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Separation of benz[*f*]isoindole derivatives of amino acid and amino acid amide enantiomers on a β-cyclodextrin-bonded phase

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ABSTRACT

A liquid chromatographic method for the determination of amino acid enantiomers and related amino acid amide enantiomers is presented. Fluorescent derivatives were formed by reaction with naphthalene-2,3-dicarboxaldehyde–cyanide reagent. The resulting benz[*f*]isoindole amino acid enantiomers and amino acid amide enantiomers were separated on a β-cyclodextrin-bonded phase. The separations were performed with ammonium nitrate buffer containing methanol as mobile phase. The influence of methanol, pH and buffer concentration in the mobile phase on retention, enantioselectivity and resolution were examined and are discussed. Remarkable differences in retention behaviour were found for the amino acids and amino acid amides studied on changing mobile phase parameters such as buffer concentrations and pH. With this method, the separation of an amino acid, together with the corresponding amino acid amide, into their enantiomers can be achieved in a single isocratic run. The method is suitable for monitoring the enantiomeric purity of an amino acid obtained by enantioselective hydrolysis of the corresponding acid amide using an aminopeptidase.

INTRODUCTION

Optically pure α-amino acids are important intermediates for the production of pharmaceuticals, food chemicals and agrochemicals. One of the routes to optically pure α-amino acids is through organic synthesis of racemic α-amino acid amides followed by an enantioselective hydrolysis using a peptidase with relaxed substrate specificity to achieve resolution on a large scale. In conjunction with this synthesis, analytical methods are required for the control of the enantiomeric purity of both α-amino acids and α-amino acid amides.

Several high-performance liquid chromatographic (HPLC) methods for the enantiomeric resolution of α-amino acids have been described. These methods can be divided into three categories: (a) enantioseparation on chiral stationary phases (CSPs) directly or by using precolumn derivatization; (b) resolution of the enantiomers by means of a chiral mobile phase, *e.g.*, ligand-exchange methods; and (c) precolumn derivatization with a chiral reagent, followed by chromatographic analysis of the resulting diastereomers.

The aim of this work was to evaluate the potential of a β-cyclodextrin (β-CD)-bonded stationary phase for the enantioseparation of α-amino acids and α-amino acid amides obtained by enantioselective synthesis.

In earlier studies [1], it was shown that β-CD-bonded phases can form inclusion complexes with derivatized and underivatized α-amino acids. However, derivatized α-amino acids containing two or more aromatic rings are separated the most effectively. The enantioselectivity of the following types of α-amino acid derivatives has been studied on β-CD phases: dansyl-α-amino acids [2–4], α-amino acid β-naphthylamides [2–4], α-amino acid β-naphthyl esters [2–4], isoindole α-amino acids [5], N-(3,5-

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dinitrobenzoyl)-α-amino acids [6] and N-(2,4-dinitrophenyl)-α-amino acids [7]. Except for the N-(3,5-dinitrobenzoyl) and N-(2,4-dinitrophenyl) derivatives, all of the above-mentioned derivatives contained two rings.

The reaction of α-amino acids with naphthalene-2,3-dicarboxyaldehyde (NDA) and cyanide results in the formation of N-substituted 1-cyanobenz[*f*]isoindole (CBI) derivatives [8], which can subsequently be analysed by reversed-phase chromatography.

In this paper, we report on the use of a β-CD phase for the enantioseparation of CBI derivatives of several α-amino acids and α-amino acid amides. The effects of pH, methanol and buffer concentration in the mobile phase and the structural features of the solutes on the retention, enantioselectivity and resolution were examined.

