Journal of Chromatography, 332 (1985) 287–295
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 639

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF PEPTIDES ON AN ASAHIPAK GS-320 COLUMN PACKED WITH HYDROPHILIC POLYMER GEL

KEIKO YASUKAWA*, MASAO KASAI, YUZO YANAGIHARA and KOHJI NOGUCHI
Technology and Development Department, Ion Exchange Membrane Administration, Asahi Chemical Industry Co., Ltd., 1-3-2, Yakoo Kawasaki-ku, Kawasaki-shi 210 (Japan)
(First received April 8th, 1985; revised manuscript received May 28th, 1985)

SUMMARY

Investigation of the chromatographic characteristics of various amino acids and peptides on an Asahipak GS-320 column, developed for high-performance gel filtration chromatography, revealed that these substances, which possess hydrophobic sites, are retained by adsorption and eluted in order of increasing hydrophobicity. The use of organic solvents as eluents was studied and practical separations of amino acids and peptides by isocratic elution were obtained.

INTRODUCTION

The analysis of peptides by high-performance liquid chromatography (HPLC) is generally performed on reversed-phase columns containing chemically bonded silica gels[1]. In previous papers[2,3] we described practical separations of sera, urine and erythrocyte components without prior deproteination on the Asahipak GS series of HPLC columns, packed with hydrophilic polymer gels, which were developed for the analysis of physiological substances[4,5]. We also reported the retention mechanism to be mainly gel filtration for hydrophilic substances of high molecular weight, such as proteins, polysaccharides and nucleic acids, and adsorption for hydrophobic substances of low molecular weight, such as nucleobases and nucleosides[6]. This paper describes the chromatographic characteristics of amino acids and peptides for an Asahipak GS-320 column.

EXPERIMENTAL

Chromatography was performed with a Hitachi 638-50 high-speed liquid chromatograph, equipped with a Jasco Uvidec-100-IV UV detector operated at 210 nm. Asahipak GS-320 (500 × 7.6 mm I.D.) or GS-320H (250 × 7.6 mm I.D.) columns (Asahi Chemical Industry) were employed. The flow-rate was 1.0–2.0 ml/min and the other chromatographic conditions were varied.

Amino acids were obtained from Kyowa Hakkou Kogyo, peptides from Pep-

0021-9673/85/$03.30 © 1985 Elsevier Science Publishers B.V.

tide Institute or Serva and proteins from Sigma. Other reagents were obtained from Wako. The organic solvents used were of HPLC grade.

The effect of alkali solution on the column performance was determined by measurement of the number of theoretical plates (N) and peak asymmetry (A_s) of the creatinine peaks obtained with 0.1 M sodium phosphate (pH 7.0) containing 0.3 M NaCl as eluent at 1 ml/min and 30°C, before and after the passage of 0.01 M NaOH solution (pH 12.1) containing 0.1 M NaCl at 1 ml/min for 16 h through the GS 320 column (500 × 7.6 mm I.D.).

The resistance of the column to degradation by organic solvents was determined by measurement of the N and A_s of the ethylene glycol peaks using distilled water as the eluent at 1 ml/min and 30°C, before and after the passage of an aqueous organic solution or an organic solvent at 1 ml/min for 2 h through the GS-320 column (500 × 7.6 mm I.D.).

N (4σ) and N (5σ) were calculated by the equations $N(4\sigma) = 16/1.7^2 (\bar{V}_R/W_{0.5})^2$ and $N(5\sigma) = 5^2 (\bar{V}_R/W_{0.044})^2$, where $W_{0.5}$ and $W_{0.044}$ are the width of the band at half-height and 4.4% height of the peak, respectively.

Peak asymmetries were calculated by the equation $A_s = (b/a)^2$, where b is the distance at 4.4% peak height from the tailing end to the perpendicular from the peak maximum to the baseline, and a is the distance from the front end of the peak to the perpendicular.

The protein and peptide recoveries were determined by comparison of the eluted peak areas obtained by passage of isocratic eluent through the GS-320H column (250 × 7.6 mm I.D.) or the GS-320 column (500 × 7.6 mm I.D.) at 1 ml/min and 30°C with those obtained when the same amount (10–25 μg) of each protein or peptide was injected under the same conditions into aproximately 10 m of tightly coiled 0.5 mm I.D. PTFE tubing.

