Journal of Chromatography, 629 (1993) 169–179
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 638

High-performance liquid chromatographic enantioseparation of glycyl di- and tripeptides on native cyclodextrin bonded phases

Mechanistic considerations

J. Zukowski, M. Pawlowska, M. Nagatkina and D. W. Armstrong

Department of Chemistry, University of Missouri-Rolla, 142 Schrenk Hall, Rolla, MO 65401-0249 (USA)

(First received July 16th, 1992; revised manuscript received September 1st, 1992)

ABSTRACT

Di- and tripeptides containing at least one glycine moiety were separated into enantiomers on α-, β- and γ-cyclodextrin bonded phases after their precolumn derivatization with 9-fluorenylmethyl chloroformate (FMOC-Cl). It is shown that the choice of a suitable cyclodextrin bonded phase used with a nonaqueous polar mobile phase offers a wide range of possibilities to optimize enantioselectivity. Use of the FMOC derivative greatly enhances sensitivity, stability and enantioselectivity.

It is postulated that under reversed-phase conditions the inclusion complex formation between the hydrophobic aromatic part of the FMOC-functionalized peptides and non-polar cyclodextrin cavity interior is the major factor contributing to retention. However, under these conditions there seems to be insufficient interaction between the hydrophilic peptide chain and cyclodextrin hydroxyls. Hence no chiral recognition and enantiomeric separation is observed. In systems operating with polar organic mobile phases the inclusion complex is suppressed as the cyclodextrin cavity is largely occupied by the mobile phase. The enantioselectivity observed is caused by hydrogen bonding between peptide chain and the hydroxyl groups at the mouth of the cyclodextrin. The stereospecific interactions depend very strongly on the cyclodextrin size, the length of the peptide chain and the mobile phase composition.

INTRODUCTION

As an extension of prior work [1], we report the separation of enantiomeric peptides having a glycine moiety in their structures. Several small peptides are known to show significant biological activity. Many of them are used as substrates in the study of transport, hydrolysis and other processes connected with absorption of protein digestion products [2,3]. Others may be precursors in the synthesis of larger peptides. All of these small peptides (except those bearing only glycine moieties) are optically active. Therefore, a technique for the easy and sensitive determination of enantiomeric purity of peptides can be useful.

Previously, several studies concerning the resolution of enantiomeric dipeptides using HPLC have been reported [4–6]. Amino acids and small peptides are not easy compounds to detect and elute. Often, these polar compounds require non-traditional mobile phases and detection techniques [7,8]. Frequently amino acids and peptides are derivatized in order to make them more suitable for chromatographic separation and/or detection. Generally the derivatizing group is a highly absorbant or fluorescent moiety. Fluorimetry is a well-known analytical

Correspondence to: D. W. Armstrong, Department of Chemistry, University of Missouri-Rolla, 142 Schrenk Hall, Rolla, MO 65401-0249, USA.

0021-9673/93/$06.00

method with high selectivity and sensitivity which enables the detection of small quantities of many important compounds.

Recently we reported the facile determination of trace enantiomeric impurities of imino acids after 9-fluorenylmethyl chloroformate (FMOC-Cl) derivatization [1]. The current work shows the result of enantiomeric resolution of glycine di- and tripeptides on native α-, β- and γ-cyclodextrin bonded phases after their precolumn derivatization with FMOC reagent. Furthermore, these results provide an opportunity to examine the separation mechanism of chiral peptides on cyclodextrin bonded stationary phases.

EXPERIMENTAL

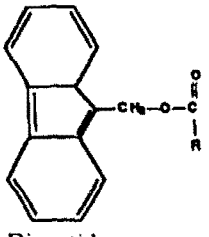
Apparatus

The HPLC system consisted of two pumps (LC-6A, Shimadzu, Kyoto, Japan), a system controller (SCL-6B, Shimadzu), Chromatopac (CR 601, Shimadzu), fluorescence detector (RF-535, Shimadzu) operated with $\lambda_{ex} = 266$ nm and $\lambda_{em} = 315$ nm and 0.2-μl injector valve (Valco, Houston, TX, USA). The columns were 250 × 4.6 mm I.D. and were packed with α-, β- and γ-cyclodextrin bonded to 5-μm spherical silica gel (Astec, Whippany, NJ, USA).

TABLE I

SEPARATION DATA FOR A NUMBER OF FMOC-DIPEPTIDES ON THREE DIFFERENT CYCLODEXTRIN BONDED PHASES

The mobile phase used with the γ- and β-cyclodextrin columns was (by volume): acetonitrile–triethylamine–acetic acid (1000:12:3). The mobile phase used with the α-cyclodextrin column was (by volume): acetonitrile–methanol–triethylamine–acetic acid (850:150:12:3). The methanol was used to shorten the long retention times. The flow-rate was 1 ml/min. All compounds were resolved as racemates or nearly racemic D,L-mixtures. When available the pure enantiomer was also run in order to determine the retention order.



Dipeptide	γ-Cyclodextrin column			β-Cyclodextrin column			α-Cyclodextrin column		
	First eluted enantiomer	k'^{a}	α^{b}	First eluted enantiomer	k'^{a}	α^{b}	First eluted enantiomer	k'^{a}	α^{b}
Gly–Abu	L	2.23	1.07		2.48	1.00		2.56	1.00
Gly–Ser	L	2.07	1.08		2.76	1.00	D	2.73	1.00
Gly–Thr	L	1.30	1.07		1.61	1.00	D	2.38	1.00
Gly–Leu	L	2.29	1.15	D	4.79	1.06	L	2.77	1.10
Gly–Phe	D	2.33	1.09	D	4.27	1.29	L	2.20	1.14
Gly–Asn	L	6.04	1.18	L	7.54	1.04	D	5.48	1.03
Gly–Met	L	1.74	1.19		2.71	1.00	L	2.72	1.03
Gly–Ala	L	2.41	1.07		3.35	1.00		2.27	1.00
Gly–Asp		10.33	1.00	D	11.75	1.22	D[c]	21.0[c]	1.05[c]
Gly–Val		2.15	1.00	D	2.81	1.05		2.25	1.00
Gly–Nva	L	2.17	1.08		2.77	1.00	L	3.79	1.17
Gly–Nle	L	2.07	1.08	D	3.41	1.05	L	4.16	1.24
Ala–Gly	D	2.60	1.06		2.87	1.00		2.12	1.00
Leu–Gly	L	2.22	1.12		2.58	1.00		1.83	1.00

[a] The capacity factors shown designate the first eluted enantiomer.
[b] α-Values of 1.00 mean that no separation of enantiomers occurred.
[c] Acetonitrile–methanol–triethylamine–acetic acid (850:150:14:1).