EXPERIMENTAL

Materials

Butyrine (But), valine (Val), norvaline (NVal), *p*-hydroxyphenylglycine (HPG), phenylglycine (PG) and phenylalanine (Phe) were obtained from Sigma (St. Louis, MO, USA). The corresponding acid amides were synthesized in our laboratory [9]. For each compound, both the racemic form and at least one optically pure enantiomer were available. Naphthalene-2,3-dicarboxyaldehyde was supplied by Polysciences (Warrington, PA, USA). Water was purified with a Milli-Q system (Millipore). HPLC-grade methanol was obtained from Merck and used as supplied. All other chemicals were of analytical-reagent grade.

Instrumentation

HPLC was performed using a Gilson (Villiers-le-Bel, France) Model 302 pump and a Gilson Model 231-401 autosampling injector for derivatization and injection. The injection loop had a 20-μl capacity. The β-cyclodextrin-bonded column (Cyclobond I, 250 × 4.6 mm I.D.) was obtained from Advanced Separation Technologies (Whippany, NJ, USA). The column was thermostated at 22°C. The derivatives were monitored with a Hitachi (Tokyo, Japan) Model F-1050 fluorescence detector using an excitation wavelength of 420 nm and an emission wavelength of 520 nm.

Mobile phases and derivatization procedure

Ammonium nitrate buffers were prepared by dissolving the required amount of ammonium nitrate in water. Mobile phases were prepared by mixing the buffer solutions with the required amount of methanol.

For derivatization, NDA reagent was prepared by dissolving 0.2 mg of NDA per ml of methanol. For the cyanide reagent, 0.2 mg of potassium cyanide was dissolved per ml of 0.4 *M* potassium borate buffer (pH 9.4). α-Amino acids and α-amino acid amides were dissolved in water. Derivatization was performed automatically with a Gilson Model 231-401 system. The reaction mixture consisted of 200 μl of NDA reagent, 200 μl of cyanide reagent and 20 μl of the analyte solution. The reaction mixture was allowed to stand for at least 2 min at room temperature before an aliquot was injected into the HPLC system.

RESULTS AND DISCUSSION

The enantioselectivity of the CBI derivatives of a series of aliphatic and aromatic α-amino acids and α-amino acid amides on a β-CD-bonded phase column was examined. In Table I, the capacity factors

TABLE I

SEPARATION DATA FOR THE ENANTIOMERS OF CBI-AMINO ACIDS AND AMINO ACID AMIDES

Chromatographic conditions: flow-rate, 0.8 ml/min; mobile phase, 0.04 *M* ammonium nitrate solution (pH 7.0) containing 45% (v/v) methanol. For other conditions, see Experimental.

α-amino acid or amide	k'^a	α	R_s
But	13.35	1.05	0.94
But-NH_2	3.71	1.07	0.96
Val	15.76	1.05	0.85
Val-NH_2	4.46	1.10	1.31
NVal	14.06	1.03	<0.50
NVal-NH_2	3.88	1.04	<0.50
HPG	17.72	1.07	1.26
HPG-NH_2	4.75	1.05	<0.50
PG	30.97	1.06	1.13
PG-NH_2	6.02	1.04	0.54
Phe	20.03	1.09	1.59
Phe-NH_2	4.73	1.09	1.18

[a] Capacity factor of the first-eluted enantiomer.

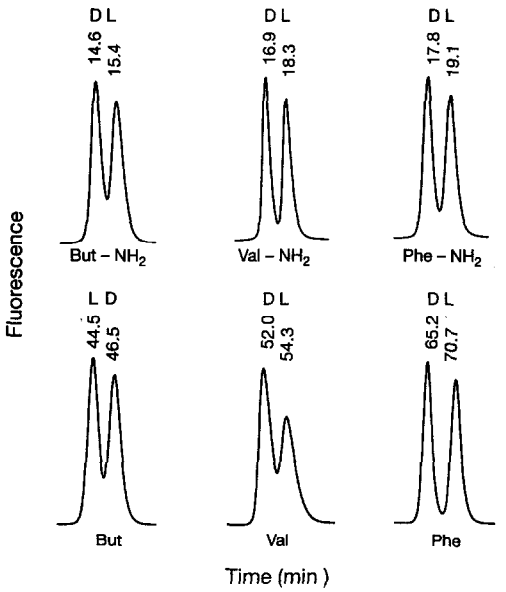

Fig. 1. Enantiomeric separation of the CBI derivatives of But, But-NH_2, Val, Val-NH_2, Phe and Phe-NH_2. For conditions, see Table I.

