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Note

Use of benzoin as pre-column fluorescence derivatization reagent for the high-performance liquid chromatography of angiotensins

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Angiotensins I, II and III are physiologically important peptides for blood pressure control[1] and they contain an arginyl residue in their molecules. Renin, an endopeptidase, releases angiotensin I from endogenous angiotensinogen. Angiotensin II, one of most potent vasoconstrictors known, is generated from angiotensin I by angiotensin-converting enzyme. In addition, an aminopeptidase removes the amino-terminal aspartic acid residue from angiotensin II yielding angiotensin III.

These peptides have generally been measured by radioimmunoassay (RIA), since these methods are sensitive enough to permit the determination of as little as 5–10 fmole amounts of the peptides[2–4]. However, high-performance liquid chromatography (HPLC) coupled with highly sensitive detection may be more suitable for either determination of angiotensins or for a study of the enzyme kinetics in the renin–angiotensin system, because the HPLC method permits simultaneous determination of angiotensins I, II and III and other related peptides. Although conventional peptide analyses with HPLC are generally performed by ultraviolet (UV) absorption detection at wavelengths between 200 and 230 nm[5–7], post-column fluorescence detection[8–10] or electrochemical detection[11], the sensitivities of the HPLC methods are still three to four orders of magnitude lower than those of the RIA methods.

We have developed recently a pre-column fluorescence derivatization method in HPLC for guanidino compounds, such as methylguanidine and guanidinosuccinic acid, by using a fluorogenic reagent, benzoin[12]. Benzoin reacts with the guanidino moiety of compounds in an alkaline medium, in the presence of 2-mercaptoethanol (to stabilize the fluorescent product) and sodium sulphite (to suppress blank fluorescence)[13], and it yields highly fluorescent derivatives, *viz.* 2-substituted amino-4,5-diphenylimidazoles[14]. Therefore, it is conceivable that arginine-containing peptides are also converted into the corresponding fluorescent derivatives by the benzoin reaction.

This paper describes a simple and sensitive HPLC method by means of the pre-column derivatization with benzoin for the simultaneous determination of angiotensins I, II and III. The HPLC method allows the quantitative determination of the peptides at the femtomole level. In the investigations, four other arginine-containing peptides, namely, tuftsin, substance P, luteinizing hormone-releasing hormone (LH-RH) and leupeptin acid were also examined with regard to the derivatization conditions.

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EXPERIMENTAL

Chemicals and solutions

Deionized and distilled water was used. The following peptides were purchased from the Protein Research Foundation (Osaka, Japan) or Sigma (St. Louis, MO, U.S.A.): 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), tuftsin (Thr-Lyr-Pro-Arg), substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Ile-Met-NH_2) and LH-RH (pyro-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2). Leupeptin acid (acetyl-Leu-Leu-Arg) was a gift from Nihon Kayaku (Tokyo, Japan). Tris(hydroxymethyl)aminomethane (Tris; Wako, Osaka, Japan) was recrystallized from aqueous 60% methanol to remove fluorescent impurities. Other chemicals were of reagent grade.

The reagent solutions used for the fluorescence derivatization were prepared as described previously[12].

Derivatization procedure

A 100-μl portion of peptide aqueous solution was placed in a test tube, to which were added 50 μl each of 5 m*M* benzoin (in methylcellosolve) and an aqueous solution containing 0.1 *M* 2-mercaptoethanol and 0.2 *M* sodium sulphite, and 100 μl of 0.8 *M* potassium hydroxide, with cooling in ice–water. The mixture was heated in a boiling-water bath for 90 sec, and then a 100-μl portion of an aqueous acidic solution containing 0.8 *M* hydrochloric acid and 0.5 *M* Tris–hydrochloric acid buffer (pH 8.5) was added. A 100-μl portion of the final reaction mixture was used for HPLC.

