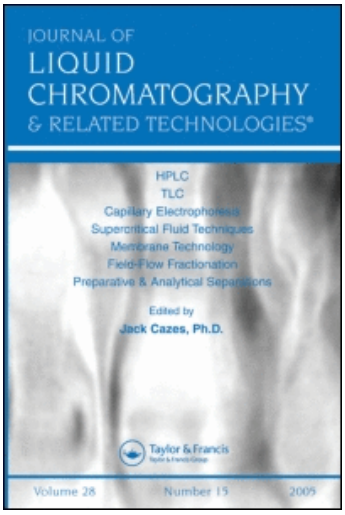

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 13 January 2005

To cite this Article Yamasaki, Yosuke , Uchida, Kanae , Tomizawa, Hiroshi , Ishikawa, Osamu and Inouye, Kuniyo(1999) 'SEPARATION OF AMINO ACIDS AND PEPTIDES BY HIGH PERFORMANCE HYDROPHOBIC INTERACTION CHROMATOGRAPHY', Journal of Liquid Chromatography & Related Technologies, 22: 13, 1965 — 1974
To link to this Article: DOI: 10.1081/JLC-100101778
URL: http://dx.doi.org/10.1081/JLC-100101778

J. LIQ. CHROM. & REL. TECHNOL., 22(13), 1965–1974 (1999)

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ABSTRACT

Chromatographic behaviors of amino acids and peptide derivatives were examined by high performance hydrophobic interaction chromatography(HIC) on two different packings, TSKgels Phenyl-5PW and Ether-5PW. Most amino acids hardly retained and eluted at an elution volume being the same as the column volume even with an initial buffer for hydrophobic interaction chromatography; aromatic amino acids showed weak retention. On the other hand, peptides retained in the column and their elution was roughly in the order of the molecular mass, although some of them showed extraordinary elution behaviors. Kunitz bovine pancreas trypsin inhibitor showed weak retention in spite of having larger molecular mass, which might be due to the steric conformation.

Some peptides showed considerably stronger retention even under ordinary conditions, and the addition of organic modifier (20 % acetonitrile) in eluent was required for their elution. HIC using less hydrophobic packings examined was found to be adequate to separate peptides quantitatively, and this might be useful in removing peptides from contaminants of amino acids and proteins.

INTRODUCTION

High performance hydrophobic interaction chromatography (HIC) is nowadays a powerful tool for separation of proteins from various biological fluids like culture media,[1-3] ascites,[4-6] plasma,[7-9] urine,[10] and others.[11-13] HIC, as well as reversed-phase chromatography (RPC), is performed according to hydrophobicity of samples, and shows quite different selectivity from size exclusion chromatography and ion-exchange chromatography (IEC). In comparison with RPC, HIC requires a much milder eluent such as a buffer containing ammonium sulfate which is commonly used in salting-out fractionation of proteins and is known to stabilize protein structures.

There are some reports on the fundamental evaluation of HIC for proteins.[14-18] However, only a few reports have been published to evaluate separation of amino acids and peptides by HIC.[19-21] In this paper, we have examined the elution behaviors of amino acids and peptides by two different HIC packings, TSKgels, Phenyl-5PW and Ether-5PW.

EXPERIMENTAL

Materials

All amino acids were purchased from Ajinomoto (Tokyo, Japan). Peptides listed in Table 1, *H*-Y-G-G-F-*OH*, Leu-enkephalin, CCK-tetrapeptide, CCK-octapeptide, angiotensin II, angiotensin I, neurotensin I, glucagon were purchased from Peptide Institute (Osaka, Japan). Lutenizing hormone releasing hormone (LHRH) and insulin were purchased from Sigma (St. Louis, MO).

Other peptides were purchased from Serva Feinbiochemica(Germany). All other reagents of special grades were purchased from Nakarai Tesque (Kyoto, Japan).

Table 1

List of Peptides Derivatives for Evaluation

Peptide	Mr	Sequence
H-Y-G-G-F-*OH*	442	*H*-Y-G-G-F-*OH*
Suc-a-A-A-*pNA*	451	*Suc*-A-A-A-*pNA*
Leu-enkephalin	556	Y-G-G-F-L
CCK-tetrapeptide(30-33)	597	W-M-D-F-NH_2
PZ-P-L-G-P-*D*-R	777	*PZ*-P-L-G-P-*D*-R
AngiotensinII	1046	D-R-V-Y-I-H-P-F
Bradykinin	1060	R-P-P-G-F-S-P-F-R
CCK-octapeptide	1063	D-Y-M-G-W-M-D-F-NH_2
LHRH[a]	1182	*Py*-H-W-S-Y-G-L-R-P-G-NH_2
Actinomycin C	1280	-------
Angiotensin I	1297	D-R-V-Y-I-H-P-H-H-L-*2AcOH*
Neurotensin	1673	*Py*-L-Y-E-N-K-P-R-R-P-Y-I-L
Angiotensinogen (1-14)	1759	D-R-V-Y-I-H-P-F-H-L-V-I-H
Glucagon	3485	30 Amino acid redidues
Insulin β-chain	3494	30 Amino acid redidues
Insulin	5808	51 Amino acid redidues
Trypsin inhibitor[b]	6500	58 Amino acid redidues

[a] LHRH: Lutenizing hormone releasing hormone.
[b] Kinitz bovine pancreas trypsin inhibitor.

