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BONDED PEPTIDE STATIONARY PHASES FOR THE SEPARATION OF AMINO ACIDS AND PEPTIDES USING LIQUID CHROMATOGRAPHY

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SUMMARY

Bonded optically active tripeptides have been applied as stationary phases for liquid chromatography. Significant retention variations are shown for some UV absorbing amino acids when compared to a silica gel column using the same mobile phase. The separation of certain isomeric dipeptides has been accomplished using the bonded optically active tripeptide stationary phases. It is shown that these separations are superior to those possible on silica gel, although the efficiencies of the column are low. Phenylthiohydantoin (PTH)-amino acids have also been analyzed using the bonded tripeptide L-Val–L-Ala–L-Ser on silica gel CT and 1% citric acid–water as the mobile phase. No two PTH-amino acids showed the same capacity ratio out of 25 tested and the separation of 15 in one isocratic run is presented. The implications and future for the use of bonded optically active peptides as stationary phases for liquid chromatography are discussed.

INTRODUCTION

Bonded stationary phases, when applied to chromatographic analysis, have provided a highly stable and efficient means for the resolution of many difficult separations. This is especially true of modern high-speed liquid chromatography where bonded phases, such as the widely used reversed-phase column (ODS), have eliminated most of the problems encountered in liquid–liquid partition chromatography. Presently only a few column types are applied with a high success rate to a wide variety of separation problems. One of the difficulties is associated with the solubilities of solutes in mobile phases. One area where this is so is the separation of amino acids, peptides and associated derivatives: a recent review[1] aptly describes the various solvent systems which have been applied in liquid chromatography (LC) and thin-layer chromatography (TLC). In general, gradient elution is applied, and often derivatization is a necessity. There is still a need for a simple and selective method of separating amino acids and peptides. It would seem that the most advantageous approach to such analysis would be to use stationary phases which are similar in structure to the

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solutes, in other words peptides. One of the first such applications took place in gas–liquid chromatography (GLC) where Gil-Av and co-workers[2,3] separated trifluoroacetic acid (TFA) amino acid esters on TFA dipeptide esters as stationary phases. The use of such optically active stationary phases showed excellent D,L resolution of several amino acid derivatives. Grushka and Scott[4] reasoned that similar systems could be applied to LC without the inherent temperature, stability and derivatization problems involved with GLC analysis. Their preliminary results using poly-Gly stationary phases showed the potential of bonded peptide phases. The idea of using bonded peptide is not new, *cf. e.g.* the work of Losse and Kuntze[5] and references therein. However, silica gel was not used as the support matrix.

The direct analysis of free amino acids and dipeptides has been a virtual impossibility for GLC. Many papers have appeared and continue to appear regarding derivatization techniques for the analysis of such compounds by GLC (see for example refs. 6–9). Amino acids and dipeptides have been extensively analyzed using ion-exchange chromatography and two dimensional TLC (*viz.*, refs. 10–15). Dinitrophenyl derivatives of dipeptides have been studied by TLC[16] and derivatives of isomeric dipeptides[17] have been separated by LC. Most notably, diastereomers of peptides have been resolved by TLC[18] and ion exchange[19]. Phenylthiohydantoin (PTH)-amino acids which result from the Edman degradation[20] of proteins and peptides have been analyzed by a variety of chromatographic methods. In general, gradient elution has been used to separate a number of PTH-amino acids. Haag and Langer[21] managed to separate fourteen PTH-amino acids in one run. Matthews *et al.*[22] claimed to have separated all but PTH-arginine and PTH-histidine on a silica gel column using gradient elution. However, only 13 PTH-amino acids were shown on one chromatogram. Using isocratic elution Graffeo *et al.*[23] have separated up to 6 PTH-amino acids, while Frank and Strubert[24] have shown the separation of nine derivatives. DeVries *et al.*[25] have shown a chromatogram with 8 PTH-amino acids.

The above few examples are typical of the progress made in the analysis of amino acids and peptides by LC and they also point out what still needs to be done.

