Journal of Chromatography, 392 (1987) 309–316
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 342

ON-LINE POST-COLUMN FLUORESCENCE DERIVATIZATION OF ARGININE-CONTAINING PEPTIDES IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received September 25th, 1986; revised manuscript received December 8th, 1986)

SUMMARY

A selective detection system based on on-line post-column fluorescence derivatization is described for the analysis of arginine-containing peptides by high-performance liquid chromatography. The peptides are automatically converted into fluorescent derivatives with benzoin, a fluorogenic reagent for guanidino compounds, after separation on a reversed-phase column (TSKgel ODS-120T) and detection in an ultraviolet absorption detector. The system permits fluorescence detection at 435 nm emission with irradiation at 325 nm for arginine-containing peptides in as little as picomole amounts. The chromatogram obtained with fluorescence detection only shows peaks corresponding to arginine-containing peptides. The facile detection of arginine-containing fragments in the tryptic digest of β-melanocyte stimulating hormone as a model compound could be achieved by comparison with a chromatogram obtained with ultraviolet absorption detection at 215 nm.

INTRODUCTION

Several physiologically important peptides having arginyl residues in the molecules, such as angiotensins[1], bradykinin[2], kyotorphin[3], substance P[4] and melanocyte stimulating hormone[5], occur in body fluid. These peptides have been measured by many methods, including radioimmunoassay[6], bioassay[7] and high-performance liquid chromatography (HPLC)[8,9]. Radioimmunoassay generally offers a high sensitivity for a peptide. However, a separate assay must be carried out for each peptide[6], and also it cannot detect unknown peptides. Bioassay usually does not have sufficient precision for peptides[7]. HPLC is advantageous for the simultaneous detection of various arginine-containing peptides. However, HPLC methods[8,9] remain a problem for complex biological samples because it is still difficult to separate all kinds of biogenic peptides. Thus, specific detection in HPLC is necessary for the determination of the arginine-containing peptides; conventional detection methods such as ultraviolet absorption (UV) detection at a wavelength between 200 and 230 nm[9] and fluorescence detection by means of on-line post-column fluorescence derivatization with a fluorogenic reagent (fluorescamine[10] or *o*-phthalaldehyde[11]) are not selective for peptides.

0021-9673/87/$03.50

We have developed a reversed-phase HPLC method coupled with on-line post-column fluorescence detection for the selective detection of arginine-containing peptides, in which benzoin was used as a fluorogenic reagent. Benzoin reacts selectively and rapidly with the guanidino moiety of compounds including arginine-containing peptides in an alkaline solution[12,13] to produce highly fluorescent derivatives, 2-substituted amino-4,5-diphenylimidazoles[14] (Fig. 1).

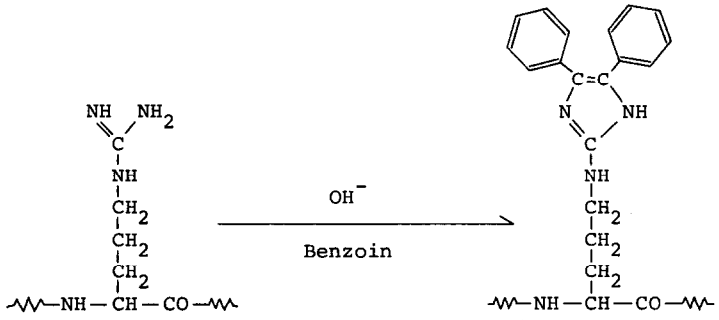

Fig. 1. Fluorescence derivatization of arginine-containing peptides with benzoin.

EXPERIMENTAL

Chemicals and solutions

All chemicals were of analytical-reagent grade, unless specified otherwise. Deionized, distilled water was used.

Benzoin (Wako, Osaka, Japan) was recrystallized from absolute methanol. Tris(hydroxymethyl)aminomethane (Tris) (Wako) was recrystallized from 60% aqueous methanol.

