Effects of mobile phase composition on the reversed-phase separation of dipeptides and tripeptides with cyclodextrin-bonded-phase columns[a]

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ABSTRACT

The effects of mobile phase composition on the reversed-phase separation of several dipeptides and tripeptides with a γ-cyclodextrin-bonded-phase column have been studied. The addition of organic modifier (*i.e.* methanol) into the aqueous buffer (pH 4.65) mobile phase causes a minimum capacity factor value to be observed for each peptide. This is interpreted to result from two retention mechanisms involved in the separation. The adsorption process causes the retention time to decrease as the water content in the mobile phase is increased. The inclusion process acts in the opposite fashion. The presence of Cu(II) salt in the mobile phase allows further modifications of separation selectivity. This is because the peptide conformation changes upon Cu(II) complexation which in turn alter the hydrophobicity and/or inclusion stability of the peptide. The effects of mobile phase pH (3.6–5.6) and ionic strength (0.001–0.06) were not significant in the present application. Studies with a β-cyclodextrin column show similar results.

INTRODUCTION

Stable cyclodextrin (CD)-bonded phases were recently developed to be used in a traditional reversed-phase mode for the separation of enantiomers [1–8]. Examples of compounds resolved include dansyl and naphthyl amino acids, several aromatic drugs, steroids, alkaloids, metallocenes, binaphthyl crown ethers, aromatic acids, aromatic amines and aromatic sulfoxides. It seems that aromatic groups are necessary for effective enantioselective separations by an inclusion process on cyclodextrin columns. The degree of inclusion determines retention and resolution, which in turn can be controlled by altering the amount of organic modifiers (*e.g.*, methanol, 2-propanol and acetonitrile) in the mobile phase. However, aromatic groups are not necessary for inclusion complex formation or extensive retention. Alkanes and

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hydrocarbons all bind tightly to cyclodextrins but are probably too flexible to show enantioselectivity.

In addition to chiral separations, CD-bonded phases have been used as conventional column packings for the separation of organometallic compounds, polynuclear aromatic hydrocarbons, substituted phenols, benzoic acids, and anilines, and styrene polymers, in both normal- and reversed-phase modes [9–15]. The use of a β-CD-bonded column for the separation of a selected group of dipeptides was explored [16]; however, detailed study was lacking.

To further test the versatility of CD-bonded phases as "universal" packings, we have performed their separations of dipeptides and tripeptides under various mobile phase conditions. In particular, a study is performed with the presence of copper(II) ions in the mobile phase which allows further modifications of retention behaviors. The results are reported in this paper.

EXPERIMENTAL

A Beckman Model 332 gradient liquid chromatographic system was used for the CD-bonded column separation. This system was equipped with two Model 110A pumps, a Model 210 sample injector valve and a Model 420 system controller. A Waters Model 440 absorption detector (254 nm) and an Omniscribe Model D5000 recorder were also applied.

The CD-bonded-phase columns (both β-CD and γ-CD) were obtained from Advanced Separation Technologies. The peptide analytes were obtained from Sigma and used without further purification. HPLC-grade solvents were obtained from Fisher Chemical. A total of 18 dipeptides and tripeptides were selected for this study. All have aromatic ring(s) which can form inclusion complexes with the cyclodextrin and can be easily detected at 254 nm. Cu(II) acetate (Aldrich) was used to prepare mobile phase solutions. The mobile phase was prepared using aqueous buffer and HPLC-grade methanol. Aqueous buffer solutions were prepared using acetic acid and ammonium acetate. Ionic strength was 0.01 M for most mobile phases unless otherwise stated. Before the separation experiments, the columns were pre-equilibrated for about 3 h using the mobile phase. After equilibrium was achieved, a flow-rate of 1 ml/min was used in the chromatographic process. For each separation, the peptides (0.1 mM) were dissolved in a solution containing the mobile phase. A back pressure of about 2000 p.s.i. was usually observed. All data points were collected by averaging more than three reproducible separations.