The present paper reports initial results obtained on three columns containing bonded optically active peptides as stationary phases. Three classes of compounds have been studied. The first class contained free amino acids. This study was limited to phenylalanine (Phe), histidine (His), tryptophan (Try) and tyrosine (Tyr) since they are the only ones which could be monitored with UV detectors. The second class consisted of some isomeric dipeptides. The analysis of isomeric dipeptides is important in peptide synthesis, sequencing operations, protein degradation analysis, and many physiological processes. The last class consisted of the PTH derivatives of amino acids.

EXPERIMENTAL

Instrumentation

The liquid chromatograph consisted of a Waters Assoc. (Milford, Mass., U.S.A.) Model 6000A pump, U6K injector and a Model 440 detector capable of monitoring at 254 and 280 nm simultaneously. Data were recorded on a dual-pen strip-chart recorder (LDC, Riviera Beach, Fla., U.S.A.). Columns were made of precision-bore 1/4-in. stainless-steel tubing, using zero-dead-volume fittings. Injections

of solutes were made via a Pressure Lok 25-μl syringe (Precision Sampling, Baton Rouge, La., U.S.A.). Solutes dissolved in an appropriate solvent in the amount of 0.25–25 μg were injected into the system, with 1 μg being typical.

Reagents

Water, for the mobile phases used, was distilled before use. All other chemicals and solvents used in mobile phases and in synthesis, unless otherwise stated, were obtained from Fisher Scientific (Fairlawn, N.J., U.S.A.). Amino acids were purchased from Sigma (St. Louis, Mo., U.S.A.). PTH-amino acids were bought from Pierce Chemical (Rockford, Ill., U.S.A.) as was *tert.*-butylazidoformate. Dioxane was received from J. T. Baker (Phillipsburgh, N.J., U.S.A.) and passed through a column of alumina to remove peroxides. The initial bonding reagent 1-trimethoxysilyl-2-(4-chloromethylphenyl)-ethane (Y-5918) was received from Union Carbide (Tarrytown, N.Y., U.S.A.). The coupling reagent used for peptide synthesis, N,N-dicyclohexylcarbodiimide, was obtained from Aldrich (Milwaukee, Wisc., U.S.A.). BioSil A (20–44 μm) was obtained from BioRad Labs. (Richmond, Calif., U.S.A.), and silica gel CT (11 μm) from Whatman (Clifton, N.J., U.S.A.).

Procedure

Four columns were prepared for the study. Column I, the reference column, was 30 cm × 3 mm I.D. packed with BioSil A. Column II (30 cm × 3.8 mm I.D.) consisted of a tripeptide Gly–L-Val–L-Phe bonded to Biosil A through reagent Y-5918. Analysis showed 0.66% (w/w) of nitrogen on the support. Column III (30 cm × 4 mm I.D.) consisted of the tripeptide L-Val–Ala–L-Val bonded to BioSil A in a manner similar to column II. Final analysis showed 0.74% nitrogen. Columns II and III were both hand packed. Column IV (25 cm × 3.8 mm I.D.) consisted of the tripeptide L-Val–L-Ala–L-Ser bonded onto silica gel CT. Final analysis showed 1.16% of nitrogen. This column was packed by suspending the packing in an ethyleneglycol–methanol (1:1) solution via an ultrasonic bath. This slurry was placed in a 70-ml packing chamber, and pressurized with a Tracor Model 5000 chromatographic pump.

Synthesis

The initial chloride groups for peptide attachment were placed on the silica gel surfaces in a manner described before[4,26]. BioSil A (10 g) and 12 g of reagent Y-5918 were placed in 50 ml of benzene and shaken overnight. The material was then rinsed with benzene and chloroform and dried. Analysis showed 10% bonded C and 2.69% Cl. The silica gel CT (3.5 g) was placed along with 7 g of Y-5918 in 50 ml of dry benzene overnight. It was then refluxed for 3 h. This material was washed with benzene and dried. *tert.*-Boc-amino acids were prepared by the well known Schwyzer method[27]. A mixture consisting of 0.03 *M* *tert.*-butylazidoformate, 0.02 *M* of amino acid and 1.6 g MgO in 90 ml dioxane–water (2:1) was stirred at 41° for 24 h. The Boc-amino acid products were then extracted and purified using the methods suggested by Stewart and Young[28].