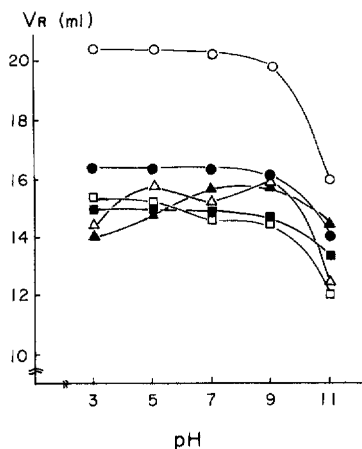


Fig. 1. Influence of pH on elution volume of amino acids. column: Asahipak GS-320 (500 × 7.6 mm I.D.). Sample: ○, phenylalanine; ●, leucine; □, glutamic acid; ■, serine; △, arginine; ▲, histidine, 10 μl (10 μg). Mobile phase: 50 mM sodium phosphate buffer (pH 3.0, 5.0, 7.0, 9.0 and 11.0). Flow-rate: 1 ml/min. Detection: UV at 210 nm. Temperature: 30°C.

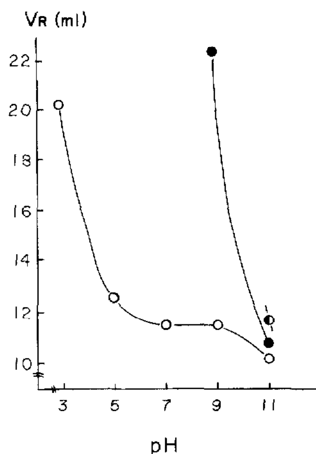


Fig. 2. Influence of pH on elution volume of insulin, insulin A chain and insulin B chain. Sample: ●, insulin; ◑, insulin B chain; ○, insulin A chain. Chromatographic conditions as in Fig. 1.

RESULTS

Influence of pH on retention

The relationship between the eluted volume (V_R) of amino acids and the pH of the eluents on the GS-320 column is shown in Fig. 1. The hydrophobic amino acids, phenylalanine and leucine, were more strongly retained. The elution volume of each amino acid decreased with increasing pH. The basic amino acids, arginine and histidine, were eluted early at both low and high pH.

The effects of eluent pH on the retention of insulin, insulin B chain and insulin A chain are shown in Fig. 2. The insulin and the insulin B chain, which have strong hydrophobic sites, were adsorbed and not eluted at low pH, but were eluted at high pH.

The primary structures of angiotensin I, II and III are shown in Fig. 3. Their early elution at both low and high pH (Fig. 4) may be closely related to their relatively high content of basic amino acids, such as arginine and histidine.

Throughout the investigation of the effect of pH on the eluted volume of amino acids and peptides, with eluent solutions ranging from low to high pH, no degradation of the column or significant changes in the chromatograms were observed.

Table I shows the N and A_s values before and after the passage of alkali solution (pH 12.1). As the results show, alkali solutions had no adverse effect on the column performance.

Angiotensin I	Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu
Angiotensin II	Asp–Arg–Val–Tyr–Ile–His–Pro–Phe
Angiotensin III	Arg–Val–Tyr–Ile–His–Pro–Phe

Fig. 3. Primary structures of angiotensin I, II and II.

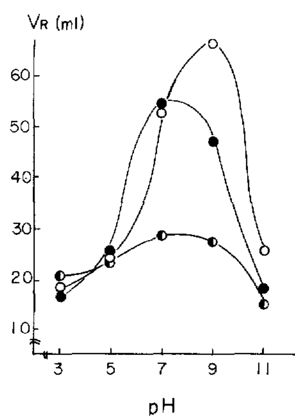


Fig. 4. Influence of pH on elution volume of angiotensin I, II and III. Sample: ●, angiotensin I; ◐, angiotensin II; ○, angiotensin III. Chromatographic conditions as in Fig. 1.

Influence of acetonitrile concentration on retention

The influence of the acetonitrile concentration on retention is shown in Fig. 5. The retention of peptides generally decreased drastically with the addition of acetonitrile to 50 m*M* phosphate buffer (pH 7.0). The insulin B chain, which exhibits high hydrophobicity and was not eluted without acetonitrile, was eluted in approximately 20 min on addition of 5% of acetonitrile to the buffer.

The elution of these peptides approached the polyethylene glycol calibration graph values with high concentrations of acetonitrile. However, for the effective separation of peptides of similar molecular weight, an acetonitrile concentration of several to 20% appears sufficient.

