Journal of Chromatography, 195 (1980) 369–377
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,831

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND FIELD DESORPTION MASS SPECTROMETRY OF HYPOTHALAMIC OLIGOPEPTIDES

D. M. DESIDERIO*, J. L. STEIN, M. D. CUNNINGHAM and J. Z. SABBATINI
Stout Neuroscience Mass Spectrometry Laboratory and Department of Neurology, University of Tennessee Center for the Health Sciences, 800 Madison Avenue, Memphis, TN 38163 (U.S.A.)
(First received November 12th, 1979; revised manuscript received March 14th, 1980)

SUMMARY

A high-performance liquid chromatographic system employing a chemically bonded alkyl phase and a tetraalkylammonium phosphate buffer has been evaluated for separation of synthetic mixtures of hypothalamic oligopeptides. Separation of two peptides in the mixture having very similar adjusted retention times has been achieved. Sensitivity down to 5 ng is attained. Peptides range in size from three to 31 amino acids. Field desorption mass spectra illustrate novel structural elucidation methods for individual peptides and signal a quantification method.

INTRODUCTION

This paper describes a high-performance liquid chromatographic (HPLC) method to separate mixtures of biological oligopeptides in a fast and facile manner, optimizing sensitivity, speed and resolution of the separation to provide a purified peptide fraction for quantification and structural elucidation studies. Towards this end, the triethylamine phosphate (TEAP)–acetonitrile system is used with a μBondapak C_{18} HPLC column. Synthetic mixtures of hypothalamic oligopeptides containing from three to 31 amino acids are employed. A field desorption (FD) spectrum of an underivatized hexapeptide illustrates the utility of the novel ionization method in peptide chemistry to provide molecular ions of underivatized oligopeptides and for quantification.

Reversed-phase (RP) HPLC using chemically bonded alkyl stationary phases has become an important and powerful tool in peptide separation chemistry. Long chain alkyl silanes are chemically bonded to silanol groups located on the silicon surface of the packing material. An end-capping reaction follows where trimethylsilyl groups are chemically bonded to most of the remaining unreacted silanol groups. Various authors reviewed HPLC column technology and provided a theoretical basis for separations afforded by RP columns[1–3]. HPLC retention indices related to compound lipophilicity were estimated[4]. Horváth and co-workers[5–7] show separation on RP columns is a function of increasing hydrophobicity of the peptide–paired-ion–solvent hydration complex.

0021-9673/80/0000–0000/$02.25

The number of ionic equilibria in which an oligopeptide can participate[8] requires paired-ion chromatography. A buffer system provides ions to pair with charges on a peptide at a pH value sufficiently low to suppress ionization of carboxyl groups. Ion-pair HPLC is extensively reviewed by Tomlinson *et al.*[9]. For purposes of peptide chemistry, an extensively studied, elaborated and used buffer system is the TEAP buffer system. Hancock and co-workers[10–18] studied this system and applied it to various separations. Rivier *et al.*[19] used the TEAP system for separation of peptides and Rivier[20] studied use of trialkylammonium phosphate buffers in RP-HPLC for high resolution and high recovery of peptides. A radioactive peptide was quantitatively recovered from a column. Resolution, flow-rate, temperature, buffer composition, reproducibility and sensitivity were studied.

Other authors expanded the role of HPLC in separation of peptides and proteins including neurohypophyseal proteins[21], polypeptide antibiotics[22], bacitracin[23] polypeptides and proteins[24,25], neuroendocrine peptides[26], amino acids, peptides and derivatives[27], peptide diastereoisomers[28,29] and analysis of the purity of commercial peptides[30]. Other studies include fluorogenic detection of oligopeptides using *o*-phthalaldehyde, fluorescamine and ninhydrin[31], with an aim towards increasing sensitivity of HPLC detection using fluorometric detectors.

MATERIALS AND METHODS

Apparatus

The Waters (Milford, MA, U.S.A.) HPLC system employed consisted of two 6000A solvent delivery systems, U6K injector, R401 detector, 660 solvent programmer, 450 variable-wavelength UV detector and M420 fluorescence detector. A μBondapak C_{18} column (30 × 0.4 cm) (Registry number 068733) was employed. All experiments were performed at ambient temperature. The 0.5-μm filters were purchased from Waters Assoc. A Precision Sampling pressure lock syringe, Series B110, 10 μl (Supelco, Bellefont, PA, U.S.A.) was employed.