(k'), selectivities (α) and resolutions (R_s) obtained for these compounds are shown. Some typical chromatograms are shown in Fig. 1.

From Table I, it can be seen that enantioselectivity is obtained for all compounds listed. For the aliphatic α-amino acid amides (But-NH_2, Val-NH_2, NVal-NH_2), higher α values are obtained compared with the corresponding α-amino acids. On the other hand, the α values for the aromatic α-amino acid amides (HPG-NH_2, PG-NH_2, Phe-NH_2) are lower than or equal to those of the α-amino acid analogues. The elution order of the enantiomers was as follows: D before L for But-NH_2, Val, Val-NH_2, NVal, NVal-NH_2, Phe and Phe-NH_2; L before D for But, HPG, HPG-NH_2, PG and PG-NH_2. For all the α-amino acids studied, higher k' values were obtained in comparison with the corresponding α-amino acid amides.

An explanation for this phenomenon may be found in the presence of the amide functionality in the α-amino acid amides and the carboxylic group present in the α-amino acids. For the latter type of compounds, it may be expected that they exist in the form of anions at pH 7.0. As both the amide group and the carboxylate anion can form hydrogen bonds with the hydroxyl groups of the CD, the higher k' values for the α-amino acids may result from stronger hydrogen bonding of the carboxylate function compared with the amide function. This, however, does not result in a better enantioselectivity with regard to the α-amino acid amides.

The benz[*f*]isoindole group is considered to form an inclusion complex with the CD cavity and is therefore assumed to be a major factor in determining the retention and enantioselectivity of the analytes studied. The R substituent in the side-chain of the α-amino acid is also considered to play a role in the retention [10].

From Table I, it can be seen that the k' values of the CBI derivatives of the α-amino acids and α-amino acid amides increased, in general, with increasing hydrophobicity of the R substituent (ethyl < propyl < isopropyl < benzyl). This can be explained in terms of a decrease in mobile phase solubility of the CBI derivatives with increasing hydrophobicity of the R substituent, and hence increasing attraction to the CD stationary phase. However, the retention observed for PG and PG-NH_2 cannot be explained by the above-mentioned mechanism. The high k' values for PG and PG-NH_2 can be explained if both the benz[*f*]isoindole group and the phenyl substituent of PG of PG-NH_2 are considered to be included in the CD cavity. If the R group and the derivative group were both included to the same extent, no chiral recognition would occur [10]. From Table I, it can be seen that enantioselectivity is obtained for PG and PG-NH_2, but less than for Phe and Phe-NH_2. A partial inclusion of the phenyl substituent of PG and PG-NH_2 would therefore provide a reasonable explanation for the retention behaviour and enantioselectivity observed.

Effect of methanol

The influence of the methanol content of the mobile phase on the retention and resolution of CBI derivatives of α-amino acid and α-amino acid amide enantiomers has been studied. In all instances, a decrease in the capacity factor of the enantiomers was observed as the percentage of methanol increased.

In addition to the k' values, both the separation factors and resolutions also decreased. Typical plots of k' and R_s *versus* methanol concentration for some CBI derivatives of α-amino acids and α-amino acid amides are shown in Figs. 2 and 3. The effect of