The first amino acid was attached to the previously modified surface in a manner discussed before[4,26]. The silica gel and Boc-amino acid were placed into 150 ml of absolute ethanol containing 5 ml of triethylamine. The mixture was refluxed with constant stirring by heating in an oil bath at 90°. The reflux continued for 48 h.

The material, after washing and drying, was transferred to a 5 g Merrifield-type reaction vessel[29] where the *tert.*-Boc protecting group is removed by 4 *N* HCl–dioxane. The second and third Boc-amino acids are attached via a standard diimide coupling schedule as outlined by Stewart and Young[28] with appropriate washing and cleaning procedures.

RESULTS AND DISCUSSION

Column I was used to determine the retention of some amino acids on a regular silica matrix. Table I shows the data obtained. The retention order, when water is used as the mobile phase, is similar to the order observed by Grushka and Scott[4] on both a Corasil II and Porasil column. Table II gives the data obtained on column II. A comparison between Tables I and II reveals several important differences. The retention order on column I (silica gel) is Tyr $<$ Try $<$ Phe $<$ His, using water as the mobile phase while on column II the retention order is His $<$ Tyr $\approx$ Phe $<$ Try. From this data it is evident that the bonded tripeptide not only serves as a deactivator of the silica surface, but it also modifies the retention order significantly. Histidine shows a 76-fold decrease in the capacity ratio while Try shows approximately a 4-fold increase in k' value. On either column I or II with any of the mobile phases attempted, no D,L resolution of amino acids has been observed. Significant variations in retention orders between columns I and II are obvious for both the 1% sodium citrate–water mobile phase and the 1% citric acid–water mobile phase. On column II His tends to exhibit long retention times when using an acidic mobile phase, and short retention times when using a basic mobile phase. The largest α value for Tyr

TABLE I

k' VALUES OF VARIOUS COMPOUNDS ON COLUMN I

Compound	*Temperature (°C)*		
	*22°**	*21°***	*20°****
His	19	0.37	0.54
Tyr	0.93	0.39	0.46
Phe	2.0	1.18	1.2
Try	1.43	0.58	0.70
L-Try–L-Phe	—	0.55	0.88
L-Val–L-Phe	—	1.90	1.34
L-Phe–L-Val	—	1.90	1.52
L-Try–L-Tyr	—	0.39	0.62
L-Try–L-Try	—	0.55	0.86
L-Phe–Gly	—	0.92	1.32
Gly–L-Phe	—	0.88	0.96
L-Tyr–Gly	—	0.36	0.46
Gly–L-Tyr	—	0.40	0.44
L-Try–Gly	—	0.38	0.80
Gly–L-Try	—	0.56	0.70

* Distilled water.
** 1% sodium citrate–water (pH $\approx$ 7.4).
*** 1% citric acid–water (pH $\approx$ 2.5).

TABLE II

k' VALUES OF VARIOUS COMPOUNDS ON COLUMN II

Solvent systems: 1 = distilled water; 2 = 1% sodium citrate–water (pH ≈ 7.4); 3 = 1% citric acid–water (pH ≈ 2.5); 4 = 1% NaCl–1% acetic acid–water (pH ≈ 5.0); 5 = 1/4% KH_2PO_4–1/4% $(NH_4)_2CO_3$–water (pH ≈ 7.6); 6 = 1/2% citric acid–1/2% sodium citrate–water (pH ≈ 4.2); 7 = methanol–water (1:3, v/v)–3/4% (w/w) sodium citrate.

