHCOOH | NHR	3.42	1.17	2.57	25:75	1.50
DNP-DL-ethionine	$CH_3CH_2SCH_2CH_2$–CHCOOH | NHR	7.22	1.17	2.37	30:70	2.50
DNP-DL-citrulline	$H_2NCONH(CH_2)_3$–CHCOOH | NHR	1.75	1.07	0.80	30:70	0.80
DNP-DL-glutamic acid	$HOOCCH_2CH_2$–CHCOOH | NHR	10.20	1.06	0.70	30:70	0.90

[a] R in the structures represents 2,4-dinitrophenyl.
[b] The capacity factor of the first eluted enantiomer.
[c] The number represents the volume ratio of methanol to TEAA buffer (0.3% TEAA, pH 5.5).
[d] Data from ref. 24.

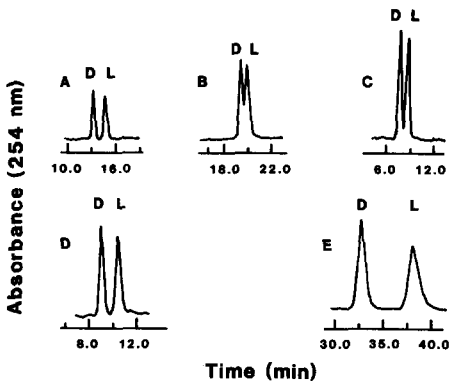


Fig. 5. Chromatograms for the resolution of DNP-DL-amino acids. (A) DNP-DL-ethionine; (B) DNP-DL-glutamic acid; (C) DNP-DL-methionine; (D) DNP-DL-norleucine; (E) DNP-DL-α-amino-*n*-caprylic acid. Column, (*S*)-(−)-α-methylbenzylamine-modified β-cyclodextrin-bonded column (250 × 4.6 mm I.D.); the mobile phase composition is given in Table IV.

Enantiomeric separation of dansylamino acids

1-Dimethylaminonaphthalene-5-sulfonyl amino acids (dansylamino acids) occupy a key position in the structural investigation of proteins and peptides, and the quantitative analysis of amino acids. Dansyl chloride reacts with free amino acids and is increasingly used in determining the amino-terminal residues of protein and peptides [16].

In this work, the enantiomeric separation of ten dansylamino acids has been achieved on the methylbenzylamine-modified β-cyclodextrin stationary phases using methanol–TEAA buffer solution (0.3% TEAA) as the mobile phase. Table III shows the optical resolution data for these ten dansylamino acids. All ten chiral compounds can be optically separated with resolution factors from 0.74 to 1.30. However, for most dansylamino acids, the resolution factor is lower than that achieved on the unmodified β-cyclodextrin column. The reason, as mentioned before, is that dansylamino acids cannot form strong inclusion complexes with modified β-cyclodextrin stationary phases.

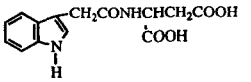
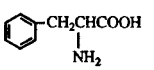
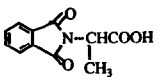
Fig. 4 shows some typical chromatograms for the solutions of these racemic dansylamino acids. The L-enantiomers are eluted first for all the dansylamino acids.

Enantiomeric separations of dinitrophenylamino acids

Dinitrophenylamino acids (DNP-amino acids) have also been used for the quantitative amino acid estimation and the structural investigation of pro-

TABLE V

OPTICAL RESOLUTION OF THE ENANTIOMERS OF OTHER AMINO ACIDS

Solute	Structure	β-CD–NHCH(CH$_3$)–C$_6$H$_5$			
		k'^a	α	R_s	Mobile phase
3-Indoletactylaspartic acid		11.1	1.18	1.36	20% Methanol, 0.3% TEAA, pH 7.0
Phenylalanine		2.06	1.17	1.50	20% Methanol, 0.3% TEAA, pH 5.5
Carbobenzyloxyalanine		3.68	1.09	0.75	30% Methanol, 0.3% TEAA, pH 5.5
N-Phthaloylalanine		1.45	2.56	2.80	50% Methanol, 0.3% TEAA, pH 5.5

[a] The capacity factor of the first-eluted enantiomer.

teins since they were first applied by Sanger[17] to the determination of the N-terminal residues of insulin. The peptides or proteins react with fluoro-2,4-dinitrobenzene in alkaline solution to give DNP derivatives. On hydrolysis the peptide chain is broken to free amino acids but the original N-terminal amino acid remains largely in the form of its DNP derivatives which can then be extracted with diethyl ether and fractioned for identification [18]. Like the dansylamino acids, DNP-amino acid derivatives are also important in the protein chemistry.