HPLC apparatus and conditions

The HPLC system consisted of a Hitachi 735A high-pressure pump, a Rheodyne 7120 syringe-loading sample injector (100-μl loop) and a Hitachi F1000 HPLC fluorescence spectrometer fitted with a 12-μl flow-cell. The fluorescence of the eluate was monitored at 425 nm (emission wavelength; excitation at 325 nm). A reversed-phase column (15 × 4 mm I.D.; packing material, LiChrosorb RP-18; particle size, 5 μm; Japan Merck, Tokyo, Japan) was used for isocratic elution with aqueous acetonitrile or methanol containing 0.05 *M* phosphate buffer (pH 8.5) as mobile phase. The column temperature was ambient (20 to 27°C) and the flow-rate was 0.8 ml/min.

RESULTS AND DISCUSSION

An important condition for the fluorescence derivatization of peptides is to form single derivatives without hydrolysis of the peptide bonds. Under the optimized conditions[12] for the fluorescence derivatization of guanidino compounds with benzoin, *i.e.*, heating in 0.67 *M* potassium hydroxide at 100°C for 5 min, most of the peptides were partially hydrolyzed during the reaction, so that several fluorescent peaks from a peptide were observed on the chromatogram. However, an attempt to conduct the benzoin reaction under conditions milder than those of the previous one gave good results for the peptides.

Fig. 1 shows the effects of the reaction time and the concentration of potassium

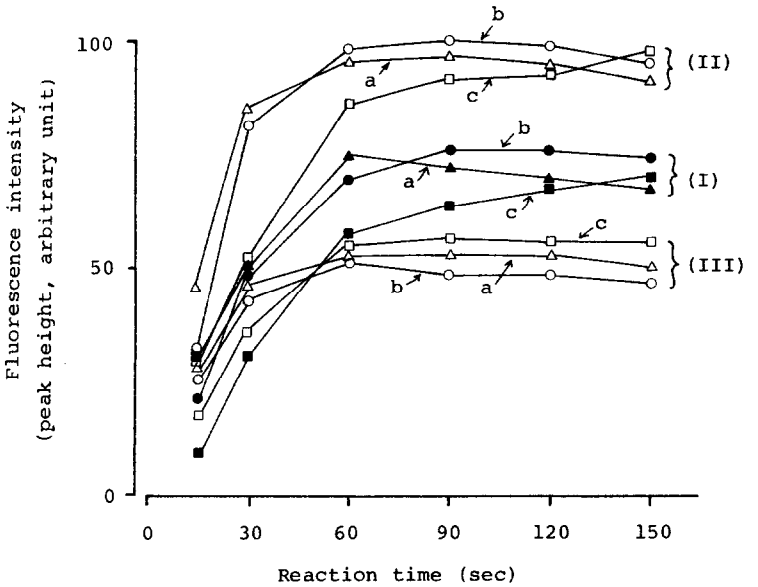

Fig. 1. Effects of reaction time and potassium hydroxide concentration on the formation of single fluorescent derivatives from angiotensins I, II and III. Portions (100 μl) of the peptide solutions were treated as in the derivatization procedure using different concentrations of potassium hydroxide. Peptide solutions (pmole per 100 μl); (I), angiotensin I (16); (II), angiotensin II (6); (III), angiotensin III (16). Concentration of potassium hydroxide (*M*) in the reaction mixture: a, 0.33; b, 0.27; c, 0.17. HPLC conditions: mobile phase, acetonitrile–0.05 *M* phosphate buffer (pH 8.5) (31:69); other conditions as in the text.

hydroxide in the reaction mixture on the formation of single fluorescent derivatives from angiotensins I, II and III. The data demonstrate that the peptides are very quickly derivatized with benzoin in 0.17–0.33 *M* of the alkali solution at 100°C. When the reaction temperature is 60°C, the reaction period needs to be much longer than 5 min, and the yield of the derivative from each peptide is 75% or less than that at 100°C. It should be also noted that the benzoin reagent requires slightly different conditions depending on the species of peptides for the derivatization. For instance, the peptides such as LH-RH and substance P of which both the C-terminal amino acids are amide type, require a much shorter reaction period (5–10 sec) in 0.27 *M* alkali solution to form the single fluorescent derivatives. The above reactivity of the peptides with benzoin was studied by separating the resulting derivatives on a reversed-phase column (LiChrosorb RP-18).

