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HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF AMINO ACIDS AND DIPEPTIDES ON A TRIPEPTIDE BONDED STATIONARY PHASE

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SUMMARY

A bonded tripeptide (L-valyl-L-phenylalanyl-L-valine) phase has been used as a stationary phase in liquid chromatography. Unique retention orders and retention variations are shown for phenylthiohydantoin (PTH) derivatives of amino acids and isomeric dipeptides using acidic and basic mobile phases. It is shown that the 25 PTH-amino acids studied here have different capacity ratios when 1 % citric acid in water (*ca.* pH 2.5) is used as the mobile phase. The analysis time for these amino acid derivatives could be shortened without a loss in the resolution by adding 5 % methanol to the above mobile phase. Of particular importance is the fact that the basic amino acids are eluted first. Isomeric dipeptides have been resolved by using deionized water (pH 5.5), and 1 % sodium citrate in water (pH 7.9), as mobile phases. The data show that the nature of the mobile phases, the pH, and the ionic strength are the important factors affecting the selectivity and efficiency of the separations of amino acids and dipeptides.

INTRODUCTION

Bonded stationary phases have increased the versatility of high-pressure liquid chromatography (HPLC), and they are now used routinely to achieve various separations. Tailor-designed bonded phases have the added advantage of selectivity for a desired class of compounds. The development and growth of affinity chromatography is one example of the power and the utility of chromatographic specificity.

A great deal of effort has been directed at the separation of amino acids, peptides, and their derivatives; see, for example, the recent reviews by Deyl[1,2]. These solutes have been separated by gas chromatography (GC), ion exchange, thin-layer chromatography and HPLC[3-24].

Specific solute–stationary phase interactions should be selective and thus should enhance the resolution. The study described here has been concentrated on designing a peptide bonded phase for the specific HPLC separation of amino acids and peptides. Peptides as stationary phases have been used in GC, among others, by

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Gil-Av and by Rogers (see, for example, refs. 25 and 26 and references therein) who separated amino acids enantiomers. In liquid chromatography (LC), Losse and Kuntze[27] have used bonded peptides. However, their supports could not withstand high pressure. Bonded amino acids were used for the separation of some D,L-amino acid enantiomers by Davankov, Rogozhin and their co-workers[28] and by Baczuk et al.[29]. Grushka and Scott[30] have shown that a simple tripeptide, namely triglycine, can be used in the separation of amino acids. In a more recent work, Kikta and Grushka[31] have studied the chromatographic behavior of several bonded tripeptides.

In our previous work[31] it was shown that the bonded peptide determines the retention times of amino acids, phenylthiohydantoin (PTH) derivatives of amino acids, and isomeric dipeptides. Such bonded phases, then, can be used to elucidate structures of proteins and complex peptides. For example, the products of Edman degradation can be analyzed on the peptide column. The present communication discusses in detail the chromatographic behavior of the stationary phase L-valyl-L-phenylalanyl-L-valine bonded to silica gel, in an effort to ascertain the nature of the interaction between the solutes and the bonded phase.

EXPERIMENTAL

Apparatus

The LC system was described previously[31]. The stainless-steel column used was 250 × 2.1 mm I.D.

Reagents

Deionized water was obtained by passing distilled water through an ion-exchanger treatment column. The pH (5.5) of the water was measured after degassing by boiling. Amino acids were obtained from Sigma (St. Louis, Mo., U.S.A.); PTH-amino acids standard kit was purchased from Pierce (Rockford, Ill., U.S.A.). Dioxane was obtained from J. T. Baker (Phillipsburg, N.J., U.S.A.) and Fisher Scientific (Fairlawn, N.J., U.S.A.). Peroxides from dioxane were removed by passing the reagent-grade solvent through a column of alumina just before use. Methanol (reagent grade) was distilled before use. Partisil-10 (11 μm) was obtained from Whatman (Clifton, N.J., U.S.A.). The bonding reagent 1-trimethoxysilyl-2-chloromethyl-phenylethane (Y-5918) was obtained from Union Carbide (Tarrytown, N.Y., U.S.A.). The coupling reagent used in the peptide synthesis, N,N-dicyclohexylcarbodiimide, was obtained from Aldrich (Milwaukee, Wisc., U.S.A.). All other chemicals and solvents used in this investigation were obtained from Fisher Scientific.