Compound	*Temperature (solvent system)*						
	26° (1)	*21° (2)*	*22° (3)*	*27° (4)*	*27° (5)*	*23° (6)*	*23° (7)*
His	0.25	0.40	—*	—*	0	—*	0.28
Tyr	1.4	0.51	0	0.30	0.25	0.41	0.12
Phe	1 4	0.59	0.18	0.46	0.50	0.53	0.14
Try	5.6	2.39	0.70	1.68	2.63	1.88	0.85
L-Tyr–L-Phe	—	—*	0	—	—	2.18	3.89
L-Val–L-Phe	—	2.55	0.05	—	—	0.86	0.97
L-Phe–L-Val	—	4.05	0.05	—	—	0.82	1.20
L-Try–L-Tyr	—	—*	0.25	—	—	7.59	10.7
L-Try–L-Try	—	—*	2.02	—	—	28.4	32.3
L-Phe–Gly	—	1.61	0.29	—	—	—	—
Gly–L-Phe	—	1.01	0.43	—	—	—	—
L-Tyr–Gly	—	1.48	0.24	—	—	—	—
Gly–L-Tyr	—	1.00	0.33	—	—	—	—
L-Try–Gly	—	4.87	0.55	—	—	—	—
Gly–L-Try	—	3.93	0.79	—	—	—	—

* Retention very long, peak could no longer be seen.

and Phe, 2, is obtained when using 1/4% KH_2PO_4–1/4% $(NH_4)_2CO_3$–water, the most basic of the mobile phases (pH ≈ 7.6). For the aqueous buffer used Try shows a general increase in retention as pH increases.

Striking differences are again noted when comparing the dipeptide data presented in Tables I and II. The isomeric dipeptides L-Val–L-Phe, L-Phe–L-Val show an interesting behavior. When using 1% citric acid–water (pH ≈ 2.5) as the mobile phase, no differences in k' value are observed on column II and the retention times are very short, while on column I a significant retention occurs, and an α value of 1.1 is observed. With high-efficiency columns the separation of these dipeptides is easily attained. When a 1% sodium citrate–water mobile phase (pH ≈ 7.4) was used, no separation was observed on column I for this pair, while on column II an α value of 1.6 was obtained, allowing the separation to occur. It should be noted that for all the bonded peptide columns used in this study, the efficiencies were poor when using amino acids and peptides as solutes. When other solutes with k' values similar to those of amino acids or dipeptides were injected, the efficiencies were 5–6 times higher.

The retention orders of the dipeptides isomers, with one exception, were inverted between column I and II. This was true for the citric acid mobile phase as well as for the citrate one.

Data for column III are given in Table III. Unfortunately, the column became plugged before any dipeptides could be studied. In general, when comparing columns II and III for similar mobile phases, retention orders seem to be very similar though absolute magnitudes vary. One significant difference is that with water as the mobile phase a difference in retention exists between Try and Phe on column III which did

TABLE III

k' VALUES OF VARIOUS COMPOUNDS ON COLUMN III

—, Retention too long, compound could not be seen. Solvent systems: 1 = distilled water; 4 = 1% NaCl–1% acetic acid–water (pH ≈ 5.0); 5 = 1/4% KH_2PO_4–1/4% $(NH_4)_2CO_3$–water (pH ≈ 7.6); 8 = methanol–water (1:1); 9 = methanol; 10 = chloroform–*n*-heptane (1:7); 11 = dichloromethane; 12 = methanol–dichloromethane (3:1).

Compound	*Temperature (solvent system)*							
	26° (1)	*27° (4)*	*22° (5)*	*23° (8)*	*21° (9)*	*20° (10)*	*21° (11)*	*22° (12)*
His	0.25	—	0.37	0.53	—	—	—	—
Tyr	1.27	0.72	0.63	0.40	2.2	—	—	2.75
Phe	1.5	0.95	0.79	0.45	2.07	—	—	2.33
Try	4.9	2.52	2.55	0.95	2.6	—	—	4.0

not exist on column II. No D,L resolutions of amino acids were observed on column III.

Table IV shows data for column IV. The different nature of this column is evident when examining the retention data. On column IV His is no longer the first amino acid to elute when water is the mobile phase. The α value for the dipeptide pair L-Val–L-Phe, L-Phe–L-Val, using 1% sodium citrate–water is one on column IV. The above cited differences are the most dramatic of several which can be seen when comparing Tables I–IV. With 1% citric acid–water as the mobile phase several interesting isomeric dipeptide separations, with adequate resolution not obtainable on the previous columns, were achieved. Fig. 1 shows the separation of some Gly based dipeptides, while Fig. 2 shows the separation of some Phe based dipeptides. Fig. 3 shows the separation of some Try dipeptides. These data compare favorably with the results of other workers[14–16]. The resolution of isomeric dipeptides is

TABLE IV

k' VALUES FOR VARIOUS COMPOUNDS ON COLUMN IV

Solvent systems: 1–6, see Table II; 13 = 2% citric acid–water (pH ≈ 2.0).