Peptides, chemicals, buffer

Triethylamine (Lot no. 03059.24) was purchased from Pierce (Rockford, IL, U.S.A.), phosphoric acid (Lot no. 790884) from Fisher Scientific (Pittsburgh, PA, U.S.A.), acetonitrile from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.); thyrotropic releasing hormone (TRH), met-enkephalin, bradykinin, angiotensin, eledoisin-related peptide, Tyr^{11} and Tyr^{1} SS and β-endorphin from Bachem (Torrance, CA, U.S.A.), substance P and somatostatin (SS) from Sigma (St. Louis, MO, U.S.A.). Water was obtained from a laboratory distillation apparatus. Ionic strength of the TEAP buffer was 76 m*M*. TEAP buffer was prepared by titrating a 0.25 *N* phosphoric acid solution to pH 3.2 with distilled triethylamine. Buffers and solvents were filtered through 0.5-μm Millipore filters to remove solid particles and degas solutions to avoid bubble formation upon decompression after the column.

Field desorption mass spectrometry

FD spectra were obtained on a Varian (Bremen, G.F.R.) 731 mass spectrometer. Initial experiments were done at the National Bureau of Standards (Gaithersburg, MD, U.S.A.) and later experiments on a Varian 731 in our laboratory. A

5-μl volume of a solution containing 1 μg/μl of the underivatized oligopeptide was applied to the benzonitrile-activated FD emitter wire with assistance from a micromanipulator–microscope–syringe system. This volume of solution corresponds to 6.65 nmol peptide (Fig. 2) and sufficed for several FD mass spectral magnetic scans recorded with an oscillographic recorder. Source temperature: 70°C. Extraction lens voltage: 3 kV. Accelerating voltage, 8 kV. Resolution: 1000.

RESULTS

Results obtained in this study include an HPLC chromatogram illustrating separation of the synthetic oligopeptide mixture, a FD mass spectrum of an underivatized hexapeptide, a table collecting chromatographic and statistical parameters for an HPLC reproducibility study and a list of integrals of Rekker's hydrophobic fragmental constants for constituent amino acids for ten hypothalamic oligopeptides.

Fig. 1 contains the HPLC chromatogram illustrating separation of a synthetic mixture of ten hypothalamic peptides. A 2-μl volume of a solution containing 1 μg of each peptide per μl TEAP was injected onto the column. Size of the peptides range from a tripeptide TRH to β-endorphin which contains 31 amino acids. Separation of this complex mixture of peptides occurred within 78 min. Peak shapes are sharp, symmetrical and narrow. Excellent resolution occurs between Tyr^{11} somatostatin and substance P, these peptides having t_A values 45.40 and 46.02, respectively. A linear flow program (Waters curve 6) from 2% to 70% solution B in 78 min was employed. Solution A was TEAP, pH 3.2; solution B was TEAP–acetonitrile (40:60 v/v).

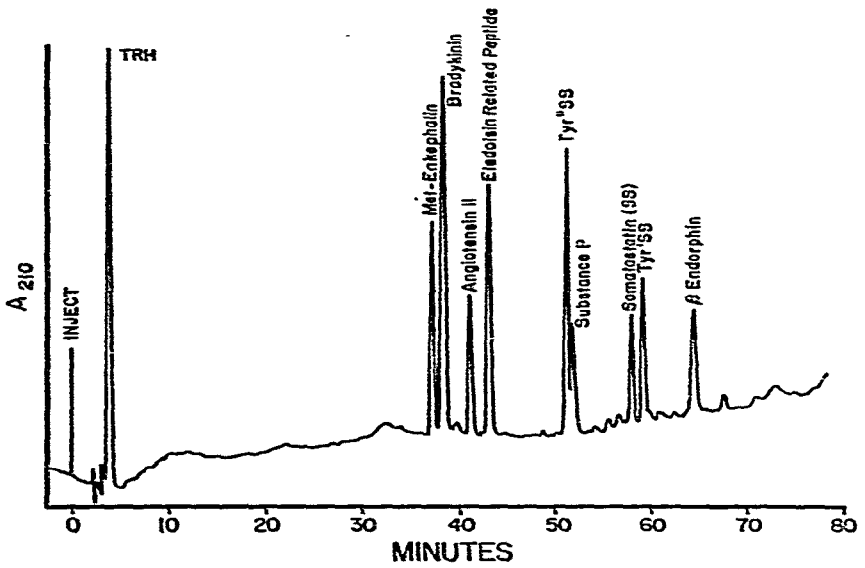

Fig. 1. HPLC chromatogram of a mixture of peptides on a single μBondapak C_{18} column (30 × 0.4 cm). Back pressure: 2000 p.s.i. Flow-rate: 1.5 ml/min. Linear flow program: 2% to 70% B in 78 min. Solutions: A, triethylamine–phosphoric acid (TEAP), pH 3.2; B, TEAP–acetonitrile (40:60). A 2-μl volume of a solution of 1 μg of each peptide per μl TEAP is injected. 0.1 a.u.f.s., 210 nm; chart speed 0.2 in./min.