Procedure

The tripeptide L-Val-L-Phe-L-Val was synthesized on the Partisil-10 through the reagent Y-5918. The synthesis is similar to that described previously[30,31]. Analysis showed 1.64% nitrogen and 17.3% carbon. Calculation indicated that about 0.26 mmoles of the peptide was bonded per gram of the Partisil-10. The peptide bonded to silica gel is shown schematically as I. R is the silica gel matrix.

$$R-Si-O-Si-(CH_2)_2-C_6H_4-COC-C-N-C-C-N-C-C-NH_2$$
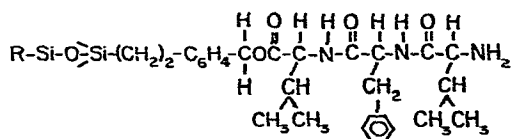
I

RESULTS AND DISCUSSION

Column stability

The reproducibility in the retention times and the elution orders with the various phases used in this study seem to indicate that the bonded tripeptide is stable. Although the acidic mobile phase (pH *ca.* 2.5) could conceivably cause the loss of amino acids from the peptide, the mild conditions used here and the consistency of the results tend to indicate that, at least during the course of this study, the peptide was stable. After a month of use the initial pressure, for a constant flow-rate, began to increase rapidly, which indicated drastic changes in the packed bed. The reasons for these changes are not known at present.

Analysis of free amino acids

Table I shows the k' values of several UV-absorbing free amino acids. The retention times increase with the pH. No changes in the retention order as a function of pH were found. In our previous work[31] on Gly-L-Val-L-Phe peptide, the k' values were smallest with the acidic buffer mobile phase and largest with the distilled deionized water mobile phase. The retention order, however, was the same as here. Moreover, the retention order is similar to that discussed by Moore and Stein[32] in ion-exchange systems, and by Niederwieser (ref. 33 and references therein) in adsorption chromatography. Unlike the works of Moore and Stein and of Niederwieser, however, acidic mobile phases could be used to rapidly elute the three amino acids. The terminal amino group of the bonded peptide is protonated at low pH, and can form a weak ion exchanger. This is the reason for the fast analysis time with the acidic mobile phase. The interactions between the solutes and the mobile phase relative to those between the solutes and the stationary phase should be studied carefully in order to fully understand the magnitude and order of retention. This was

TABLE I

k' VALUES FOR SOME AMINO ACIDS

D,L-*Amino acid*	Mobile phase		
	1% Citric acid in water, pH ca. 2.5	1% Sodium citrate in water, pH ca. 7.9	Distilled water, pH ca. 5.5
Tyrosine	0.48	2.28	1.25
Phenylalanine	0.68	2.44	1.46
Tryptophan	1.15	5.56	4.38

discussed thoroughly by Scott[34]. The interactions of the amino acids with the bonded peptide seem to be stronger when a basic mobile phase is used.

Analysis of PTH derivatives of amino acids

The capacity ratios of 25 PTH-amino acids are shown in Table II. Distilled deionized water (pH *ca.* 5.5) eluted only three of the PTH derivatives in reasonable times. Using methanol mobile phase, most of the PTH-amino acids eluted very fast (all k' values were less than 1).

