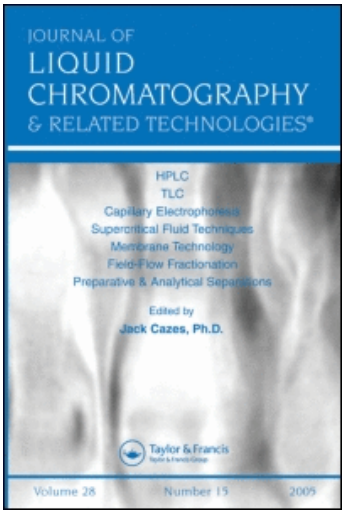

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF ARGININE-CONTAINING NEUROPEPTIDES BY PRECOLUMN DERIVATIZATION AND FLUORIMETRIC DETECTION

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ABSTRACT

A previously developed fluorescence method for determining leupeptin and the angiotensins is shown to be applicable to assaying other small arginine-containing peptides. In this procedure arginine residues are derivatized by reaction with benzoin. Femtomole sensivity is obtainable with this method. However, it cannot be used with larger arginine-containing peptides because those fragment upon derivatization.

INTRODUCTION

A variety of opioid (1) and tachykinin (2) peptides act as neurotransmitters. They are involved in assorted neuronal functions, including sensing pain (3). Neurons utilizing different peptides may be simultaneously active. Thus, to

explore the actions of neuropeptides, methods for measuring a number of peptides with high sensitivity in a single analysis are needed.

High-performance liquid chromatography (HPLC) is a powerful method for separating complex mixtures of peptides in biological tissues and fluids (4,5). However, detection is a problem. Ultraviolet detection lacks sensitivity. Radioreceptorassay (RRA) has been used as an off-line detector for members of the three families of opioid peptides following separation by HPLC (4,6,7), and a more specific RRA for δ-receptor-preferring peptides has been developed (8). However, radioreceptorassay also lacks adequate sensitivity for some applications and is expensive and cumbersome. Radioimmunoassay (RIA) is sensitive but expensive, and each assay can detect only one or a few peptides. The combination of HPLC, RRA, RIA and mass spectrometry/mass spectrometry can be used to fully characterize very small amounts of neuropeptides (9,10). However, this approach is laborious and not suited to routine assays. A high sensitivity, on-line method for detecting peptides of interest as they elute from an HPLC column would be ideal.

Many opioid peptides and their N- or C-terminal extensions contain arginine (1). Extended forms contain arginine and/or lysine at cleavage sites for peptide processing. Many other neuropeptides, such as the tachykinins, neurotensin, somatostatin and adrenocorticotropin also contain arginine. Kai and coworkers (11) developed a high sensitivity, fluorescence method for detecting guanidine-containing compounds by reacting this group with benzoin. They detected the peptides leupeptin and the angiotensins with femtomole sensitivity (12,13). In the present study, we extend Kai's method to separating and determining a variety of arginine-containing peptides, especially opioid peptides.

MATERIALS AND METHODS

Materials

Methionine enkephalin-arginine (ME-Arg), arginine-methionine enkephalin (Arg-ME) and dynorphin A fragments 1-6 (dyn 1-6) and 1-7 (dyn 1-7) were purchased from Peninsula (Torrance, CA). Methionine enkephalin-arginine glycine leucine (ME-ArgGlyLeu), methionine enkephalin-arginine phenylalanine (ME-ArgPhe), dynorphin B (dyn B), and arginine-vasopressin (Arg-vasopressin) were purchased from Sigma Chemical Co. (St. Louis, MO), tuftsin from Calbiochem (San Diego, CA) and L-arginine from Pierce Chemical Co. (Rockford, IL).

Derivatization of Peptides

Derivatives were prepared by slight modifications of the method of Kai et al. (11,12,13). The peptides were dissolved in distilled deionized water in concentrations of 0.25 - 0.5 µg/µl. Before use, these samples were diluted in glass tubes and cooled by placement in ice. The following solutions were added in succession to the samples (units are volumes per volume of sample solution): 0.5 volumes of benzoin dissolved in methyl cellosolve (4 mM), 0.5 volumes of an aqueous solution containing 0.1 M 2-mercaptoethanol and 0.2 M sodium sulfite, and one volume of 0.8 M potassium hydroxide. This mixture was heated in a hot water bath and then cooled in an ice bath for 2 min. The pH was lowered by adding one volume of a solution containing 0.8 M HCl and 0.5 M Tris-HCl (1:1 v/v). There was 2.5 ng/µl of each peptide in the final solution.

