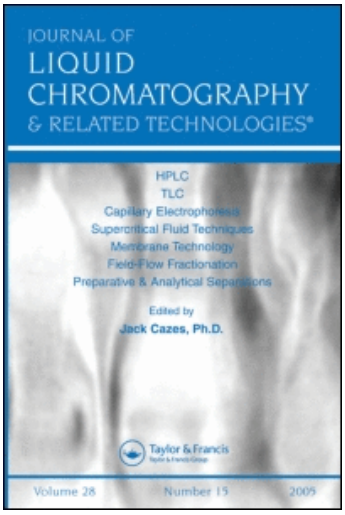

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Effect of Column Type and Experimental Parameters on the HPLC Separation of Dipeptides

Cynthia D. Ridlon[a]; Haleem J. Issaq[a]
[a] NCI-Frederick Cancer Research Facility, Program Resources, Inc., Frederick, Maryland

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EFFECT OF COLUMN TYPE AND EXPERIMENTAL PARAMETERS ON THE HPLC SEPARATION OF DIPEPTIDES*

Cynthia D. Ridlon and Haleem J. Issaq**

Program Resources, Inc.
NCI-Frederick Cancer Research Facility
P. O. Box B
Frederick, Maryland 21701

ABSTRACT

The separation of underivatized phenylalanine dipeptides by high performance liquid chromatography using a beta-cyclodextrin bonded silica and a reversed-phase C-18 column was evaluated. Parameters such as per cent organic modifier, pH, buffer type, and temperature were shown to have various effects on the dipeptides separation. The results revealed that the separation of dipeptides was possible using the beta-cyclodextrin column. Changing the percent methanol in the mobile phase from 15 to 10 percent improved the separation only slightly. Changing the pH of the mobile phase affected the peak shape and retention times depending on the amino acid in

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**To whom correspondence should be addressed.

the dipeptide. It was found that pH changes affected the retention times of Phe-Asp, Phe-Glu, and Phe-Trp more than the other dipeptides analyzed. Two buffer systems were evaluated, triethylammonium acetate (0.1%, pH 4.0) and ammonium acetate (0.01 M, pH 4.5); both systems gave comparable separations. The effect of temperature on the separation of the phenylalanine dipeptides was also studied. The only observed effects in varying the temperature from ambient to 57°C were the decrease in retention times and the peaks were sharper at higher than lower temperatures.

A comparison between using the beta-cyclodextrin column and the reversed-phase C-18 column for the separation of the phenylalanine dipeptides and other nonaromatic dipeptides indicated that both columns work equally well, with the C-18 requiring a higher concentration of methanol in the mobile phase. A comparison of the separation of a group of stereoisomers showed that the beta-cyclodextrin and C-18 columns gave comparable results with the C-18 requiring different concentrations of methanol in the mobile phase.

INTRODUCTION

The most widely used supports for high pressure liquid chromatography to date are the silica bonded-phases. With bonded-phase chromatography columns the packing contains a stationary phase which is chemically bound to the silica support. In this study, two bonded-phase columns are compared relative to their ability to separate dipeptides. The one column of particular interest is the beta-cyclodextrin bonded-phase which is a cyclic oligosaccharide containing seven glucopyranose units, having an alpha-1,4 linkage, arranged in the shape of a hollow truncated cone. Cyclodextrins have been used in the past as mobile phase modifiers (1), as stationary phases in the form of polymerized gels in column chromatography (2), and linked using ethylenediamine linkages to silica gel (3-4). The beta-cyclodextrin column used was developed by Armstrong in which the linkage of

the cyclodextrin molecules to the silica matrix was by a six to ten atom spacer (5). The advantages of the beta-cyclodextrin in the separation of isomers which have previously been difficult to separate was shown by the works of Armstrong and coworkers where they have separated a variety of diastereo- (6), structural, optical, and geometrical isomers (7). They also separated mycotoxins, polycyclic aromatic hydrocarbons, quinones, and heterocyclic compounds (8). More recently, it has been shown by Issaq (9) that the separation of a selected group of dipeptides could be achieved using the beta-cyclodextrin column.

The mechanism of separation on a cyclodextrin column is through inclusion complex formation. With the relatively hydrophobic interior of the cyclodextrin cavity, water soluble and insoluble compounds of varying size, shape, and chemistry form different strength inclusion complexes. The stability of inclusion complex formation is determined by hydrophobic interactions, hydrogen bonding, solvent interactions, and the degree to which the solute molecule fills the cavity (10).

Dipeptides consist of two amino acids linked by a peptide bond where the carboxyl group of one amino acid is bonded to the amino group of another amino acid. The analysis of dipeptides has been performed using ion-exchange (11-12), paper and thin-layer chromatography (13-14), gas chromatography and combined gas chromatography-mass spectrometry (15). Some of these methods require derivatization of the dipeptides prior to separation and identification. Also, analysis of underivatized dipeptides using high pressure liquid chromatography has been reported. A tripeptide bonded stationary phase was used by Grushka and co-workers (16-17) to separate some dipeptides which contained aromatic amino acids and sequence isomeric pairs. Molnar and Horvath (18) have separated dipeptides using non-polar stationary phases. Lundanes and Greibrokk (19) used four different reversed phases to separate underivatized dipeptides. A strong cation-exchanger was used by Nakamura and coworkers (20) to separate

dipeptides containing histidine and a weak anion-exchanger was used by Dizdaroglu and Simic (21) to separate underivatized dipeptides including sequence isomeric and diastereoisomeric dipeptides.

The importance of a simple method for the separation of dipeptides is demonstrated by the peptide sequencing generation of dipeptides by DAP I. Having the broadest substrate specificity, DAP I catalyzes the removal of dipeptide units, in consecutive order, from the unsubstituted amino termini of the peptide chain (22) and has certain advantages over the chemical methods (23).