TABLE I

ADJUSTED RETENTION TIMES FOR BIOLOGIC PEPTIDES

Data obtained on a μBondapak C_{18} column (30 × 0,4 cm). Back pressure: 2000 p.s.i. Flow-rate: 1.5 ml/min. Linear flow program: 3% to 60% B in 65 min. Chromatogram was run from 2% to 70% in 78 min. Solutions: A, triethylamine-phosphoric acid (TEAP), pH 3.2; B, TEAP–acetonitrile (40:60). A 2-μl volume of a solution of 1 μg of each peptide per μl of TEAP is injected. 0.1 a.u.f.s., 210 nm, μ = 0.08 *M*. I_A = Mean, σ = standard deviation, V = coefficient of variation = $100\sigma/I_A$. Adjusted retention (t_A) = $t_R - t_0$.

Peptide	*Chromatogram*							I_A	σ	V
	1	*2*	*3*	*4*	*5*	*6*	*7*			
TRH	1.06	1.63	1.73	0.77	0.61	0.89	1.57	1.18	0.46	38.98
Met-enkephalin	—	32.91	31.85	32.32	31.85	32.40	33.21	32.42	0.55	1.70
Bradykinin	32.91	33.78	32.44	33.05	32.83	33.05	34.25	33.19	0.62	1.86
Angiotensin II	35.76	36.36	35.31	33.05	35.49	36.16	36.93	35.58	1.24	3.49
Eledoisin-related peptide	37.32	38.21	36.97	37.56	37.26	37.85	38.97	37.73	0.68	1.80
Tyr^{11} SS	—	—	44.64	45.22	44.88	45.85	46.41	45.40	0.73	1.61
Substance P	44.98	46.89	45.27	45.94	45.53	46.56	46.97	46.02	0.80	1.74
Somatostatin (SS)	50.53	52.54	50.98	51.59	51.16	52.48	52.73	51.72	0.87	1.68
Tyr^1 SS	—	—	—	—	52.26	53.60	53.88	53.25	0.87	1.63
β-Endorphin	56.32	57.56	56.85	57.58	57.06	58.80	58.89	57.58	0.97	1.68

Sensitivity of the UV detector for the data reported was 0.1 absorbance units full scale (a.u.f.s.). The wavelength selected for this study (210 nm) reflects experience of other workers[20,25] to optimize sensitivity towards oligopeptides rather than proteins where in the former case, the probability increases that an aromatic residue is not present.

Retention time (t_0) for a completely unretained compound was determined in two ways. First, the position of the center for the first baseline disturbance[32–34] in the chromatogram in Fig. 1 was measured as 2.05 min. Multiple injections ($n = 8$) yielded 2.01 $\pm$ 0.08 min for t_0. In the second method, t_0 is calculated from column geometry as $t_0 = Ld/1.5\ F$ where d is the inside column diameter (0.39 cm), F the solvent flow-rate (1.5 ml/min) and $L = 30$ cm. A t_0 value of 2.03 min is calculated.

Adjusted retention time (t_A) is defined as observed retention time (t_R) minus t_0[34]. Multiple injections of this mixture of oligopeptides yielded data assembled in Table I: adjusted retention times for individual injections; average of the multiple injections ($\bar{t}_A$); σ (standard deviation) and V (coefficient of variation) defined as $100\sigma/t_A$. Standard deviations range from 0.46 to 1.24 and yield coefficients of variation (excluding the first eluting TRH peak) between 1.6 and 3.5%. This reproducible system provides a potential method of identification from t_A values alone.

Table II collects adjusted retention time (t_A) values *versus* Rekker integrals[35] of the five most hydrophobic values (RI_5) on one hand, and *versus* all residues (RI) on the other hand.

