Measurement of Plasma Angiotensin II by Radioimmunoassay

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FUKUCHI, S. and KATSUSHIMA, I. *Measurement of Plasma Angiotensin II by Radioimmunoassay*. Tohoku J. exp. Med., 1970, 103 (2), 185-193 — A radioimmunoassay for determining angiotensin II in plasma has been developed using antibodies produced in rabbits immunized with porcine-gamma-globulin/angiotensin II-amide complex. The method can detect 10 pg of angiotensin II, and is not influenced significantly by angiotensin I. The mean level of angiotensin II in subjects with normal blood pressure and no significant abnormality in water and electrolyte balance, was 53 pg per ml. ——— angiotensin II; radioimmunoassay

Renin, secreted by the juxtaglomerular apparatus of the kidney, acts on a plasma substrate with the release of the decapeptide angiotensin I. This is then cleaved by a converting enzyme in plasma to give the physiologically active octapeptide angiotensin II. Available evidence suggests that angiotensin II is concerned with the control of aldosterone secretion and plays a major role in the regulation of body fluid and electrolytes in health and disease. However, study of renin-angiotensin system has been hampered by the lack of a convenient method of sufficient sensitivity and specificity to permit precise measurement of angiotensin II. So a number of investigators have attempted to apply the technique of radioimmunoassay to this problem. Radioimmunoassay is a more sensitive and specific method than bioassay and capable of measuring angiotensin II from a relatively small amount of plasma.

In this report the details of a simple, rapid radioimmunoassay capable of measuring angiotensin II extracted from 2 ml of human plasma are given. In addition, the abnormalities observed in various hypertension and in certain other conditions are discussed.

METHODS

Angiotensin antibodies. Synthetic angiotensin II amide was coupled to porcine gamma globulin by the carbodiimide-condensation reaction as described by Goodfriend *et al.* (1964). Aspartyl-1-valyl-5-angiotensin II amide (20 mg) was combined with porcine gamma globulin (10 mg) and dissolved in 0.5 ml distilled water. Two hundred mg of CDI reagent (1-ethyl-3 (3-dimethylaminopropyl) carbodiimide hydrochloride) in 0.25 ml distilled water was added and the mixture was stirred at room temperature for 30 minutes. The mixture was dialyzed in No. 8 Visking tubing against 2 l of distilled water at 4°C for 24 hrs, with one change of the dialysis fluid. The resulting complexes were

emulsified in complete Freund's adjuvant and administered to the toe-pads of rabbits followed by intramuscular injections at intervals of 2 weeks.

Preparation of tracer ^{131}I -angiotensin II amide: Iodination of angiotensin II amide was performed by the Chloramine T method of Hunter and Greenwood (1962). Ten μl (containing 5 μg) of synthetic aspartyl-1-valyl-5-angiotensin II amide was added to 20 μl of 0.05 M phosphate buffer, pH 7.4, in a small test tube. Two to 3 mc of carrier-free ^{131}I was transferred with a microsyringe to the test tube. Then, 25 μl of the oxidizing agent Chloramine T (0.8 mg/ml in 0.05 M phosphate buffer) was added, and the solution was mixed quickly. After 45 to 60 seconds, 50 μl of sodium metabisulfite (1.2 mg/ml in 0.05 M phosphate buffer) was added to stop the reaction. To protect the angiotensin from excess radiation damage or loss on the glassware, 10 μl normal human serum albumin (5.0%) was then added, which was then diluted with 300 μl of phosphate buffer. The iodination mixture was applied to a small (70 $\times$ 5 mm) column of Amberlite CG-400 (Rohm & Haas), equilibrated with 0.05 M sodium acetate/HCl, pH 5.0, and eluted with the same buffer. Each three drops of unabsorbed radioactivity was collected into a test tube containing 1.0 ml of 0.5% bovine serum albumin in 0.05 M phosphate buffer, pH 7.4 (BSA diluent) and stored frozen.

Extraction of angiotensin from plasma: Blood samples were collected with a syringe containing 0.2 ml of 8% EDTA disodium and promptly chilled in ice. The plasma was removed, aliquoted, and stored at -20°C until analyzed. No plasma sample was stored longer than 1 week during which no detectable decrease in angiotensin level was observed.