Chemicals

Amino acids di- and tripeptides were purchased from different sources (ICN, Irvine, CA, USA), (Sigma, St. Louis, MO, USA), and (Novobiochem, La Jolla, CA, USA). Acetonitrile, methanol, acetic acid and triethylamine were of OmniSolv grade and supplied from EM Science (Gibbstown, NJ, USA).

Procedure

The derivatizing agent, FMOC-Cl, was purchased from Sigma (St. Louis, MO, USA). Derivatization was performed according to ref. 9. FMOC-glycine chloride (FMOC-Gly-Cl) was prepared according to ref. 10 and was used for derivatization of amino acids and dipeptides. The derivatization procedure was performed in 0.2 *M* boric acid buffer–acetonitrile solution (1:1, pH 7.7). After derivatization samples were acidified with a 50% acetic acid solution to stabilize the FMOC group. Following acidification, the samples were diluted 10–50 times with acetonitrile prior to injection.

RESULTS AND DISCUSSION

Selectivity

Table I gives retention data for a number of glycine dipeptides that were resolved on α-, β- and γ-cyclodextrin bonded phase columns. As indicated, two standard eluents containing acetonitrile with the same amount of triethylamine and acetic acid were used for the enantioseparation of the 15 racemates listed in Table I. In the case of β- and γ-cyclodextrin bonded stationary phases, the use of neat acetonitrile proved to be an optimal eluent for the enantioseparation of a number of FMOC-imino acids [1]. Under the same mobile phase conditions, most of the FMOC-dipeptides investigated in this study elute in reasonable amount of time. In general the retention times obtained on the β-cyclodextrin column were slightly higher than those found on the γ-cyclodextrin column. For glycyl aspartic acid (Gly–Asp) and glycyl aspargine (Gly–Asn) which contain, respectively, carboxylic acid and amide groups in their side chains, the analysis times were significantly longer. At equivalent mobile phase compositions, the α-cyclodextrin column retained peptides longer than the β- and γ-cyclodextrin columns. In this case, the addition of a small percentage of methanol to the acetonitrile mobile phase decreases the analysis time to practical levels. It is apparent from this data (Table I) that retardation and enantioselectivity are closely related to cyclodextrin size.

In general, the γ-cyclodextrin column was the most selective stationary phase since it resolved 13 of the 15 investigated racemic mixtures. The only two exceptions were glycyl valine (Gly–Val) and Gly–Asp. Surprisingly these racemates are resolved on the β-cyclodextrin column, which exhibited rather limited enantioselectivity towards the other analytes investigated. Fig. 1 shows the base line resolution of glycyl norvaline (Gly–Nva) on an α-cyclodextrin column and Gly–Asp on a β-cyclodextrin column.

The data in Table I shows a selection of suitable cyclodextrin bonded phases which, in many cases, enables one to choose the elution order for a given pair of enantiomers. This is also illustrated in Fig. 2, where the enantiomers of glycyl phenylalanine (Gly–Phe) are shown to have a reversal in elution order on the α-cyclodextrin and β-cyclodextrin columns. This is of great practical importance in the trace analysis of enantiomers since the detectability and accuracy of the determination is much better when the minor component is eluted first [1,11]. Thus the choice of a suitable cyclodextrin stationary phase offers a wide range of possibilities to optimize enantioselectivity.

Previously reported chromatographic separations in analogous systems exhibiting enantioselectivity are optimized by changing two independent factors:

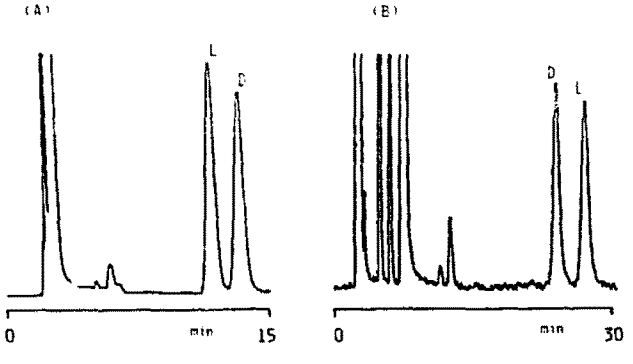


Fig. 1. Enantiomeric resolution of glycyl dipeptides on cyclodextrin bonded phases under optimal experimental conditions. (A) FMOC-Gly–Nva. Stationary phase: α-cyclodextrin bonded phase. Eluent: acetonitrile–methanol–triethylamine–acetic acid (850:150:12:3, v v). (B) FMOC-Gly–Asp. Stationary phase: β-cyclodextrin bonded phase. Eluent: acetonitrile–methanol–triethylamine–acetic acid (900:100:6:1.5, v v). Flow-rate: 1 ml min.

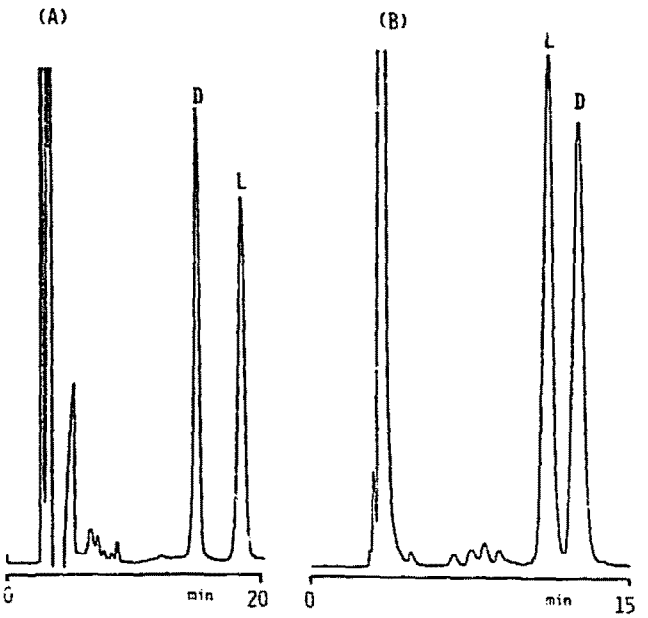


Fig. 2. Change of elution order for FMOC-Gly–Phe enantiomers obtained on β- and α-cyclodextrin bonded phases. (A) Stationary phase: β-cyclodextrin bonded phase. Eluent: acetonitrile–triethylamine–acetic acid (1000:12:3, v/v/v). (B) Stationary phase: α-cyclodextrin bonded phase. Eluent: acetonitrile–methanol–triethylamine–acetic acid (850:150:14:1, v/v). Flow-rate: 1 ml min.