The following peptides were purchased from Peptide Institute (Osaka, Japan) or Sigma (St. Louis, MO, U.S.A.): kyotorphin (Tyr-Arg), kallidin (Lys-Arg-Pro-Pro--Gly-Phe-Ser-Pro-Phe-Arg), bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), 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), substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2), β-melanocyte stimulating hormone (β-MSH) (Asp-Glu-Gly-Pro-Tyr-Lys-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp) and methionine-enkephalin (Tyr-Gly-Gly-Phe-Met).

Mobile phase for HPLC. Eluents A and B were mixtures of acetonitrile and 0.2 *M* sodium phosphate buffer (pH 2.3) (1:19 and 2:3, respectively). The eluents were degassed before use.

Reagent solutions for post-column derivatization. Reagent A was prepared by mixing equal volumes of 6 m*M* benzoin (in methyl-Cellosolve), 4.8 *M* potassium hydroxide and 2.1 *M* β-mercaptoethanol (concentrations 0.002, 1.6 and 0.7 *M*, respectively), and reagent B by mixing equal volumes of 1.0 *M* Tris and 4.2 *M* hydrochloric acid. The reagent solutions were thoroughly degassed before use.

Chromatograph and its operation

Fig. 2 is a schematic diagram of the HPLC system constructed for the analysis of arginine-containing peptides.

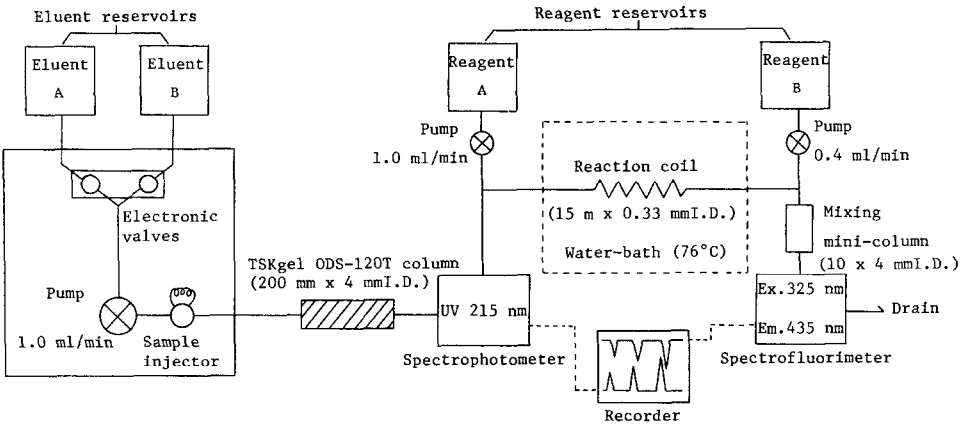

Fig. 2. Schematic diagram of post-column fluorescence derivatization HPLC for arginine-containing peptides.

Sample solution was injected in the volume range 10–50 μl. Peptides in the sample were separated on a 200 × 4 mm I.D. reversed-phase column packed with TSKgel ODS-120T, particle size, 5 μm (Toyo Soda, Tokyo, Japan) with a gradient elution using mixtures of acetonitrile and 0.2 *M* sodium phosphate buffer (pH 2.3) (eluents A and B). The mobile phase was pumped at a flow-rate of 1.0 ml/min by a Hitachi 638–30 high-performance liquid chromatograph fitted with a programming electronic controller. The acetonitrile concentration in the mobile phase was 5% for the first 5 min, then increased linearly from 16% to 27.5% for the next 45 min (see Fig. 5). The column temperature was ambient (*ca.* 24°C).

The column eluate was passed through a Hitachi 638-41 UV detector equipped with a flow-cell (13 μl), operating at 215 nm, and conducted to a fluorescence reactor system. In the system, reagent A was added to the eluate stream by a Hitachi 633 reagent pump at a flow-rate of 1.0 ml/min, then the mixture was passed through a PTFE reaction coil (15 m × 0.33 mm I.D.) immersed in a water-bath at 76°C. Reagent B was added to the reaction mixture at a flow-rate of 0.4 ml/min by the same type reagent pump as for reagent A, and the final reaction mixture was passed through a stainless-steel mini-column (10 × 4 mm I.D.) packed with glass-wool (*ca.* 40 mg) for mixing.