The purpose of this study was to examine the separation of available underivatized phenylalanine dipeptides by HPLC. The beta-cyclodextrin column was chosen as little work has been done regarding dipeptide separation on this column. Although it has been shown previously to possess the ability to separate closely related compounds including dipeptides, no one to date has made a comprehensive investigation of parameters such as percent organic modifier, pH, buffer type, and temperature on the separation. The effects of these parameters on the separation of the phenylalanine dipeptides selected will be considered. The analysis of the phenylalanine dipeptides on beta-cyclodextrin will be compared with those obtained on a reversed-phase C-18 column. The optimum conditions for dipeptide separation on both columns will be compared with literature data, primarily reversed-phase C-18, since these are the most popular columns used today. Separation of a select group of stereoisomeric and nonaromatic dipeptides will also be compared on both columns.

EXPERIMENTAL

Apparatus

The separation of dipeptides was performed using a Waters Associates, Inc. (Milford, MA, USA) M-6000A pump, a Waters Wisp 710B autosampler, and Waters Model 720 system controller, Kratos Analytical Instrument (Westwood,

NJ, USA) GM 770R variable wavelength detector, and a Houston Instrument (Austin, TX, USA) OmniScribe recorder to monitor the column effluents. For the temperature study, using the cyclodextrin column, it was necessary to use a second HPLC system. This system consisted of a Hewlett Packard (Avondale, PA, USA) HP1090 Liquid Chromatograph equipped with a heater, a photo diode array detector, an HP 85B system controller, and an HP 3392A integrator. The mobile phase solvents were filtered using a Millipore filter holder (Milford, MA, USA) with Millipore filters having a pore size of 0.5 um organic and 0.45 um aqueous. The pH meter used to adjust the pH of the mobile phases was a Fisher Accumet Model 320 (Fair Lawn, NJ, USA). The samples were dissolved in water or 0.1 N HCl, then sonicated. Dipeptide separations were carried out on a 250 mm x 4.6 mm Cyclobond I column by Advanced Separation Technologies, Inc. (Whippany, NJ, USA) which has a beta-cyclodextrin bonded to 5u spherical silica particles and a Waters 150 mm x 3.9 mm Nova pak C-18 reversed-phase column with 5u spherical silica packing.

Materials

Dipeptides were obtained from Sigma (St. Louis, MO, USA), Chemical Dynamics Corp. (South Plainfield, NJ, USA), and U.S. Biochemical Corp. (Cleveland, OH, USA). The following were purchased from Fisher (Fair Lawn, NJ, USA): triethylamine, 0.1 N HCl, ammonium acetate, 1 N sodium hydroxide, and glacial acetic acid. The triethylammonium acetate (TEAA) buffer was prepared by making a 0.1% TEAA and titrating with glacial acetic acid or sodium hydroxide to obtain the desired pH. To prepare a mobile phase using this buffer, a v/v methanol/0.1% TEAA was made. The ammonium acetate buffer used was prepared by weighing 0.77 grams of ammonium acetate in approximately 700 ml of Milli-Q water. The pH was adjusted to 4.5 using glacial acetic acid and the v/v methanol/water adjusted accordingly to one liter. After the v/v methanol/buffer was made to one liter, the mobile phase was then filtered and vacuum degassed before use. The methanol, HPLC

grade, was purchased from Burdick and Jackson Labs (Muskegon, MI, USA). The buffers were prepared using water from a Millipore (Milford, MA, USA) Milli-Q water system.

Methods

The operating conditions of all analyses were at ambient temperature with a flow rate of 1 ml/min and a chart speed of 0.5 cm/min unless otherwise specified. The absorption of the effluent was monitored at 254 nm for the aromatic dipeptides, since this group contains a pi bond, and 220 nm for the nonaromatic dipeptides. The TEAA solution used in this study was a 0.1% prepared volumetrically with a pH 4.0 unless noted otherwise.

RESULTS AND DISCUSSION

I. Effect of percent methanol on separation

a. Beta-cyclodextrin column

The first experimental approach for the separation of the dipeptides on the cyclodextrin column was to prepare a mobile phase based on the literature. The results of Issaq (9) demonstrated that a mobile phase of methanol/water (16:84) with a beta-cyclodextrin column would separate a select group of dipeptides. It was recommended by Armstrong (24) that 0.1% triethylamine acetate (TEAA) buffer at pH 4.0 be used in place of water in the methanol/water system for derivatives of amino acids and peptides. Therefore, a mobile phase of 15% MeOH/85% TEAA was used.

The percent organic modifier in the mobile phase had a noticeable effect on the retention times of certain dipeptides. A comparison of retention times (RT) of phenylalanine dipeptides using a mobile phase of 15% MeOH/85% TEAA and 10% MeOH/90% TEAA showed that Phe-Glu eluted after Phe-Tyr with 15% MeOH and before Phe-Tyr with 10% MeOH (Table I). At 15% MeOH, Phe-Leu and Phe-Ile, which are structural isomers, were resolved by about one minute while at 10% MeOH they were separated by 1.2 minutes. Resolution of the dipeptides did not always improve by decreasing the percentage of

Table I. Retention time values of selected aromatic dipeptides using a beta-cyclodextrin column. Conditions: solvent system - A: 15% MeOH/85% TEAA, B: 10% MeOH/90% TEAA.