Plasma samples were thawed on ice and the pH of plasma was adjusted to 2.0 with 2 N HCl after the addition of 2 drops of 5% DFP. Then 2 ml of the plasma were pipetted into a centrifuge tube containing equal volume of distilled water. The sample was then heated in a boiling water bath for 5–10 min, cooled and centrifuged at 3,000 rpm for 10 min. The supernatant was transferred to another test tube, the precipitate was washed with 2 ml of distilled water and the mixture was centrifuged to collect the supernatant. The last precipitate was discarded.

The collected supernatant was applied on a column (0.5 $\times$ 1.0 cm) of Dowex 50W-X4 (100–200 mesh, H^+ form) at room temperature.

Before application to column one hundred grams of Dowex 50W-X4 was washed with distilled water followed with 2 l of 2 N NaOH, 2 l of distilled water, 2 l of 2 N hydrochloric acid and finally with 2 l of distilled water. The washed Dowex 50W-X4 was stored in distilled water at 4°C until the day of use.

The column was washed with 2 ml of 0.2 M acetate buffer, pH 6.0. Then the column was washed with 3.0 ml of distilled water. These effluents were discarded. Finally, angiotensin was eluted from the column with 1.5 ml of 0.025 N NaOH into a small graduated tube containing 1 drop of 0.5 N HCl. The eluate was adjusted to pH 7.4, of which 0.5 ml was diluted with the same volume of 1.0 % BSA diluent. The eluate was stored in a deep-freezer until the radioimmunoassay.

Radioimmunoassay: This was performed in small glass tubes. To each tube were added 1 ml of unknown or standard angiotensin II amide in BSA diluent, 0.1 ml of ^{131}I -tracer angiotensin II amide in BSA diluent (about 5,000 cpm), and 0.1 ml of antiserum diluted with BSA diluent (1:10,000–100,000).

This gave a total volume of 1.2 ml in each tube. The tubes were shaken and stored at 4°C for 18 hours. Next morning 0.1 ml of dextran-coated charcoal were added to each of the tubes which were then shaken for 25 min and centrifuged at 3,000 rpm for 3 min. The dextran-coated charcoal was diluted 10 times by BSA diluent from the stock solution which was previously made by mixing of equal volume of 0.5% dextran 80 and 5% charcoal.

The supernatant was discarded and the precipitate was counted in a well-type scintillation counter. The figure of 'percentage free radioactivity' was used as a quantitative

indicator of the standard or unknown samples of angiotensin which reduce the binding of ^{125}I tracer angiotensin II amide to its antibody in proportion to the amount of unlabeled angiotensin present. Since the dextran-coated charcoal binds 'free' angiotensin but not 'antibody-bound' angiotensin, the charcoal count or 'percentage free radioactivity' rises with the addition of increasing quantities of angiotensin (Fig. 1).

Plasma angiotensin levels in various hypertension and normal controls: Blood samples were obtained in the early morning from fasting recumbent subjects, who were fed on unrestricted diet with no special medications which might have influence on body water or electrolyte balance.

RESULTS

Production of antibodies. Antibodies to angiotensin were detected about 3 months and became to be useful in radioimmunoassay 6 to 10 months after the first injection, with a final antiserum concentration of 1:10,000 to 100,000. The usual dilution of the antiserum was 1: 20,000, giving a sensitivity of 10 pg for each assay. Further injections of angiotensin II amide-porcine gamma globulin did not produce any rise in anti-angiotensin titer (Fig. 2).

Fig. 1. Standard curve of angiotensin by radioimmunoassay. ^{125}I -angiotensin II and various amounts of angiotensin II standards with 1/20,000 antiserum.

Fig. 2. Titer of anti-angiotensin serum (1: 10,000). Changes in the titer of antiserum from a rabbit immunized with angiotensin II amide/porcine gamma globulin complex.

^{131}I -angiotensin. The ^{131}I tracer angiotensin II amide could be used as long as 10 days after iodination, if stored in deep freezer. The mean specific activity of the iodinated angiotensin was approximately 200 mc/mg.

