Enzyme Immunoassay of Angiotensin II in Human Plasma

Fumitaka YASUNAGA,* Ayumi KAWANO, Norifumi MORIKAWA and Masaharu TAKEYAMA

Department of Hospital Pharmacy, Medical College of Oita, Hasama-cho, Oita-gun, Oita 879-55, Japan. *Received October 2, 1991*

We established a highly sensitive double-antibody enzyme immunoassay (EIA) for angiotensin (ANG) II. For competitive reactions, the ANG II-antibody was incubated with ANG II standard (or sample) and β-D-galactosidase-labeled ANG III (delayed addition). Free and antibody-bound labeled antigen were separated using an anti-rabbit immunoglobulin G (IgG) coated immunoplate. The enzyme activity on the plate was fluorometrically determined. The present immunoassay allows detection of 0.4 to 72 fmol/well of ANG II. Using the present EIA, ANG II-like immunoreactivity (-LI) in human plasma was determined. The level of ANG II-LI in human plasma from 10 healthy volunteers was 33.3 ± 10.4 pmol/l.

Keywords angiotensin II-like immunoreactivity; human plasma; highly sensitive EIA; β-D-galactosidase-labeled antigen; fluorogenic substrate

The renin-angiotensin system (RAS) plays an important role in the regulation of blood pressure and extracellular volume. Within this system, the enzyme, renin, which is the starting point of the system, acts upon angiotensinogen to form antgiotensin (ANG) I. The decapeptide, ANG I, which is inactive, is then cleaved by ANG converting enzyme (ACE) to form active ANG II. The octapeptide, ANG II, is a potent vasoconstrictor and aldosterone secretagogue,[1] and its structure of equine was determined by Skeggs *et al.*[2] in 1957. The structure of human ANG II[3] was later demonstrated to be identical to that of equine ANG II.

Radioimmunoassay (RIA) has been widely used to establish the identity of ANG II in biological samples, but in terms of safety, sensitivity and ease of manipulation, the RIA method is still less than satisfactory.

In 1989, Jose *et al.*[4] reported an enzyme immunoassay (EIA) for ANG II using ANG II-antiserum-coated microplates and horseradish peroxidase-linked ANG II as a conjugated enzyme. However, they did not describe the EIA method in detail and their method had some shortcomings. In the present study, we wish to report a highly sensitive and specific EIA for ANG II using β-D-galactosidase-labeled ANG III as a marker antigen, a second antibody-coated immunoplate and 4-methylumbelliferyl β-D-galactopyranoside as fluorogenic substrate.

Materials and Methods

Materials ANG I, II and III and tetragastrin were purchased from Peptide Institute Inc. (Osaka, Japan). Synthetic porcine secretin,[5] neuromedin-C[6] and vasoactive intestinal polypeptide (VIP)[7] were kindly provided by Dr. H. Yajima. Bovine serum albumin (BSA), polyoxyethylene sorbitan monolaurate (Tween 20), *N*-(ε-maleimidocaproyloxy)succinimide (EMC-succinimide) and 4-methylumbelliferyl β-D-galactopyranoside (MUG) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). β-D-Galactosidase (β-Gal from *Escherichia coli*) and goat anti-rabbit immunoglobulin (IgG) were purchased from Boehringer Mannheim Corp. (Mannheim, Germany) and Organon Teknika Corp. (Westchester, PA, U.S.A.), respectively.

Antiserum to ANG II (AB619) was purchased from Chemicon International Inc. (El Segundo, CA, U.S.A.). The lyophilized ANG II-antiserum was reconstituted to 200 ml with an assay buffer (0.05 M phosphate buffer, pH 7.4, containing 0.5% BSA, 1 mM $MgCl_2$ and 250 KIU/ml aprotinin). All other chemicals were of analytical reagent grade.

TABLE I. Structures of Angiotensin Peptides

ANG I	(Human)	H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-OH
ANG II	(Human)	H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-OH
ANG III	(Human)	H-Arg-Val-Tyr-Ile-His-Pro-Phe-OH

Preparation of Plasma Extract Human plasma samples were obtained from 10 healthy volunteers. Blood was collected in a chilled tube containing 500 KIU/ml aprotinin and 1.2 mg/ml ethylenediaminetetraacetic acid (EDTA). After centrifugation (3000 rpm, 4 °C, 15 min), plasma was collected and stored at −40 °C until use. Human plasma (1 ml) was diluted fivefold by 4% acetic acid (AcOH), pH 4.0, and loaded on reversed phase C18 cartridges (Sep-pak C18, Waters Co. Inc., Milford, MA. U.S.A.). After washing with 4% AcOH (10 ml), the immunoreactive ANG II was eluted with 70% acetonitrile (MeCN) in 0.5% AcOH, pH 4.0 (2 ml). The eluates were concentrated by spin-vacuum evaporation, lyophilized, reconstituted to 100 μl with the assay buffer and subjected to EIA and to high performance liquid chromatography (HPLC) analysis, respectively. Recovery of this extraction procedure was 92 ± 10% ($n=6$).