Compound	*Temperature (solvent system)*					
	21° (1)	*22° (2)*	*18° (6)*	*19° (3)*	*17° (13)*	*21° (5)*
His	1.69	0.73	0.04	0	0	0.67
Tyr	0.46	0.47	0.21	0.24	0.25	0.65
Phe	0.46	0.54	0.26	0.35	0.33	0.69
Try	3.25	2.13	1.42	1.03	1.0	2.34
L-Tyr–L-Phe	6.13	3.42	1.96	0.38	0.34	3.22
L-Val–L-Phe	1.06	1.04	0.43	0.06	0.10	0.96
L-Phe–L-Val	1.06	1.04	0.43	0.06	0.03	0.86
L-Try–L-Tyr	11.9	10.3	5.89	1.07	0.52	9.39
L-Try–L-Try	21.8	31.1	28.3	4.80	4.67	41.7
L-Phe–Gly	0.34	0.63	0.28	0.03	0.07	0.64
Gly–L-Phe	0.34	0.63	0.41	0.10	0.14	0.52
L-Tyr–Gly	0.25	0.46	0.23	0	0.02	0.67
Gly–L-Tyr	0.20	0.46	0.29	0.01	0.07	0.52
L-Try–Gly	1.81	2.0	1.43	0.20	0.24	2.22
Gly–L-Try	1.61	2.0	1.99	0.43	0.39	1.97

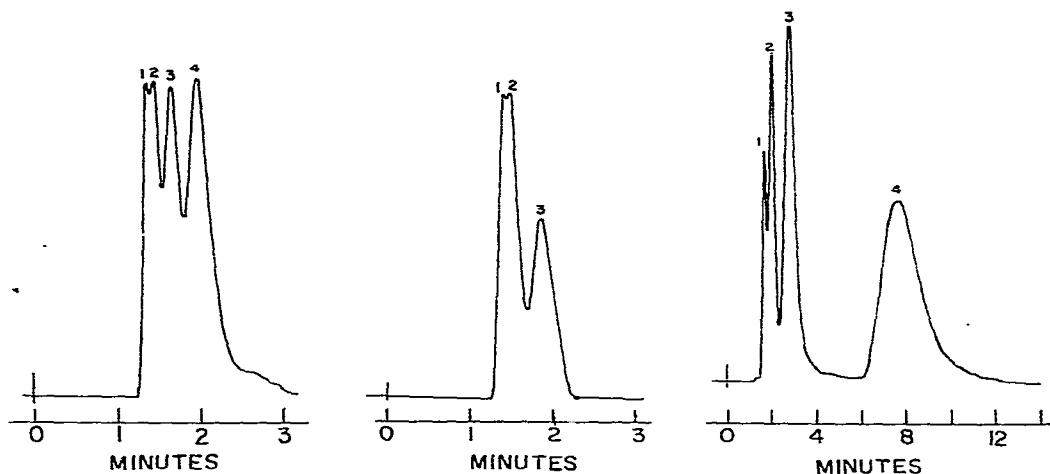

Fig. 1. Separation of glycine-based dipeptides. Conditions: column IV mobile phase, 1% citric acid–water, 19°, pH 2.5, 0.05 a.u.f.s., 1 ml/min. Peaks: 1 = L-Phe–Gly; 2 = Gly–L-Phe; 3 = L-Try–Gly; 4 = Gly–L-Try.

Fig. 2. Separation of Phe-based dipeptides. Same conditions as in Fig. 1. Peaks: 1 = L-Phe–Gly; 2 = Gly–L-Phe; 3 = L-Tyr–L-Phe.