Applicable concentrations of organic solvents

The efficiency (capacity) of the Asahipak GS-320 column was evaluated from changes in N (4σ, 5σ) and A_s of ethylene glycol before and after the use of organic solvents, with the results shown in Table II. No significant change in column performance was observed for most of the solvents, which are indicated by ticks in Table II. Where a decline in performance did occur, with the solvents indicated by crosses in Table II, it appeared to be the result of changes in the packing conformation rather than any chemical change in the gel particles. These results suggest that the column

TABLE I

CHANGE IN COLUMN EFFICIENCY WITH THE USE OF ALKALINE SOLUTION AS ELUENT

Column, Asahipak GS-320 (500 × 7.6 mm I.D.); eluent, 0.1 m*M* sodium phosphate containing 0.3 *M* sodium chloride (pH 7.0); flow-rate, 1 ml/min; sample, creatinine, 20 μl (20μg); detection, UV at 250 nm; temperature, 30°C.

Alkali solution	*Column pressure (kg/cm²)*	N (4σ)	N (5σ)	A_s
Before	33	19800	15800	1.32
After	34	19800	15800	1.32

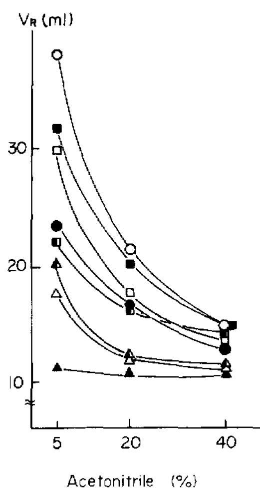


Fig. 5. Effect of acetonitrile concentration on peptide elution volume. Sample: ○, met-enkephalin; ●, leu-enkephalin □, angiotensin I; ◧, angiotensin II; ■, angiotensin III; △, insulin; ◭, insulin B chain; ▲, insulin A chain. Mobile phase: acetonitrile–50 mM sodium phosphate buffer (pH 7.0) (5:95, 20:80 and 40:60). Other conditions as in Fig. 1.

efficiency deteriorates when organic solvents that cause gel shrinkage are used, but that it is maintained when organic solvents of relatively high viscosity are used or when an increased column pressure occurs as a result of gel swelling.

With the Asahipak GS-320 column it was thus found possible to use methanol, ethanol, acetonitrile and n-propanol, the first two at concentrations of up to 100% and the last two at up to 50% (aqueous solutions). The acetonitrile concentration appropriate for the adsorption chromatography of peptides on this column is 20% or less, and no problem is expected in the practical use of organic solvents, including acetonitrile and tetrahydrofuran, with the column. All of these are commonly used in the analysis of peptides.

Influence of temperature on peptide analysis

Rapid, isocratic separations of α-, β- and γ-endorphin standards were obtained, as shown in Fig. 6. The separations were performed at 30, 40 and 50°C. To reduce the time necessary for analysis, a short column and an eluent flow-rate of 2 ml/min were utilized. Improved chromatograms were obtained at the higher temperatures, owing to decreased peak widths.

Recovery of proteins and peptides

The GS 320 column gave excellent recoveries of proteins and peptides, as indicated by the results in Tables III and IV.

Practical applications

As one practical application, the concurrent isocratic separation of various hydrophilic and hydrophobic dipeptides, such as Arg–Asn and Leu–Tyr, was carried out, as shown in Fig. 7. It was also found possible to separate isomers, such as Trp–Glu and Glu–Trp.

TABLE II

CHANGE IN COLUMN EFFICIENCY WITH THE USE OF ORGANIC SOLVENTS AS ELUENT

Column, Asahipak GS-320 (500 × 7.6 mm I.D.); low-rate, 1-ml/min; temperature, 30°C.