HPLC Gradient Separation and Fluorimetric Detection

Analyses were carried out on a Waters ALC-202 liquid chromatograph equipped with a Kratos FS970 fluorescence detector (excitation wavelength 325 nm; emission 418 nm cutoff filter). A 25 cm X 4.6 mm, 5 µm Vydac C18 column (Alltech, Deerfield, IL)

was employed. A tube packed with silica gel was placed between the pump and the injector to saturate the mobile phase with silica to retard dissolution of the column particles. A C-4 guard column (Phenomenex, Rancho Palos Verdes, CA) was placed between the injector and the analytical column to protect the latter from deposition of precipitated material. The aqueous component of the mobile phase was TEAP buffer (0.25 N phosphoric acid to which enough triethylamine was added to raise the pH to 8.5); the organic component was acetonitrile. The HPLC was operated in both isocratic and gradient modes.

RESULTS AND DISCUSSION

Optimum Reaction Times and Temperatures

These studies were carried out on ME-Arg, Arg-ME, ME-ArgGlyLeu, ME-ArgPhe, and Dyn 1-6. Reaction parameters were chosen based on peak heights obtained following different reaction times and temperatures. These results (Table 1) demonstrated that different peptides had different optimum reaction times and temperatures. When the temperature was 100°C, ME-ArgGlyLeu and ME-ArgPhe fragmented extensively to two peaks, and the other three peptides decomposed to a small degree. Fragmentation increased with reaction time at 100°C. The best compromise conditions among the peptides studied was 80°C and 3 min.

The reaction conditions described above for the opioid peptides were not optimum for peptides such as dynorphin 1-8, dynorphin 1-9 and dynorphin A that contain more than one arginine residue; such peptides fragmented to several pieces under these conditions. This problem might be solved by postcolumn derivatization in which fragmentation would not matter.

TABLE 1

Optimization of Conditions for Peptide Derivatization

Temperature (°C)	Reaction time (min)	Peak height (cm/12.5 ng peptide) dyn 1-6	Arg-ME	ME-Arg	ME-ArgGlyLeu	ME-ArgPhe
	1.5	2.1	1.45	1.2	1.2	0.9
50	3	4.3	2.1	1.9	2.3	2.3
	5	5.1	3.4	2.5	3.4	3.3
	1.5	7.4	2.8	0.9	2.7	2.5
60	3	7.5	4.4	2.1	3.7	4.4
	5	4.3	5	3.3	4.8	5.0
	1.5	5.3	2.9	1.9	2.8	3.7
70	3	7.5	3.3	2.4	5.2	6.8
	5	11.2	3.4	2.0	3.7	6.0
	1.5	3.5	3.5	*3.6	4.4	*7.0
80	3	*13.2	*4.7	2.8	4.4	6.8
	5	12.2	4.1	2.6	3.8	5.3
	1.5	10.4	3.8	2.7	*5.5	5.6
85	3	10.0	4.6	2.7	4.1	6.0
	5	10.0	4.0	2.6	3.2	4.3
	1.5	*14.6	4.3	1.8	2.9	4.3
100	3	10.3	4.1	2.5	1.8	3.8
	5	7.0	4.0	1.8	1.5	2.7

The asterisk indicates the best conditions for the individual peptides.