HPLC

HIC was performed with high-pressure gradient equipment consisting of a dual pump CCPM, a solvent mixer Static Mixer A, a UV detector UV-8020 with micro-flow cell(2μL), an autosampler AS-8020 installed with a 10 or 100μL sample loop on an injector (Rheodyne model 7125) and a data processor by SC-8020. All equipment were purchased from Tosoh(Tokyo, Japan).

Separation of amino acids was performed by isocratic elution with 0.1 M sodium phosphate buffer (pH 7.0), with or without 1.5 M ammonium sulfate at a flow-rate of 0.3 mL/min. A 10μL of the amino acid solution was applied to HPLC(100μg/mL). Peptides were separated with a linear gradient of ammonium sulfate from 1.5 M to 0 M in 0.1 M sodium phosphate buffer (pH 7.0) at a flow-rate of 1.0 mL/min. The gradient was generated at the time of sample injection (time 0 min) over 30 min. As an organic modifier, 20 % acetonitrile was added to the elution buffer when the recovery of peptides was

Table 2

Elution Behavior of Amino Acids by Hydrophobic Interaction Chromatography

	Capacity Factor (k')[a]			
	Phenyl-5PW		Ether-5PW	
	Eluent A[b]	Eluent B[c]	Eluent A[b]	Eluent B[c]
Glycine	1.02	0.99	1.00	1.00
Alanine	1.02	1.00	1.00	1.00
Valine	1.03	1.01	1.00	1.01
Leucine	1.04	1.04	1.02	1.04
Isoleucine	1.04	1.03	1.01	1.03
Serine	1.02	0.99	1.00	0.99
Threonine	1.02	1.00	1.00	1.00
Aspartic acid	1.02	0.99	0.99	0.99
Glutamic Acid	1.02	0.98	1.00	0.97
Aspargine	1.02	0.99	1.00	0.99
Glutamine	1.02	1.00	1.00	1.00
Lysine	1.02	0.97	1.00	0.97
Arginine	1.03	0.99	1.00	0.99
Cysteine	1.07	1.00	1.02	1.02
Methionine	1.04	1.03	1.02	1.03
Phenylalanine	1.04	1.11	1.06	1.10
Tyrosine	1.29	1.49	1.27	1.48
Histidine	1.03	1.00	1.01	1.00
Proline	1.03	1.00	1.00	1.00
Water dip (blank; V_0)	1.00	1.00	1.00	1.00

[a] $k' = (V_1-V_0)/V_0$.
[b] Eluent A: 0.1 M sodium phosphate buffer (pH 7).
[c] Buffer B: 0.1 M sodium phosphate buffer (pH 7) containing 1.5 M ammonium sulfate.

low. All samples were eluted at 25°C and were detected by absorbance at 220 nm. The samples of 10 to 100μg each dissolved in 100μL were injected. The sample volumes injected of amino acids and peptides were 10 and 100 μL, respectively. Recovery of peptides was calculated by peak area in comparison to that obtained without elution through a column. High performance HIC was carried out by using TSKgel Phenyl-5PW and TSKgel Ether-5PW (Tosoh, Tokyo, Japan). The column size was 7.5 mm inner diameter by 7.5 cm length.

RESULTS AND DISCUSSION

Separation of amino acids by HIC was evaluated by the capacity factor (k'), which is the index for interaction of sample with packings. The k' value is defined as V_t/V_0, where V_t is the elution volume of a sample and V_0 is that of water-dip. The k' values of amino acids determined by HIC using TSKgels Phenyl-5PW and Ether-5PW were listed in Table 2. The respective amino acids were eluted in an isocratic mode with two eluent buffer systems: buffer A, 0.1 M sodium phosphate buffer pH 7.0, and buffer B, buffer A containing 1.5 M ammonium sulfate. Buffer B is favorably used for adsorption of proteins to the Phenyl-5PW and Ether-5PW columns, and buffer A is for their desorption from the columns.

