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Liquid chromatographic separation of the enantiomers of dinitrophenyl amino acids using a β-cyclodextrin-bonded stationary phase

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ABSTRACT

A β-cyclodextrin-bonded chiral stationary phase was used for the liquid chromatographic resolution of racemic amino acid derivatives. Ten pairs of dinitrophenyl amino acid enantiomers were separated by this technique. Methanol–triethylammonium buffer solutions were used as the mobile phases. The effects of pH, methanol and triethylammonium acetate buffer concentration in the mobile phase, and the structural features of the solutes on the retention and enantio-selectivity were examined and discussed in terms of the overall retention mechanism.

INTRODUCTION

Enantiomeric separation of amino acids is important in many fields, such as peptide synthesis, asymmetric syntheses in organic chemistry, amino acid biochemistry, the studies of food processing and protein degradation processes in humans [1–3] and the dating of archaeological materials [4]. For the last several years, there existed two general approaches to the high-performance liquid chromatographic separations of the enantiomers of amino acids. One is ligand-exchange chromatography using a chiral ligand immobilized on a solid support as the stationary phase. The other technique employs active chelates in the mobile phase. Recently, cyclodextrin-bonded stationary phases developed by Armstrong and co-workers [5,6] were also successfully used for the enantiomeric resolution of amino acids.

Cyclodextrins (CDs) are oligosaccharides in which glucose units are joined together to form a toroidal structure with a hydrophobic cavity and hydrophilic exterior faces. The main property of CDs which allows them to affect chiral separation is their ability to form enantio-selective inclusion complexes with guest molecules. It is believed that chiral recognition is caused by inclusion complex formation between the cavity of CD and the hydrophobic moiety of the solute, and by hydrogen bonding between the polar functional groups of the solute in the vicinity of its chiral center and the hydroxyl groups of the CD [5]. The enantiomers of tryptophan, phenylalanine, tyrosine and analogues have been separated on an α-CD bonded phase column [6]. The

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separation of some racemic 5-dimethylamino-1-nahthalenesulphonyl (dansyl) amino acids using a β-CD bonded phase column have also been reported [5]. To date, however, there has been no report on the enantiomeric separation of any 2,4-dinitrophenyl (DNP) amino acids on any CD-bonded stationary phase.

In this paper we describe a method for separating D- and L-DNP-amino acids using a β-CD bonded stationary phase with traditional aqueous-organic mobile phases. The effects of pH, the mobile phase composition, buffer concentration and the structural features of solutes on the retention time and enantiomeric resolution will be discussed in terms of the retention mechanism.

EXPERIMENTAL

Apparatus

Chromatography was performed using a liquid chromatographic system which consisted of a Model 590 pump (Waters Assoc., Milford, MA, U.S.A.), a Model 7125 injector containing a 10-μl loop (Rheodyne, Cotati, CA, U.S.A.) and a Model 440 UV detector (Waters). The chromatograms were recorded on a Model SE120 strip chart recorder (Goerz Electro, Austria). The column temperature was controlled through a HETO 623 water bath (Bach-Simpson, London, Canada).

A Cyclobond I, 250 × 4.6 mm I.D. column was purchased from Advanced Separation Technologies (Whippany, NJ, U.S.A.). The Cyclobond I column is β-CD molecules chemically bonded to spherical silica gel through a five-atom, non-nitrogen-containing spacer. When not in use, the column was stored in 100% methanol.

Chemicals

All D- and L-DNP-amino acids were obtained from Sigma (St. Louis, MO, U.S.A.). HPLC-grade methanol and triethylamine were purchased from Fisher (Fair Lawn, NJ, U.S.A.). Glacial acetic aid was obtained from Allied Chemical (Pointe Claire, Canada). Water was deionized by passing distilled water through a Barnstead water-purification system.