Specificity. The high specificity of this immunoassay was demonstrated by the fact that large excess of bradykinin, vasopressin and purified renin substrate did not displace labeled angiotensin from antibody. Experiments were also carried out to examine the possibility that degradation products of angiotensin II present in the plasma or formed during the incubation period might contribute significant interference in the system. A known amount of standard angiotensin II (100 pg) was added to heparinized plasma and incubated at 37°C and pH 7.4 to allow degradation of the octapeptide by angiotensin activity. It was found by radioimmunoassay that more than 50% of the angiotensin II was destroyed by only 30 minutes incubation. These results suggest that no significant interference with the immunoassay by degradation products occurred.

Evidence for the specificity of the procedure was also obtained by determining the inhibitory effect of four different angiotensins on the binding of ^{131}I -angiotensin amide by an antiserum. The standard inhibition curves obtained with angiotensin amide, synthetic asp-1-val-5-angiotensin II, and asp-1-leu-5-angiotensin II were similar, whereas about 100 times more natural angiotensin I was required to yield a comparable degree of inhibition (Fig. 3).

Sensitivity. The lower limit of sensitivity of the assay was designed by the amount of labeled peptide used. Namely using 5 pg of labelled peptide, it was possible to detect about 10 pg. Sensitivity may easily be increased by extraction of larger plasma volumes.

Fig. 3. Specificity of the assay; Various amounts of three kinds of peptides were incubated with the same amounts of ^{125}I -angiotensin II amide and antiserum.

TABLE 1. Reproducibility of plasma angiotensin II measurement

Date of assay	Reproducibility (pg/ml)
2/8	90
2/11	98
2/12	112
2/14	104
2/16	96

Range 90-112

Mean =100

S.D. = ± 7.5

The blood sample from a subject was divided into aliquots and assayed consecutively as a measure of reproducibility.

Recoveries and reproducibility. Recovery rate was tested by addition of known amounts of angiotensin II amide to the plasma of a patient with primary aldosteronism, previously dialyzed overnight. The mean and standard deviation was $87.0 \pm 7.6\%$. Table 1 shows the results of 5 repeated measurements on the same plasma sample. Estimated values ranged from 90 to 112 (mean 100 ± 7.5) pg/ml.

Fig. 4. Effect of plasma protein on the standard curve. The presence of 20% dialyzed plasma in the incubation mixture resulted in some interference with binding of antigen and antibody.

Factors affecting the standard curve. It was observed that the presence of serum protein in the incubation mixture exerted a significant influence on the binding of angiotensin II by its homologous antibody. Fig. 4 represents an experiment in which standard curves were set up in BSA diluent containing 20% of dialyzed plasma.

The presence of dialyzed pooled plasma resulted in some interference with binding of antigen and antibody, and greater amounts of plasma (40 to 50%) significantly interfered with the reaction. Marked decrease was induced in the sensitivity, if unextracted plasma was used to the radioimmunoassay. Therefore, protein-free angiotensin extracted from plasma was finally used in the immunoassay.

Inhibition of angiotensinase activity. Inhibitory effects of pH, EDTA and DFP against angiotensinase activity were studied from the recovery rate of angiotensin II, which had been added to the plasma at the blood sampling. The angiotensinase activity was inhibited most effectively with addition of EDTA disodium at the sampling and of DFP before Dowex column chromatography.

Fig. 5. Measurements of plasma angiotensin II in arterial blood of normal subjects and patients with various kinds of diseases.

Clinical observations (Fig. 5). Fig. 5 presents the plasma values drawn in the morning for 20 normal subjects aged 22 to 60 years, 11 of whom were male. For this group angiotensin concentrations ranged from 18 to 106 pg/ml, average 53.2 pg/ml, with a standard deviation of 29.3.

Plasma angiotensin levels in 12 of 13 cases of essential hypertension were within normal range, and in only one of them, slightly elevated. In most of 16 cases of chronic glomerulonephritis angiotensin was within normal range. In 6 of 10 cases of renovascular hypertension angiotensin was elevated to 180–700 pg/ml, but was within normal limits in remaining 4 cases. In contrast, angiotensin in 11 cases of primary aldosteronism was suppressed to subnormal levels. In 5 cases of Cushing's syndrome and 2 of pheochromocytoma, angiotensin was relatively decreased. In one of 2 cases of Addison's disease, angiotensin showed normal range and in the remaining one, slightly elevated.