Eluent	*Concentration (%)*	*η (cP)*	*Pressure drop (kg/cm²)*	*Column efficiency*		*Fitness for use*
				Before use	*After use*	
Methanol	50	1.34	63	18900 (N 4σ) 14100 (N 5σ) 1.7 (A_s)	19900 (N 4σ) 14400 (N 5σ) 1.7 (A_s)	V
	100	0.56	24	18900 (N 4σ) 14100 (N 5σ) 1.7 (A_s)	19900 (N 4σ) 14400 (N 5σ) 1.7 (A_s)	V
Ethanol	50	2.02	136	23300 (N 4σ) 17100 (N 5σ) 0.7 (A_s)	23200 (N 4σ) 16200 (N 5σ) 0.7 (A_s)	V
	100	1.00	56	23300 (N 4σ) 17100 (N 5σ) 0.7 (A_s)	23200 (N 4σ) 16200 (N 5σ) 0.7 (A_s)	V
Acetonitrile	50		47	21900 (N 4σ) 12600 (N 5σ) 0.2 (A_s)	21200 (N 4σ) 11500 (N 5σ) 0.3 (A_s)	V
	100	0.33	14	24100 (N 4σ) 17100 (N 5σ) 0.5 (A_s)	24400 (N 4σ) 6700 (N 5σ) 0.1 (A_s)	×
Tetrahydrofuran	100	0.47	22	25300 (N 4σ) 19800 (N 5σ) 0.7 (A_s)	21600 (N 4σ) 11000 (N 5σ) 0.3 (A_s)	×
Acetone	100	0.29	11	21800 (N 4σ) 13800 (N 5σ) 0.4 (A_s)	23700 (N 4σ) 4800 (N 5σ) 0.1 (A_s)	×
n-Propanol	50		104	24600 (N 4σ) 16200 (N 5σ) 1.4 (A_s)	24500 (N 4σ) 18200 (N 5σ) 0.9 (A_s)	V
Distilled water	—	0.80	34	> 16000 (N 4σ) > 1000 (N 5σ) 0.5–3.0 (A_s)	standard value	V

TABLE III

RECOVERY OF PROTEINS

Column, Asahipak GS-320H (250 × 7.6 I.D.); eluent, 0.1 *M* sodium phosphate containing 0.3 *M* sodium chloride (pH 7.0); flow-rate, 1 ml/min; sample, 25 μl (25 μg) standard; detection, UV at 210 nm; temperature, 30°C.

Protein	*Recovery (%)*
Ovalbumin	93
Myoglobin	92
Lysozyme	95
Cytochrome C	86

TABLE IV

RECOVERY OF PEPTIDES

GS-320 column for insulin, insulin A and insulin B and GS-320H column for leu- and met-enkephalin and angiotensin I, II and III; 50 m*M* sodium phosphate (pH 7.0)–methanol (80:20) eluent for insulin, insulin A and insulin B, 50 m*M* sodium phosphate (pH 7.0)–*n*-propanol (80:20) eluent for leu- and met-enkephalin and 50 m*M* sodium phosphate (pH 3.0) for angiotensin I, II and III; flow-rate, 1 ml/min; sample, 10 μl (10 μg); detection, UV at 210 nm; temperature, 30°C.

Peptide	*Recovery (%)*
leu-Enkephalin	82
met-Enkephalin	91
Angiotensin I	97
Angiotensin II	98
Angiotensin III	98
Insulin A	95
Insulin B	94
Insulin	106

Another application was the analysis of RNase F_1 digested with trypsin and chymotrypsin, using 0.1 *M* acetic acid solution as the eluent[7]. The results are shown in Fig. 8. RNase F_1 was digested with trypsin for 14 h at 37°C and then with chymotrypsin for 1 h at 37°C. The faster eluted peak (A) represents trypsin and chymotrypsin and the shoulder peak (B), indicated by the vertical arrow, represents undigested RNase F_1. It was therefore possible to analyse the mixture of peptides and proteins on this column with a single injection and no prior deproteination.

The performance of the column was found to be highly stable even with repeated direct injections of mixed protein solutions, without a guard column. No significant change appeared in the chromatograms of as many as 400 directly injected samples of the above mixed protein solutions. Similarly, no observable change was found in the chromatograms for 300 injections of 20-μl samples of undiluted and otherwise untreated human blood serum with 0.1 *M* sodium phosphate containing 0.3 *M* NaCl as the (pH 7.0).

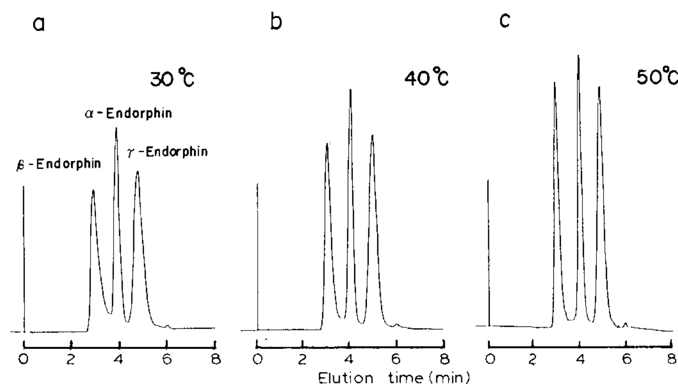
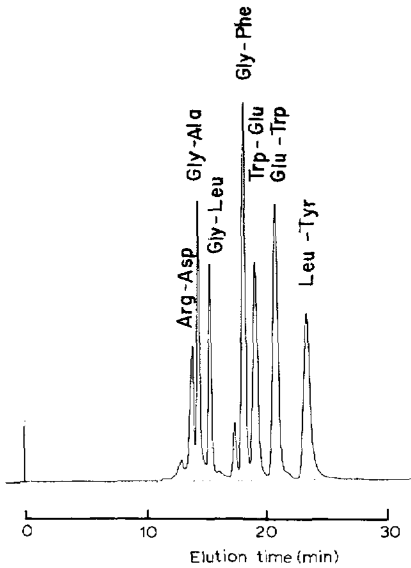