The fluorescence intensity of each arginine-containing peptide in the last eluate was monitored at 435 nm emission against 325 nm excitation (both spectral bandwidths 10 nm) using a Hitachi 650-10LC spectrofluorimeter equipped with a flow-cell (18 μl).

RESULTS AND DISCUSSION

Conditions of post-column fluorescence derivatization

In the post-column reaction system, the mixture of benzoin and potassium hydroxide as reagent A was introduced to the column eluate with a reagent pump. The reagent solution in the presence of 0.7 *M* β-mercaptoethanol or 0.17 *M* sodium sulphite was usable for *ca.* 24 h. In its absence, however, the reagent did not give constant fluorescence intensities from arginine-containing peptides (Fig. 3); 0.7 *M* β-mercaptoethanol gave the largest peak heights for the peptides and is recommended in the procedure for reproducible results. Concentrations of 2 m*M* and 1.6 *M* of benzoin and potassium hydroxide, respectively, in the solution gave almost the maximum fluorescence intensity for each arginine-containing peptide.

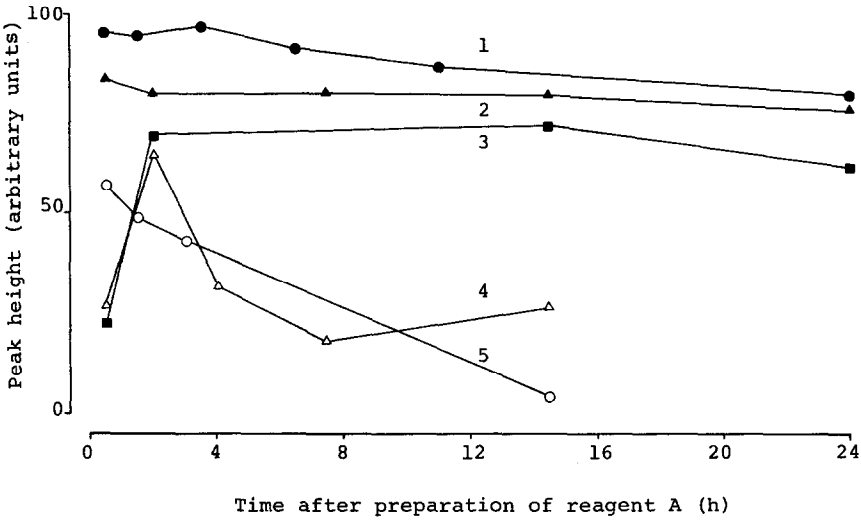

Fig. 3. Effect of various reductants on the stability of benzoin and potassium hydroxide solution used for post-column fluorescence derivatization of kallidin in the reactor system. Reductants: 1, 0.7 *M* β-mercaptoethanol; 2, 0.17 *M* sodium sulphite; 3, 0.7 *M* sodium hypophosphite; 4, 0.7 *M* disodium phosphite; 5, none. A 50-μl volume of 20 nmol/ml kallidin solution was injected in to the chromatograph. The reductant was added to a mixture of 2 m*M* benzoin and 1.6 *M* potassium hydroxide.

An elevated reaction temperature was required for the development of fluorescence from all the arginine-containing peptides tested (Fig. 4). Maximum and constant fluorescence intensities were obtained at temperatures between 70 and 80°C; 76°C was selected.

The length of the reaction coil, ranging from 5 to 25 m, also affected the fluorescence intensities of the arginine-containing peptides. Maximum and constant intensities were obtained by use of a coil longer than 10 m. However, the peaks were broadened with increased length of the coil. For example, the peak widths obtained with a 25-m coil were approximately 1.3 times greater than those with a 10-m coil. A 15-m reaction coil was tentatively employed in the system; the reaction time was *ca.* 40 s.

