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SENSITIVE ASSAY FOR SERUM ANGIOTENSIN-CONVERTING ENZYME AND SEPARATION OF ANGIOTENSIN ANALOGUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

YASUHIRO SAKAMOTO, TAKASHI MIYAZAKI, MASAAKI KAI and YOSUKE OHKURA*

Faculty of Pharmaceutical Sciences, Kyushu University 62, Maidashi, Higashi-ku, Fukuoka 812 (Japan)

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SUMMARY

A high-performance liquid chromatographic method is described for the assay of angiotensin-converting enzyme in human serum and for the separation of angiotensins and their analogues after pre-column fluorescence derivatization with benzoin. Angiotensin II, formed enzymatically from angiotensin I, is converted into a fluorescent derivative which is then separated isocratically from the substrate and biological substances in the enzyme reaction mixture on a reversed-phase column (TSK gel ODS-120T). The lower limit of detection for angiotensin II is 0.66 pmol per enzyme assay tube. The method is simple and sensitive, and requires as little as 5 μl of human serum. Angiotensin analogues can also be separated and quantified by the chromatographic technique, and thus this method permits the use of the analogues of angiotensin I as substrates.

INTRODUCTION

Angiotensin (ANG)-converting enzyme (ACE; E.C. 3.4.15.1) mediates the cleavage of the carboxy-terminal His-Leu of the decapeptide ANG I, generating the powerful vasocontracting octapeptide ANG II [1].

The ACE activity in serum has commonly been assayed by a radiochemical method [2], high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [3] or a spectrofluorimetric method [4] with artificial substrate, hippuryl-His-Leu or hippuryl-Gly-Gly. Other methods that use natural substrate ANG I are based on radiochemical [5] or spectrofluorimetric [6] determination of the released dipeptide His-Leu, or radioimmunoassay [7]

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of the released ANG II. These methods, however, do not distinguish the product from its degradation fragments, which may result from further hydrolysis by some peptidases present in the crude sample. Thus, HPLC methods have been proposed for the separation and quantification of ANG I, II and III and related peptides, so as to assay the enzymes in the renin–angiotensin system [8, 9]; however, UV detection of the HPLC methods is less sensitive for the ANGs.

We have recently developed a HPLC method with pre-column fluorescence derivatization using benzoin as a reagent for the sensitive determination of synthetic ANGs at the femtomole level [10]. This reagent reacts with the guanidino moiety and gives highly fluorescent derivatives, 2-substituted amino-4,5-diphenylimidazoles [11, 12]. Therefore, ANGs that retain an arginyl residue in the molecule are derivatized selectively to give fluorescent compounds by the benzoin reaction (Fig. 1). This paper describes a practical application of the method for the separation of ANGs and their analogues, and a simple and sensitive assay of ACE activity in human serum.

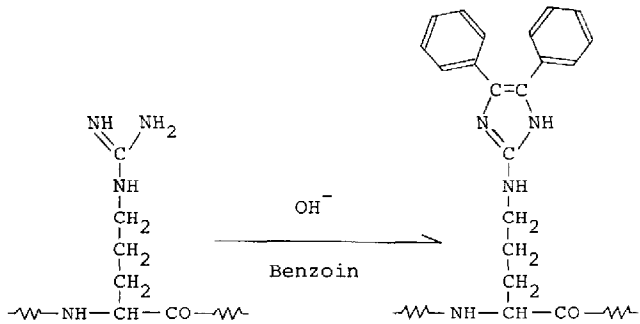

Fig. 1. Fluorescence derivatization with benzoin.

EXPERIMENTAL

Chemicals and solutions

The following peptides were purchased from the Protein Research Foundation (Osaka, Japan): ANG I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu), ANG II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), ANG III (Arg-Val-Tyr-Ile-His-Pro-Phe), [Val^5]-ANG I, [Val^5]-ANG II, [Asn^1, Val^5]-ANG I, [Asn^1, Val^5]-ANG II and hippuryl-His-Leu. Deionized and distilled water was used. Tris(hydroxymethyl)aminomethane (Tris; Wako, Osaka, Japan) was recrystallized from methanol–water (60:40) to remove fluorescent impurities. Other chemicals were of reagent grade. The synthetic peptides were dissolved in water and stored at −80°C until use. Prior to use, the stability of the solutions (1 μmol/ml for each peptide) was checked using the present HPLC method. The solutions were usable for at least two weeks. Serum specimens were obtained from healthy volunteers in our laboratory. The activity of ACE in serum was constant for at least three days when the serum was stored at −20°C. The

reagent solutions used for the fluorescence derivatization were prepared as described previously [11].