RESULTS AND DISCUSSION

Effects of organic modifier on peptide retention

Table I lists the values of capacity factors (k') of the dipeptides and tripeptides at various binary (methanol–aqueous buffer, pH 4.65) mobile phase compositions using a γ-CD column. It is seen that for each peptide there is a solvent composition that gives the lowest k' value, *i.e.*, the plot of k' *vs.* increasing organic modifier concentration is parabolic in shape which is in contrast to the linear relationship (plotted on a logarithmic scale) for non-cyclodextrin type columns [17]. This solvent composition is, in most cases, methanol–buffer (20:80). These minimum k' values increase when the

TABLE I

DEPENDENCE OF k' ON MOBILE PHASE COMPOSITION FOR DI- AND TRIPEPTIDES USING A γ-CD COLUMN

An asterisk indicates where the lowest k' value is observed.

Peptide	k'							
	Aqueous buffer–methanol							
	20:80	30:70	40:60	50:50	60:40	80:20	95:5	100:0
L-Trp–L-Phe	1.937		1.689		1.254*	1.305	2.365	3.698
L-Phe–L-Ala	1.273		1.216		0.749	0.444*	0.511	0.810
L-Ala–L-Trp	0.937		0.486		0.413	0.378*	0.533	0.765
L-Ala–L-Tyr	0.832		0.349		0.216	0.105*	0.105	0.133
L-Val–L-Trp	1.387		0.762		0.663	0.571*	0.917	1.429
L-Val–L-Phe	1.241		0.603		0.537	0.387*	0.578	0.968
Gly–L-Phe	0.873		0.387		0.356	0.219*	0.287	0.419
Gly–D-Phe	0.873		0.375		0.355	0.167*	0.268	0.397
Gly–L-Tyr	0.759		0.260		0.257	0.095	0.079*	0.105
L-Val–L-Tyr	1.241		0.556		0.454	0.206*	0.210	0.295
Gly–L-Trp	1.010		0.492		0.476	0.365*	0.476	0.683
Gly–L-Phe–L-Phe	0.686	0.508		0.371	0.369*	0.483	1.397	2.365
DL-Leu–Gly–DL-Phe	0.667	0.476		0.289*	0.308	0.365	1.11, 1.15	2.02, 2.16
L-Tyr–Gly–Gly	1.476	1.086		0.581	0.546	0.460	0.415*	0.765
L-Val–L-Tyr–L-Val	0.863	0.670		0.365	0.327	0.257*	0.400	0.635
L-Trp–Gly–Gly	1.762	1.210		0.508	0.470	0.283	0.206*	0.222
Gly–Gly–L-Phe	1.254	0.711		0.340	0.289	0.229*	0.292	0.429
Gly–L-Phe–L-Ala	0.829	0.505		0.317	0.244	0.210*	0.343*	0.552

methanol content in the mobile phase is either increased or decreased. Similar phenomena were also observed when substituted phenols and anilines were separated using CD colunns except that the solvent composition provided the lowest k' values contained more organic modifier, i.e., 2-propanol–water (80:20) [13].

A tentative rationalization of this observation can be provided if it is assumed that at least two mechanisms are involved in the retention of solutes. The first retention mechanism is the adsorption process which occurs between the peptides and the polar groups at the surface of the stationary phase. The major interactions of the adsorption process are the hydrogen bonding and ion–dipole interactions. These interactions gradually diminish as the water content in the mobile phase is increased. This also causes a concomitant decrease of peptide k' values.

Another retention mechanism is the inclusion of the peptides into bonded cyclodextrins. The degree of inclusion can be mediated by the amount of organic modifier. In general, inclusion is favored if the mobile phase is more hydrophilic. Thus, the peptides are held more tightly when the water content in the mobile phase is higher, i.e., greater k' values.