Under acidic conditions all the PTH-amino acids have different k' values, although some are close to one another (*e.g.* PTH-L-glutamine and PTH-glycine). The order of elution is of particular interest. Using an acidic mobile phase the reten-

TABLE II

k' VALUES OF PTH-AMINO ACIDS AT VARIOUS MOBILE PHASES

PTH-amino acid	1% Citric acid in water, pH 2.5	5% Methanol in pH 2.5	10% Methanol in pH 2.5	1% Sodium citrate in water, pH 7.4	Distilled water pH 5.5	Methanol
PTH-L-histidine monohydro-chloride	0.13	0.13	0.10	10.8	4.88	0.33
PTH-L-arginine**	0.25	0.13	0.10	5.86	0.00	0.00
PTH-D,L-threonine**	4.86	3.80	3.10	5.75	—	0.50
PTH-D,L-serine	6.11	4.36	3.95	4.35	—*	0.70
PTH-L-asparagine	6.88	5.34	4.05	4.65	12.1	0.75
PTH-S-methyl-L-cysteine	7.21	5.13	4.15	3.80	—	—*
PTH-L-glutamine**	8.40	6.05	4.75	5.23	—*	0.70
PTH-glycine	8.75	7.10	5.75	7.44	—	0.75
PTH-D,L-alanine**	12.1	9.38	7.28	7.56	—	0.63
PTH-L-hydroxyproline	13.8	9.38	8.75	9.58	—	0.50
PTH-L-methionine sulfone	14.0	9.80	8.00	7.98	—*	0.88
PTH-L-glutamic acid**	24.6	16.6	14.3	15.1	—*	—*
PTH-D,L-valine	28.0	18.5	14.2	29.8	—*	0.50
PTH-L-proline	34.6	24.5	17.3	22.5	—*	0.63
PTH-D,L-aspartic acid**	36.2	25.0	22.2	9.16	—*	—*
PTH-D,L-methionine	39.0	27.0	19.7	28.7	—*	0.63
PTH-L-tyrosine**	46.2	31.3	23.3	39.8	—*	0.60
PTH-isoleucine	49.5	31.6	22.9	40.1	—*	0.38
PTH-L-leucine	54.8	33.0	25.5	62.1	—*	0.38
PTH-norleucine**	65.7	42.4	31.8	54.6	—	0.50
PTH-D,L-phenylalanine**	85.9	58.5	44.0	107.0	—	0.63
PTH-(S-carboxymethyl)-L-cysteine	143.0	85.1	68.8	13.9	—	—
PTH-D,L-tryptophan**	198.0	145.0	103.0	224.0	—	0.75
PTH-(ε-phenylthiocarbamyl)-L-lysine	385.0	217.0	162.0	285.0	—	0.93
PTH-L-cysteic acid (K salt)	—*	—*	—*	9.22	—	—*

* No peak observed in a reasonable length of time.
** Indicate PTH derivatives that could be easily separated with 5% methanol.

tion order of the PTH-amino acids obtained here is in close correlation with the order of the adsorption coefficients of the free amino acids on neutral polystyrene resin from 0.1 N hydrochloric acid solution[35]. When an acidic mobile phase is used, the monohydrochloride salt of PTH-histidine and PTH-arginine elute very close to the void volume. Such is not the case in reversed-phase chromatography[22], in chromatography on silica gel with organic solvents[2,23] or on cation exchangers[32]. The low k' values of these two solutes are not surprising since at the pH of 2.5 they have a charge of +2 while the bonded peptide phase has a charge of +1. Electrostatic consideration then indicates that the solutes would favor interaction with the mobile phase and, therefore, would elute fast. Lysine, being a polar basic amino acid, should also elute fast. In the present study. however, the lysine was in the form of ε-phenylthiocarbamyl derivative. The resultant molecule is non-polar, and it was retained in the column for a very long time.

The two acidic amino acids, glutamic acid and aspartic acid, were eluted before most of the non-polar amino acids.

The hydrophobic, non-polar, PTH-amino acids, eluted in the following order when acidic mobile phase was used: Ala < Val < Pro < Met < Ile < Leu < Phe < Trp. This elution order, in general, can be correlated with the molecular weight, and inversely with the solubility in water. The retention order found here is rather different from that found in reversed-phase chromatography[22], or in adsorption chromatography on silica gel[18,23].