the composition of the polar organic solvents used in mobile phases and/or the relative ratio of triethylamine and acetic acid used [1]. Fig. 3 shows how the triethylamine and acetic acid concentration influence the resolution of Gly–Asn enantiomers. This behavior was also used to optimize enantioseparation of Gly–Phe (see Fig. 2B). The ratio of 14:1 of triethylamine to acetic acid improves enantioseparation ($\alpha = 1.18$) whereas utilizing the conditions in Table 1 slightly decreases resolution ($\alpha = 1.14$) without significantly effecting the retention time.

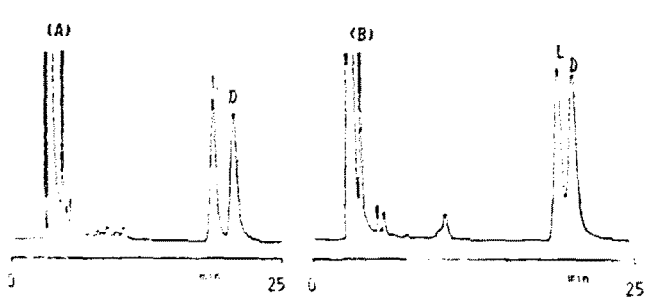


Fig. 3. Effect of relative amounts of triethylamine and acetic acid in the eluent consisting of neat acetonitrile on the resolution of FMOC-Gly-Asn enantiomers. Stationary phase: γ-cyclodextrin bonded phase. Eluent: acetonitrile–triethylamine–acetic acid: (A) 1000:9:6 (v/v/v); (B) 1000:6:9 (v/v/v).

Neat acetonitrile has proved to be an optimal solvent for many analogous separations [1,12]. However, some separations (as mentioned above for the α-cyclodextrin column) require the addition of methanol to decrease retention times. The separation of Gly–Asp on the β-cyclodextrin column, which exhibits a very long analysis time under the conditions listed in Table I, was optimized by changing the concentration of polar organic solvents as well as the amount of amine and acid modifiers (see Fig. 1B). Note that the experimental conditions in Fig. 1B have been optimized to provide the best analytical separation. These conditions are different from those in Table I which summarizes data generated

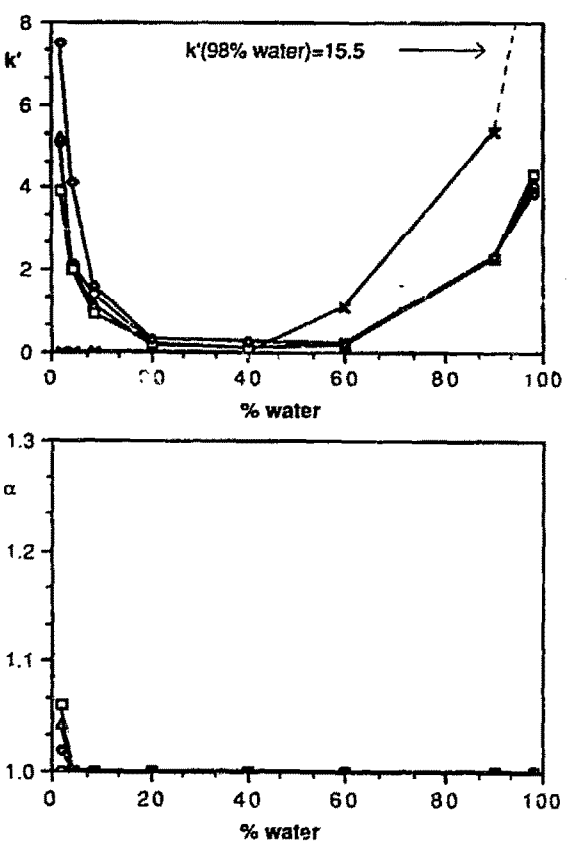


Fig. 4. Influence of water concentration in the mobile phase on the retention characteristics of selected FMOC-derivatized dipeptides and fluorene obtained on α-cyclodextrin bonded phase. Eluent: acetonitrile–water–1.2% triethylamine–0.3% acetic acid (v/v). Test compounds: × = fluorene; ○ = FMOC-Gly-Abu; ◇ = FMOC-Gly-Leu; □ = FMOC-Gly-Phe; △ = FMOC-Gly-Asn. Flow-rate: 1 ml min.

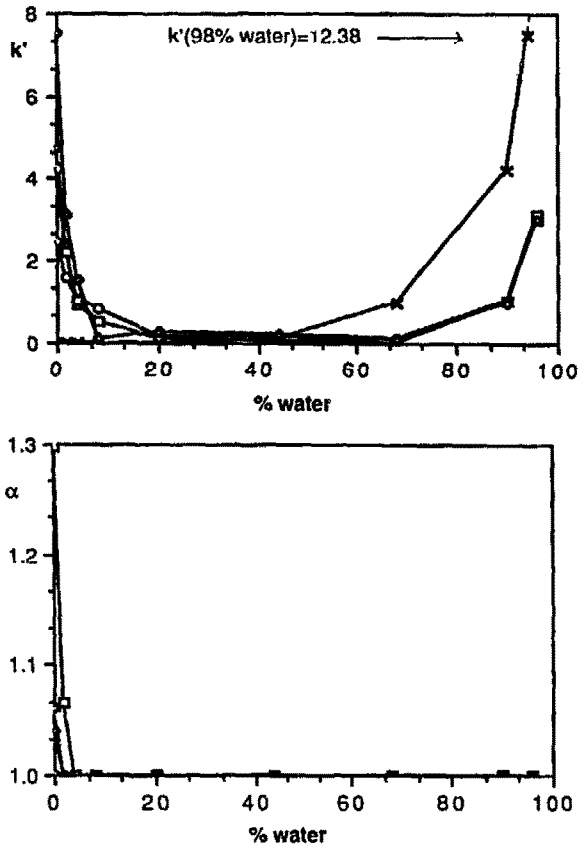


Fig. 5. Influence of water concentration in the mobile phase on the retention characteristics of selected FMOC-derivatized dipeptides and fluorene obtained on β-cyclodextrin bonded phase. Other conditions as in Fig. 4.

under identical conditions so that valid comparisons could be made.