Dipeptide	A RT (min)	B RT (min)
Phe-Ala	4.5	4.5
Phe-Gly	5.0	4.8
Phe-Val	5.8	5.6
Phe-Met	7.5	7.2
Phe-Ile	8.6	8.4
Phe-Leu	9.6	9.6
Phe-Tyr	11.8	12.0
Phe-Glu	12.0	11.0
Phe-Asp	12.2	11.0
Phe-Phe	17.6	16.0
Phe-Trp	18.0	16.6

organic modifier in the mobile phase, for example, in the case of Phe-Ala and Phe-Gly, separation was depressed by 0.2 minutes. An increase or decrease in resolution may be attributed to the solubility of that pair of dipeptides in the mobile phase. In the case of Phe-Leu and Phe-Ile, the solubility (retention) of Phe-Leu did not change between 15% and 10% MeOH (RT = 9.6 minutes) whereas that of Phe-Ile increased by changing from 15% (RT = 8.6 minutes) to 10% (RT = 8.4 minutes). There were cases where both dipeptides retention was affected by the change of percent methanol, for example, in 15% MeOH Phe-Phe (RT = 17.6 minutes) and Phe-Trp (RT = 18.0 minutes) were different than at 10% MeOH, Phe-Phe (RT = 16.0 minutes) and Phe-Trp (RT = 16.6 minutes) which means that both were more soluble in 10% MeOH, but to different degrees, which improved the separation by 0.2 minutes. A 5% MeOH/95% TEAA and a 1% MeOH/99% TEAA were investigated using

Phe-Gly and Phe-Ala. At 5% MeOH/95% TEAA the retention time of Phe-Gly was 5.6 minutes and that for Phe-Ala was 5.2 minutes. When a 1% MeOH/99% TEAA was used, the retention time of Phe-Gly became 6.6 minutes and that for Phe-Ala 5.8 minutes. Since Phe-Asp and Phe-Glu coeluted, 5% MeOH was used in the mobile phase. The results were a separation of these two dipeptides: Phe-Glu eluting in 9.5 minutes and Phe-Asp in 15.6 minutes. Comparing the retention times of Phe-Ala and Phe-Gly at one, five and ten percent methanol, a progressive decrease in the retention time was observed. This has been explained by Armstrong and DeMond (7) as a result of the competition of the organic modifier for the preferred site in the hydrophobic cyclodextrin cavity. Both 15% and 10% gave similar results in terms of retention time. Since an improvement in the separation of Phe-Tyr and Phe-Glu was seen at 10%, this percent methanol was chosen for the mobile phase used to study the effect of other parameters on the separation.

b. Reversed-phase column

In an effort to compare the results from the beta-cyclodextrin column with the more commonly used reversed-phase column, the mobile phase selected was that used by Lundanes and Greibrokk (19), containing 10% MeOH/0.01 M ammonium acetate buffer, pH 6.6. Double peaks were observed for some of the dipeptides tested and it appeared that Phe-Asp and Phe-Glu were eluting too fast. To increase the retention times of Phe-Asp and Phe-Glu, the concentration of methanol was lowered to 5%. With 5% MeOH/0.01 M ammonium acetate Phe-Glu eluted at 2.3 minutes while Phe-Asp had a retention time of 4.0 minutes. However, the other dipeptides analyzed either gave double peaks or did not elute from the column at this percent methanol.

As recommended (19), the pH was controlled in order to eliminate the double peaks observed. Without pH control the 5% MeOH/0.01 M ammonium acetate has a pH of 6.6. Glacial acetic acid was used to lower the pH to 4.5. The results at 5% MeOH/0.01 M ammonium acetate, pH 4.5 with acetic

Table II. Retention time values of selected aromatic dipeptides using a reversed-phase C-18 column. Conditions: Solvent system - A: 10% MeOH/0.01 M ammonium acetate, pH 4.5, B: 20% MeOH/0.01 M ammonium acetate, pH 4.5, C: 20% MeOH/0.1 M potassium phosphate.

Dipeptide	A RT (min)	B RT (min)	C RT (min)
Phe-Asp	2.7	2.2	2.0
Phe-Glu	3.0	2.2	2.0
Phe-Ala	3.6	2.1	2.4
Phe-Gly	4.8	2.0	2.6
Phe-Val	5.4	3.8	3.6
Phe-Tyr	12.4	4.3	4.2
Phe-Met	12.6	6.0	6.1
Phe-Ile	21.4	8.0	7.7
Phe-Leu	45.8	13.8	14.0
Phe-Trp	61.0	19.6	21.0
Phe-Phe	64.0	22.0	22.4

acid, gave only single peaks for the dipeptides, however, the larger dipeptides such as Phe-Ile, Phe-Leu, and Phe-Met were retained too long on the column.

To reduce the retention time of the larger dipeptides, the volume of methanol was increased to 10%. Table II shows the observed retention times of the 11 dipeptides tested. It was clear from these results that increasing the percent methanol provided a reasonable analysis time for Phe-Ile and Phe-Val. However, for Phe-Leu, Phe-Trp, and Phe-Phe it was necessary to increase the methanol volume further. A 20% MeOH/0.01 M ammonium acetate buffer, pH 4.5, was used. The results (Table II) revealed that Phe-Leu eluted in 13.8 minutes, Phe-Trp in 19.6 minutes and Phe-Phe in 22.0 minutes. In order to achieve a better separation for the smaller size

dipeptides, a 5% MeOH/0.01 M ammonium acetate, pH 4.5 was used. This proved to be very useful in separating Phe-Ala and Phe-Gly with retention times of 5.6 and 8.1 minutes respectively, whereas Phe-Asp and Phe-Glu were separated by 0.5 minutes.