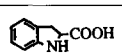
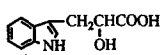
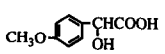
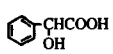
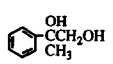
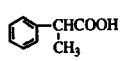
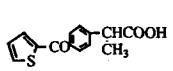
Table IV lists the resolution data for the enantiomers of DNP-amino acids. Some typical chromatograms are shown in Fig. 5. In these cases, the D-enantiomers are eluted first.

Enantiomeric separation of some other chiral compounds

It was also found that the modified β-cyclodextrin stationary phase exhibited a very good enantioselectivity for certain aromatic amino acids and other chiral compounds which contain only one benzyl ring in their molecules. The enantiomers of these chiral compounds, including phenylalanine, phthaloylalanine, tyrosine, carboxylic acids, and some pharmaceuticals, were separated on the methylbenzylamine- and naphthylethylamine-modified β-cyclodextrin columns with methanol–TEAA buffer (0.3% TEAA) as the mobile phase. To date there have been no reports on the resolution of any enantiomers of these chiral compounds on a unmodified β-cyclodextrin column.

TABLE VI

OPTICAL RESOLUTION OF THE ENANTIOMERS OF CHIRAL COMPOUNDS

Solute	Structure	β-CD–NHCH(CH$_3$)–C$_6$H$_5$			
		k'^a	α	R_s	Mobile phase
Indoline-2-carboxylic acid		1.84	2.08	3.0	20% Methanol, 0.25% TEAA, pH 7.0
3-Indoletactic acid		11.1	1.18	1.36	25% Methanol, 0.25% TEAA, pH 6.2
4-Methoxymandelic acid		3.77	1.27	1.01	25% Methanol, 0.25% TEAA, pH 6.2
Mandelic acid		0.42	2.54	3.33	30% Methanol, 0.3% TEAA, pH 5.5
2-Phenylpropanediol		1.0	1.19	1.01	30% Methanol, 0.3% TEAA, pH 5.5
2-Phenylpropionic acid		1.65	1.24	1.26	30% Methanol, 0.3% TEAA, pH 5.5
Suprofen		5.25	1.11	0.90	40% Methanol, 0.25% TEAA, pH 6.0

[a] The capacity factor of the first-eluted enantiomer.

Table V shows the optical resolution data for some aromatic amino acids which have only one benzene ring in the molecule. As can been seen, good resolution has been achieved for these D/L pairs with R_s values as large as 2.80 for phthaloyl-alanine.

Table VI summarizes the optical resolution data for other chiral compounds. Most of them are carboxylic acids with only one benzene ring in the molecule. Very good resolutions have been achieved on the methylbenzylamine-modified stationary phase with R_s values greater than 3.00 in several cases.

Table VII shows the optical resolution data for chiral phenothiazine drugs on a naphthylethylamine-modified β-cyclodextrin-bonded phase column. Although the enantiomers of these compounds can be separated on the unmodified β-cyclodextrin column, the resolution factors are much lower than these values.

Fig. 6 shows some typical chromatograms of the chiral compounds.

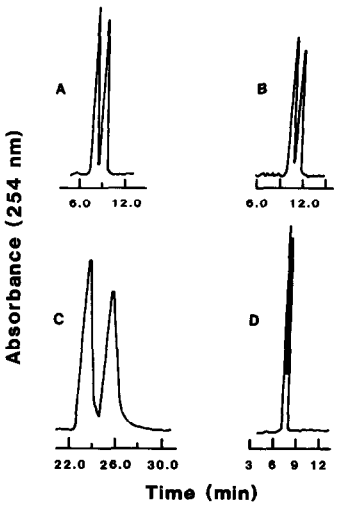


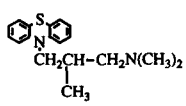
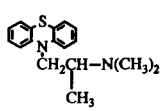
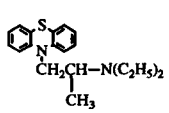
Fig. 6. Chromatograms for the enantiomeric separation of some other chiral compounds. (A) 2-phenylpropionic acid; (B) phenylalanine; (C) trimeperazine; (D) 2-indoletactylaspartic acid. Column, (*S*)-(−)-α-methylbenzylamine-modified β-cyclodextrin-bonded column (250 × 4.6 mm I.D.); mobile phase, methanol–TEAA buffer (30:70) (0.3% TEAA, pH 5.5).