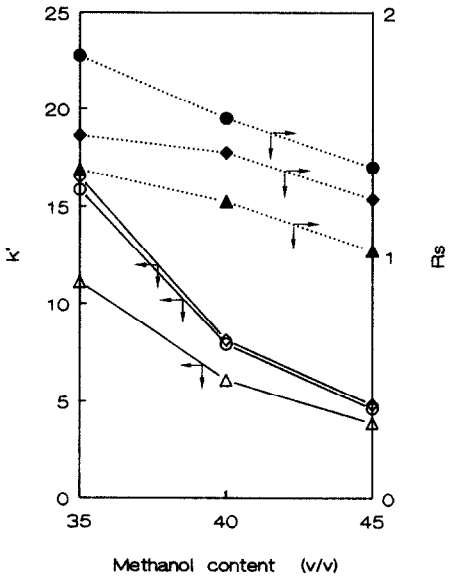

Fig. 2. Influence of methanol content in the mobile phase on the capacity factor (solid lines) and resolution (dotted lines) of the CBI derivatives of (△,▲)But-NH_2, (○,●)Val-NH_2 and (◇,◆) Phe-NH_2. Mobile phase, 40 m*M* ammonium nitrate buffer (pH 5.4); flow-rate, 0.8 ml/min. *k'* is the capacity factor of the first-eluted enantiomer.

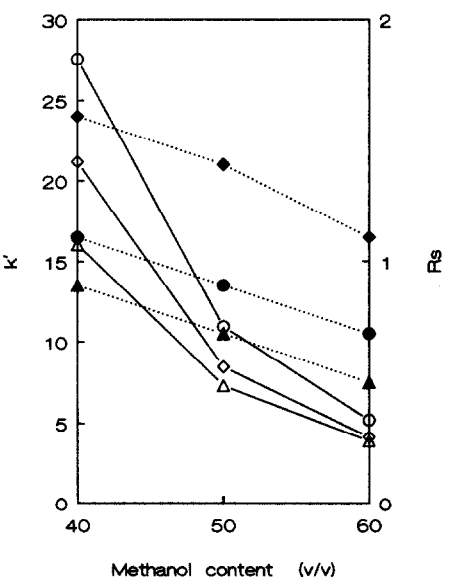

Fig. 3. Influence of methanol content in the mobile phase on the capacity factor (solid lines) and resolution (dotted lines) of the CBI derivatives of (△,▲)But, (○,●)PG and (◇,◆)Phe. Mobile phase, 80 m*M* ammonium nitrate buffer (pH 5.3); flow-rate, 1.0 ml/min. *k'* is the capacity factor of the first-eluted enantiomer.

methanol content on the retention of CBI-α-amino acids shows the same tendencies as those observed with dansyl-α-amino acids [3,10] and DNP-α-amino acids [7]. With respect to the effect on the resolution, our data agree well with those for dansyl-α-amino acids [3] and DNP-α-amino acids [7]. However, in another study on dansyl-α-amino acids [10] an increase in resolution was observed when the methanol content was increased.

Effect of pH

The effect of pH on the retention and resolution was investigated by varying the pH of the mobile phase from 4.0 to 7.0. For the CBI derivatives of Val, But and Phe and the corresponding acid amides, the results are shown in Figs. 4 and 5. In the case of the CBI-α-amino acids (Fig. 5), a decreased retention with increase in pH is observed. The same observations were made for DNP-α-amino acids [7] and dansyl-α-amino acids [10].

Because of the electron-withdrawing properties of the benz[*f*]isoindole group, the pK_a values of the CBI-α-amino acids studied are expected to be lower