Chiral recognition mechanism

It has been reported that the chiral recognition mechanism for a number of FMOC-derivatized imino acids on *R*(−)-1-(1-naphthyl)ethylcarbamoylated-β-cyclodextrin bonded phase is dependent on the mobile phase composition [1]. The retardation and selectivity of the system were found to be very sensitive to the water content of the eluent. Similar behavior was observed in this study for native α-, β- and γ-cyclodextrin bonded phases.

Figs. 4–6 show the change in retention characteristics for selected glycyl dipeptides obtained on cyclodextrin bonded phases. The eluents consisted of acetonitrile–water mixtures and included small amounts of triethylamine and glacial acetic acid. The chromatographic sorption on the native cyclodextrin bonded phases as well as carbamoylated-β-cyclodextrin bonded phases, occur under two operating conditions (*i.e.*, in the reversed-phase mode with water-rich eluents and under unconventional conditions using non-aqueous polar organic mobile phases). However, in contrast to the carbamoylated-β-cyclodextrin bonded phase, the native α-, β- and γ-cyclodextrin columns exhibit enantioselectivity towards FMOC-derivatized dipeptides *only* when operated with non-aqueous polar organic solvents.

Aqueous systems

In the reversed-phase mode, the separation mechanism on native cyclodextrins phases is thought to be the result of the inclusion complex formation between the hydrophobic moiety of the analyte and the relatively non-polar interior of the cyclodextrin

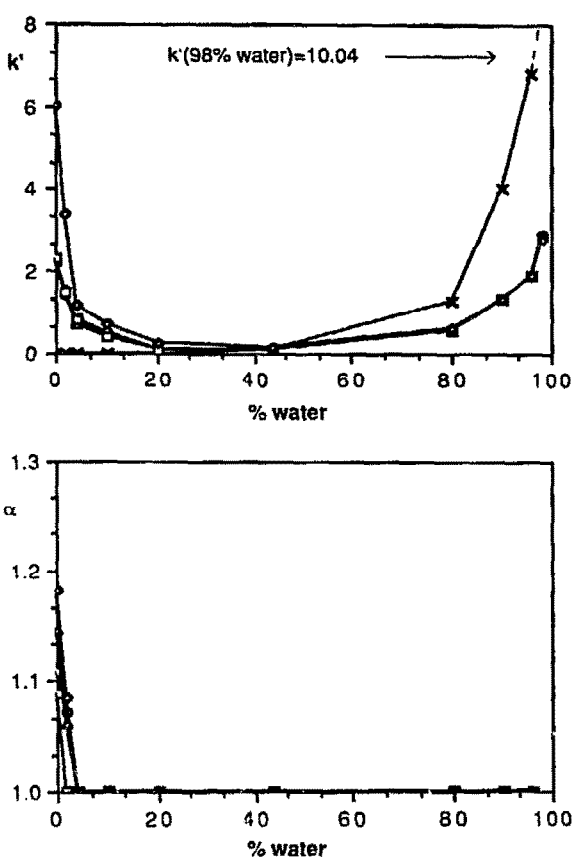


Fig. 6. Influence of water concentration in the mobile phase on the retention characteristics of selected FMOC-derivatized dipeptides and fluorene obtained on γ-cyclodextrin bonded phase. Other conditions as in Fig. 4.

cavity [13,14]. The retention behavior of FMOC functionalized glycyl dipeptides and the fluorene probe molecule shown in Figs. 4–6 confirm this behavior. The plots obtained on α-, β- and γ-cyclodextrin stationary phases are essentially identical with respect to retardation and selectivity. For eluents containing more then 50% (v/v) water, all stationary phases exhibit affinity not only towards FMOC-derivatized dipeptides but also toward the fluorene molecule (Figs. 4–6). Also note that only the fluorene probe molecule is not retarded in water poor systems. Moreover, no enantioselectivity is observed under reversed-phase conditions for all stationary phases investigated. The lack of enantioselectivity in the reversed-phase mode could be due to the FMOC-dipeptide totally interacting with the achiral linkage chain which connects the cyclodextrin to the silica gel. This could result in retention without enantiomeric selectivity. However, this particular scenario is not likely to occur. In fact it is apparent that the retardation of analytes depends very strongly on cyclodextrin cavity size. The larger size of the β-cyclodextrin and γ-cyclodextrin cavity result in inefficient binding due to the loose nature between the arene and cyclodextrin. This, in turn, reduces the retention time of the analytes compared to those obtained on the α-cyclodextrin column. It is most likely that the fluorene group is included in the cyclodextrin cavity and the dipeptide chain is located outside the cavity and is solvated by the hydrophilic mobile phase.

It should be mentioned that the data on inclusion complex formation between cyclodextrin and polyaromatic hydrocarbons of different sizes are not consistent and often diverse. Some literature reports that the naphthalene molecule is too large to be included into the α-cyclodextrin cavity [15,16] as are anthracene molecules for the β-cyclodextrin cavity [15]. On the other hand, an induced circular dichroism study [17] has provided evidence that β-cyclodextrin accommodates one pyrene molecule with axial inclusion while γ-cyclodextrin includes two pyrene molecules of "S" helicity. This study has been confirmed by comparative spectroscopic binding studies (absorption spectral characteristics, circular dichroism and fluorescence evidence), where benz[*a*]pyrene was shown to form an inclusion complex of 1:1 host–analyte ratio with the β-cyclodextrin and a 1:2 host analyte ratio with γ-cyclodextrin [18]. Additionally there are several chromatographic studies reporting the retardation of large polyaromatic hydrocarbon molecules (pyrene, chrysene) on β- and γ-cyclodextrin [19,20] and more recently on α-cyclodextrin columns [21]. Moreover, NMR study [22], conformational (optical rotation) analysis [23] and X-ray crystallographic data [24] provide evidence that the bonds of α-cyclodextrin are relatively weaker than β- and γ-cyclodextrins. These can lead to induced fits of larger molecules into the α-cyclodextrin cavity by deformation of its structure under high aqueous buffer conditions.