Fig. 3. Separation of Try-based dipeptides. Same conditions as in Fig. 1. Peaks: 1 = L-Try–Gly; 2 = Gly–L-Try; 3 = L-Try–L-Tyr; 4 = L-Try–L-Try.

extremely interesting since it can be useful in the determination of peptide sequences. A lower pH citrate buffer, down to pH 2, did not seem to improve the resolution. On the other hand, no significant resolution for isomeric dipeptides could be obtained at pH higher than 2.5 on column IV. It is interesting to note that for the 1% sodium citrate–water system (pH 7.4) no differences in k' could be seen for the isomeric dipeptides, while for a similar pH system (7.6) with 1/4% $(NH_4)_2CO_3$-1/4% KH_2PO_4–water buffer differentiation of k' was evident, even though no resolution could be obtained due to excessive zone broadening. Changes in retention behavior due to the nature of the buffer is typical of ion-exchange systems. It is possible that one of the retention mechanisms here is indeed that of ion exchange, which is due to the terminal amine group on the bonded peptide. The effects of ionic strength and various buffers will have to be investigated further in order to explain many of the observed retention differences.

Table V gives the retention data for 25 PTH-amino acids. The amino acids are arranged in the increasing order of retention on column IV using a 1% citric acid–water mobile phase. It should be noted from the data that when using this mobile phase no two PTH-amino acids have the same capacity ratio, although some are close in magnitude. Thus given a sufficiently efficient column all PTH-amino acids could be resolved isocratically. In the present case, as seen in Fig. 4, 15 could be separated with sufficient resolution for identification using the same mobile phase. In a practical sense column IV would probably be sufficient to identify the major PTH-amino acid from an Edman degradation[20] since only a few amino acid residues are present per

TABLE V

k' VALUES OF PTH AMINO ACID DERIVATIVES

Compound	*Column I*	*Column IV*			
	1% Citric acid (pH = 2.5)	*1% Citric acid–water (pH = 2.5)*	*1/2% KH_2PO_4–water (pH = 4.8)*		
	18°	*20°*	*23°*	*21°*	*18°*
PTH-D,L-methionine sulfone	0.70	0.00	—	1.22	—
PTH-L-histidine·HCl	1.46	0.40	3.14	—	—
PTH-L-arginine	1.72	0.50	—	5.25	—
PTH-D,L-serine	0.50	4.06	—	3.25	—
PTH-D,L-threonine	0.62	4.20	—	2.80	—
PTH-L-asparagine	0.50	4.78	—	2.83	—
PTH-glycine	0.60	5.84	4.00	4.40	4.52
PTH-L-glutamine	0.68	6.00	—	4.71	—
PTH-L-alanine	0.70	7.72	5.58	—	—
PTH-L-glutamic acid	0.46	10.2	—	10.9	—
PTH-D,L-aspartic acid	0.40	10.7	—	7.47	—
PTH-L-hydroxyproline	0.72	11.9	—	7.44	—
PTH-D,L-valine	1.20	15.5	11.3	—	—
PTH-S-methyl-L-cysteine	0.96	19.8	—	—	18.5
PTH-D,L-methionine	1.30	21.2	—	16.5	—
PTH-L-proline	2.20	22.4	—	16.8	—
PTH-L-tyrosine	0.44	27.0	22.2	—	—
PTH-L-leucine	2.10	30.1	21.6	—	—
PTH-L-isoleucine	2.00	30.3	20.4	—	—
PTH-norleucine	2.42	35.3	26.6	—	—
PTH-(S-carboxymethyl)-L-cysteine	0.48	37.4	—	—	30.9
PTH-D,L-phenylalanine	1.06	47.5	36.8	—	—
PTH-D,L-tryptophane	0.70	99.0	77.3	—	—
PTH-L-cysteic acid K salt	0.20	119	—	—	9.55
PTH-(ε-phenylthiocarbamyl)-L-lysine	2.64	193	—	—	106

sequencing step with the major component usually present in excess. Twelve of the solutes eluted in about 40 min. The fact that only 15 PTH-amino acids can be resolved is due to poor efficiency. As far as we know this is the largest number of PTH-amino acids resolved in an isocratic mode, and this also compares very favorably with separations previously cited[21–25] which generally make use of two-dimensional TLC or gradient elution system.