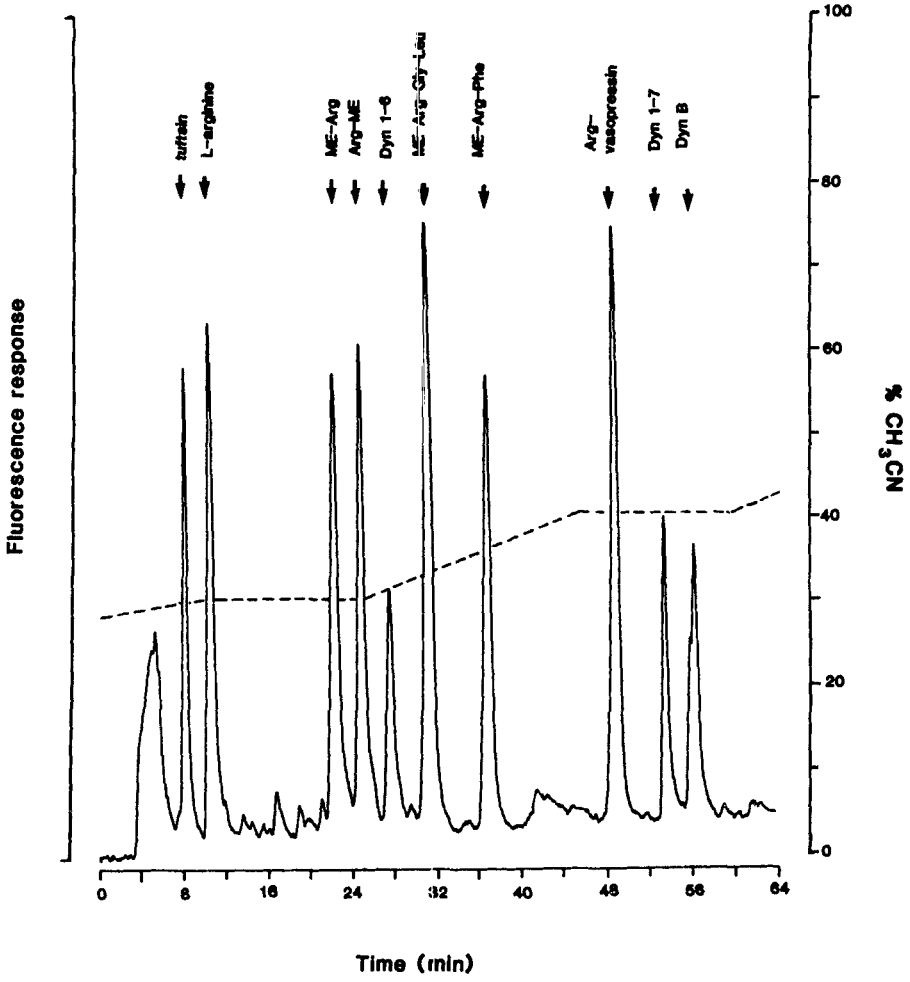

FIGURE 1. Chromatogram of derivatives of 9 different peptides and L-arginine. Derivatizing conditions are given in the text. Amounts of each peptide injected were tuftsin 25 ng, L-arginine 10 ng, ME-Arg 62.5 ng, Arg-ME 100 ng, dyn 1-6, 30 ng, ME-ArgGlyLeu 125 ng, ME-ArgPhe 125 ng, arg-vasopressin 500 ng, dyn 1-7 250 ng, dyn B 375 ng. Each peak is identified near its top. The gradient program used to develop the chromatogram in percentages of acetonitrile was 28 - 30% in 10 min, 30 % for 10 - 25 min, 30% to 40% from 25 to 45 min, running isocratically at 40% acetonitrile for 45 to 60 min, and to 50% from 60 to 80 min.

TABLE 2

Detection limits for opioid peptides

Peptide	Detection limit (fmol)
Dyn 1-6	175
ME-ArgGlyLeu	278
ME-ArgPhe	285
ME-Arg	342
Arg-ME	342

Reverse-Phase HPLC Separation of Neuropeptides

Fig. 1 displays a chromatogram of derivatives of 9 different peptides and L-arginine. These benzoin-labeled compounds were well separated. Except for a small shoulder on the peak for dyn B, all of the peptides produced single peaks. Thus, this method is useful for small, single arginine-containing peptides. Kai et al. (13) noted that C-terminal amidated peptides, such as substance P, require a much shorter reaction time than angiotensin. Consistent with this, derivatization of Arg-vasopressin for 10 seconds at 70°C produced a very nice peak.

Detection Limits

The detection limits for 5 opioid peptides are given in Table 2. The threshold for detection was a signal-to-noise ratio of 2:1. Excellent sensitivity, in the femtomole range, was obtained for each peptide. Isocratic elution at 30% to 35% acetonitrile was used when optimizing reaction conditions and determining detection limits.

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