Once it was determined that this group of dipeptides, having phenylalanine in the first position, required various percent methanol concentrations to be eluted optimally on reversed phase, a gradient elution system was attempted. A gradient profile (System I) using a 20 minute linear curve from 100% A (0% MeOH/0.01 M ammonium acetate, pH 4.5) followed by ten minutes of 100% B (20% MeOH/0.01 M ammonium acetate, pH 4.5) appeared to be the optimum gradient profile for separating all the dipeptides in this group except for Phe-Phe which had a retention time greater than 40 minutes (Table II). Another gradient profile (System II) involved a linear curve from 100% A (5% MeOH/0.01 M ammonium acetate, pH 4.5) at 0.5 ml/minute to 100% B (20% MeOH/0.01 M ammonium acetate, pH 4.5) at 1 ml/min in 15 minutes followed by 15 minutes of 100% B. System II appeared to be another good system for separating this group of dipeptides (Table III). Although Phe-Asp and Phe-Glu did not separate as well as they did with gradient System I, System II, however, maintained a more stable baseline throughout the experiment.

II. Effect of Mobile Phase pH on Separation

a. Beta-cyclodextrin column

To observe what effect the pH would have on the separation and retention time of the dipeptides, the pH of the 0.1% TEAA was adjusted using glacial acetic acid to the desired pH value. The mobile phase consisted of 10% MeOH/90% TEAA. The effect of the pH on the phenylalanine dipeptides is shown graphically in Figure 1. The range of pH values were within those acceptable for this column, as stated in the literature provided by the manufacturer of the column.

Table III. A. Gradient Elution System I. System A - 0% MeOH/0.01 M ammonium acetate, pH 4.5; Solvent System B - 20% MeOH/0.01 M ammonium acetate, pH 4.5; linear, 20 min. from 100% A to 100% B, then 10 min. of 100% B; flow rate 1 ml/min.

B. Gradient Elution System II. Solvent System A - 5% MeOH/0.01 M ammonium acetate, pH 4.5; Solvent System B - 20% MeOH/0.01 M ammonium acetate, pH 4.5; linear, 15 min. from 100% A to 100% B, then 15 min of 100% B; flow rate linear from 0.5 to 1.0 ml/min in 15 min.

Dipeptide	A RT (min)	B RT (min)
Phe-Asp	3.6	5.2
Phe-Glu	5.2	5.5
Phe-Ala	8.6	7.2
Phe-Gly	10.6	8.9
Phe-Val	14.8	11.7
Phe-Tyr	17.4	14.0
Phe-Met	19.9	15.2
Phe-Ile	22.0	17.4
Phe-Leu	27.4	22.3
Phe-Trp	34.2	29.4
Phe-Phe	>40.0	30.2

The results demonstrated that by an increase in pH from 4.0 to 7.0, Phe-Glu and Phe-Asp were separated in three minutes, whereas at all other pH values they eluted at the same time or within less than 0.5 minutes. At pH 6.0, the isoelectric point for most amino acids, there was a negative charge on the side chain of the acidic amino acids of the dipeptides and they coeluted. At pH 7.0, there were two negative charges on these dipeptides and separation was enhanced. At a pH of 3.5 to 5.0, Phe-Ala eluted from the column first and from pH 6.0 to 7.4 Phe-Gly eluted first.

Chromatography of the dipeptides at pH 3.5, resulted in sharp peaks and fast elution (less than 13 minutes). At pH 4.0, optimal peak shape and

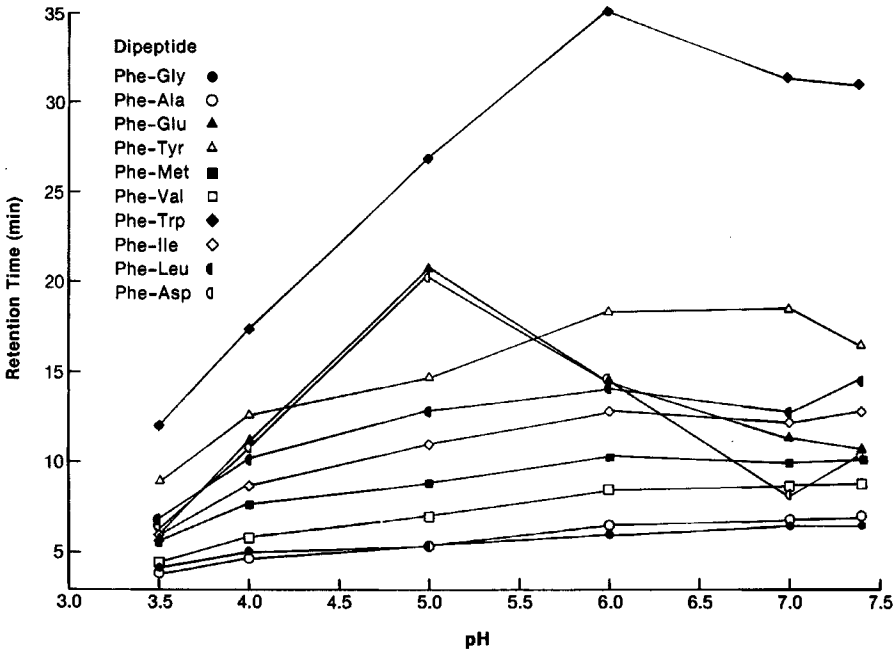

Figure 1. Effect of solvent system pH on the retention time values of selected aromatic dipeptides using a beta-cyclodextrin column. Solvent system: 10% MeOH/90% TEAA (0.1%, pH 3.5, 4.0, 5.0, 6.0, 7.0, 7.4).

separation between the majority of the dipeptides of phenylalanine was observed. The peaks were slightly broader at pH 5.0 and retention was increased. At pH 6.0, the peak width and the retention time increased except for the dipeptides Phe-Glu and Phe-Asp, which decrease in retention time. Although Phe-Asp and Phe-Glu could be separated at pH 7.0, Phe-Asp and Phe-Val which were separating at all other pH values were now eluting close together. The peak shape at pH 7.0 does not appear as broad as at pH 6.0. At pH 7.4, Phe-Glu, Phe-Asp and Phe-Met eluted close together and the peak shape was similar to those at pH 7.0. From Figure 1 it was determined that pH 4.0 was the optimal pH of the mobile phase for separating the majority of the dipeptides in this group.

b. Reversed-phase column

The effect of pH on the analysis of peptides using reversed-phase has been shown previously (25). As mentioned earlier, pH control was necessary in order to eliminate the double peaks observed.