Procedures

Mobile phase was prepared by mixing methanol with triethylammonium acetate (TEAA) buffer. The mobile phase was degassed by bubbling helium into it for about 10 min before use. Sample solutions were prepared by dissolving each raceme in methanol to give a concentration of about 1 mg/ml. Typically, 2 μl of sample solution were injected. The chromatography was performed at a flow-rate of 1.0 ml/min. Absorbance of the column effluent was monitored at a wavelength of 254 nm.

All data points on graphs were obtained by averaging at least three separate determinations. A careful reproducibility study involving five injections revealed a relative standard deviation of less than 2% in capacity factors, and of less than 6% in resolution factors.

RESULTS AND DISCUSSION

In this investigation, the optical separations of ten DNP-amino acids were examined using a β-CD bonded phase column. Table I lists the optical separation data

TABLE I

OPTICAL RESOLUTION OF THE ENANTIOMERS OF DNP-AMINO ACIDS

The separation was done on a 250 × 4.6 mm I.D. β-cyclodextrin-bonded phase column. In the structures, *R* represents 2,4-dinitrophenyl. k' = Capacity factor of the first eluted enantiomers; α = enantioselectivity; R_s = resolution.

No.	Solutes	Structure	k'	α	R_s	Mobile phase[a]
1	DNP-DL-α-Amino-*n*-butyric acid	CH_3CH_2–CHCOOH NHR	3.00	1.04	0.60	20:80
2	DNP-DL-Norvaline	$CH_3CH_2CH_2$–CHCOOH NHR	4.0	1.06	0.80	10:90
3	DNP-DL-Norleucine	$CH_3(CH_2)_3$–CHCOOH NHR	2.67	1.28	2.45	25:75
4	DNP-DL-α-Amino-*n*-caprylic acid	$CH_3(CH_2)_5$–CHCOOH NHR	11.3	1.30	3.40	25:75
5	DNP-DL-Methionine sulphoxide	$CH_3SOCH_2CH_2$–CHCOOH NHR	6.67	1.05	0.8	5:95
6	DNP-DL-Methionine sulphone	$CH_3SO_2CH_2CH_2$–CHCOOH NHR	1.32	1.05	0.9	5:95
7	DNP-DL-Methionine	$CH_3SCH_2CH_2$–CHCOOH NHR	1.88	1.14	1.50	25:75
8	DNP-DL-Ethionine	$CH_3CH_2SCH_2CH_2$–CHCOOH NHR	5.00	1.18	2.50	25:75
9	DNP-DL-Citrulline	$H_2NCONH(CH_2)_3$–CHCOOH NHR	1.90	1.05	0.80	5:95
10	DNP-DL-Glutamic acid	$HOOCCH_2CH_2$–CHCOOH NHR	3.60	1.06	0.90	10:90

[a] The numbers represents the volume ratio of methanol to TEAA buffer (0.5% TEAA, pH 6.20).

obtained, together with the structures of these amino acid derivatives. The L-isomers are eluted first for all the DNP-derivatized amino acids. The same elution order has been reported for dansyl-amino acids [5]. Some typical chromatograms are shown in Fig. 1. As can be seen, the β-CD column exhibits high enantioselectivity toward the DNP-amino acids. The enantiomers of these DNP-amino acids could be separated on a 250 × 4.6 mm I.D. β-CD bonded phase column with resolution factors from 0.6 up to 3.40.

Effect of structural features on enantioselectivity

From our experience of working with a β-CD bonded phase column and to the best of our knowledge, no enantiomers of underivatized amino acids can be separated with the β-CD column. It is believed that the size of unsubstituted amino acids is too small to bind tightly with the CD cavity to form a strong inclusion complex [7,8], a prerequisite for the chiral recognition. The results of this study show that the DNP substituent of the amino acids plays an important role in chiral recognition. This is not