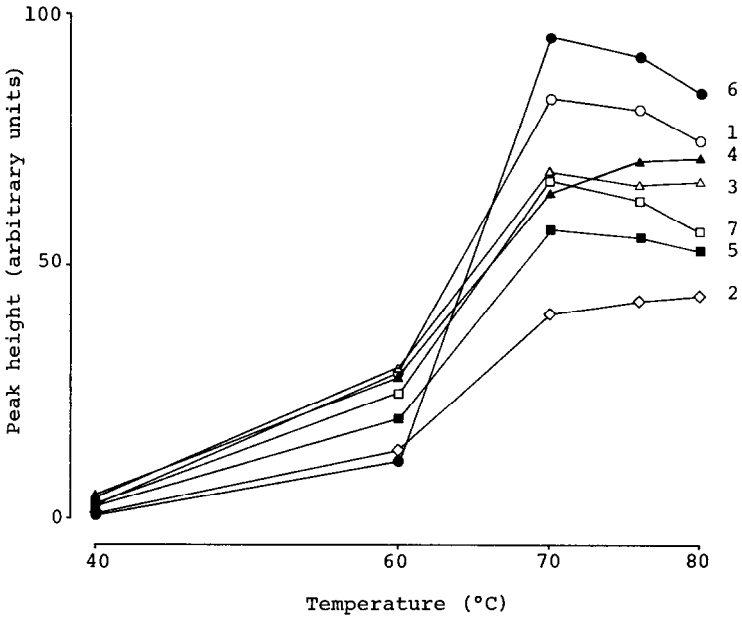

Fig. 4. Effect of reaction temperature on the fluorescence development from peptides in the reactor system. Peptides (nmol per 50-μl injection volume in parentheses): 1, kallidin (2.0); 2, bradykinin (0.5); 3, angiotensin II (0.3); 4, angiotensin III (1.2); 5, angiotensin I (0.3); 6, substance P (0.8).

A mixture of Tris and hydrochloric acid was added after the fluorescence reaction in order to adjust the pH of the reaction mixture to 8–10. The final mixture was mixed well by passing through a short column packed with glass-wool. The fluorescence intensities produced from arginine-containing peptides were approximately 20 times higher than those obtained without pH adjustment. The excitation and emission maxima of the fluorescence from all the arginine-containing peptides tested were around 325 and 435 nm, respectively, and no change in the maximum wavelengths was observed even when the volume ratio of acetonitrile and 0.2 *M* sodium phosphate buffer (pH 2.3) in the mobile phase was altered during the gradient elution.

Determination of arginine-containing peptides

Peptides with molecular weights between 500 and 3000 have been separated well on a reversed-phase column by elution with a mobile phase composed of acetonitrile and phosphate or perchlorate buffer (pH 2–4)[15]. Therefore, the separation of the arginine-containing peptides tested (molecular weight 300–2000) was carried out under similar conditions to those in conventional HPLC.

Fig. 5 shows chromatograms obtained by post-column fluorescence detection and UV detection in reversed-phase HPLC for seven arginine-containing peptides (kyotorphin, kallidin, angiotensin I, II and III, substance P and β-MSH), an arginine-free peptide (methionine-enkephalin) and eight biological substances other than peptides. Fluorescence detection utilizing the benzoin reaction gave a sufficient speci-