Apparatus

The HPLC system consisted of a Hitachi 635A high-pressure pump, a Rheodyne 7125 syringe-loading sample injector (100-μl loop) and a Hitachi F1000 HPLC fluorescence spectrometer fitted with a 12-μl flow-cell. A reversed-phase column (250 × 4 mm I.D.; packing material, TSK gel ODS-120T; particle size, 5 μm; Toyo Soda, Tokyo, Japan) was used.

Assay procedure

A mixture of 5 μl of serum, 70 μl of 0.2 *M* phosphate buffer (pH 7.5) containing 30 m*M* sodium chloride, 20 μl of 0.8 μmol/ml ANG I or its analogous peptides, and 10 μl of water was incubated at 37°C for 15 min. The enzyme reaction was stopped by adding 100 μl of 0.5 *M* perchloric acid. The mixture was centrifuged at 800 *g* for 5 min. For the enzyme blank, the same procedure was carried out but without incubation.

A 100-μl volume of the resulting supernatant was placed in a test tube, to which were added 50 μl of 5 m*M* benzoin (in methylcellosolve), 50 μl of an aqueous solution containing 0.1 *M* 2-mercaptoethanol and 0.2 *M* sodium sulphite, and 100 μl of 0.8 *M* sodium hydroxide, with cooling in ice—water. The mixture was heated in a boiling water-bath for 90 s. Then, a 100-μl volume of an acidic solution containing 0.5 *M* Tris—HCl buffer (pH 8.5) and 0.6 *M* hydrochloric acid was added to adjust the pH to ca. 8.5.

A 100-μl aliquot of the final reaction mixture was used for HPLC. The fluorescent derivatives of the ANGs and/or their analogues were separated on the TSK gel ODS-120T column by isocratic elution using a mixture of acetonitrile and 48 m*M* phosphate buffer (pH 8.5) (33:67). The fluorescence of the benzoin derivatives of the peptides in the eluate was monitored using an excitation wavelength of 325 nm and an emission wavelength of 435 nm [10]. Peak heights were used for the quantification. The amount of ANG II or its analogous peptides produced enzymatically was quantified by the standard addition method: each standard peptide (0.1—2 nmol) was added to the supernatant after enzyme reaction or the enzyme blank.

RESULTS AND DISCUSSION

Separation and quantification of ANGs and their analogues

Fig. 2 shows a chromatogram of the reaction mixture spiked with ANGs I, II and III and their analogous peptides without incubation. The fluorescent derivatives of the peptides other than [Val^5]-ANG I or [Asn^1, Val^5]-ANG II were separated within 24 min on the reversed-phase column by isocratic elution with 33% acetonitrile in 48 m*M* phosphate buffer (pH 8.5). At acetonitrile concentrations below 33%, the separation of the derivatives was better than that shown in Fig. 2. A complete separation of the seven peptides was achieved with a mobile phase containing 31% acetonitrile in 46 min. Higher acetonitrile concentrations decreased the retention times of all derivatives. However, at 34% acetonitrile, the [Val^5]-ANG II or ANG II peak overlapped with peak 2

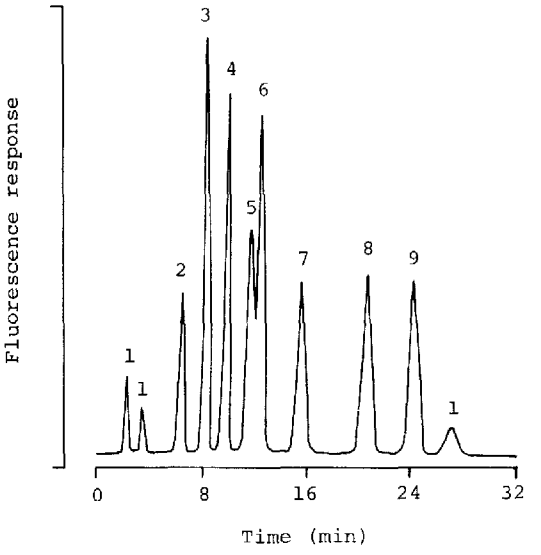

Fig. 2. Chromatogram of the benzoin derivatives of ANGs and related peptides added to ACE assay tube of the enzyme blank. ANGs I, II and III and their analogues (1 nmol each) were added, instead of the substrate of ANG I, to the incubation mixture with no incubation, and treated as described in the Experimental section. Peaks: 1 = reagent blank; 2 = endogenous substance in serum; 3 = [Val^5]-ANG II; 4 = ANG II; 5 = [Val^5]-ANG I; 6 = [Asn^1, Val^5]-ANG II; 7 = ANG I; 8 = [Asn^1, Val^5]-ANG I; 9 = ANG III.