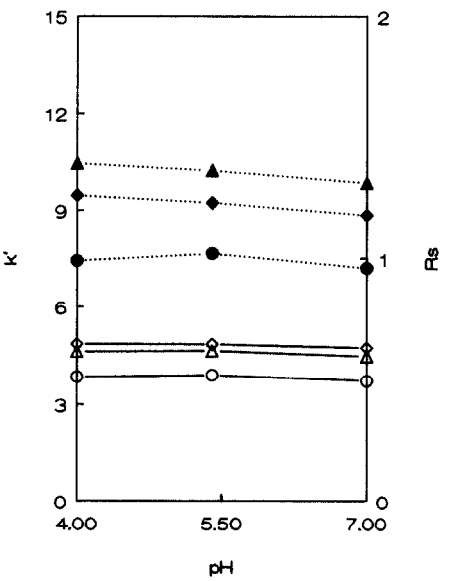

Fig. 4. Influence of pH of the mobile phase on the capacity factor (solid lines) and resolution (dotted lines) of the CBI derivatives of (△,▲)Val-NH_2, (○,●)But-NH_2 and (◇,◆)Phe-NH_2. Mobile phase, 40 m*M* ammonium nitrate buffer containing 45% (v/v) methanol; flow-rate, 0.8 ml/min. pH measured in the aqueous part of the mobile phase. *k'* is the capacity factor of the first-eluted enantiomer.

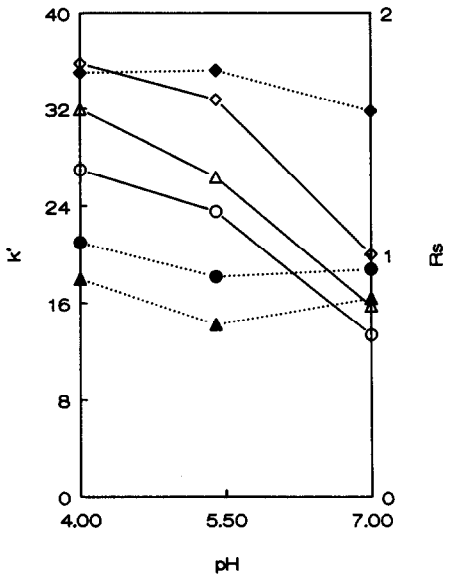

Fig. 5. Influence of pH of the mobile phase on the capacity factor (solid lines) and resolution (dotted lines) of the CBI derivatives of (△,▲)Val, (○,●)But and (◇,◆)Phe. Conditions and definitions of pH and k' as in Fig. 4.

than those of the native α-amino acids. Therefore, no significant change in the carboxylic acid/carboxylate anion ratio is expected in the pH range studied; the CBI-α-amino acids will be mainly present as anions. Hence, the binding strength of the CBI-α-amino acids with the CD stationary phase should not be affected by changes in pH. For DNP-α-amino acids, the decrease in k' has been explained by assuming competition between the DNP-α-amino acid anions and the OH^- ions for binding with the hydroxyl groups of the CD [7]. This mechanism may also explain the data obtained for CBI-α-amino acids. The increase in OH^- ions that occurs when the pH is increased will weaken the binding strength of the carboxylate anions and hence that of the CBI-α-amino acid solutes. Consequently, a decrease in k' with increase in pH is observed. On changing the pH, no significant change in the R_s values of the CBI-α-amino acids was observed.

In contrast to the CBI-α-amino acids, the k' values of the CBI-α-amino acid amides remained constant in the pH range 4.0–7.0. This may be explained by assuming that in the presence of OH^- and buffer anions, the hydrogen-bonding ability of the neutral acid amide group is too small to bind to the hydroxyl groups of the CD. Consequently, the overall interaction of the CBI-α-amino acid amides with the CD cavity will not be affected by a change in pH.

Effect of buffer concentration

The ammonium nitrate concentration in the eluent was varied in order to study its influence on the retention and resolution of both CBI-α-amino acid and CBI-α-amino acid amides. The results for Val, But, HPG and the corresponding α-amino acid amides are presented in Figs. 6 and 7. For the CBI-α-amino acid amides (Fig. 6), no significant changes in either the k' or the R_s values were observed with varying buffer concentration. For the CBI-α-amino acids, on the other hand (Fig. 7), the k' values decreased with increasing buffer concentration. With respect to the resolution, there was a slight increase for HPG, whereas a slight decrease occurred for But and Val.