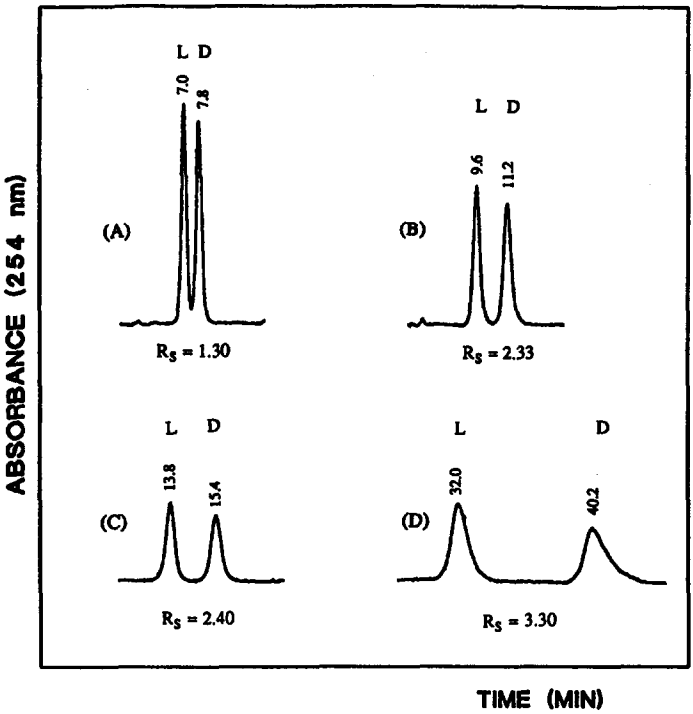

Fig. 1. Chromatograms showing the resolutions of DNP-D,L-amino acids. (A) DNP-DL-Methionine; (B) DNP-DL-norleucine; (C) DNP-DL-ethionine; (D) DNP-DL-α-amino-*n*-caprylic acid. Column, 250 × 4.6 mm I.D. Cyclobond I; mobile phase, 30:70 methanol–TEAA buffer (0.5%, pH 6.2) (30:70); temperature, 20°C; flow-rate, 1 ml/min.

surprising since it had been previously reported that the nitrophenyl group could tightly bind to the β-CD cavity to form a strong inclusion complex [9,10]. The introduction of a DNP substituent into the amino acid molecules provides the strong binding site required for the chiral recognition.

A comparison of the α values obtained for these DNP-amino acids indicates that the enantioselectivity is also affected by the size of the alkyl substituents around the chiral center. As can be seen from Table I, the enantioselectivities (α) for α-amino-*n*-butyric acid, norvaline, norleucine and α-amino-*n*-caprylic acid are 1.04, 1.06, 1.28 and 1.30, respectively. The only difference between the structures of these solutes is the size of the alkyl substituent on the chiral center. The results obtained in this study show that the enantioselectivity increases with the size of alkyl substituent. This is true for the enantioselectivities of DNP-DL-methionine ($\alpha = 1.14$) and DNP-DL-ethionine ($\alpha = 1.18$) as well. It seems that the alkyl substituent plays an important role of steric hindrance, which weakens the strength of inclusion complexation and/or hydrogen bonding for one of the enantiomers.

Effect of methanol content

The effects of the methanol content on the retention and resolution were investigated by changing the methanol-water ratio in the mobile phase. Fig. 2 shows

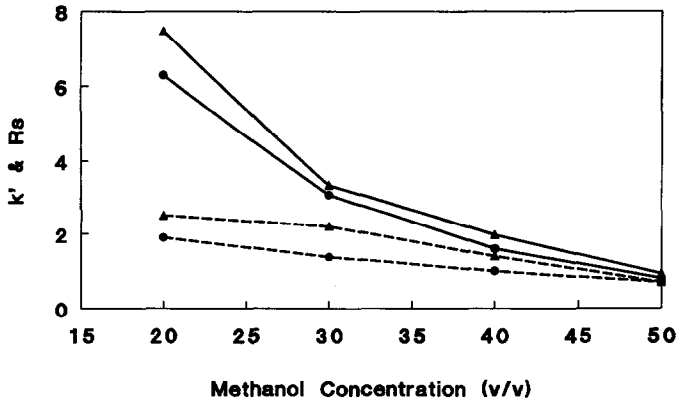

Fig. 2. Effect of methanol concentration in the mobile phase on the retention (solid lines: capacity factors of the first eluted enantiomers, k') and resolution (broken lines: R_s) of (▲) DNP-DL-ethionine and (●) DNP-DL-norleucine. Column, 250 × 4.6 mm I.D. Cyclobond I; mobile phase, 0.5% TEAA and pH 5.0; temperature, 20°C; flow-rate, 1 ml/min. k' is the capacity factor of the first eluted enantiomer.