in Fig. 2. Therefore, a concentration of 33% acetonitrile in the mobile phase was chosen for the assay. Peaks 1 in Fig. 2 were always detected, even when the benzoin reaction was carried out in the absence of peptide and serum. They were attributed to fluorescent compounds from benzoin itself. Peak 2 was formed only when serum was reacted with benzoin; it had the same retention time as the benzoin derivative of synthetic arginine, even when chromatographed under different conditions. The arginine content in human serum is relatively high: ca. 0.6 nmol in 5 μl of serum [13, 14]. Thus, peak 2 is due to an endogenous substance in human serum, having a guanidino moiety and probably due to arginine.

Calibration graphs for the synthetic peptides tested were all linear up to at least 10 nmol in the incubation mixture (105 μl). The lower limits of detection were in the range 0.66—2.4 pmol in the incubation mixture, which corresponds to amounts of approximately 80—300 fmol in a 100-μl injection volume, at a signal-to-noise ratio of 2. The endogenous ANGs I, II and III present in serum cannot be detected by the present HPLC method for ACE assay, since their amounts are ca. 0.25 fmol or less in 5 μl of human serum [15].

Determination of ACE activity in human serum

The chromatograms shown in Fig. 3 demonstrate that ANG II is apparently formed from ANG I by catalysis of ACE present in human serum. Under the conditions of the enzyme assay, ACE in serum was most active at pH 7.5 in 0.2 *M* phosphate buffer containing 30 m*M* sodium chloride. The concentration of ANG II formed, increased proportionally with increasing serum volume up

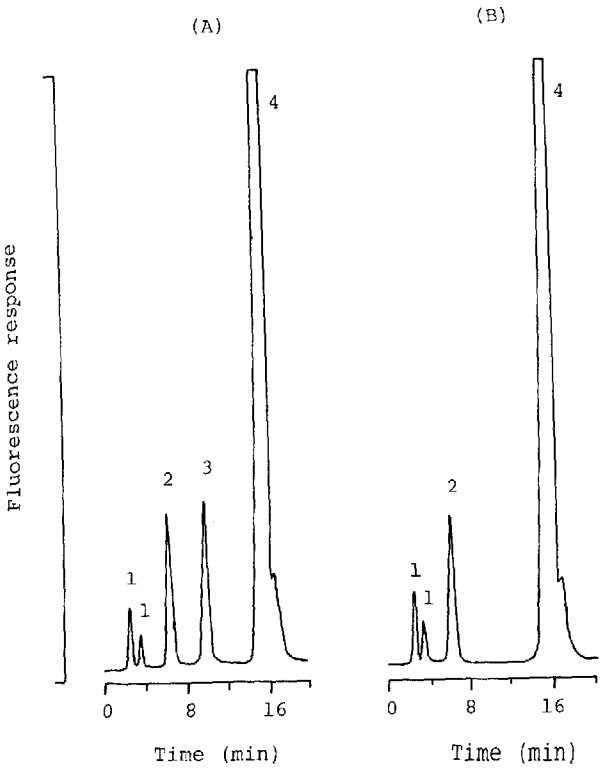

Fig. 3. Chromatograms showing (A) the ACE reaction of ANG I as the substrate and (B) enzyme blank without incubation. The conditions for the enzyme reaction and HPLC are described in the Experimental section. Peaks: 1 = reagent blank; 2 = endogenous substance in serum; 3 = ANG II; 4 = ANG I.

to at least 10 μl, and with incubation time up to 60 min at 37°C. The recovery of synthetic ANG II (1.0 nmol) added to the incubation mixture was 98.5 ± 2.6% (mean ± S.D., n = 5).