III. Effect of Buffer Type on Separation

a. Beta-cyclodextrin column

Although our buffer system of 0.1% TEAA pH 4.0 was recommended, an effort was made to see what effect a change in the buffer would have on the separation of these dipeptides. A 0.01 M ammonium acetate buffer, pH 4.5 was chosen. Ammonium acetate, a common reversed-phase buffer, was reported by Lundanes and Greibrokk (19) to separate some dipeptides by reversed-phase mode. A comparison of the results is summarized in Table IV.

Table IV. A comparison between the beta-cyclodextrin solvent system and the reversed-phase solvent system for selected dipeptides using the beta-cyclodextrin column. Retention time in minutes.

Dipeptide	10% MeOH/90% TEAA 0.1%, pH 4.0	10% MeOH/NH4Ac 0.01 M, pH 4.5
Phe-Ala	4.7	4.5
Phe-Gly	5.1	4.8
Phe-Val	5.8	5.6
Phe-Met	7.6	6.9
Phe-Ile	8.7	8.2
Phe-Leu	10.2	9.1
Phe-Asp	11.0	10.9
Phe-Glu	11.0	10.5
Phe-Tyr	12.6	11.1
Phe-Phe	16.6	14.2
Phe-Trp	17.4	17.4

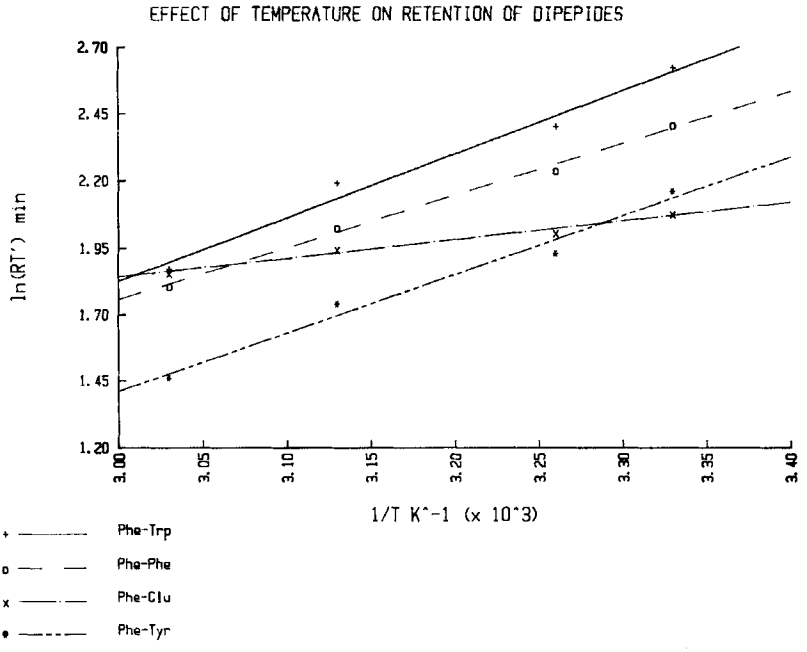

Figure 2. Effect of temperature on retention of a selected group of dipeptides, using a beta-cyclodextrin column and a mobile phase of 10% MeOH/0.01M ammonium acetate; pH 4.5.

The results in Table IV show that both buffer systems worked equally well in separating the dipeptides. Although Issaq (9) was able to show that a group of dipeptides could be separated using a beta-cyclodextrin column with a simple methanol/water mobile phase. It was discovered that without a buffer system the peaks were broad. Adjusting the pH with a small percentage of TEAA (0.1%) to pH 4.0 resulted in an improved peak shape as illustrated in Figure 3.

b. Reversed-phase column

In order to investigate the effect a change in the buffer system used in the mobile phase would have on retention and resolution using a C-18

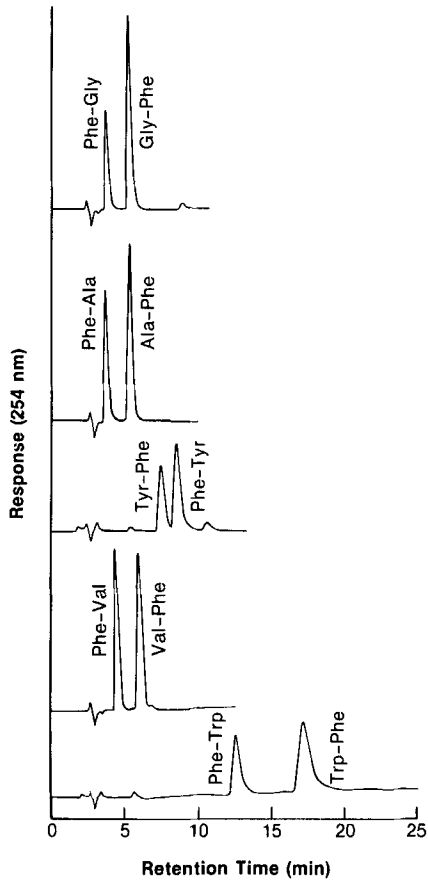

Figure 3. Separation of selected aromatic dipeptide stereoisomers using a beta-cyclodextrin column. Solvent system: 10% MeOH/90% TEAA.

column, a 20% MeOH/0.1 M potassium phosphate mobile phase was used. The pH of this system was 4.8. The results are shown in Table II (C).