the typical plots of capacity factors and resolution factors *versus* the methanol contents. The TEAA buffer concentration is 0.5%, and the pH of the mobile phase is 5.0 in this set of experiments.

It is found that the effect of methanol content on retention and optical resolution of DNP-amino acids gives almost the same tendencies as those observed with dansyl-amino acids [5], that is, an increase in the methanol content results in both a decreased retention and a decreased enantioselectivity or resolution factor. For some of these DNP-amino acids, the optical separation can be achieved only at very low methanol content (< 10%). It is not surprising since it is known from the CD-binding studies that an increase in organic content in the solvent will weaken the strength of inclusion complexation between guest molecules and β-CD [11]. In this case, the increase of methanol content in the mobile phase diminishes the extent of inclusion complexation between the DNP substituent and β-CD, resulting in a decreased retention and a decreased optical resolution factor.

Effect of TEAA buffer concentration

Fig. 3 shows the influence of TEAA buffer concentration in the mobile phase on the retention and resolution. It is found that an increase in the TEAA concentration in the mobile phase results in a decreased retention of both enantiomers in all instances. However, the effect of TEAA concentration on the optical resolution is somewhat more complex. As the TEAA concentration in the mobile phase varies from 0.1% to 1.0%, two types of behavior can be observed. The resolution increases with the increasing TEAA concentration and then decreases when TEAA is greater than 0.5%. Resolution maxima are observed at TEAA concentrations of about 0.5%.

These facts can be explained by considering the effect of TEAA on both the column separation efficiency (N) and the enantioselectivity (α) of β-CD column. It has been found that the addition of TEAA buffer in the mobile phase substantially increased the separation efficiency of a β-CD bonded phase column. A TEAA buffer

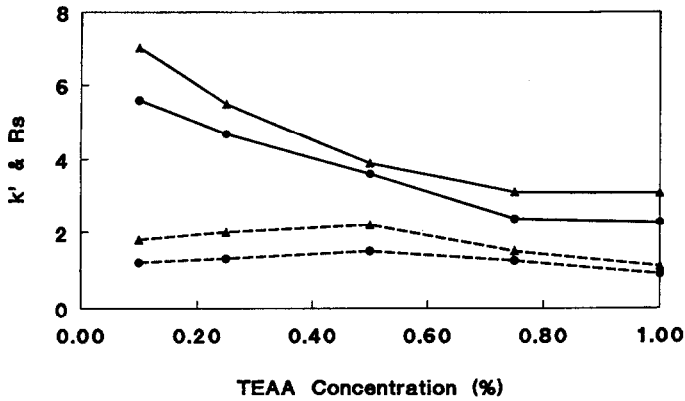

Fig. 3. Effect of TEAA concentration in the mobile phase on the retention and resolution of (▲) DNP-DL-ethionine and (●) DNP-DL-norleucine. Conditions: column, 250 × 4.6 mm I.D. Cyclobond I; mobile phase, methanol–TEAA buffer solution (30:70); pH 5.0; temperature, 20°C; flow-rate, 1 ml/min. Solid and broken line as in Fig. 2.

(0.02 *M* and pH 5.0) substituted for water in a methanol–water (40:60) system can produce a three- to four-fold increase in the column efficiency for the separation of phenothiazine derivatives [12]. The increasing separation efficiency will result in an increase in the resolution. On the other hand, the TEAA molecule, as an organic modifier, can include in the β-CD cavity and there it competes with solute. The addition of TEAA in the mobile phase will weaken the strength of inclusion complexation between the DNP substituent and the β-CD 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 optical resolution. As the TEAA concentration increases in the mobile phase, the enantioselectivity (α) becomes the limiting factor, and the resolution decreases with the increasing TEAA concentration. When the TEAA concentration increases to 1.5%, no optical separation can be observed for most of these amino acid derivatives.