The results of the present study indicate that the fluorene molecule can penetrate into all cyclodextrin cavities and that the inclusion complex formation between the hydrophobic aromatic part of the analytes and apolar cyclodextrin cavity is the major factor contributing to the retention of FMOC-functionalized dipeptides under reversed-phase conditions. The hydrophilic part of the guest molecule remains outside the cavity. The most striking conformation of this picture is the finding that the retention of different types of FMOC-dipeptides is nearly identical (on a given column) and always lower than retention of the fluorene molecule. The fact that no enantioselectivity is observed in any of these cases would seem to indicate that the simultaneous, optimum interaction of the fluorene moiety with the cyclodextrin cavity and between the peptide moieties and the cyclodextrin hydroxyls does not occur with these analytes.

The model postulated for the retention mechanism based on inclusion complex formation is further supported by the kinetic study presented below. Fig. 7 shows the dependence of the plate height, H, on the linear flow velocity, u, for the fluorene molecule and different FMOC-derivatized glycyl dipeptides obtained on γ- and β-cyclodextrin columns. The dashed line represents H values obtained for fluorene on cyclodextrin columns operated with neat acetonitrile. No retardation of fluorene was observed in this system and therefore the dashed line can be considered the limit for column efficiency due to the packing quality and extra column effects. As can be seen in Fig. 7 the characteristics presented are very similar for γ- and β-cyclodextrin column, which enables teh comparison of the results obtained on these two columns.

According to the general non-equilibrium theory

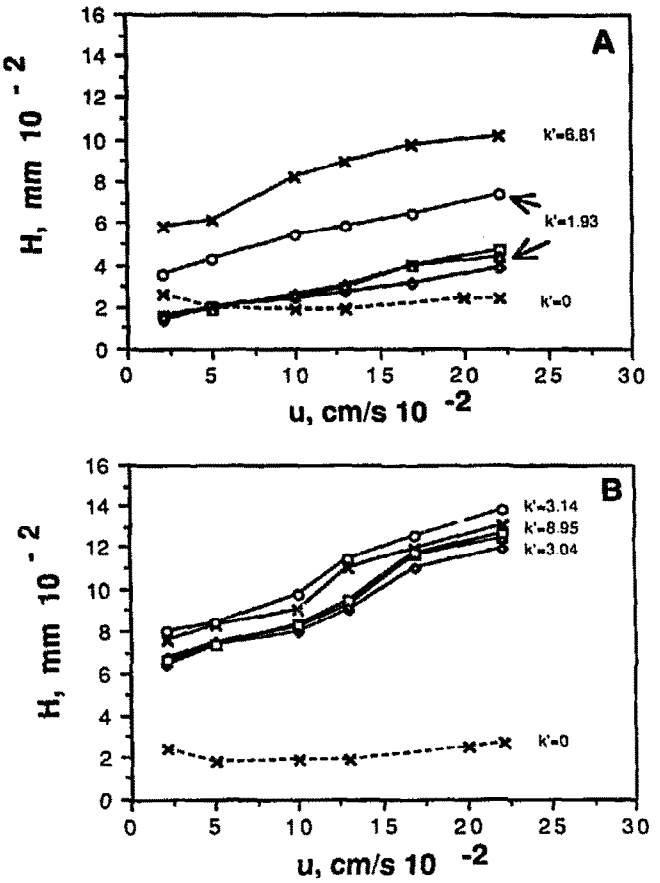


Fig. 7. The dependence of height equivalent to a theoretical plate (HETP) values on linear velocity for fluorene and selected FMOC-derivatized dipeptides in aqueous systems. Eluent: water–acetonitrile–triethylamine–acetic acid (960:40:12:3, v/v). Test compounds: × = fluorene, ◇ = Asn, △ = Abu, □ = Leu, ○ = Phe. Stationary phase: (A) γ-cyclodextrin bonded to 5-μm spherical silica gel, (B) β-cyclodextrin bonded to 5-μm spherical silica gel. The dashed line represents HETP values obtained for fluorene in systems operated with neat acetonitrile as the mobile phase.

developed by Giddings [25] the plate height, H, results from three independent contributions: flow pattern effects (A), longitudinal diffusion (B) and mass transfer effects (C).

$$H = B/u + C_k u + \sum 1/(1/A + 1/C_m u) \quad (1)$$

where the terms C_k and C_m indicate adsorption–desorption kinetics and diffusion controlled kinetics originating in the stationary and mobile phase, respectively.

The equation in the coupling form represents the major sources of zone spreading in practical liquid chromatography (LC) for a very broad range of the flow velocities. The terms in eqn. 1 have a different dependence on the flow velocity. Ordinary molecular diffusion in the flow direction is inversely proportional to flow velocity and contributes significantly only at very low flow velocities. The sorption–desorption kinetics provides a term proportional to flow velocity. The coupling terms arise from inequalities of flow velocity in the mobile phase. Over the limited flow range used in LC some of these terms (A) are believed to be constant and some proportional to velocity (C_m).

As shown in Fig. 7, the dependence of the plate height on the flow-rate of all analytes investigated is approximately linear within the range of flow-rates applied. This indicates that the zone spreading is mainly caused by mass transfer effects in the stationary phase. Moreover, although linear velocities as low as $2 \cdot 10^{-2}$ cm/s were used, no minimum of H, u_{min}, was found (although the minimum of H for the unretained analyte on the same column was observed at $u > 5 \cdot 10^{-2}$ cm/s). Generally the values of u_{min} and H_{min} depend on the magnitude of the C_k term and therefore can differ between different solutes. Extremely high contribution of C_k shifts the minimum in H to very small values of u.

Since the mass transfer effect can be expressed as:

$$C_k = \frac{2q}{k_d} \cdot \frac{k'}{(1 + k')^2} \quad (2)$$

were: q is the geometrical parameter and k_d is the desorption rate constant; the two findings discussed above indicate that the overall plate height of a solute is determined by the sorption–desorption kinetics in the stationary phase.