Most of the amino acids examined eluted at the same time as the water-dip eluted (i.e., k' = 1). Tryptophan showed the high k' value, suggesting a considerably retarded elution as previously reported by K. Inouye.[22] With regard to hydrophobicity of packings, no clear difference in the k' values between the two columns was recognized, although the Phenyl-5PW gel is more hydrophobic than the Ether-5PW.[7] Amino acids seem to be more hydrophilic compared with proteins. Although Gehas et al.[19] reported considerable retardation of dansyl-derivatized amino acids at more than 0.3 M ammonium sulfate in an eluent with SynChropak propyl column, non-derivatized amino acids were found to be eluted without serious retardation even at 1.5 M ammonium sulfate in the buffer (Table 2), which was enough for protein adsorption.[8,16] It is therefore suggested that amino acids can be separated from proteins in the buffer containing ammonium sulfate.

Table 3 summarizes the elution and recovery of various peptides by high-performance HIC on TSKgel Phenyl-5PW which was appropriate packings for proteins.[7,16,23,24] All peptides except for actinomycin C were well retained and separated on the column although some peptides, CCK-octapeptide, actinomycin C, glucagon, and insulin, required the organic modifier in the buffer for elution. Elution of peptides seemed to be independent of their molecular masses of the peptide. The largest peptides, Kunitz bovine pancreas trypsin inhibitor(6500 Da) among those examined showed surprisingly weak retention, suggesting that this peptide might have a steric conformer being more hydrophilic in its surface area.

Recovery of peptides was almost quantitative when the organic modifier was added in the buffer. As a result, actinomycin C was not eluted out nor recovered at all from Phenyl-5PW, which suggests that this peptide is higher in the hydrophobic property than general proteins, presumably due to its phenoxazone moiety, and the moiety may strongly interact with phenyl-functional groups in the packings.

Table 3

Recovery of Peptides by Hydrophobic Interaction Chromatography on TSK Gel Phenyl-5PW

Peptide	Mr	Elution Volume (mL)	Recovery %
H-Y-G-G-F-*OH*	442	43	95
Suc-A-A-A-*pNA*	451	14.0 (11.7)	87 (86)
Leu-Enkephalin	556	7.9	126
CCK-tetrapeptide(30-33)	597	19.4	90
PZ-P-L-G-P-*D*-R	777	30.6 (29.6)	27 (95)
Angiotensin II	1046	8.4	95
Bradykinin	1060	6.1 (6.22)	109 (107)
CCK-octapeptide	1063	N.D. (23.4)	N.D. (125)
LHRH[a]	1182	18.6	97
Actinomycin C	1280	N.D. (N.D.)	N.D. (N.D.)
Angiotensinogen(1-14)	1759	31.4 (24.5)	88 (90)
Glucagon	3485	N.D. (26.5)	N.D. (95)
Insulin β-chain	3494	31.5 (25.2)	89 (92)
Insulin	5808	N.D. (24.6)	N.D. (127)
Trypsin inhibitor[b]	6500	8.9 (8.9)	82 (82)

[a] LHRH: Lutenizing hormone releasing hormone.
[b] Kunitz bovine pancreas trypsin inhibitor.
N.D. : Not detected.
Parenthesis indicates separation with final buffer containing 20% acetonitrile.

TSKgel Ether-5PW was reported to show better results for the separation of proteins, which has oligo-ethylene glycol as functional groups and less hydrophobic than Phenyl-5PW packings.[5,7] Table 4 shows the elution volume and recovery of peptides on Ether-5PW. All peptides except for actinomycin C were eluted with the ordinary buffer. Much hydrophobic actinomycin C, however, was eluted with the buffer containing an organic modifier(20 % acetonitrile) from the column.

The elution order seemed independent of the molecular mass, and the selectivity of peptides was slightly different from that obtained with Phenyl-5PW especially for *H*-Y-G-G-F-*OH*, Leu-enkephalin, and Kunitz bovine pancreas trypsin inhibitor). It should be noted angiotensinogen (1-14), *PZ*-P-L-G-P-*D*-R and insulin β-chain eluted at almost the same positions although they eluted separately on an ODS column by RPC (data not shown).

Table 4

Recovery of Peptides by Hydrophobin Interaction Chromatography on TSK Gel Phenyl-5PW

Peptide	Mr	Elution Volume (mL)	Recovery %
H-Y-G-G-F-*OH*	442	4.4	100
Suc-A-A-A-*pNA*	451	11.8	86
Leu-enkephalin	556	7.5	133
CCK-tetrapeptide (30-33)	597	16.6	91
PZ-P-L-G-P-*D*-R	777	21.6 (23.3)	24 (87)
Angiotensin II	1046	6.1	93
Bradykinin	1060	3.8	110
CCK-octapeptide	1063	271.(19.0)	59 (44)
LHRH[a]	1182	11.6	94
Actinomycin C	1280	N.D. (27.4)	N.D. (92)
Angiotensinogen (1-14)	1759	21.1	97
Glucagon	3485	24.8	8.5
Insulin β-chain	3494	20.7	88
Insulin	5808	21.9	119
Trypsin inhibitor	6500	2.5	102

[a] LHRH: Luteinizing hormone releasing hormone.
[b] Kunitz bovine pancreas trypsin inhibitor.
N.D.: Not detected.
Parenthesis indicates separation with final buffer containing 20% acetonitrile.