Effect of mobile phase composition on the retention and resolutions

In this work, several polar solvents, such as water, methanol, ethanol, acetonitrile and tetrahydrofuran, were investigated as the potential mobile phase for these modified β-cyclodextrin stationary phases. It was found that most chiral compounds of

TABLE VII

OPTICAL RESOLUTION OF THE ENANTIOMERS OF PHENOTHIAZINES

Solute	Structure	β-CD–NHCH(CH$_3$)–			
		k'^a	α	R_s	Mobile phase
Trimeperazine		7.77	1.14	1.21	30% Methanol, 0.7% TEAA, pH 5.5
Promethazine		7.74	2.40	3.67	30% Methanol, 0.3% TEAA, pH 5.5
Ethopropazine		10.1	2.79	4.00	30% Methanol, 0.3% TEAA, pH 5.5

[a] The capacity factor of the first-eluted enantiomer.

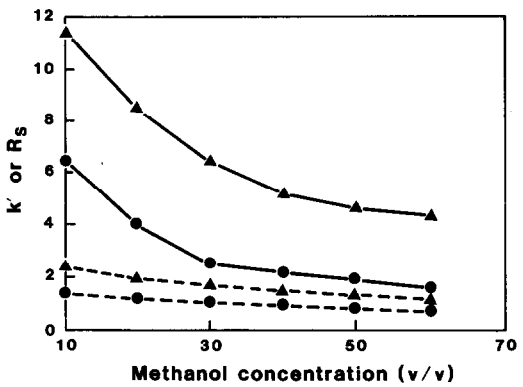


Fig. 7. Effect of methanol concentration on the retention and resolution. Column, (*S*)-(−)-α-methylbenzylamine-modified β-cyclodextrin-bonded column; mobile phase, methanol–TEAA buffer (0.5% TEAA, pH 5.5); flow-rate, 1 ml/min. ▲ = DNP-2-aminocaprylic acid, ● = 4-methoxymandelic acid. —— = k', – – – = R_s.

interest cannot be eluted within a reasonable time from the modified β-cyclodextrin columns using water alone as the mobile phase. The use of 100% methanol, ethanol, acetonitrile or tetrahydrofuran gives no resolution at all. The methanol–water and ethanol–water mobile phases were found to provide much better selectivity than acetonitrile–water and tetrahydrofuran–water mobile phases. The methanol–water system was chosen as the mobile phase.

In order to find the optimum conditions for good resolution, the effects of methanol concentration, TEAA buffer, and pH on the retention time and resolution were studied.

Effect of methanol concentration in the mobile phase

The effect of methanol concentration in the mobile phase on the retention and resolution was investigated by changing the methanol/water ratio in the mobile phase from 10:89.5 to 60:39.5 (v/v). TEAA concentration was 0.5%, and the pH was controlled at 5.5. It was found that the effect of methanol content on the retention and optical resolution gave almost the same tendencies as those observed on the unmodified β-cyclodextrin column. An increase in the methanol concentration resulted in a decrease in boh retention time and resolution factor (see Fig. 7). The effect of methanol concentration on the resolution was almost linear. When the methanol concentration reached 75%, no resolution could be observed for almost all the chiral compounds.