It can be concluded from plots in Fig. 7 that the rate of the adsorption–desorption process for functionalized dipeptides is usually higher than for fluorene molecule (the only possible exception would be Phe on the β-cyclodextrin column). The kinetic data presented provide strong support for the postulated model. The strength of the interaction between the solute and the stationary phase (*i.e.*, retention and to some extent sorption–desorption kinetics) are a result of the inclusion complexation process. The presence of the hydrophilic chain of FMOC-derivatized dipeptides results in shorter retention times and faster mass transfer effects compared with values found for the fluorene molecule itself. The

differences observed in adsorption–desorption rate between fluorene and FMOC-derivatized dipeptides are especially significant for processes on the γ-cyclodextrin stationary phase, which owing to the large cavity, forms relatively loose complexes with the analytes. For the β-cyclodextrin column the sorption–desorption processes are significantly slower for all analytes investigated compared with the values obtained on γ-cyclodextrin phase that is undoubtedly due to the stronger interactions between the smaller cyclodextrin cavity and the aromatic part of the molecule.

The different magnitudes of band spreading processes for the different dipeptide chains can be explained by taking into account additional factors, *e.g.*, the structure of the side chains. The side chains as shown in Figs. 4–6 do not influence the retention in the reversed-phase mode. However, they can contribute to the band broadening in two ways: (1) by changing the individual diffusion velocity of the solute in the mobile phase, which would contribute to C_m term ($C_m = wd_p^2/D_m$, where: w is dimensionless constant, d_p particle size, D_m diffusion coefficient) and (2) by affecting the degree of solvation of the analyte in the bulk mobile phase (*i.e.*, increasing or decreasing the rate of mass transfer effect between stationary and mobile phases).

As can be seen in Fig. 7, the H values for Leucine (Leu) and α-aminobutyric acid (Abu), having aliphatic side chains, are almost identical for the same column. In case of Gly–Asn the presence of the hydrophilic side chain increases the rate of mass transfer of the solute between cyclodextrin stationary phase and hydrophilic mobile phase resulting in slightly lower H values. The hydrophobic aromatic group in Gly–Phe increases the tendency of the analyte to be excluded from the water rich eluent to the vicinity of cyclocdextrin moiety. This results in significantly slower kinetics for both columns investigated as well as a slightly higher retention time for FMOC-Gly–Phe on β-cyclodextrin column.

It should be pointed out that the analytes investigated form symmetrical peaks in the reversed-phase mode. Only Gly–Phe shows a slightly leading peak (fronting) suggesting a slight concave form of its adsorption isotherm, which might result from a second layer (or multi layer) adsorption of other molecules onto the adsorbed aromatic ring of the side chain.

Non-aqueous systems

It is evident from retention characteristics shown in Figs. 4–6 that a different retention mechanism is involved for systems operating with polar organic mobile phases. This is in direct contrast to the traditional reversed-phase mode. First, the fluorene molecule is not retarded on any native cyclodextrin columns. Apparently there is no inclusion complex formation between cyclodextrin and hydrophobic part of the analyte in acetonitrile. It is also highly unlikely that the hydrophilic peptide chain would penetrate into apolar cyclodextrin cavity. Moreover, it has been reported previously that acetonitrile exhibits an appreciable affinity for the cyclodextrin cavity [26,27] and in polar organic solvents (above 80% acetonitrile) the formation of a conventional inclusion complex is not likely since the cyclodextrin cavity is occupied by the mobile phase [28]. This is in a good agreement with results presented in this study and leads to the assumption that the enantioselectivity observed is caused by hydrogen bonding between the analyte and the hydroxyl groups at the mouth of the cyclodextrin.

The results in Table I demonstrate how the retardation of the peptides are closely related to the size of cyclodextrin. The k' values of dipeptides increase when the molecular size of cyclodextrin used is decreased. In the case of α-cyclodextrin, the addition of 15% (by volume) of methanol is needed to decrease the retardation time to a practical level. In order to compare the data in a meaningful way, the results shown in Figs. 4–6 were obtained using the same mobile phase for all cyclodextrin stationary phases. Because of the long retention times ($k' > 15$) the data for α-cyclodextrin operated in conjunction with neat acetonitrile are not included.

Table II and Fig. 8 show dependence of k' values and selectivities on the length of the peptide chain. In general, with the exception of leucine, the native cyclodextrins exhibit no enantioselectivity towards FMOC-functionalized amino acids in spite of the presence of the functional (carboamide and carboxylic) groups available for hydrogen bonding. This could be due either to steric hindrance of the adjacent, bulky fluorene moiety which may effectively prevent the access of hydroxyl groups of cyclodextrin molecule to the appropriate functional groups of the analyte, or it could result from the inability of the FMOC-deactivated nitrogen to form

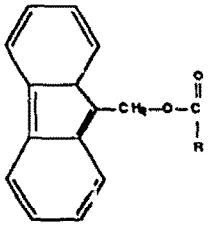
strong hydrogen bonds. Extending the chain length and introducing new hydrogen bonding groups enables stereoselective interaction between the peptide "tail" and the hydroxyl groups of the cyclodextrin moiety and results in an increase in retention and enantioselectivity. The dependence demonstrated in Tables I and II and Figs. 4–6 is strong evidence that hydrogen bonding formation between hydrophilic chain of the FMOC-functionalized peptides and the external part of cyclodextrin molecules (outer sphere complex) is the major factor contributing to the chiral recognition on native cyclodextrin phases operated with polar organic mobile phases. Proper geometric conditions must be met for stereoselective interaction between the analyte and the hydroxyls at the mouth of the cyclodextrin moiety. The "small" diameter of α-cyclodextrin properly match the length of the di- and tripeptide chain. Carboamide and carboxylic groups in the solute can interact with several hydroxyl groups on the both sides of the cyclodextrin mouth which results in large increases in retention time compared with the results obtained on β- and γ-cyclodextrins.

Fig. 9 presents the kinetic data for L-enantiomers of selected FMOC-functionalized amino acids, dipeptides and tripeptides obtained on the most selective γ-cyclodextrin column, operated with the non-aqueous eluent. Unlike the kinetic behavior found for the reversed-phase model (*i.e.*, inclusion complex formation), the plate height with polar organic mobile phases is simply correlated with the capacity factors of the analytes [29,30]; the stronger the interaction between the stationary phase and the solute (which results in the strong retardation) the slower the mass transfer is between stationary and mobile phases. The strength of interaction and the kinetic behavior depends on the structure of the analyte. The presence of the polar carboamide group in the Asn molecule significantly increases the retention and decreases the rate of sorption–desorption process. Extending the peptide chain and thereby introducing additional sites for hydrogen bonding causes a similar effect; the retardation tends to increase and the adsorption–desorption rate decreases. No significant differences were found in the sorptiona–desorption kinetics for enantiomers. The mass transfer was always slightly slower for the longer retained stereoisomer. The peak shapes for all analytes investigated were found to be highly symmetrical. This indicates that the adsorption sites are

TABLE II

A COMPARISON OF SEPARATION DATA FOR RELATED FMOC-AMINO ACIDS, DIPEPTIDES AND TRIPEPTIDES

The mobile phase composition and the flow-rate of these columns are identical to that in Table I.