A comparison was made between the 20% MeOH/0.01 M ammonium acetate buffer, and the 20% MeOH/0.1 M potassium phosphate, monobasic, buffer. The results, shown in Table II (B and C), indicated that both systems were comparable in terms of elution time. The advantage of the phosphate system over the acetate was that the phosphate mobile phase needed no pH adjustment since the pH was already at 4.8.

IV. Effect of Column Temperature on Separation

a. Beta-cyclodextrin column

In order to study the effect of temperature on the retention time of dipeptides on a beta-cyclodextrin column an HPLC system equipped with a column heater for temperature control was needed. The dipeptides were analyzed at ambient temperature first in order to obtain a baseline of retention times using this HPLC system. Retention times were recorded at 27, 37, 47 and 57 degrees centigrade. The effect of temperature on the retention times of the dipeptides is shown in Table V.

Increase in temperature of the column resulted in a decrease in the retention times of all the dipeptides analyzed. In the case of Phe-Tyr, the retention time decreased most, between 27°C and 37°C a 17.8% change and

Table V. Effect of temperature on the retention time, in minutes, of selected dipeptides using a beta-cyclodextrin column. Solvent System - 10% MeOH/0.01 M ammonium acetate, pH 4.5.

	Temperature				
Dipeptide	Ambient	27°C	37°C	47°C	57°C
Phe-Ala	4.5	4.1	4.0	3.9	3.7
Phe-Gly	4.8	4.3	4.1	4.0	3.8
Phe-Val	5.6	4.9	4.7	4.5	4.3
Phe-Met	6.9	6.1	5.6	5.2	4.7
Phe-Ile	8.2	7.2	6.6	6.1	5.4
Phe-Leu	9.1	8.0	7.3	6.7	5.8
Phe-Glu	10.5	9.4	8.9	8.4	7.8
Phe-Asp	10.9	9.5	9.0	8.6	7.9
Phe-Tyr	11.1	10.1	8.3	7.1	5.7
Phe-Phe	14.2	12.6	10.8	9.0/9.6	7.5
Phe-Trp	17.4	15.2	12.4	10.3	7.9

between 27°C and 47°C a 29.7% change was observed, respectively. The most dramatic change for Phe-Tyr was between 27°C and 57°C where the percent difference in retention time was 43.6%. For Phe-Trp, the same type of pattern was seen to occur. The percent change between 27°C and 57°C was 47.9%. Another interesting phenomenon was that Phe-Phe gave two peaks at 47°C. Phe-Phe, like Phe-Tyr and Phe-Trp, also showed a larger percent decrease in retention time with increasing temperature than most of the dipeptides. The peaks were sharper with increase in temperature, however, it did not appear to improve the separation between members of this group of dipeptides significantly except for Phe-Asp and Phe-Tyr. The peaks were sharper and eluted faster at higher temperature because the dipeptides were more soluble, the viscosity of the mobile phase decreased and mass transfer increased.

Another interesting phenomenon of the effect of temperature is given in Figure 2, which shows that the optimum temperature for the separation of a group of dipeptides (Phe-Trp, Phe-Phe, Phe-Glu and Phe-Tyr) is 44°C. Note that although Phe-Trp and Phe-Glu were separated by 7 minutes at room temperature they coeluted at 57°C. On the other hand Phe-Tyr and Phe-Glu which were resolved by 0.6 minutes at room temperature were separated by 2 minutes at 57°C. This means that in certain instances column temperature can be selectively used to improve the separation.

b. Reversed-phase column

The effect of temperature of reversed-phase columns on peptide separation has been reported previously (25).

V. Separation of dipeptide stereoisomers

a. Beta-cyclodextrin column

As illustrated in Figure 3, baseline separation of five pairs of stereoisomeric dipeptides was accomplished using the 10% MeOH/90% TEAA

Table VI. A comparison between the beta-cyclodextrin solvent system and the reversed-phase solvent system for selected stereoisomeric dipeptides on a beta-cyclodextrin column.

Dipeptide	10% MeOH/90% 0.1% TEAA pH 4.0	10% MeOH/0.01 M NH_4Ac pH 4.5
Phe-Gly/Gly-Phe	4.1/5.6	4.7/6.5
Phe-Val/Val-Phe	4.6/6.2	5.5/7.4
Phe-Ala/Ala-Phe	3.9/5.4	4.6/6.3
Phe-Tyr/Tyr-Phe	7.8/8.8	9.5/11.2
Phe-Trp/Trp-Phe	12.7/17.4	17.3/22.0

mobile phase. Comparing these results with those using 10% MeOH/0.01M ammonium acetate, pH 4.5 (Table VI), it was clear that either system was capable of separating this group of stereoisomeric dipeptide pairs. The 10% MeOH/90% TEAA could separate the pairs in a shorter elution time than the 10% MeOH/0.01 M ammonium acetate, pH 4.5. The separation of underivatized dipeptides by Disdaroglu and Simic (21) showed that with dipeptides containing both aliphatic and aromatic amino acids, the dipeptide with the aliphatic side chain in the first position would elute first. However, the results in Figure 3, for example, revealed that Phe-Val eluted before Val-Phe using this mobile phase system on the beta-cyclodextrin column. This was also true using the reversed-phase column where Phe-Val eluted before Val-Phe (Figure 5).