The retention order of PTH amino acids with uncharged polar groups, under acidic conditions, is Thr < Ser < Asn < S-methyl-Cys < Gln < Gly < Tyr. With the exception of tyrosine, these amino acids eluted before most of the non-polar hydrophobic ones. Although no direct correlation could be found between the retention times and the solubility of these amino acids, threonine, which is the most soluble in aqueous phases, eluted before all other amino acids in the group, and tyrosine, the least soluble, retained the longest. Again, the retention order of the PTH amino acids with uncharged polar group is unlike that obtained with the more conventional stationary phases[18,22,23].

With the peptide bonded phase reported here, under acidic conditions the addition of a hydroxyl group decreases the retention time, e.g. the k' values of hydroxyproline and of tyrosine are less than that of proline and phenylalanine, respectively. The hydrogen bonding capabilities of the hydroxyl-containing amino acids with the mobile phase, decrease their hydrophobicity. A similar observation was made by Niederwieser and Giliberti[36] in their separation of 5-hydroxytryptophan from tryptophan by adsorption on neutral polystyrene resins.

Fig. 1 shows a chromatogram of seven PTH-derivatives eluted with a citric acid buffer (pH ca. 2.5). Most of the PTH-amino acids shown are the polar ones. By using a 5% methanol in the citric acid buffer, eleven PTH-amino acids could be separated isocratically (Fig. 2). The derivatives shown in Fig. 2 are marked in Table II. No attempts were made to optimize the resolution and the number of PTH-amino acids separated. However, it is clear from Figs. 1 and 2 that a methanol gradient is required for a rapid separation of most of the 25 PTH derivatives studied here.

When a basic buffer is used to elute the PTH-amino acids, several changes in the retention order occur. At a pH of 7.4, the terminal amine on the bonded peptide is mostly unprotonated. The imidazolium group in histidine is less than 10% pro-
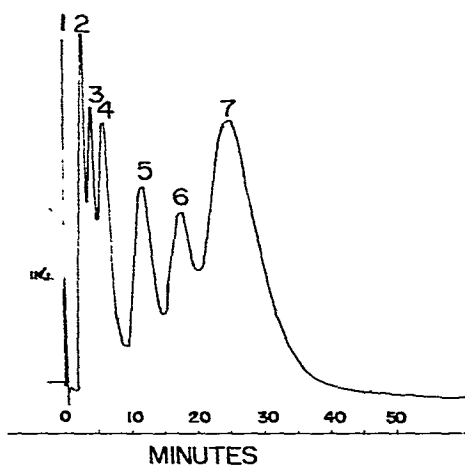
Fig. 1. Separation of some PTH-amino acids. Mobile phase, 1 % citric acid in water (pH *ca.* 2.5); flow-rate, 1.0 ml/min; $\lambda = 254$ nm, 0.2 a.u.f.s. 1 = PTH-L-Arginine; 2 = PTH-D,L-serine; 3 = PTH-glycine; 4 = PTH-D,L-alanine; 5 = PTH-D,L-glutamic acid; 6 = PTH-D,L-aspartic acid; 7 = PTH-L-tyrosine.
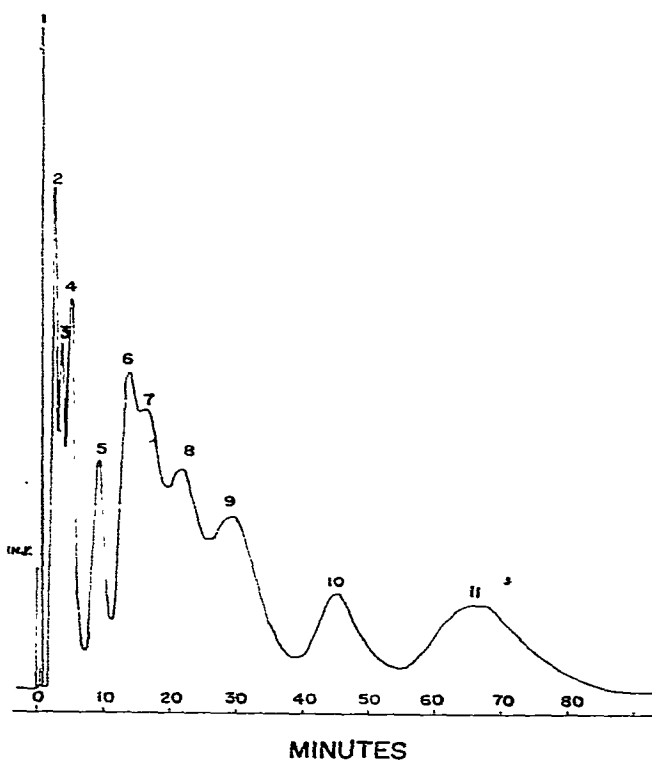