Compound	α-Cyclodextrin column			β-Cyclodextrin column			γ-Cyclodextrin column		
	First eluted enantiomer	k'^a	α	First eluted enantiomer	k'^a	α	First eluted enantiomer	k'^a	α
Ala		1.11	1.00		1.45	1.00		1.42	1.00
Gly–Ala		2.27	1.00		3.35	1.00	L	2.41	1.07
Gly–Gly–Ala	L	3.27	1.05		7.61	1.00	L	7.29	1.06
Leu		1.16	1.00	D	1.03	1.07		1.03	1.00
Gly–Leu	L	2.77	1.10	D	4.79	1.06	L	2.29	1.15
Gly–Gly–Leu	L	5.12	1.14	D	14.10	1.31	L	7.54	1.14

[a] The capacity factors shown designate the first eluted enantiomer.

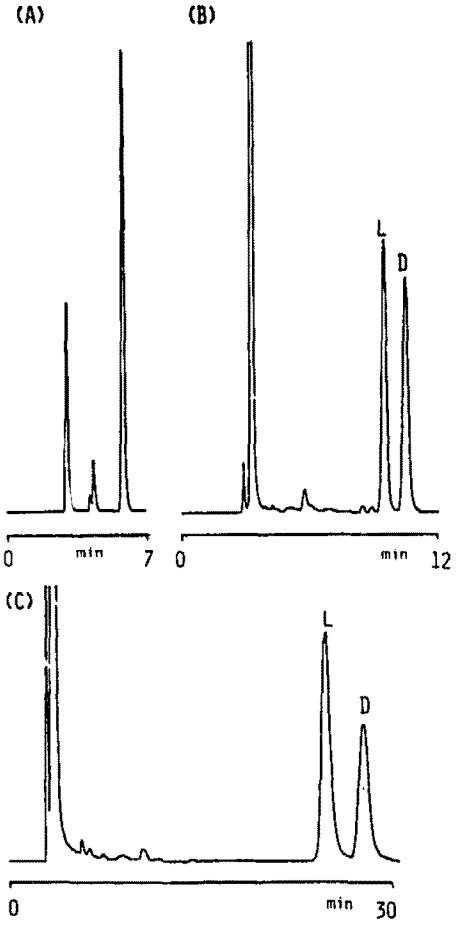


Fig. 8. Influence of the length of peptide chain on retention and enantioselectivity obtained on γ-cyclodextrin bonded phase. Eluent: acetonitrile–triethylamine–acetic acid (100:12:3, v/v/v). (A) FMOC-Leu; (B) FMOC-Gly-Leu; (C) FMOC-Gly-Gly-Leu. Flow-rate: 1 ml/min.

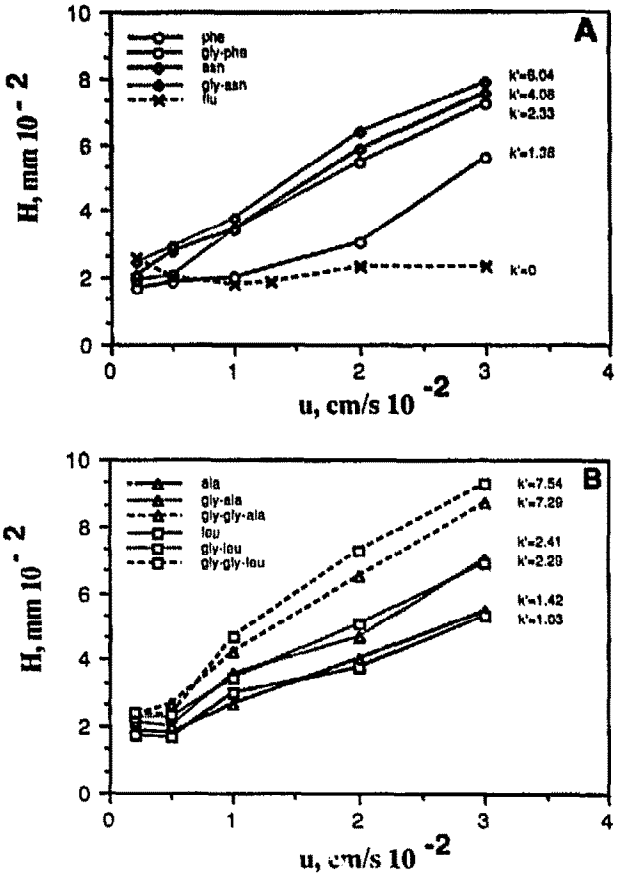


Fig. 9. The dependence of HETP values on linear velocity for fluorene and selected FMOC-derivatized amino acids, di- and tripeptides in non-aqueous system. Eluent: acetonitrile–triethylamine–acetic acid (1000:12:3, v/v/v). Stationary phase: γ-cyclodextrin.

essentially of a single type, *e.g.*, homogeneous in adsorption energies [25,31]. Therefore the type and strength of the interaction between the sites and the analytes depend mainly on the analyte structure and configuration. These data provide consistent support for the model of external adsorption on the hydroxyl groups at the mouth of cyclodextrin cavity as proposed for these non-aqueous systems.