Effect of pH

The influence of pH on the retention and resolution of DNP-DL-amino acids was investigated by changing the pH of the mobile phase from 4.5 to 7.0 using 0.5% TEAA buffer. Some typical plots of the capacity factor and resolution *versus* pH values are shown in Fig. 4. The retention timse of all the DNP-amino acids decreased with increasing pH. This fact can be explained by considering the effect of pH on the form of the amino acids and on the bonding strength of the carboxylic acid function to the hydroxyl group of β-CD.

By N-substitution, the DNP-amino acids have lost the dipolar ion character of the parent amino acids, and hence can be considered as carboxylic acids with a –NHDNP substituent mostly at α-position. Since the –NHDNP group is a strong electron-withdrawing group, the pK_a values of DNP-amino acids should be much

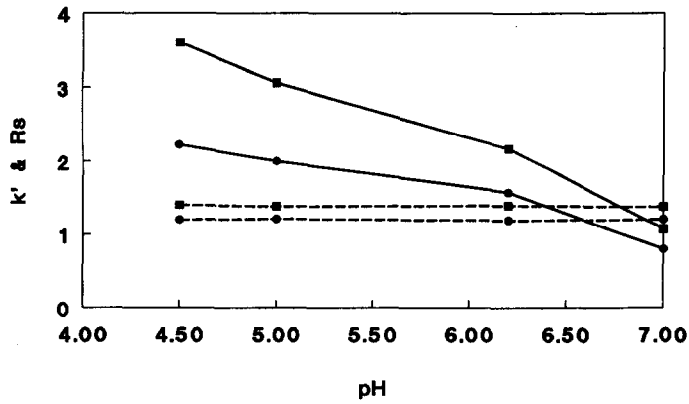

Fig. 4. Effect of pH on the retention and resolution of (■) DNP-DL-ethionine and (●) DNP-DL-methionine. Conditions: column, 250 × 4.6 mm I.D. Cyclobond I; mobile phase, methanol–TEAA buffer (0.5% TEAA) (30:70); temperature, 20°C; flow-rate, 1 ml/min. Solid and broken lines as in Fig. 2.

lower than that of carboxylic acids, even much lower than the pK_a value (3.71) of N-acetylglycine ($CH_3CONHCH_2COOH$) [13]. Therefore, it can be expected that the DNP-amino acids exist mainly in the form of anions in the pH range of 4.5 to 7.0. Thus, the strength of the inclusion complexation between the DNP substituent and the cavity of cyclodextrin should not be affected by changing pH. However, it had been reported that the OH^- ion had a high hydrogen bonding ability to the hydroxyl groups of ROH molecules [14,15]. The existence of OH^- in the mobile phase will compete with the carboxylate group of DNP-amino acids to interact with the hydroxyl group of cyclodextrin. With the increase of OH^- concentrations in the mobile phase, caused by the increasing pH, the bonding strength between the carboxylate group of the solute and the hydroxyl group of the cyclodextrin, and thus the overall interaction of solute with β-CD, will be weakened. Therefore, a decreased retention time with the increase of pH is observed.

Fig. 4 also shows that the optical resolutions are not affected by changing pH. This suggests that the interaction between the polar groups of solute and the hydroxyl groups of CD is not the main factor for the chiral recognition.

CONCLUSIONS

It has been demonstrated that the β-CD bonded phase column exhibits a high enantioselectivity for the DNP-amino acid derivatives. The effects of methanol and TEAA concentration in the mobile phase, pH, and the structural features on the retention and resolution suggest that the inclusion complex formation between the cavity of CD and the DNP substituent, and the steric hindrance of alkyl substituents around the chiral center of the solutes are the important factors in the chiral recognition.

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