Fig. 2 contains the low resolution FD mass spectrum of a synthetic model hexapeptide Lys-Glu-Thr-Tyr-Ser-Lys, molecular weight 754 at an emitter heating current (e.h.c.) of 21 mA. The only ions recorded in this mass spectrum occur at m/z 755, 756 and 757 and represent $(M + H)^+$, $(M + 2H)^{+\cdot}$ and $(M + 3H)^+$, respectively.

DISCUSSION

Interactions between hydrophobic side chains of oligopeptides and chemically bonded C_{18} phases reduce molecular surface area exposed to an aqueous solvent and provide the basis for strong bonding between a peptide and the reverse phase. The high surface tension of water contributes to enhancing retention of a peptide to hydrophobic stationary phases. As the percentage of the organic modifier increases during gradient elution, intermolecular peptide–phase interactions decrease and increasingly hydrophobic oligopeptides elute from the column. TEAP plays a rôle in this process by decreasing hydrophilicity of polar peptides by ion-pairing and consequently reducing overall charge of the peptide–ion-pair complex[36]. This model for paired-ion chromatography elution of oligopeptides on RP-HPLC is supported by the correlation observed in Table II between each oligopeptide's integral of Rekker's hydrophobic fragmental constants (Rekker integral, RI) of each constituent amino acid[25,35] and t_R in the RP-HPLC system. Empirical improvement in this correlation was observed by O'Hare *et al.*[25], where they considered only the five most hydrophobic residues Trp, Phe, Leu, Ile and Tyr. In the RI column, Table II, two pairs of peptides (met-enkephalin, 32.42 *vs.* bradykinin, 33.19; and Tyr^{11} SS, 45.40 *vs.* substance P, 46.02) are reversed and do not obey the correlation. In the RI_5 column, one pair (bradykinin and angiotensin) is reversed.

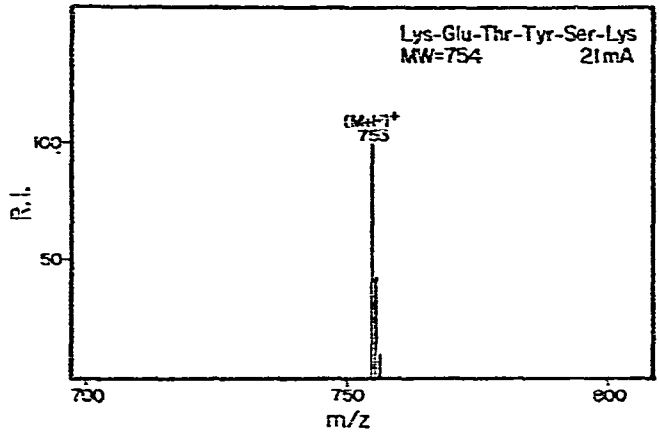

Fig. 2. FD mass spectrum of underivatized hexapeptide, Lys-Glu-Thr-Tyr-Ser-Lys.

TABLE II

ADJUSTED RETENTION TIMES *vs.* REKKER INTEGRALS FOR FIVE MOST HYDROPHOBIC VALUES (RI_5) AND ALL VALUES (RI)

Pyrollidone carboxylic acid (PCA) estimate = — 0.06.

Peptide	$\bar{t}_A$	RI_5	*RI*
TRH	1.18	0	0.72
Met-enkephalin	32.42	3.94	5.02
Bradykinin	33.19	4.48	4.47
Angiotensin II	35.58	3.94	7.05
Eledoisin-related peptide	37.73	6.22	8.72
Tyr^{11} SS	45.40	6.25	10.15
Substance P	46.02	6.47	9.01
Somatostatin (SS)	51.72	6.79	10.69
Tyr^{1} SS	53.25	9.10	12.39
β-Endorphin	57.58	14.14	12.80
		$r^2 = 0.76$ $y = 0.20x - 1.78$	$r^2 = 0.86$ $y = 0.22x - 0.78$

The RI_5 data yield a coefficient of determination value (r^2) of 0.76 while the RI method yields a value of 0.86. This present study statistically favors calculating the sum of all Rekker constants as opposed to the sum of only the five most hydrophobic residues. Nonetheless, it is apparent in either case reversals are noted and therefore it is likely that other factors such as conformation, charge, etc., play a role in the RP-HPLC in addition to hydrophobicity. It is interesting to note in passing that the concept of relating RP-HPLC elution with increasing hydrophobicity closely parallels the situation of correlating gas charomatographic (GC) elution with integrals of retention index increments of amino acid residues in oligopeptide-derived O-trimethylsilylated perfluorinated dideuteroalkyl polyamino alcohols[37].