It is clear that several other factors contribute to the retention behavior observed in this study. It has been reported [32,33] that the peptide bond has the ability to enhance the acidity of neighboring groups due to an inductive effect. Thus, considering this effect, it is apparent that the N-terminal peptide bond of the tripeptide will exhibit an N–H bond of substantially enhanced acidity compared with the dipeptide, which can significantly change the interaction between the cyclodextrin and the analyte. It is also possible that the interaction of the analyte with cyclodextrin can significantly alter the structure of the peptide chain in cases where strong hydrogen bonding is involved. This can also lead to a better "fit" of the extended chain with the relatively static structure of the cyclodextrin molecule. Moreover, short peptide chains and even cyclic peptides in certain solvents from definite structures [34]. The nature of the solvent has a great influence on the strength of non-covalent interactions. This could explain the very narrow range of mobile phase compositions where the enantioselectivity towards

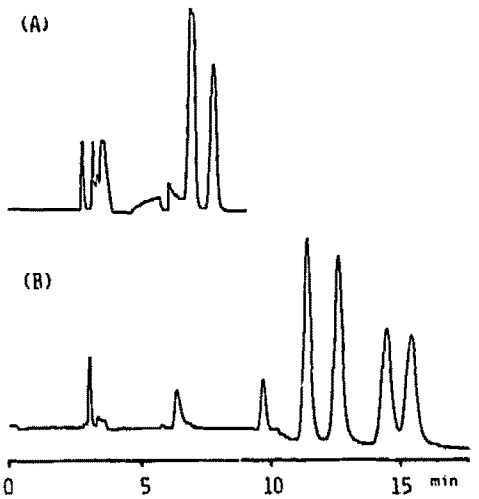


Fig. 10. Stereoisomeric resolution of Ala-Val on γ-cyclodextrin bonded phase. (A) Derivatization with FMOC-Cl; (B) derivatization with FMOC-Gly-Cl. Eluent: acetonitrile–triethylamine–acetic acid (1000:12:3, v/v/v). It is apparent that both pairs of enantiomers are resolved in chromatogram "B", whereas there is only slight resolution in chromatogram "A".

small peptides is observed. Clearly the addition of small amounts of water to the system can totally destroy enatioselectivity. This must be the result of the solvation of the peptide chain and cyclodextrin as well as any induced structural changes caused by the water solvation.

The dependence of enantioselectivity on peptide chain length shown in this study for glycyl di- and tripeptides seems to be a general trend for enantioseparations on native cyclodextrin phases. Fig. 10 shows the chromatographiv behavior of FMOC-derivatized dipeptides having two chiral centers in the peptide chain: the addition of glycyl residue significantly improves the enantioselectivity. Further study on this subject is in progress.

ACKNOWLEDGEMENT

Support of this work by the National Institute of General Medical Science (BMT 2R01 GM36292-04) is gratefully acknowledged.

REFERENCES

1 J. Zukowski, M. Pawlowska and D. W. Armstrong, *J. Chromatogr.*, 623 (1992) 33.
2 K. Yasumoto and K. Sugiyama, *Agric. Biol. Chem.*, 44 (1980) 1339.
3 D. A. Priestman and J. Butterworth, *Biochem. J.*, 231 (1985) 689.
4 M. H. Hyun, I. K. Baik and W. H. Pirkle, *J. Liq. Chromatogr.*, 11 (1988) 1249.
5 M. Katoh, T. Ishida, Y. Baba and H. Kiniwa, *J. Chromatogr.*, 473 (1989) 241.
6 J. Florance and Z. Konteatis, *J. Chromatogr.*, 543 (1991) 299.
7 I. Molnar and Cs. Horváth, *J. Chromatogr.*, 142 (1977) 623.
8 T. Shibo, T. Yamaguchi, K. Nishimura and M. Sugiura, *J. Chromatogr.*, 405 (1987) 145.
9 S. Einarsson, B. Josefsson and S. Lagerkvist, *J. Chromatogr.*, 382 (1983) 609.
10 L. A. Carpino, B. J. Cohen, K. E. Stephens, Jr., S. Y. Sadat-Aalaee, J. H. Tien and D. C. Langridge, *J. Org. Chem.*, 51 (1986) 3734.
11 J. A. Perry, J. D. Rateike and T. J. Szczerba, *J. Chromatogr.*, 389 (1987) 57.
12 D. W. Armstrong, S. Chen, C. Chang and S. Chang, *J. Liq. Chromatogr.*, 15 (1992) 545.
13 D. W. Armstrong, W. DeMond and B. P. Czech, *Anal. Chem.*, 57 (1985) 481.
14 D. W. Armstrong, *J. Liq. Chromatogr.*, 7 (1984) 353.
15 J. Szejtli, *Cyclodextrins and Their Inclusion Complexes*, Akademiai Kiado, Budapest, 1982.
16 J. Emest, D. Kodali and R. Catena, *J. Chem. Soc. Chem. Commun.*, (1981) 758.
17 N. Kobayashi, R. Saito, Y. Hino, A Veno and T. Osa, *J. Chem. Soc. Chem. Commun.*, (1982) 706.
18 R. Woodberry, S. Ranson and V. Fu-Ming, *Anal. Chem.*, 60 (1988) 2621.
19 D. W. Armstrong, A. Alak, W. DeMond, W. L. Hinze and T. E. Riehl, *J. Liq. Chromatogr.*, 8 (1985) 261.
20 M. Olsson, L. E. Sander and S. A. Wise, *J. Chromatogr.*, 477 (1989) 277.
21 *Cyclobond™ Handbook*, ASTEC, Whippany, NJ, 1992.
22 D. J. Wood, F. E. Hruska and W. Saenger, *J. Am. Chem. Soc.*, 99 (1977) 1735.
23 D. A. Ress, *J. Chem. Soc. B.*, (1970) 877.
24 K. Harata and H. Uedaiva, *Nature (London)*, 253 (1975) 190.
25 J. C. Giddings, *Dynamics of Chromatography*, Dekker, New York, 1965
26 L. T. Ward and D. W. Armstrong, *J. Liq. Chromatogr.*, 9 (1986) 407.
27 S. M. Han and D. W. Armstrong, *J. Chromatogr.*, 389 (1987) 257.
28 P. R. Fielden and A. J. Packhann, *J. Chromaogr.*, 516 (1991) 355.
29 Cs. Horváth and H. J. Lin, *J. Chromatogr.*, 149 (1978) 43.
30 E. Katz, K. L. Ogan and P. W. Scott, *J. Chromatogr.*, 270 (1983) 51.
31 A. M. Rizzi, *J. Chromatogr.*, 478 (1989) 71.
32 S. A. Bernhard, A. Berger, J. H. Carter, E. Katchalski, M. Sela and Y. Shalitin, *J. Am. Chem. Soc.*, 84 (1962) 2421.
33 P. DeMontigny, C. M. Riley, L. A. Sternson and J. F. Stobaugh, *J. Pharm. Biomed. Anal.*, 8 (1990) 419.
34 H. D. Jakubke and H. Jeschkait, *Amino Acids, Peptides and Proteins*, Wiley, New York, 1977, p. 68.