b. Reversed-phase column

Consistent with the beta-cyclodextrin column study, the group of five pairs of stereoisomers were analyzed using a reversed-phase column. Table VII gives the results of the dipeptide pairs analyzed. Figure 4 and Figure 5 show the chromatograms for each pair. A comparison of the separation of

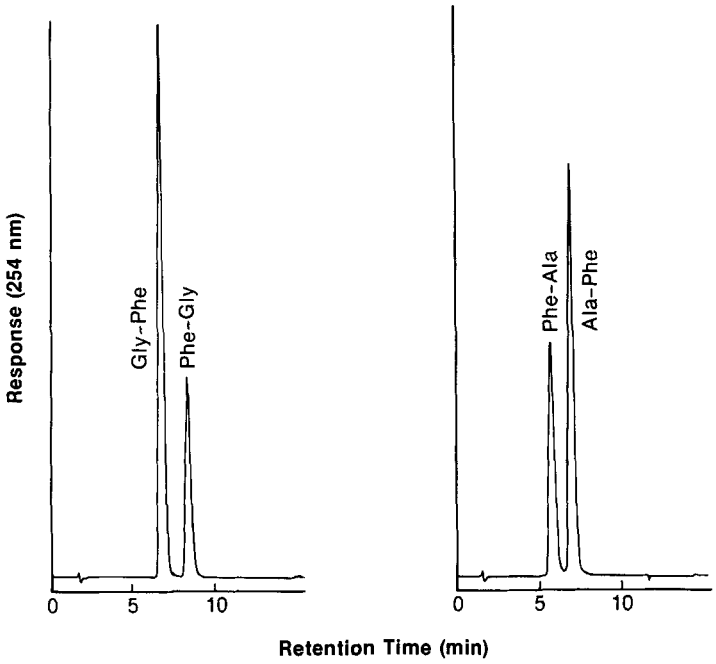

Figure 4. Separation of selected aromatic dipeptide stereoisomers using a reversed-phase C-18 column. Solvent system: 5% MeOH/0.01M ammonium acetate, pH 4.5.

these stereoisomers using beta-cyclodextrin and C-18 columns with the optimum mobile phases, Tables VI and VII, showed that the C-18 column gave better resolution.

VI. Separation of Nonaromatic Dipeptides

b. Beta-cyclodextrin column

A group of nonaromatic dipeptides was selected to run at 220 nm, in order to detect the peptide bond which absorbs at this wavelength. Table VIII shows the results using a mobile phase of 5% MeOH/95% TEAA and Figure 6B shows the separation of a pair of nonaromatic stereoisomers. Selected phenylalanine dipeptides were also analyzed at 220 nm using a 5% MeOH/95% TEAA and a 10% MeOH/90% TEAA.

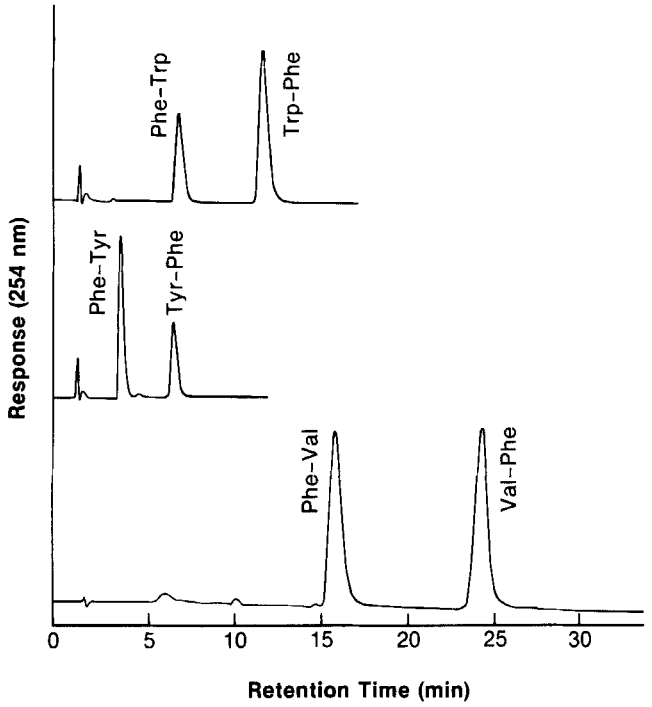

Figure 5. Separation of selected aromatic dipeptide stereoisomers using a reversed-phase C-18 column. Conditions: Phe-Trp/Trp-Phe - 30% MeOH/0.01 M ammonium acetate, pH 4.5; Phe-Tyr/Tyr-Phe - 20% MeOH/0.01 M ammonium acetate, pH 4.5; Phe-Val/Val-Phe - 5% MeOH/0.01 M ammonium acetate, pH 4.5.

Table VII. Separation of a selected group of stereoisomers using a reversed-phase C-18 column. Conditions: solvent systems - A: 5% MeOH/0.01 M ammonium acetate, pH 4.5; B: 20% MeOH/0.01 M ammonium acetate, pH 4.5; C: 30% MeOH/0.01 M ammonium acetate, pH 4.5.

Dipeptide	RT (mixture)	Mobile Phase
Phe-Ala/Ala-Phe	5.8/7.1	A
Phe-Gly/Gly-Phe	8.5/6.8	A
Phe-Val/Val-Phe	16.2/24.8	A
Phe-Tyr/Tyr-Phe	3.9/6.9	B
Phe-Trp/Trp-Phe	7.0/12.0	C

Table VIII. Retention time values of selected nonaromatic dipeptides on A: beta-cyclodextrin column, Solvent System - 5% MeOH/95% TEAA and B: reversed-phase C-18 column, Solvent System - 10% MeOH/0.01 M ammonium acetate, pH 4.5.