It is believed that when the methanol content is high, the adsorption process is dominant, which causes the peptide k' value to decrease with increasing water content in the mobile phase. This decrease in k' value is stopped when a lowest k' value is reached. Upon further increase of water content in the mobile phase, the inclusion process prevails and the k' value increases.
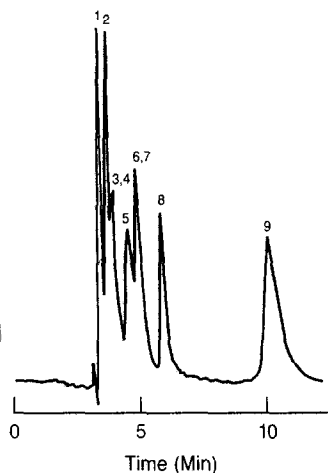
Fig. 1. Chromatogram for the separation of several dipeptides, γ-CD column, methanol–aqueous buffer (5:95), pH 4.65, flow-rate 1 ml/min. Peaks: 1 = Gly–L-Tyr; 2 = L-Val–L-Tyr; 3 = Gly–L-Phe; 4 = Gly–D-Phe; 5 = L-Phe–L-Ala; 6 = L-Ala–L-Trp; 7 = L-Val–L-Phe; 8 = L-Val–L-Trp; 9 = L-Trp–L-Phe.
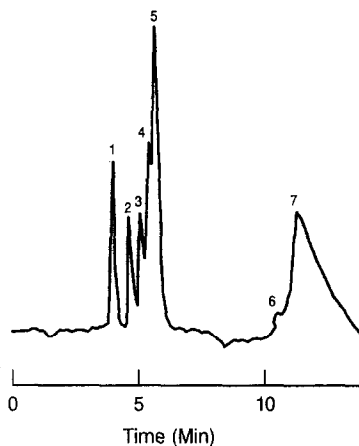
Fig. 2. Chromatogram for the separation of several tripeptides, γ-CD column, 100% aqueous buffer, pH 4.65, flow-rate 1 ml/min. Peaks: 1 = L-Trp–Gly–Gly; 2 = Gly–Gly–L-Phe; 3 = Gly–L-Phe–L-Ala; 4 = L-Val–L-Tyr–L-Val; 5 = L-Tyr–Gly–Gly; 6 = DL-Leu–Gly–DL-Phe; 7 = Gly–L-Phe–L-Phe.

The presence of the inclusion process can be further substantiated when a peptide contains more hydrophobic functional groups, e.g., Gly–L-Phe–L-Phe and DL-Leu–Gly–DL-Phe. A much greater increase of k' value (as compared to those peptides with less hydrophobic functional groups) is observed when the water content is increased in the mobile phase. This is evident particularly when the water content is high, e.g., 80%.

Figs. 1 and 2 show the chromatograms of several dipeptides and tripeptides at mobile phase compositions of 5% methanol in aqueous buffer and 100% aqueous buffer (pH 4.65), respectively. In the two cases, the organic modifier content is low to take advantage of substrate inclusion for chromatographic resolution. However, it is also possible to apply greater amount of organic modifier to diminish the inclusion and to increase the adsorption process for substrate resolution. For example, Fig. 3 shows the separation of a mixture of selected dipeptides and tripeptides using methanol–aqueous buffer (50:50) as the mobile phase.

Effects of mobile phase pH and ionic strength on peptide retention

The effect of mobile phase pH on peptide retention is examined with acetic acid–ammonium acetate buffer solutions at pH 5.6, 4.6 and 3.6 with an ionic strength of 0.01 M. The results shown in Table II indicate that the variation of mobile phase pH does not drastically affect the peptide retention. This is probably because the peptides in their zwitter ionic form are the dominant species in this pH range. A mobile phase pH 4.65 is chosen for further testing of the mobile phase ionic strength effect on peptide retention.
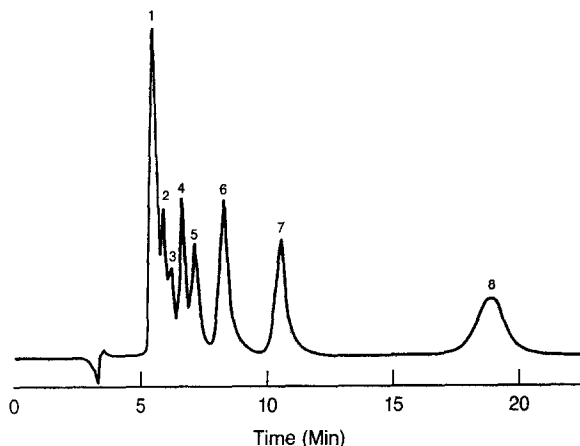
Fig. 3. Chromatogram for the separation of several dipeptides and tripeptides, γ-CD column, methanol-aqueous buffer (50:50), pH 4.65, flow-rate 1 ml/min. Peaks: 1 = Gly–L-Tyr; 2 = Gly–D-Phe; 3 = Gly–L-Tyr–L-Ala; 4 = Gly–L-Phe–L-Ala; 5 = Gly–L-Phe–L-Leu; 6 = Gly–L-Phe–L-Phe; 7 = L-Phe–Gly; 8 = L-Phe–L-Tyr.