Preparation of Enzyme-Labeled Antigen ANG III was conjugated with β-Gal by EMC-succinimide according to the method of Kitagawa *et al.*[8] ANG III (0.50 mg) in 0.05 M phosphate buffer, pH 7.0 (1 ml), was stirred with EMC-succinimide (0.55 mg) in tetrahydrofuran (50 μl) at 20 °C for 60 min. Then, obtained EMC-ANG III was purified with a Sephadex G-15 column (1.0 × 65 cm) using 0.05 M phosphate buffer, pH 7.0, as an eluate. Purified EMC-ANG III fractions were combined with β-Gal (1.30 mg) by stirring at 25 °C for 50 min. The β-Gal conjugate was then applied to a Sephacryl S-300 column (1.5 × 55 cm) and eluted with 0.05 M phosphate buffer, pH 7.0, containing 1 mM $MgCl_2$. β-Gal-labeled ANG III fractions were collected and stored at 4 °C after the addition of 0.2% BSA and 0.1% NaN_3.

Assay Procedure for ANG II For assay, the above-mentioned assay buffer and the washing buffer, 0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl and 0.05% Tween 20, were used. Second antibody-coated immunoplates were prepared as previously reported[9] using Microwell maxisorp F8 plates (Nunc, Roskilde, Denmark) and anti-rabbit IgG. A test tube containing 100 μl of the diluted solution of the ANG II-antiserum, AB619, and 100 μl of each sample solution or standard solution was incubated at 4 °C for 15 h and then 50 μl of the diluted solution of the enzyme-labeled antigen was added. The test tube was further incubated at 4 °C for 24 h. The antibody-antigen solution (100 μl) from each test tube was added to the second antibody-coated immunoplate. The plate was incubated at 20 °C for 5 h, then washed 4 times with the washing buffer. After washing, 0.1 mM MUG (200 μl) in a substrate buffer (0.05 M phosphate buffer, pH 7.0, containing 1 mM $MgCl_2$) was added to each well. The plate was again incubated at 37 °C for 2 h. The resulting fluorescence intensity (λ_{ex} 360 nm, λ_{em} 450 nm) of each well was measured with a MTP-100F microplate reader (Corona Electric, Ibaraki, Japan).

HPLC Analysis of Plasma HPLC was performed using a reversed phase C8 column (Wakosil 5C8, 4.6 × 250 mm, Wako Pure Chemical Industries Ltd., Osaka, Japan). The HPLC consisted of a model 610 dual pump system (Waters Co. Inc., Milford, MA, U.S.A.). An extract from human plasma (2 ml) by the Sep-pak C18 cartridge mentioned above was reconstituted to 100 μl with 20% methanol in 10 mM ammonium acetate buffer, pH 5.6, and applied to the column. The immunoreactive ANG II was eluted with a linear gradient of methanol (from 20% to 80% in 30 min)

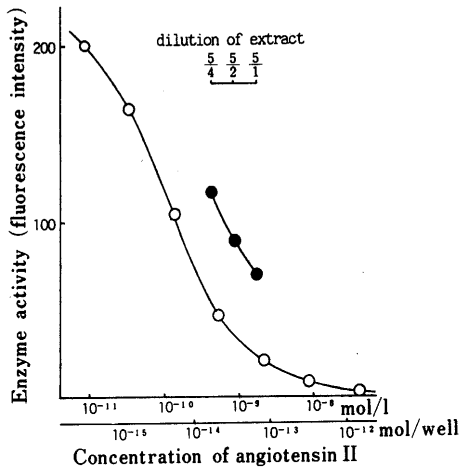

Fig. 1. Standard Curve of Synthetic ANG II and Dilution Curve of Plasma Extract Obtained with Antiserum AB619 by EIA

Synthetic ANG II (○), plasma extract (●).