The reference data in Table V for silica gel (column I) show that indeed the peptide-bonded phase controls the retention orders. Selectivities and capacity ratios vary over a very narrow range (2.64 as compared to 193 on column IV). It is important to note that several PTH-amino acids have very small α values on column I, making even an extremely efficient silica gel column less useful than a moderately efficient equivalent of column IV. Fig. 4 demonstrates that at high k' value the column is rather inefficient. The reasons for this phenomenon are not known yet, although as was mentioned previously solutes other than amino acids, with the same k' value, showed lower HETP values.

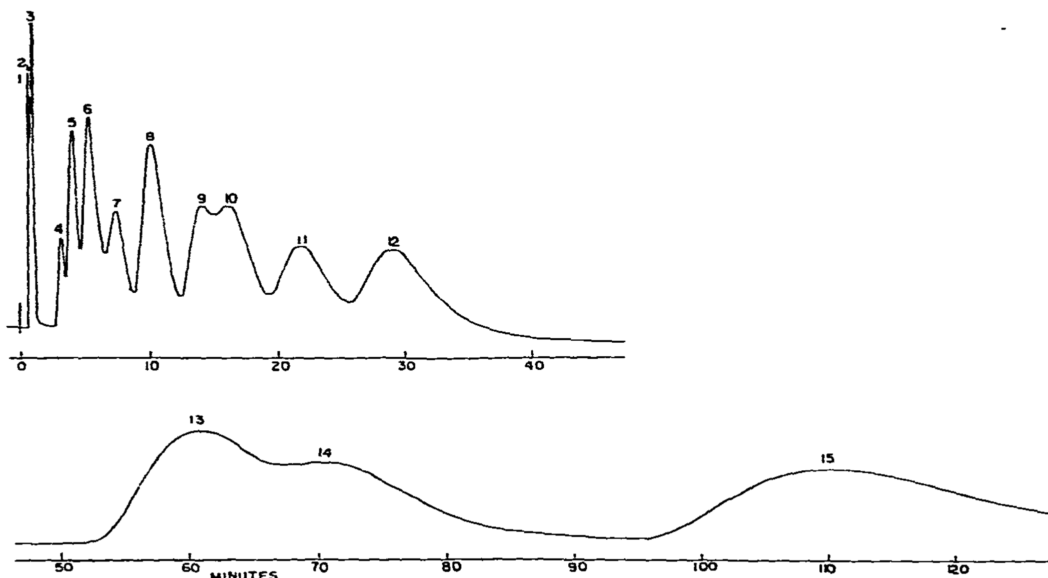

Fig. 4. Separation of PTH-amino acids. Conditions: column IV mobile phase, 1% citric acid–water, 21°, 0.1 a.u.f.s., 254 nm, 2 ml/min. 1 = PTH-D,L-methionine sulfone; 2 = PTH-L-histidine·HCl; 3 = PTH-L-arginine; 4 = PTH-D,L-serine; 5 = PTH-glycine; 6 = PTH-L-alanine; 7 = PTH-D,L-aspartic acid; 8 = PTH-D,L-valine; 9 = PTH-L-proline; 10 = PTH-L-tyrosine; 11 = PTH-norleucine; 12 = PTH-D,L-phenylalanine; 13 = PTH-D,L-tryptophan; 14 = PTH-L-cysteic acid K salt; 15 = PTH-(ε-phenylthiocarbamyl)-L-lysine.

CONCLUSIONS

It has been shown that separations of amino acids, dipeptides, isomeric dipeptides, and PTH-amino acids can be obtained using bonded peptides as stationary phases. The modification of retention orders as compared with the usual chromatographic adsorbents or other bonded phases is of great interest. Current work is aimed at producing more efficient columns with larger peptide coverages. Hopefully, optically bonded phases will lead to D,L resolution of free amino acids. It is also hoped to obtain an alternative detection system which will allow the monitoring of free amino acids.

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