The peptides derivatized by the procedure described in the experimental section can be successfully resolved on the column by isocratic elution with acetonitrile–0.05 *M* phosphate buffer (pH 8.5) (31:69), as shown in Fig. 2. In this case, the derivatives of the other peptides, LH-RH and substance P, are eluted at the retention times of 22.0 and 38.5 min, respectively. The HPLC conditions are similar to those of conventional HPLC for intact peptides[6], but the mobile phase should contain a weakly alkaline buffer (pH 8.0–10.0) since the benzoin derivatives fluoresce most intensely in the weakly alkaline solution. Either borate or Tris–hydrochloric acid buffer (pH 8.5) can be used in place of the phosphate buffer. Of the buffers, the phosphate buffer

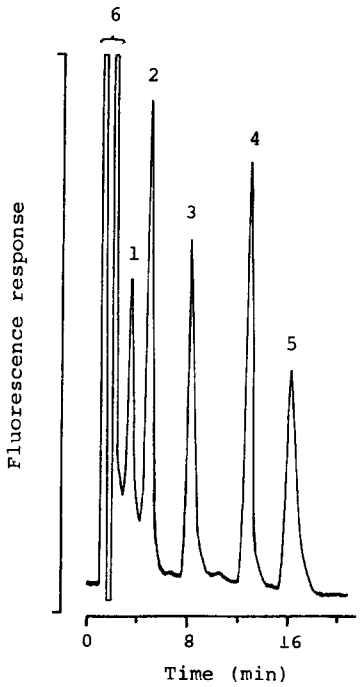

Fig. 2. Isocratic elution of the benzoin derivatives of arginine-containing peptides on the LiChrosorb RP-18 column. Peaks and amounts of the peptides (pmole per injection volume of 100 μl): 1 = taftsin (4); 2 = angiotensin II (1.5); 3 = angiotensin I (4); 4 = leupeptin acid (2.5); 5 = angiotensin III (4); 6 = reagent blank. The HPLC conditions are the same as in Fig. 1.

affords the best resolution for the derivatized peptides. When methanol is used as an organic modifier in place of acetonitrile, the peptides tested are eluted in the same order as that in Fig. 2, but a high concentration of methanol (63% in the mobile phase) is necessary to elute them at almost the same retention times. The excitation and emission maxima of the fluorescence from all the tested peptides in the phosphate buffer were *ca.* 325 and 435 nm, respectively. These maximum wavelengths are not changed by addition of acetonitrile or methanol to the mobile phase.

Calibration curves are all linear for the relationship between the peak heights and the amounts (0–0.1 nmole) of the peptides used for the derivatization. The lower limits of detection for angiotensins I, II and III are 88, 27 and 130 fmoles, respectively, in contrast to 10–100 pmoles[5–7] for the underivatized peptides by UV absorption detection. This sensitivity is also *ca.* 100 times higher than those of other HPLC methods in which fluorescamine[8], *o*-phthalaldehyde[9] or ninhydrin[10] are used in the post-column fluorescence reaction. The lower limit of this method is defined as the amount in the injection volume giving a signal-to-noise ratio of two.

The RIA method is *ca.* 10 times more sensitive than the present HPLC method, but can be used to measure only one peptide and sometimes requires separation of quite similar peptides because of the specificity depending on the antibody[4]. On the other hand, the present HPLC method can be used to determine simultaneously angiotensins I, II and III, and some fragments produced from these peptides during

the assay of the enzymes in the renin–angiotensin system may be measured. The HPLC method is thus suitable not only for the assay of the enzyme activities of renin and angiotensin-converting enzyme, but also for obtaining useful information on the enzymatic degradation of angiotensins by various other peptidases coexisting in a biological sample; this study is in progress in our laboratory.

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