Table II also shows the retention data of peptides obtained at three different mobile phase ionic strength, *i.e.*, 0.06, 0.01 and 0.001 *M*. It turns out that the mobile phase ionic strength is not very significant in changing the peptide retention times. However, for practical applications, ionic strength cannot be too high in order to avoid precipitation of the buffer salt in the column. Neither can it be very low in order to

TABLE II

RETENTION TIMES OF A SERIES OF PEPTIDES WITH pH AND IONIC STRENGTH USING A γ-CD COLUMN

Peptide	Retention time (min)					
	pH dependent: total ionic strength of buffer 0.01 *M*			Ionic strength dependent: pH 4.65		
	pH			Concentration of buffer (*M*)		
	5.55	4.65	3.65	0.06	0.01	0.001
Gly–D-Phe	4.42	4.43	4.15	4.43	4.28	4.12
Gly–L-Phe	4.47	4.53	4.12	4.53	4.32	3.98
L-Phe–L-Ala	4.55	4.77	4.35	4.77	4.57	4.70
Gly–L-Tyr	3.63	3.65	3.60	3.65	3.33	3.53
L-Val–L-Trp	6.33	6.58	5.40	6.58	6.17	6.43
L-Ala–L-Tyr	3.73	3.73	3.62	3.73	3.43	3.62
L-Trp–L-Phe	12.97	12.67	12.50	12.67	10.33	10.67
Gly–L-Phe–L-Ala	4.57	4.45	4.42	4.45	4.05	4.17
Gly–Gly–L-Phe	4.38	4.42	4.32	4.42	4.02	4.37
L-Trp–Gly–Gly	4.45	4.67	4.58	4.67	4.45	4.75
L-Tyr–Gly–Gly	3.32	3.38	3.63	3.38	3.38	3.58

avoid the decrease of buffer capacity. A mobile phase with an ionic strength of 0.01–0.05 M is recommended for general applications.

Effect of Cu(II) ion on peptide retention

The use of metal ions and their complexes in modern liquid chromatography has been demonstrated to be a powerful technique to achieve unique separation selectivities. In general, metal ions and their complexes can be classified into two categories: substitution inert and substitution labile, based on their kinetics of ligand displacement reactions [18]. For example, copper(II) ion and its complexes are considered to be substitution labile when chromatography time scale is concerned. Depending on whether the metal ion or complex is fixed on the stationary phase or when it is moved along the column in the mobile phase, one can distinguish two types of chromatography: (1) the chromatography of ligands in which the metal ion is held by the stationary phase via strong complex formation or adsorption [19,20]; (2) the chromatography of complexes in which the metal ion is bound more strongly towards the ligands in the mobile phase [21,22]. Both approaches have been applied in the novel separation of enantiomers, e.g., amino acids.

In the present study, copper(II) ions can form complexes with peptide ligands in the mobile phase. The Cu(II) ion complex formation can change the peptide conformation and therefore, their retention behavior. In Table III are listed the k' values of several peptides in the absence and presence of various concentrations of copper(II) ion in the mobile phase using a γ-CD column (pH 4.65). At the present experimental condition, a Cu(II) concentration of $1.25 \cdot 10^{-3}$ M or higher is required for significantly different separation selectivity. A separate spectrophotometric experiment also confirmed the presence of Cu(II)–peptide complex formation when