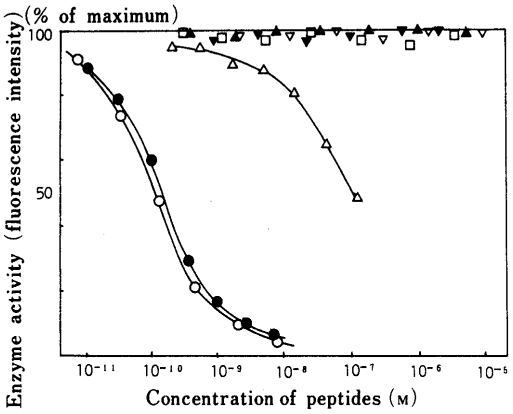

Fig. 2. Inhibition Curves of Various Peptides in EIA by Competition between β-D-Gal-Labeled ANG III and Various Peptides toward Antiserum AB619

ANG II (○), ANG III (●), ANG I (△), secretin (▲), neuromedin-C (▼), VIP (▽), tetragastrin (□).

in 10 mM ammonium acetate buffer, pH 5.6. The flow rate was 1 ml/min and the fraction size was 1 ml. The fractions were concentrated by spin-vacuum evaporation, lyophilized, and reconstituted to 100 μl with the assay buffer and subjected to EIA.

Results

Standard Curve for ANG II The typical calibration curve for this EIA of ANG II is shown in Fig. 1. A linear displacement of enzyme-labeled ANG III by synthetic ANG II was obtained, when plotted as a semilogarithmic function from 10 to 1800 pmol/l (0.4 to 72 fmol/well) of ANG II. The minimum amount of ANG II detectable by the present EIA system was 15 pmol/l (0.6 fmol/well). An intraassay variation of 5% and interassay variation of 9% were obtained at 110 pmol/l. Serial dilution of plasma extract showed a parallel displacement of enzyme-labeled ANG III to synthetic ANG II in this EIA.

Specificity of the Antiserum (AB619) Immunospecificity of the antiserum (AB619) was examined by EIA using β-Gal-labeled ANG III. The displacement curves of ANG peptides are shown in Fig. 2. ANG III showed approximately 80% of cross-reactivity as compared to synthetic ANG II. ANG I also inhibited the binding of β-Gal-labeled ANG III with the ANG II-antibody, but exhibited only 0.1% of cross-reactivity. Secretin, VIP, tetragastrin and neuromedin-C hardly inhibited the binding of β-Gal-labeled ANG III with the ANG II-antibody.

TABLE II. Levels of ANG II-LI in Human Plasma

Subject	Age (years)	Sex	ANG II-LI (pmol/l)
1	23	F	32.2
2	30	M	21.7
3	25	F	27.4
4	44	M	41.5
5	38	M	47.5
6	35	M	51.7
7	62	M	33.9
8	39	M	26.7
9	14	M	22.4
10	25	F	27.6
Mean ± S.D.			33.3 ± 10.4

M, male; F, female.

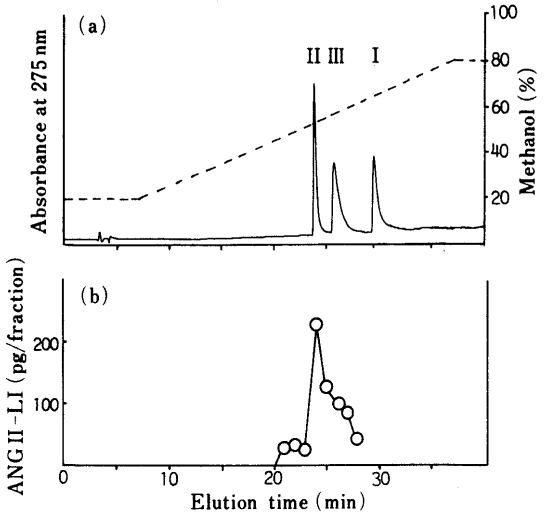

Fig. 3. Reverse Phase HPLC Profile of Plasma Extract on a C8 Column

(a) Chromatograph of ANG standards monitored at 275 nm. (b) Immunoreactive profile of human plasma (2 ml). ANG II-LI (○), Extracted plasma sample was reconstituted in 20% methanol in 10 mM ammonium acetate buffer, pH 5.6 and injected onto HPLC. The methanol gradient is indicated by the dotted line. The HPLC conditions are described in the Methods section.

Measurement of ANG II-LI in Human Plasma by EIA The proposed EIA was applied to the determination of ANGII-LI in human plasma samples from 10 volunteers (7 men and 3 women, 14 to 62 years old). Table II shows the concentrations of ANG II-LI in human plasma; the mean was 33.3 ± 10.4 pmol/l.