Fig. 2. Separation of some PTH-amino acids. Mobile phase, 1 % citric acid in water + 5 % methanol; flow-rate, 0.97 ml/min; $\lambda = 254$ nm. 1 = PTH-L-Arginine; 2 = PTH-D,L-threonine; 3 = PTH-L-glutamine; 4 = PTH-D,L-alanine; 5 = PTH-L-glutamic acid; 6 = PTH-D,L-aspartic acid; 7 = PTH-L-tyrosine; 8 = PTH-norleucine; 9 = PTH-D,L-phenylalanine; 10 = PTH-(S-carboxymethyl)-L-cysteine; 11 = PTH-D,L-tryptophan.

tonated while the guanidinium group of arginine is still positively charged. As a consequence, the retention order of histidine and arginine is the reverse of that whihc occurs at a pH of 2.5. Also they are no longer eluted first. The PTH-aspartic acid eluted before the PTH-glutamic acid. This is certainly not the order of elution in conventional amino acid analysers using a cation exhanger. With the cation-exchange system an increase in the pH causes the basic amino·acids to elute faster.

With the exception of the basic amino acids, as well as Thr, Val, Leu, Phe, and Trp, the basic mobile phase reduced the retention times of the PTH derivatives. In some cases, this reduction was rather large, e.g. S-carboxymethyl-L-cysteine and the potassium salt of cysteic acid. These two solutes, having acidic groups behaved in a similar manner to aspartic and glutamic acids.

The elution order of the hydrophobic PTH-amino acids with the basic buffer is similar to that with the acidic one. The exception is valine which is retained longer than methionine.

With the basic mobile phase, the retention order of the PTH derivatives of the amino acids with uncharged polar groups is S-methyl-Cys < Ser < Asn < Gln < Thr< Gly < Tyr. This order is rather different from that observed with acidic mobile phase. However, it is similar to the elution order observed in reversed-phase systems[22,23]. As was the case with the acidic mobile phase, the PTH derivatives of the amino acids with uncharged polar group, in general, eluted before the hydrophobic amino acids.

Separation of dipeptides

Our previous work[31] showed that peptide bonded phases can be utilized in the separation of isomeric dipeptides. Table III shows the capacity ratios of eleven dipeptides eluted with various mobile phases.

The capacity ratios of the dipeptides, when distilled and deionized water (pH *ca.* 5.5) was used as the mobile phase, are larger, with one exception, than those obtained with the acidic and basic buffers. This is most probably due to the fact that the ionic strength of the deionized water is extremely low. The retention times ob-

TABLE III

k' VALUES FOR SEVERAL DIPEPTIDES

Dipeptides	Mobile phase		
	1% Citric acid in water, pH 2.5	1% Sodium citrate in water, pH 7.9	Distilled water, pH 5.5
Gly-L-Trp	0.60	11.4	24.1
L-Trp-Gly	0.31	14.6	— *
Gly-L-Phe	0.25	3.62	5.78
L-Phe-Gly	0.08	4.62	22.5
Gly-L-Tyr	0.15	3.43	3.27
L-Tyr-Gly	0.13	4.38	10.8
L-Phe-L-Val	0.39	7.95	59.2
L-Val-L-Phe	0.43	7.90	28.0
L-Trp-L-Tyr	0.90	3.52	— *
L-Trp-L-Trp	3.41	— *	— *
L-Tyr-L-Phe	0.38	— *	— *