The minimum detectable activity of ACE was 10 pmol/min · ml serum, which corresponds to 750 fmol of ANG II formed in the incubation mixture under the conditions of the enzyme reaction with the 5-μl serum volume and 15-min incubation time. The amount of ANG II formed in the incubation mixture (Fig. 3) was 460 pmol. Therefore, ACE activity in as little as 5 μl of serum can readily be assayed by the present HPLC method. This serum size is the smallest reported so far for the assay of ACE activity in human serum. The standard deviation was 0.17 (n = 8) for a mean activity of 6.1 nmol/min · ml serum (coefficient of variation, 2.8%).

The enzyme reaction conditions are almost the same as those for the spectrofluorimetric method [6] described by Friedland and Silverstein, in which the amount of His-Leu produced from the substrate ANG I was determined by the fluorescence reaction with *o*-phthalaldehyde. The ACE activities in sera determined by the present HPLC method were compared with those determined by the spectrofluorimetric method [6] and also by the HPLC—UV method using hippuryl-His-Leu as the substrate [3] (Table I). The ACE activities, obtained

TABLE I

COMPARISON OF THE PRESENT METHOD WITH OTHER METHODS FOR ASSAY OF ACE ACTIVITY IN HUMAN SERUM

ACE activity* (nmol/min · ml)		
Present HPLC	Spectrofluorimetry [6]	HPLC—UV [3]
22.3	16.9	29.3
12.7	10.3	22.5
12.4	10.6	20.4
8.1	6.8	16.7
6.1	5.2	7.8

*The regression equations for the present method (x) against the spectrofluorimetric method (y_a) and the HPLC—UV method (y_b) are $y_a = 0.72x + 1.07$ ($r = 0.997$) and $y_b = 1.18x + 4.83$ ($r = 0.929$), respectively.

by the present HPLC spectrofluorimetric and HPLC—UV methods, represent the amounts of ANG II formed from ANG I, His-Leu formed from ANG I and hippuric acid formed from hippuryl-His-Leu, respectively. The values of the ACE activity obtained by our method agree with those obtained by the two other methods. However, the values obtained by the present HPLC method are consistently higher than those obtained by spectrofluorimetry, and lower than those by the HPLC—UV method. These discrepancies may attributed to methodological error, since the dipeptide His-Leu is further hydrolysed by peptidases in human serum during the incubation [4]. Conversely, hippuric acid is formed more rapidly from its substrate by ACE in human serum than is ANG II [6].

The proposed HPLC method permits the use of not only ANG I but also of its analogous peptides as ACE substrates. Fig. 4 shows the chromatograms of [Val^5]-ANG II and [Asn^1, Val^5]-ANG II produced enzymatically from the corresponding ANGs I, respectively, under the same conditions as those for ANG I. The rates of formation of these products by ACE in 5 μl of serum were 41 and 37 pmol/min for [Val^5]-ANG II and [Asn^1, Val^5]-ANG II, respectively. No additional degradation of the products, ANG II and [Val^5]-ANG II, occurred during incubation with serum for at least 60 min, but [Asn^1, Val^5]-ANG II was degraded at a rate of ca. 1.0%/min during the incubation time, as shown in Fig. 5. The degradation of [Asn^1, Val^5]-ANG II was not observed during the incubation time in the absence of serum. In addition, the inactivation of serum by heating at 100°C for 15 min did not result in degradation of this peptide. Therefore, [Asn^1, Val^5]-ANG II is believed to be degraded enzymatically by some peptidases such as non-specific amino- or carboxy-peptidases and/or angiotensinases [16] present in human serum. In the chromatograms of Fig. 4B and Fig. 5B and C, the height of peak 2 increased with the degradation of [Asn^1, Val^5]-ANG II. The fluorescence spectra of the eluate of peak 2, with excitation and emission maxima at 325 and 435 nm, respectively, were identical to those [10, 14] of arginine and arginine-containing peptides. Thus, the increase of peak 2 is believed to be due to arginine and/or arginine-containing peptide fragment(s), which were produced from