This is not surprising since it is known from the cyclodextrin-binding studies that methanol and other alcohols, such as ethanol, propyl alcohol, and *n*-butyl alcohol, can form inclusion complexes with β-cyclodextrin [19]. The formation constant of the β-cyclodextrin-methanol (1:1) complex has been measured by spectrophotometric examination of the effect of methanol on the association of β-cyclodextrin with sodium 4-[(4-hydroxy-1-naphthyl) azo]-1-naphthalenesulfonate and phenolphthalein [20,21], respectively. Association constants of 0.32 and 0.40 M^{-1} were reported. Assuming that the same considerations apply to our system, the following simple complexation pattern can be used to explain the results obtained:

$$G + CD = G\text{–}CD \quad (1)$$

$$K = [G\text{–}CD]/[G]\,[CD] \quad (2)$$

$$Me + CD = Me\text{–}CD \quad (3)$$

$$K_{Me\text{–}CD} = [Me\text{–}CD]/[Me]\,[CD] \quad (4)$$

Where CD, G, and Me denote β-cyclodextrin, DNP-amino acids, and methanol, respectively. G–CD and Me–CD are the inclusion complexes of β-cyclodextrin–DNP-amino acid and cyclodextrin–methanol, respectively. K and $K_{Me\text{–}CD}$ are the corresponding complex formation constants. Therefore, the true formation constant (K^*) for the inclusion complex of cyclodextrin with solute can be expressed as

$$K^* = \frac{K}{1 + K_{Me\text{–}CD}\,[Me]} \quad (5)$$

If the possible formation of higher stoichiometries between β-cyclodextrin and methanol is taken into consideration, the following equation can be derived:

$$K^* = \frac{K}{1 + K_{Me\text{–}CD}\,[Me] + K_{Me\text{–}CD}\,[Me]^2 + \ldots} \quad (6)$$

As can be seen from eqn. 6, at higher methanol concentrations, methanol can become strongly competitive for complexation with β-cyclodextrin thereby decreasing the degree of complexation between solute and β-cyclodextrin.

In addition, at high methanol concentrations, the properties of the bulk solvent begin to change substantially. Consequently, the presence of the methanol will likely make the solvent more favourable to the solute than a simple aqueous solution. The difference in hydrophobicity of the solvent and β-cyclodextrin cavity will become smaller, making inclusion complex formation between solute and β-cyclodextrin less favourable. Both of these effects could account for the decreases in retention time and resolution factor when the methanol content in the mixture is increased.

Effect of TEAA concentration in the mobile phase

Fig. 8 shows the effect of TEAA buffer concentration in the mobile phase on the retention and optical resolution. It was found that an increase in the TEAA concentration in the mobile phase results in a decreased retention time for both enantiomers. As was observed on unmodified β-cyclodextrin stationary phase, the effect of TEAA concentration on the optical resolution is somewhat more complex. When the TEAA concentration changed from 0% to 1.0%, two types of behaviour can be observed. At the beginning, the resolution increases with the increasing TEAA concentration and then it decreases when TEAA exceeds 0.3%. Resolution maxima are observed at a TEAA concentration of about 0.3%. These observations can be explained by considering the effect of TEAA on both the column efficiency (N) and the enantioselectivity (α) of β-cyclodextrin bonded phase column, and the relationship of resolution (R_s), N and α [22]:

$$R_s = \tfrac{1}{4}(\alpha - 1)\sqrt{N}\,\frac{k'}{(1 + k')} \qquad (7)$$

It has been found that the addition of TEAA buffer in the mobile phase will substantially increase the separation efficiency (N) of a modified β-cyclodextrin-bonded phase column. The increase in separation efficiency will result in an increase in the resolution factor.

On the other hand, the TEAA molecule, as an organic modifier, can include in the β-cyclodextrin cavity and there it competes with solute. The addition of TEAA in the mobile phase will weaken the strength of inclusion complexation between the solutes and the β-cyclodextrin cavity, resulting in a decrease in the enantioselectivity. The results obtained in this study indicate that at low TEAA concentrations the separation efficiency is the limiting factor for the resolution. An increase in TEAA concentration increases the column efficiency, thus increasing the resolution. As the TEAA concentration increases in the mobile phase, the enantioselectivity becomes the limiting factor, thus the resolution decreases with the increasing TEAA concentration. When TEAA concentration exceeds 2.5%, no optical resolution can be observed for most of the chiral compounds.