TABLE III

PEPTIDE RETENTION TIME AS A FUNCTION OF MOBILE PHASE COMPOSITION WITH A γ-CYCLODEXTRIN-BONDED-PHASE COLUMN

Group	Peptide	Retention time (min)				
		Mobile phase: aqueous buffer[a]–methanol				
		100:0	100:0 with $1.25 \cdot 10^3$ M Cu(II)	80:20 with $1.25 \cdot 10^3$ M Cu(II)	60:40 with $1.25 \cdot 10^3$ M Cu(II)	40:60 with $1.25 \cdot 10^3$ M Cu(II)
I	Gly–D-Phe	4.43	6.00	5.03	5.03	6.08
	Gly–L-Phe	4.53	5.88	5.27	4.97	6.13
	L-Val–L-Trp	6.58	7.20	6.33	5.87	6.58
	L-Ala–L-Tyr	3.73	9.30	7.00	5.78	6.10
	L-Trp–Gly–Gly	4.67	5.87	5.17	7.00	10.00
	L-Tyr–Gly–Gly	3.38	4.60	4.23	5.27	8.33
II	L-Phe–L-Ala	4.77	3.92	5.10	3.50	4.42
	Gly–L-Tyr	3.65	3.17	5.55	3.58	4.63
	L-Trp–L-Phe	12.67	7.67	5.27	5.25	6.00
	Gly–L-Phe–L-Ala	4.45	3.92	5.33	3.63	4.23
	Gly–Gly–L-Phe	4.42	4.10	5.13	5.03	6.83

[a] pH 4.65 acetic acid–acetate buffer, ionic strength 0.01 M; 25°C.

Cu(II) concentration is greater than $1.25 \cdot 10^{-3}$ M since the complex has a characteristic UV absorption band at 230 nm.

A careful examination of the peptide retention data using aqueous buffer in the absence and presence of $1.25 \cdot 10^{-3}$ M Cu(II) ion shows that the retention times increase for some peptides (group I) and decrease for others (group II), upon complexation with Cu(II) ion. It is still reasonable to assume that when an aqueous buffer solution is used as the mobile phase without the organic modifier, the contribution to retention by inclusion of Cu(II)–peptide complex is the greatest, as compared to those mobile phases containing some amount of organic modifier. Thus, the group I peptides which result in longer retention times upon Cu(II) ion complexation may either form stronger cyclodextrin inclusion complexes due to favorable conformational change, or be less hydrophilic than the corresponding uncomplexed ones, or be both. On the other hand, the group II complexed peptides with shorter retention times (than the uncomplexed ones) should be more hydrophilic or less readily to form cyclodextrin inclusion complexes or both, upon Cu(II) ion complexation.

Further testing of the above hypothesis can be performed by adding methanol to the mobile phase. The addition of methanol to the mobile phase should decrease the retention time for those group I peptides which are more hydrophobic and/or form stronger inclusion complex upon complexation because methanol can compete for inclusion with the cyclodextrin and also make the mobile phase more hydrophobic. This is observed as expected, as shown in Table III. The group II peptides which give shorter retention times upon Cu(II) ion complexation can be held either longer or shorter, depending on the prevailing retention mechanism in the CD column, when methanol is introduced into the mobile phase. If the shortening of retention time is due to unfavorable inclusion and increased hydrophilicity (less adsorption), the increase of methanol content in the mobile phase would make it more hydrophobic, thus results in more substrate adsorption and increased retention. It would also further reduce the inclusion, thus results in decreased retention. The net effect would be determined by the extent of contribution to retention by the two mechanisms. A quantitative prediction of retention contribution by the two proposed mechanisms is not possible at this moment. However, the experimental results indicate that four out of five peptides tested have increased retention upon addition of methanol (Table III). One peptide, L-Trp–L-Phe, shows further decrease in retention time. Tests with other peptides such as Gly–L-Phe–L-Leu and Gly–L-Phe–L-Phe also show similar decrease in retention time.