The HPLC system employed in this study for resolution of a synthetic mixture of hypothalamic oligopeptides has several important parameters. Speed of separation for such a complex mixture is increased: within 78 min the mixture of peptides

ranging from three to 31 amino acids is resolved. Speed of separation is important for a rapid, sensitive and highly resolving chromatographic system to provide metabolic profiles for biologic extracts of hypothalamic oligopeptides. Gas chromatograms are common for drug and steroid metabolic profiles in various body fluids.

Sensitivity of UV detection is sufficient for many peptide studies and options currently under study exist to further increase sensitivity. Somatostatin is one hypothalamic oligopeptide currently being investigated in many laboratories[38] and a rapid, unequivocal identification and quantification method is required. At 210 nm, 5 ng (pmol) of SS is detected at the appropriate retention time. In a single rat hypothalamus *ca.* 40 ng SS are present[39]. Thus, sufficient sensitivity exists for detection and quantification of somatostatin in one rat hypothalamus by means of UV detection. Another method under development which promises increased sensitivity includes fluorescence detection[25]. Structural assignment and quantification of oligopeptides may be afforded by FD mass spectrometry (MS) operated in the selected ion monitoring (SIM) mode using appropriate stable isotope-labeled internal standards[40].

In addition to speed and sensitivity of peptide separations, in many cases the most important parameter is resolution of peptides. The TEAP buffer used with the C_{18} column has provided continued excellent resolution of synthetic mixtures of peptides, a necessary preliminary study to looking at extracts of hypothalamic tissue. For example, t_R values of Tyr[11] somatostatin and substance P are quite close and further chromatographic refinements (such as temperature, buffer, pH, flow-rate, column) in this separation may further resolve this pair. The high resolution of this RP-HPLC system is necessary for biologic extracts and will follow either gel or Sep-pak chromatography separations of proteins from peptides in biologic extracts[41].

Reproducibility of retention times (t_R) for multiple injections of peptide mixtures becomes an important parameter for peptide identification for chromatographic systems that do not have a follow-up step such as MS for structural elucidation of individual HPLC peaks. Reproducibility of t_R values was studied in a series of multiple injections of mixtures of oligopeptides. Data in Table I collect t_R values (individual and average), standard deviation (σ) and coefficient of variation (V). Retention times (t_R) are reproducible with an average coefficient of variation of 1.91%. By injecting known amounts of standards first, followed by injection of an unknown mixture of the same compounds, it should be possible to identify and quantify individual components in a mixture of oligopeptides. For cases involving mixtures of unknown compounds, a structural elucidation method is required following HPLC separation.

MS offers an attractive option to elucidate, identify and quantify an oligopeptide in a purified HPLC fraction. FD-MS in particular provides a fast and facile method for unambiguous identification because it is possible to produce ions only in the molecular ion region. On the other hand, for unknown underivatized oligopeptides, sequence-determining fragment ions may be produced by either increasing e.h.c. or by following FD by collision activation (CA)[42,43].

The type of data one obtains with FD-MS is illustrated by the FD spectrum of an underivatized hexapeptide Lys-Glu-Thr-Tyr-Ser-Lys given in Fig. 2. In contrast to the plethora of peaks commonly found in electron impact (EI) or chemical ionization (CI) mass spectra of derivatized oligopeptides[44], the spectrum in Fig. 1 is extra-

ordinarily clean. Unambiguous identification of sequence ions by either CA or by increasing e.b.c. is corroborated by accurate mass measurement (not performed) of the protonated molecular ion, $C_{53}H_{54}N_8O_{12}$ 755.3939. Similar results are obtained with enkephalins and other oligopeptides[45].

It is clear that, in order to employ MS techniques, one must avoid a non-volatile buffer salt such as TEAP. Appropriate alternate volatile buffers which can be removed by lyophilization are TEA–formate, TEA–acetate or ammonium acetate[29]. Alternatively, individually resolved RP-HPLC peptide fractions can be desalted by employing Waters Sep-pak cartridges after HPLC and before MS analysis[41].

ACKNOWLEDGEMENT

The authors gratefully acknowledge financial assistance from NIH (RO1-GM-NS-26666), technical assistance of William Skea of Waters Assoc. and use of the Varian 731 field desorption mass spectrometer by Drs. Ed. White and Harry Hertz at the National Bureau of Standards in Gaithersburg, MD, U.S.A., during preliminary studies.

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