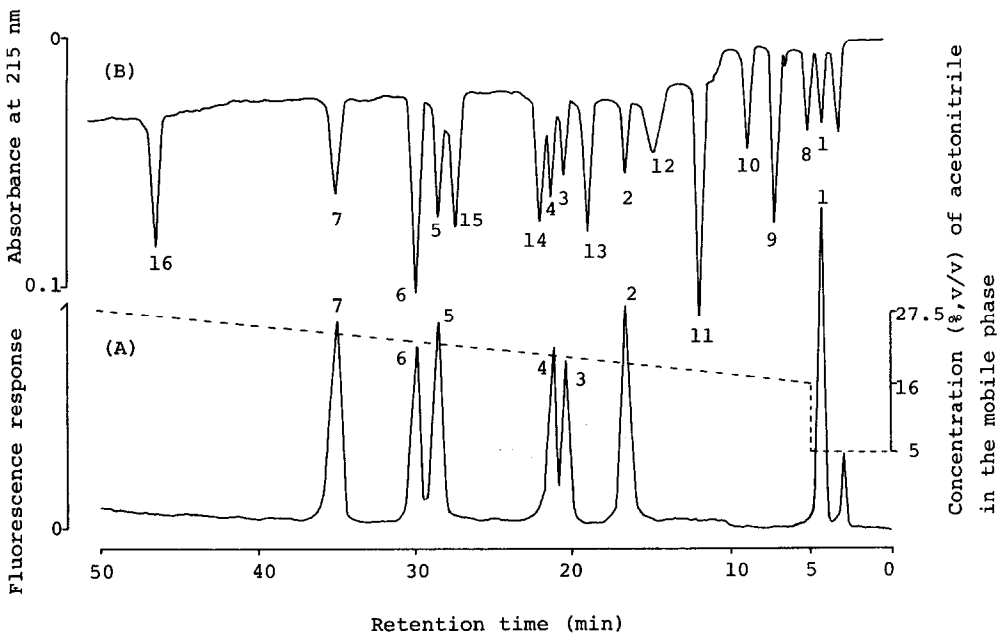

Fig. 5. Chromatograms obtained with fluorescence and UV detection in the HPLC of peptides and several biological substances. Peaks (nmol per 50-μl injection volume in parentheses): 1, kyotorphin (1.0); 2, kallidin (0.5); 3, angiotensin II (0.25); 4, angiotensin III (0.25); 5, angiotensin I (0.5); 6, β-MSH (1.0); 7, substance P (0.5); 8, tyrosine (1.0); 9, propionic acid (10.0); 10, phenylalanine (2.0); 11, tryptophan (0.5); 12, phenylpyruvic acid (10.0); 13, methionine-enkephalin (1.0); 14, sorbic acid (2.5); 15, estriol (1.5); 16, estrone-3-sulphate (3.0).

ficity for the arginine-containing peptides (Fig. 5A). On the other hand, UV detection at 215 nm, which has been widely used for peptide detection, permitted the detection of all the tested biological substances including the arginine-containing peptides (Fig. 5B). This indicates that UV detection is inadequate for the detection of arginine-containing peptides in biological samples.

All the calibration graphs of peak height *versus* amount injected (0.1–1.0 nmol of each) for the arginine-containing peptides with both detection methods were linear. The correlation coefficients (r) for all the graphs were 0.985–0.990 for fluorescence detection and 0.986–0.993 for UV detection. The limits of fluorescence and UV detection were 5–15 and 10–20 pmol, respectively, for the tested peptides, at a signal-to-noise ratio of 2. The coefficients of variation for the arginine-containing peptides tested were 3.8–10.2% for fluorescence detection and 3.7–10.0% for UV detection (n = 10 in each instance).

Detection of tryptic peptides from β-MSH

β-MSH, selected as a model peptide, was digested with trypsin and the enzyme reaction mixture was subjected to HPLC in order to demonstrate the utility of the technique for the detection of arginine-containing peptides.