HIC, therefore, is suggested to have different selectivity from RPC in terms of hydrophobicity. Recovery of most peptides were almost quantitative on Ether-5PW, and it was improved by adding 20 % acetonitrile in the buffer. Only CCK-octapeptide showed lower recovery which might be caused from its broader peak.

Figure 1 shows the chromatogram of the mixture of peptides by high-performance HIC on TSKgel Ether-5PW. The peptides were well separated in wide range as sharp peaks in 30 min, while CCK-octapeptide with the most retarded retention showed a rather broad peak. The resolution between CCK-tetrapeptide and insulin, glucagon and CCK-octapeptide, was improved by conducting a longer gradient (over 60 min) and they were separated from each other completely.

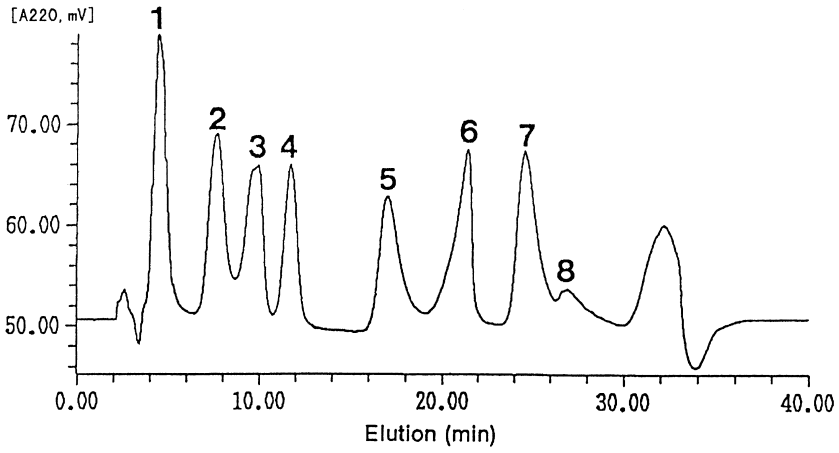

Figure 1. Separation of peptides by high performance hydrophobic interaction chromatography on TSKgel Ether-5PW. Peptides were separated by linear gradient of ammonium sulfate from 1.5 M to 0 in 0.1 M sodium phosphate buffer (pH 7.0) at a flow rate of 1.0 mL/min at 25• . The elution was detected by absorbance at 220 nm. Samples at amount from 10 to 100• g each in 100μL were injected. Samples; *H*-Y-G-G-*OH*(1), Leu-enkephalin(2), angiotensin I(3), luteinizing hormone releasing hormone(4), CCK-tetrapeptide(5), insulin(6), glucagon(7) and CCK-octapeptide(8).

We have evaluated the elution behaviors of amino acids and peptides by high performance HIC with TSKgels Phenyl-5PW and Ether-5PW which are generally applicable to protein separation. All amino acids examined eluted without substantial retention and the k' values were less than 1. It is suggested that these molecules were much less hydrophobic (i.e. hydrophilic) for HIC with the columns tested. Therefore, amino acids would be easily separated from samples, which are retained in the columns. This might be a new application of the HIC for the removal of amino acids from biological fluids containing proteins like culture media by means of HIC preferably rather than RPC or IEC.

HIC was found to be an effective tool for separation of peptides. Various peptides were well separated in wide range with sharp peaks within 30 min as well as proteins, whereas the hydrophobicity of the peptides examined would be rather higher than that of proteins. Recently, we[5,6] reported separation of fragments derived from IgM, from their parental IgM by HIC on TSKgel Ether-5PW by using the slight difference in hydrophobicity between two proteins. Less hydrophobic packings, therefore, would be applicable to separate peptides, which enables us to apply them in separation of biological samples containing peptides (e.g. recombinant peptides in cell culture media, crude synthetic peptide samples). In addition, HIC has different selectivity from RPC since

elution conditions are quite different. Fairly milder eluents are used in HIC than RPC, which can be favorable for the high recovery of activity and maintaining the correct conformation of peptide molecules during HIC. Accordingly, high-performance HIC would be one of the powerful tools for separating peptides and removing amino acids from proteins when less hydrophobic HIC packings are applied.

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Received March 10, 1998
Accepted June 17, 1998
Manuscript 4743

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