In three subjects angiotensin levels were determined in simultaneously drawn venous and arterial blood samples. In every subjects the arterial values were slightly higher than the venous values.

DISCUSSION

The main advantages of the radioimmunoassay procedure described for angiotensin II, as compared with bioassay method, are its specificity, sensitivity and simplicity.

It has previously been expected that the peptide of molecular weight 1,031 was unlikely to be antigenic. A number of methods have been explored in attempts to enhance the antigenicity of small polypeptides. The compounds have

been injected alone in adjuvant (Klein *et al.* 1966), coupled to protein (Goodfriend *et al.* 1964, Deodhar 1960) and to polyamino acid carriers, (Haber *et al.* 1965) and adsorbed to charcoal (Boyd and Peart 1968). Angiotensin/porcine-gamma-globulin complex has, in our hands, elicited the formation of satisfactory antibody in a high proportion of animals immunized. Antibodies produced in this way have been shown to cross-react with native angiotensin II, and a radioimmunoassay, based on such antibodies, has been used to determine angiotensin levels in human plasma.

Other small peptides of similar molecular weight, vasopressin, bradykinin and ACTH, did not interfere with the reaction of antiangiotensin II antibody with angiotensin II. Furthermore, interference by breakdown products of angiotensin II was undetectable. Gocke *et al.* (1969) reported that, even when extensive degradation of a large amount of angiotensin II by angiotensinase activity was allowed to occur, measurement of remaining angiotensin II by the immunoassay was in very close agreement with the measurement made by bioassay.

Another consideration in validation of this assay was demonstration of reproducibility. The data presented in Table 1 reveal that while there was some variation in measurements on aliquots of the same sample from day to day, the possible error was within acceptable limits relative to the magnitude of changes encountered in pathological states.

The recovery of known amounts of angiotensin in unextracted plasma was usually above 100% as shown in Fig. 3. The recovery suggested some interference effects of the dialyzed plasma to the observed concentration of angiotensin II. Therefore, the method by the extraction from plasma was thought to be most suitable for the immunoassay. Gocke *et al.* (1969) assayed angiotensin of plasma without extraction and compared with results against a standard curve prepared with the same amount of dialyzed pooled plasma. Angiotensin concentrations ranged from 20 to 200 pg/ml measured by this method.

Page *et al.* (1969) used columns of Dowex 50W-X2 (NH_4^+) for the extraction of angiotensin from plasma and reported relatively low average of 21 ± 10 pg/ml for normal subjects. Boyd *et al.* (1967) extracted from plasma onto 10 mg of Fuller's earth, of which recoveries ranged from 50 to 68% with a mean value of 56%. The plasma levels of angiotensin II in normal subjects ranged from less than 8 to 56 pg/ml. Catt *et al.* (1967) used the cation exchange resin, SE-Sephadex for the extraction of angiotensin II from arterial blood, with the recovery rate of 65–70% and reported normal average of 21 pg/ml. The cause of the difference among the results of these methods is thought to be depending on the method of angiotensin extraction, and not on the quality of antibody. The present method showed the highest recovery rate of 87.0% among these extraction methods and also relatively high normal average of 53.2 pg/ml.

The use of dextran-coated charcoal to separate antibody-bound and antibody-unbound (free) angiotensin in the incubation tubes has added the important factors of rapidity and simplicity.

The biologically effective level of a circulating hormone is usually best determined by measurement of its arterial concentration, representing the concentration at which the hormone is presented to its target tissue *via* the blood stream. Venous blood samples may be equally useful if the arteriovenous difference of hormone concentration is negligible, and the assay procedure is shown to be specific in both arterial and venous blood for the hormone to be measured. While venous estimations are widely used due to the convenience of blood sampling, arterial blood levels are particularly important where metabolism of the hormone to be measured may render levels fallacious. In the present experiments, showing higher values for angiotensin than venous blood, arterial blood was used for the assay.

This simple radioimmunoassay procedure for angiotensin II will allow estimations to be performed on quite large numbers of samples within each assay and so will be easily applicable in ordinary clinical laboratories.

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