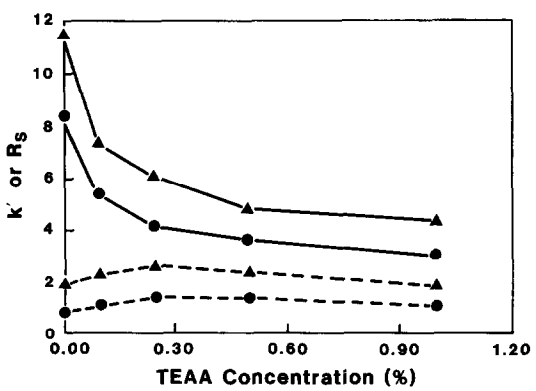


Fig. 8. Effect of TEAA concentration on the retention and resolution. Column, (*S*)-(−)-α-methylbenzylamine-modified β-cyclodextrin-bonded column; mobile phase, methanol–TEAA buffer (35% methanol, pH 5.5); flow-rate, 1 ml/min. ▲ = DNP-2-aminocaprylic acid, ● = 4-methoxymandelic acid. —— = k' − − − = R_s.

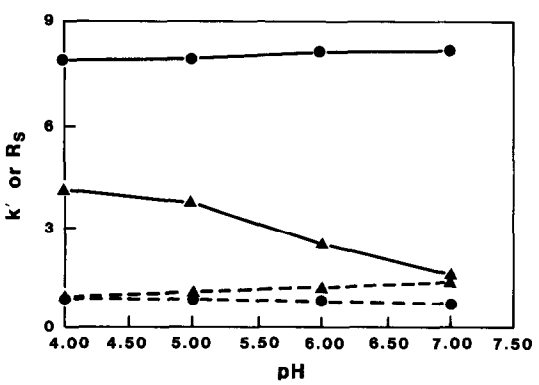


Fig. 9. Effect of pH on the retention and resolution. Column, (*S*)-(−)-α-methylbenzylamine-modified β-cyclodextrin-bonded column; mobile phase, methanol–TEAA buffer (0.3% TEAA, 35% methanol); flow-rate, 1 ml/min. ▲ = Dansyl-serine, ● = trimeperazine. —— = k', − − − = R_s.

Effect of pH

The effect of pH on the retention and resolution was investigated by changing pH of the mobile phase from 4.0 to 7.0 using a 0.5% TEAA buffer. Two typical plots are shown in Fig. 9. It was found that pH only affects the retention and resolution of those compounds which have an ionization constant in the range of 10^{-4} to 10^{-7}. For example, over this pH range the compound trimeperazine, which has a pK_a value of 9.0, will be in its molecular form. Its retention and resolution are not affected by changing pH. The other line shows the effect of pH on dansylserine. Both the retention time and resolution decreased with increasing pH. This is probably due to ionization of this compound.

In general, the effect of mobile phase composition can be summed up as follows: (i) increasing methanol content decreases the retention time and the resolution; (ii) the TEAA buffer improves column efficiency, but decreases the enantioselectivity; and (iii) neutral molecules are more readily retained than charged molecules.

CONCLUSIONS

The modified β-cyclodextrin chiral stationary phases can be seen as a combination of donor–acceptor phase and cyclodextrin phase. They have, on the molecular level, an hydrophobic cavity capable of forming inclusion complexes with the hydrophobic moiety of the solute molecule, aromatic groups capable of π–π (charge-transfer) interaction, polar hydrogen-bonds sites which can form hydrogen bonding with the polar functional groups of the solute, and bulky non-polar groups which provide steric repulsion, Van der Waals interaction and conformational control. These multiple-interaction chiral stationary phases have exhibited a high stereoselectivity toward a wide variety of chiral compounds. Enantiomeric separations of some chiral compounds which have only one benzyl ring in the molecule have been achieved without derivatization. These phases can be operated in the reversed-phase mode with mobile phase containing water and organic modifier. They are chemically and physically robust, and they have a chromatographic behaviour similar to the unmodified β-cyclodextrin column.

These multiple-interaction stationary phases have some disadvantages. (1) For some solutes the retention time is much longer than that with the unmodified β-cyclodextrin stationary phase. This may be a problem for fast analyses. (2) The stationary phases have a light brown coloration probably resulting from the formation of the nitroxide. The presence of the brown color makes the stationary phase unsuitable for thin-layer chromatography.

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