* Solute did not elute in reasonable time.·

tained with the basic buffer are longer than those obtained with the acidic mobile phase. The large differences in the k' values of the isomeric dipeptide, when distilled deionized water is the eluent, is noteworthy. Their separation is quite simple, see, e.g., Fig. 3 for Gly-Phe and Phe-Gly. As seen from Table III all isomeric dipeptides can be separated in one run. The shape of the peaks should be noticed. It was found that with water as the mobile phase the peptide peaks were rather broad and tailed severely.
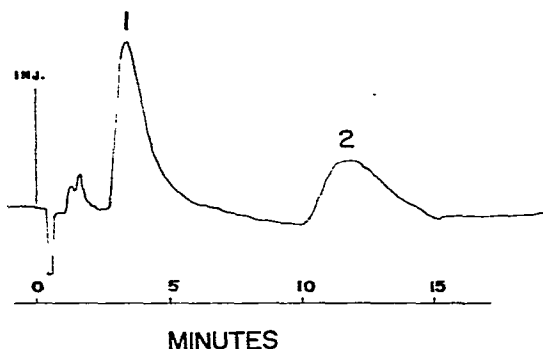

MINUTES

Fig. 3. Separation of isomeric dipeptides. Mobile phase, distilled and deionized water pH *ca.* 5.5; flow-rate, 1.0 ml/min. 1 = Gly-L-Phe; 2 = L-Phe-Gly.

The two dipeptides in each isomeric pair show different retention times irrespective of the mobile phase. The basic mobile phase allows easier separations of the dipeptides since the capacity ratios are in the optimum range of 2 to 10. As an example, Fig. 4 shows a chromatogram of the Gly-Phe, Phe-Gly isomeric dipeptide, Gly-Trp, Trp-Gly isomeric dipeptide and Phe-Val. To our knowledge this is one of the best separations of isomeric dipeptides ever reported in the literature.

The data in Table III show some interesting trends. The elution order of each isomeric pair in the acidic buffer is the reverse of that observed with the other two mobile phases. A check of our previous data[31] revealed the same trend. Recently, Pietrzyk and Chu[37] discussed the use of Amberlite XAD copolymers as a reversed-phase medium in HPLC. Among the data reported there are the retention volumes of Gly-Phe and Phe-Gly as a function of pH. Their results also show an inversion in the retention order. In addition, the elution order found by them is the same as found here.

The results in Table III show that whenever the first amino acid in an isomeric pair is glycine, that peptide elutes last with the acidic buffer and first with the other two mobile phases. When the first amino acid in a pair is phenylalanine, that dipeptide elutes first with the acidic mobile phase and last with the basic buffer. At present, it is not quite clear why the retention order reverses when the mobile phases are changed. A possible explanation is as follows: under acidic conditions, the terminal amine group is protonated. Whenever this protonated group is close to an aromatic ring, it sterically hinders the interaction between the ring and the bonded phase, resulting in shorter retention times. With basic mobile phases the carboxylic group is charged. When it is close to an aromatic ring, it will prevent strong interaction between the ring and the bonded phase. As an example, the terminal amine is close to Phe in the
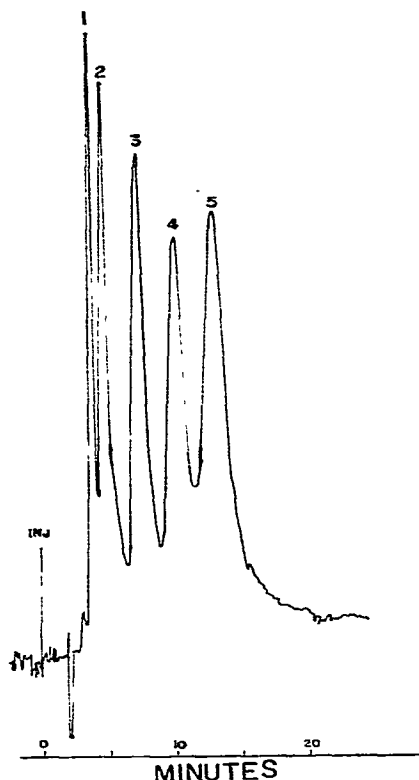
Fig. 4. Separation of several isomeric dipeptides. Mobile phase, 1% sodium citrate (pH *ca.* 7.9); flow-rate, 0.34 ml/min; λ = 254 nm. 1 = Gly-L-Phe; 2 = L-Phe-Gly; 3 = L-Phe-L-Val; 4 = Gly-L-Trp; 5 = L-Trp-Gly.