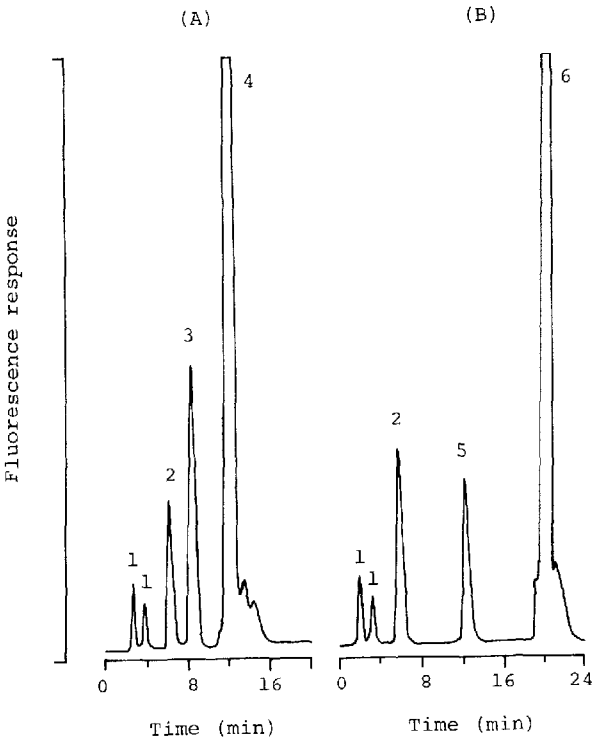

Fig. 4. Chromatograms illustrating the ACE reaction of (A) [Val^5]-ANG I or of (B) [Asn^1, Val^5]-ANG I as the substrate. Each peptide (16 nmol) was added to the incubation mixture, and treated as described in the Experimental section. Peaks: 1 = reagent blank; 2 = endogenous substance in serum and/or fragment(s) from [Asn^1, Val^5]-ANG II; 3 = [Val^5]-ANG II; 4 = [Val^5]-ANG I; 5 = [Asn^1, Val^5]-ANG II; 6 = [Asn^1, Val^5]-ANG I.

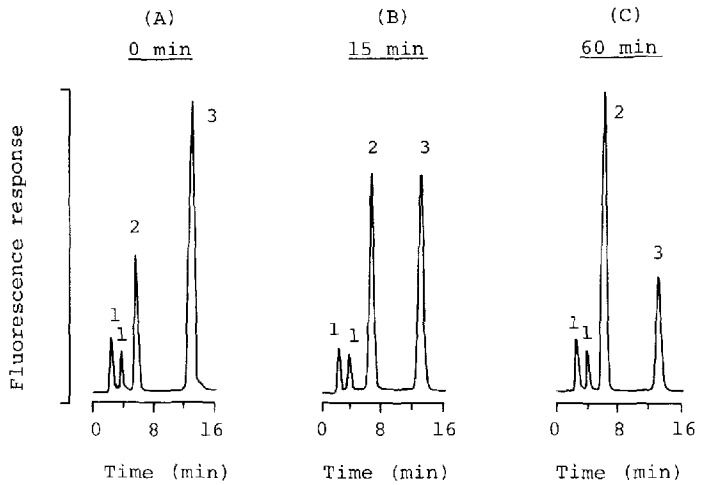

Fig. 5. Chromatograms showing the course of the enzyme reaction of [Asn^1, Val^5]-ANG II with serum. The peptide (1.0 nmol) was added instead of the substrate of ANG I to the incubation mixture, and the mixture was incubated for (A) 0 min, (B) 15 min and (C) 60 min. Other conditions are described in the Experimental section. Peaks: 1 = reagent blank; 2 = endogenous substance in serum and/or fragment(s) from [Asn^1, Val^5]-ANG II; 3 = [Asn^1, Val^5]-ANG II.

[Asn^1, Val^5]-ANG II. The chemical structure of the fragment(s) remains unknown. The above results also suggest that terminal aspartic acid of the product ANG II is an important amino acid residue for protection from degradation by peptidases present in human serum.

This study describes the first HPLC method with fluorescence detection for the assay of ACE in human serum. The proposed HPLC method is 200- or 400-fold more sensitive than conventional methods [3—6], but less sensitive than radioimmunoassay [7]. However, radioimmunoassay measures only one peptide in a single analysis and requires separation of quite similar peptides, depending on the specificity of the antibody. The pre-column derivatization with benzoin is selective for guanidino compounds, including arginine-containing peptides [10, 11, 13, 14]. The fluorescent derivatives of ANGs and related peptides can readily be separated from the various interfering substances in crude samples. The proposed HPLC method can be applied not only to enzyme assays in the renin—angiotensin system but also to investigation of enzymatic degradation of various ANGs in biological samples.

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