Both groups of complexed peptides eventually show increased retention times after minimum k' values are achieved, as explained previously (Table III). Fig. 4 is the chromatogram for the separation of several dipeptides and tripeptides using a γ-CD column with $1.25 \cdot 10^{-3}$ M Cu(II) ion in the mobile phase. It is observed that the retention orders of these peptides are indeed different from that without the presence of Cu(II) ion in the mobile phase. For example, the retention order of Gly–L-Phe–L-Leu and Gly–L-Phe–L-Ala in Fig. 4 is reversed in Fig. 3. On the other hand, the peptide peaks in the chromatogram are in general broader than those without Cu(II) ion. This indicates that the Cu(II) ion complexation equilibrium may have caused additional zone broadening in the present case.
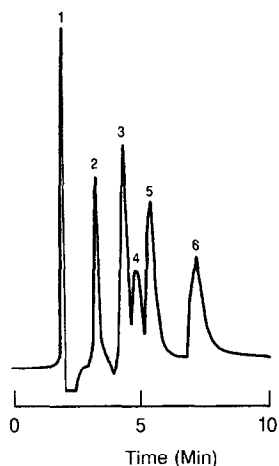
Fig. 4. Chromatogram for the separation of several dipeptides and tripeptides, γ-CD column, ammonium acetate–acetic acid buffer, [Cu(II)] = 1.25 · 10^{-3} M, pH 4.65, flow-rate 1 ml/min. Peaks: 1 = system peak; 2 = Gly–L-Tyr; 3 = Gly–L-Phe–L-Leu; 4 = Gly–L-Phe–L-Ala; 5 = L-Val–L-Tyr; 6 = L-Phe–Gly.

Comparison of β-CD and γ-CD columns

Tables IV and V list the retention times of some selected peptides in the absence and presence of 1.25 · 10^{-3} M Cu(II) ion in the mobile phase buffer solutions, respectively. The general observations for the separations using the γ-CD column are also observed for the β-CD column, *i.e.*, minimum k' values and selectivity change upon Cu(II) ion complexation. The mobile phase composition to observe minimum k' values now shifts to methanol–aqueous buffer (40:60) indicating that β-CD columns afford stronger inclusion complex formation with the peptides. This is consistent with the general notion that the binding of aromatic substrates with β-cyclodextrin is stronger than that with γ-cyclodextrin presumably because of a better cavity size fit [14]. Further comparisons are difficult because the cyclodextrin columns have different

TABLE IV

DEPENDENCE OF RETENTION TIME ON MOBILE PHASE COMPOSITION FOR SOME PEPTIDES USING A β-CD COLUMN

Peptide	Retention time (min)			
	Methanol–aqueous buffer[a]			
	0:100	20:80	40:60	60:40
L-Phe–L-Ala	4.76	4.30	5.60	7.32
L-Ala–L-Tyr	4.18	3.91	4.00	4.40
L-Val–L-Trp	6.10	5.05	5.25	5.45
Gly–L-Phe–L-Ala	4.49	4.09	4.20	4.80
L-Val–L-Tyr–L-Val	4.95	4.26	4.50	5.48
DL-Leu–Gly–DL-Phe	6.90	4.67	4.52	5.00

[a] pH 4.65 acetic acid–ammonium acetate buffer, ionic strength 0.01 M.

TABLE V

DEPENDENCE OF RETENTION TIME ON MOBILE PHASE COMPOSITION FOR SOME PEPTIDES USING A β-CD COLUMN

$[Cu(II)] = 1.25 \cdot 10^{-3}\ M$; ammonium acetate–acetic acid buffer (0.01 M), pH 4.65.

Peptide	Retention time (min)			
	Methanol–aqueous buffer			
	0:100	20:80	40:60	60:40
L-Phe–L-Ala	6.20	5.78	4.73	7.31
L-Ala–L-Tyr	6.25	5.30	4.95	6.25
L-Val–L-Trp	8.97	5.80	5.20	7.91
Gly–L-Phe–L-Ala	3.82	4.89	4.80	11.20
L-Val–L-Tyr–L-Val	7.53	3.93	3.83	7.34
DL-Leu–Gly–DL-Phe	9.78	4.60	4.20	8.33

degree of mixed surface coverage of functional groups (*i.e.*, cyclodextrin, alkyldiol and silanol groups), partly because of the difficulty of controlling the extent of surface derivatization reaction [23].

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