As cyclodextrins have the ability to bind inorganic anions such as nitrate [11], the buffer anions may compete with the CBI-α-amino acid anions for

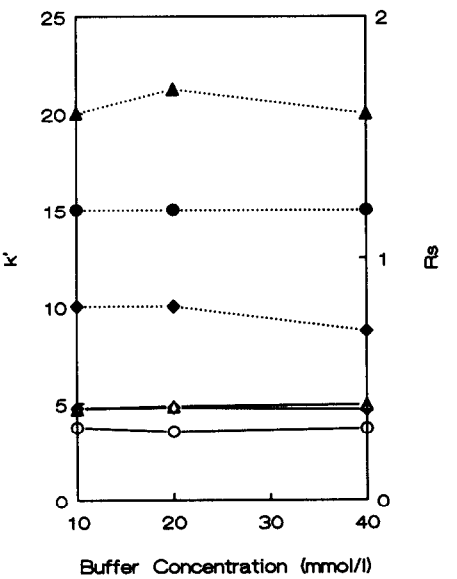

Fig. 6. Influence of buffer concentration of the mobile phase on the capacity factor (solid lines) and resolution (dotted lines) of the CBI derivatives of (△,▲)Val-NH_2, (○,●)But-NH_2 and (◇,◆)HPG-NH_2. Mobile phase, ammonium nitrate buffer (pH 5.6) containing 45% (v/v) methanol; flow-rate, 1.0 ml/min. k' is the capacity factor of the first-eluted enantiomer.

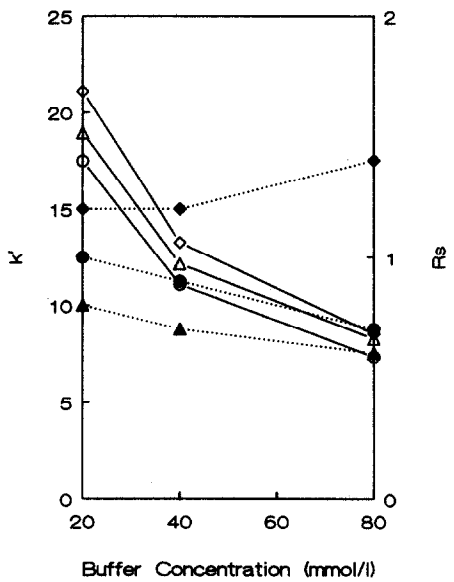

Fig. 7. Influence of buffer concentration of the mobile phase on the capacity factor (solid lines) and resolution (dotted lines) of the CBI derivatives of (△,▲)Val, (○,●)But and (◇,◆)HPG. Mobile phase, ammonium nitrate buffer (pH 5.4) containing 50% (v/v) methanol; flow-rate, 1.0 ml/min. k' is the capacity factor of the first-eluted enantiomer.

binding with the hydroxyl groups of the CD. The decrease in the retention can therefore be explained by increased exclusion of the negatively charged CBI-α-amino acid from the CD cavity at higher ionic strength of the eluent.

For the CBI-α-amino acid amides it is expected that even at the lowest buffer concentration studied (10 m*M*), the nitrate anions will interfere with the hydrogen bonding of the neutral acid amide functionality with the hydroxyls of the CD. This situation evidently does not change at higher nitrate concentrations. Analogous to the effect of OH^-, the overall interaction of the CBI-α-amino acid amides with the CD cavity will therefore not be affected by variation of the buffer concentration.

CONCLUSIONS

The results indicate that on-line derivatization of α-amino acids and the corresponding α-amino acid amides with NDA–cyanide reagent, followed by separation on a β-CD column, is a useful assay for the control of the enantiomeric purity of both α-amino acids and α-amino acid amides.

Among the mobile phase parameters studied, the methanol content in the mobile phase is the most effective parameter for adjusting the enantiomeric resolution of both α-amino acids and α-amino acid amides. By adapting the elution conditions, the method described may also be useful for the enantioseparation of other α-amino acids and related α-amino acid amides.

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