Phe-Gly dipeptide while the carboxyl group is close to Phe in the Gly-Phe dipeptide. In acidic buffer the elution order is Phe-Gly < Gly-Phy while the inverse is true in basic mobile phases. A more comprehensive study is needed in order to ascertain the reason for the retention reversal. It would be of importance to correlate the isoelectric point of isomeric dipeptides with the pH at which they are not resolved.

Whether acidic or basic mobile phases were used, it is interesting to note the retention order of dipeptides having a common amino acid: Gly-Tyr < Gly-Phe < Gly-Trp; and Tyr-Gly < Phe-Gly < Trp-Gly (note that with the acidic buffer Phe-Gly < Trp-Gly). This is the same as the order of elution of the PTH-derivatives of tyrosine, phenylalanine and tryptophan. The same behavior can be seen with other hydrophobic dipeptides, *e.g.* Gly-Phe < Val-Phe and Phe-Gly < Phe-Val, which agrees with the retention order of the PTH derivatives of glycine and valine. Niederwieser[35] pointed out the same phenomenon in his study on the adsorption of peptides from acidic solutions.

The hydrophobic dipeptides should have similar properties to the hydrophobic amino acids. It is not surprising, therefore, that the retention order is determined by the amino acid which is not common in the series of the dipeptides mentioned above.

It should be pointed out that the capacity ratios of the dipeptides are in general less than those of the single amino acids, both with the basic and acidic buffers.

There are not enough data to draw any conclusion regarding the retention orders of polar dipeptides as compared with that of the polar amino acids.

In our previous work[31] with the Gly-Val-Phe bonded phase, it was noted that under basic conditions, the k' values of the isomeric dipeptides Val-Phe and Phe-Val were rather different. In acidic buffer the two capacity ratios of the two peptides were the same and were very close to zero. The present bonded phase is Val-Phe-Val. Since the sequence of the last two amino acids in the bonded phase reported here is inverted as compared with the previous study, it was hoped that the retention times of Phe-Val and Val-Phe will also be inverted. Table III show that in the present study, these two dipeptides did have different k' values with acidic mobile phase. However, at a pH of 7.9 the difference between the k' values was very small. At a pH of 7.4, which is the same condition as reported elsewhere[31], the difference in the k' values increased somewhat, but it was not nearly as large as reported in ref. 31. The retention order of Phe-Val > Val-Phe is the same in both columns. It is not clear whether the fact that the selectivity factor for this isomeric dipeptide pair is smaller on the column studied here, is an indication of the influence of the bonded phase on the selectivity.

CONCLUSION

Bonded peptide phase can offer a useful chromatographic system for the separation of amino acids, PTH derivatives of amino acids, and isomeric dipeptides. The retention order of the PTH-amino acids is rather unique, and, as might be expected, it is a function of the pH of the mobile phase. When fast elution of the basic amino acids is required, the bonded peptide column offers a definite advantage. Elution orders of dipeptide seem to resemble closely the elution orders of their amino acid constituents. Changing the pH of the mobile phase can change the retention order of isomeric dipeptides. More work is needed in order to examine whether the elution behavior follows the titration curves of the dipeptides.

Work currently in progress shows that the bonded peptide can be used effectively to separate diastereomers of dipeptides, as well as enantiomers of dipeptides.

ACKNOWLEDGEMENT

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