Fig. 6 shows chromatograms obtained in the course of the enzyme reaction of

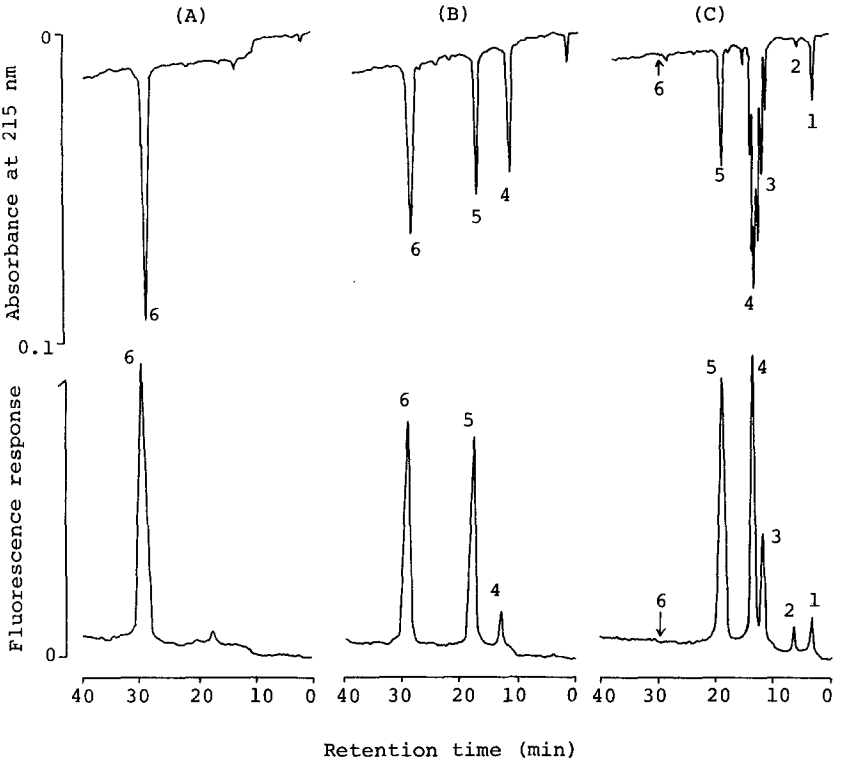

Fig. 6. Chromatograms of the peptide fragments produced by tryptic digestion of β-MSH. A 50-μl volume of 100 nmol/ml β-MSH solution was mixed with 100 μl of 0.1 *M* sodium phosphate buffer (pH 8.0) and 10 μl of 1 mg/ml trypsin solution and the mixture was incubated at 37°C. A 40-μl volume of the mixture was injected into the chromatograph at reaction times of (A) 0, (B) 4 and (C) 8 h. Peaks: 1–5, fragments from β-MSH; 6, β-MSH.

β-MSH with trypsin. Trypsin mediates the hydrolysis of peptide bonds at the carboxyl sides of arginyl and lysyl residues[16]. β-MSH has an arginyl residue and two lysyl residues in the molecule. Therefore, the following five arginine-containing fragments and also four arginine-free fragments should be produced by the tryptic digestion; the arginine-containing fragments are (a) Met-Glu-His-Phe-Arg, (b) Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys, (c) Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp, (d) Asp-Glu-Gly-Pro-Tyr-Lys-Met-Glu-His-Phe-Arg and (e) Asp-Glu-Gly-Pro-Tyr-Lys-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys. In the chromatogram (Fig. 6C) obtained on reaction for 8 h, five peaks corresponding to the benzoin-reactive fragments were clearly observed with fluorescence detection, whereas several partly overlapping peaks were found with UV detection. It is presumed that the peaks detected with UV detection but not with fluorescence detection are arginine-free fragments produced by tryptic digestion of β-MSH and by autolysis of trypsin. The fluorescent peaks 1–5 are ascribable to be the above arginine-containing fragments (a)–(e), respectively, as peptides of larger molecular size are eluted later on the reversed-phase column as compared with the hydrophobicity of peptides com-

posed of similar amino acid sequences[17]. However, the actual identification of the peaks in the chromatograms could not be carried out, because authentic samples of each fragment were not available.

The proposed HPLC method for arginine-containing peptides based on on-line post-column fluorescence detection using benzoin provides an approach for the sensitive and selective detection of arginine-containing peptides in complex samples. The technique may also be an aid in peptide chemistry related to peptide synthesis and in analyses of amino acid sequences.

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