Dipeptide	A RT (min.)	B RT (min.)
Ala-Ile	4.8	2.4
Gly-Asp	5.6	1.3
Gly-Gly	3.2	1.4
Gly-Ile	4.2	2.5
Gly-Thr	3.3	1.3
Gly-Trp	6.9	7.0
Gly-Val	3.5	1.9
Leu-Leu	6.4	19.8
Leu-Val	4.2	4.6
Pro-Ile	4.8	2.7
Trp-Gly	6.1	8.3
Trp-Ile	10.4	29.8
Val-Gly	3.4	1.8
Val-Ile	4.3	4.1
Val-Leu	4.6	5.8
Val-Val	3.4	2.3

The group of nonaromatic dipeptides were selected based on the aliphatic chain of the amino acid and their size for analysis on the beta-cyclodextrin column to see if they could elute through the beta-cyclodextrin cavity and if they could be separated. Table VIII A showed that when using a 5% MeOH/95% TEAA solvent system, the pairs of nonaromatic dipeptide stereoisomers, such as Gly-Val/Val-Gly and Val-Leu/Leu-Val could not be separated. A 1% MeOH or a 10% MeOH with the TEAA buffer did not improve the separation. However, Trp-Gly/Gly-Trp could be baseline separated, with Trp-

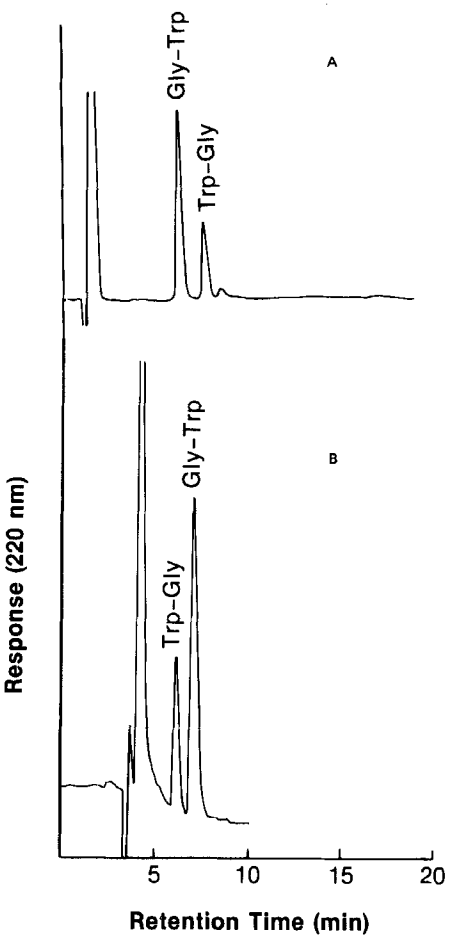

Figure 6. Separation of selected nonaromatic dipeptide stereoisomeric pairs using A: Reversed-phase C-18 column, solvent system - 10% MeOH/0.01 M ammonium acetate, pH 4.5 and B: Beta-cyclodextrin column, solvent system - 5% MeOH/95% TEAA.

Gly resulting in a retention time of 6.1 minutes and Gly-Trp, 7.1 minutes. Figure 6B illustrated these results.

In examining the group of dipeptides with Gly as the first amino acid, the order of elution on the beta-cyclodextrin column appears to be, except for Gly-Gly and Gly-Asp the result of an increase in the size of the aliphatic side chain of the second amino acid such that the longer the chain the longer the retention time which is consistent with the inclusion mechanism. With Val as the first amino acid of the dipeptide, Val-Gly and Val-Val coelute and little separation occurs between Val-Leu and Val-Ile while Leu-Val and Leu-Leu are easily separated from each other as are Trp-Gly and Trp-Ile (Table VIII).

b. Reversed-phase column

The group of nonaromatic dipeptides analyzed on the beta-cyclodextrin column were chromatographed using the reversed-phase column. Table VIII B showed that the nonaromatic dipeptides could be separated using the reversed-phase column. Comparing the results with those on the beta-cyclodextrin column, the more hydrophobic dipeptide such as Trp-Ile eluted slower on reversed-phase at 10% MeOH, 29.8 minutes than it did on beta-cylcodextrin at 5% MeOH, 10.4 minutes. Leu-Leu also exhibited the same phenomenon. Figure 6A showed that two pairs of nonaromatic stereoisomers could be separated. Since digests of proteins contain both aromatic and nonaromatic dipeptide mixtures and previous work with dipeptides has been monitored in the 200 nm to 220 nm range, detection of the aromatic dipeptides at 220 nm using beta-cyclodextrin and reversed-phase was examined. The results revealed that at 220 nm the detection of the dipeptides was more sensitive than at 254 nm. However, at 220 nm the acetate mobile phases also showed strong absorbance. Since the reversed-phase column required different percent methanol concentrations in the mobile phase in order to elute the dipeptides and the stereoisomeric

dipeptide pairs, this made the beta-cyclodextrin column more advantageous over the reversed-phase for this group of dipeptides containing phenylalanine.

CONCLUSION

The results show that the separation of underivatized phenylalanine dipeptides by HPLC using a beta-cyclodextrin or a reversed-phase column was possible. Parameters such as percent organic modifier, pH, buffer type, and temperature were shown to have various effects on the peak shape, retention time, and separation of the phenylalanine dipeptides. Comparison of the separation of the phenylalanine dipeptides and other nonaromatic dipeptides showed that both columns worked equally well although the C-18 column required a higher percentage methanol in the mobile phase. The separation of the stereoisomeric dipeptides gave comparable results on both the beta-cyclodextrin and reversed-phase columns.

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