HPLC Analysis of Plasma Human plasma extract was subjected to reversed phase HPLC in order to study the molecular variants of ANG II-LI present in human plasma. Extract from human plasma by a Sep-pak C18 cartridge was applied to reversed phase HPLC on a C8 column. The elution profile of ANG II-LI in human plasma is shown in Fig. 3. ANG II-LI was eluted as one major peak at the elution position of standard synthetic ANG II with a shoulder at that of standard synthetic ANG III.

Discussion

Using β-Gal-labeled ANG III as a marker antigen, an anti-rabbit IgG coated immunoplate and MUG as a fluorogenic enzyme substrate, we developed a highly

sensitive EIA for the quantitation of ANG II. As a marker antigen, we used the β-Gal-labeled ANG III rather than the β-Gal-labeled ANG II, because the former was about twice as sensitive than the latter.

Although RIA for ANG II has been widely used, it has some disadvantages: 1) A γ-counter is expensive and has to be used in a radioisotope room. 2) The radioligand has a short half life. 3) Radioisotopes may be health hazards. Jose *et al.*[4] recently reported an EIA of ANG II which would retain the advantages of RIA systems and minimize the disadvantages. They described a saturation sequential EIA in microplates by a two-step procedure, but they showed no standard curve and inter- and intra-assay variations of high value. The EIA reported here, using β-Gal-labeled antigen, second antibody-coated immunoplate and MUG as a fluorogenic substrate, was highly sensitive for the quantitation of ANG II, and a sharp standard inhibition curve was obtained. In addition, β-Gal-labeled antigen is stable in aqueous form at 4 °C for at least one year, unlike the iodinated form which must be made and purified once a month. The use of second antibody coated immunoplate makes the assay procedure quite simple. By an extraction procedure with Sep-pak C18 column from human plasma (1 ml) combined with this EIA, the present method made it possible to determine ANG II levels in human plasma, that is to say, the minimal detectable quantity in this method was as little as 1.5 pmol/l. The sensitivity was higher than the EIA method for ANG II reported by Jose *et al.*[4]

The levels of ANG II-LI in plasma from 10 healthy volunteers reported here were 3.3 ± 10.4 pmol/l.

The molecular heterogenicity of ANG II-LI in human plasma was also examined by reversed phase HPLC. The main peak of ANG II-LI in plasma was eluted at the same elution volume of synthetic ANG II with a shoulder at the same elution volume of synthetic ANG III, and we detected a minor peak eluted earlier at a position of ANG II, which is thought to possibly be an ANG III metabolite.

The ANG II-antiserum recognizes not only ANG II but also ANG III and the other ANG metabolite, so that by using an HPLC system combined with this simple and sensitive EIA for ANG II, we can measure the precise levels of ANG II.

Thus, this simple and sensitive EIA for ANG II should be useful for clinical research and also for studies in animal research.

References

1) M. J. Peach, *Physiol. Rev.*, **57**, 313 (1977).
2) L. T. Skeggs, J. R. Kahn, K. Lentz, and N. P. Shumway, *J. Exp. Med.*, **106**, 439 (1957).
3) K. Arakawa, M. Nakatani, A. Minohara, and M. Nakamura, *Biochem. J.*, **104**, 900 (1967).
4) M. L. Jose, R. Armando, R. Maximiliano, and M. Miguel, *Med. Sci. Res.*, **17**, 1043 (1989).
5) S. Kiyama, K. Kitagawa, T. Akita, W. Y. Chey, A. Ayalp, A. Otsuki, N. Fujii, S. Funakoshi, and H. Yajima, *Chem. Pharm. Bull.*, **33**, 3205 (1985).
6) N. Fujii, S. Futaki, K. Akaji, H. Yajima, A. Inoue, and T. Segawa, *Chem. Pharm. Bull.*, **33**, 3731 (1985).
7) M. Takeyama, K. Koyama, K. Inoue, T. Kawano, T. Tobe, and H. Yajima, *Chem. Pharm. Bull.*, **28**, 1873 (1980).
8) T. Kitagawa, T. Shimozono, T. Aikawa, T. Yoshida, and H. Nishimura, *Chem. Pharm. Bull.*, **29**, 1130 (1981).
9) M. Takeyama, K. Kondo, Y. Hayashi, and H. Yajima, *Int. J. Pept. Prot. Res.*, **34**, 70 (1989).