Fig. 6. Influence of temperature on separation of α-, β- and γ-endorphin. Column: Asahipak GS-320H (250 × 7.6 mm I.D.). Sample: mixture of α-, β- and γ-endorphin standards, 5 μl (5 μg) each. Mobile phase: 0.1% trifluoracetic acid–acetonitrile (95:5). Flow-rate: 2 ml/min. Detection: UV at 210 nm. Temperature: (a) 30°C; (b) 40°C; (c) 50°C.



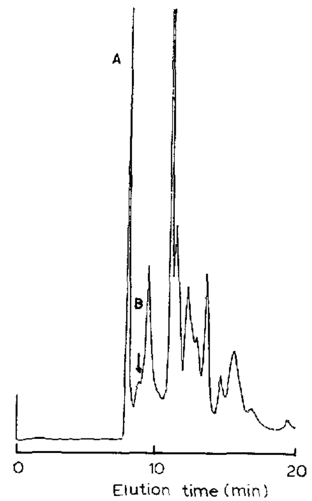


Fig. 7. Separation of a variety of dipeptides. Column: Asahipak GS-320. Sample: mixture of dipeptides, 5 μl (5 μg). Mobile phase: 50 m*M* sodium phosphate buffer (pH 7.0) (isocratic). Flow-rate: 1 ml/min. Detection: UV at 210 nm. Temperature: 30°C.

Fig. 8. Analysis of fractions of RNase F_1 digested by trypsin and chymotrypsin. Column: Asahipak GS-320. Sample: digested fractions of RNase F_1, 10 μl. Mobile phase: 0.1 *M* acetic acid. Flow-rate: 1 ml/min. Detection: UV at 280 nm. Temperature: ambient. (Chromatogram provided by courtesy of Dr. Hiroshi Yoshida).

DISCUSSION

It was found that the Asahipak GS-320 column is capable of the concurrent analysis of various peptides and proteins with molecular weights ranging from 100 to 100 000. Because of the hydrophilicity of the gel, originating from its hydroxy groups, the recovery of protein and peptides was high and hydrophobic peptides were eluted with a small addition of organic solvent. In contrast to reversed-phase columns, packed with silica-based gels, the GS-320 column remains effective with alkaline eluents, because it is packed with hydrophilic polymer gel.

When a peptide sample containing proteins was injected into the column, the proteins of high molecular weight were eluted first by gel filtration and the peptides were then separated by adsorption chromatography. It was therefore possible to analyse a mixture of peptides and proteins without prior deproteination, which we refer to as "intra-column deproteination".

Based on these chromatographic characteristics of GS-320, we conclude that it will be effective particularly in the analysis of crude samples, such as digested peptides and extracts of bacteria.

REFERENCES

1 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 255 (1983) 125–136.
2 H. Wada, K. Makino, T. Takeuchi, H. Hiroyuki and K. Noguchi, *J. Chromatogr.*, 320 (1985) 369–377.
3 K. Noguchi, N. Imai, Y. Yanagihara and M. Kasai, paper presented at the Fourth Annual Conference on Liquid Chromatography, Tokyo, 1983.
4 M. Kasai, N. Itoh, Y. Yanagihara and K. Noguchi, paper presented at the 27th Seminar of The Research Group on Automatic Liquid Chromatography, Kyoto, 1984.
5 K. Yasukawa, M. Kasai, Y. Yanagihara and K. Noguchi, paper presented at the First Mini Seminar (62nd Seminar) on Liquid Chromatography, Tokyo, 1984.
6 K. Yasukawa, M. Kasai, Y. Yanagihara and K. Noguchi, *Nucleic Acids Res.*, 12 (1984) 109–112.
7 J. Hirabayashi and H. Yoshida, *Biochem. Int.*, 7 (1983) 255–262.