A SENSITIVE RADIOIMMUNOASSAY FOR THE DETERMINATION OF PLASMA ANGIOTENSIN II IN HUMAN SUBJECTS

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A sensitive and specific radioimmunoassay for the determination of plasma angiotensin II was developed by using the antisera against synthetic angiotensin II in combination with labeled angiotensin II. This assay employs an acetone extraction procedure and detects as little as 0.8 pg per tube of angiotensin II.

The mean (±S.E.) plasma angiotensin II concentration in 19 normal subjects was 14.4 ± 1.8 pg/ml in a state of overnight fasting and recumbency. In 13 normal subjects, in whom 40 mg of furosemide was injected intravenously, plasma angiotensin II concentration before and after 30 and 120 minutes in an upright position was 14.6 ± 2.2, 56.6 ± 5.7 and 74.3 ± 9.0 pg/ml, respectively. In 6 normal subjects, an infusion of isotonic saline, angiotensin II concentration reduced from 14.1 ± 3.7 to 9.2 ± 1.7 pg/ml.

Thus, it was ascertained that the simplified radioimmunoassay method reported here using an acetone-petroleum ether extraction method was specific and highly sensitive.

ANGIOTENSIN II, an octapeptide with a molecular weight of approximately 1,000, is the vasoactive substance of the renin-angiotensin system. It is useful to determine angiotensin II concentration in circulating human plasma under various physiological and pathological conditions, though many problems regarding the role of angiotensin II in circulating human plasma as compared with plasma renin activities remain unresolved. Thus, several radioimmunoassay systems for measuring angiotensin II have been reported recently1–14. We developed a more sensitive and specific radioimmunoassay for angiotensin II by a simplified extraction procedure using an acetone-petroleum ether method15. In this report the details of this radioimmunoassay system capable of measuring angiotensin II in extracts of a small volume of plasma were described.

METHODS

Preparation of antisera. Antisera to angiotensin II were prepared by the immunization of rabbits with a conjugation of synthetic angiotensin II and porcine gamma-globulin16. The antiserum used was obtained from the Teikoku Zoki Co., Tokyo, Japan.

Iodination and purification of 125I-labeled angiotensin II. Labeling of synthetic angiotensin II (Peptide Institute Protein Research Foundation, Osaka, Japan) with 125I was performed by a modification of the chloramine-T method of Greenwood, Hunter and Glover17. The reaction mixture was applied to a 0.7 by 20 cm column chromatography of superfine Sephadex G-25. The labeled angiotensin II was eluted from the column with 0.01 M acetic acid containing 0.5 per cent bovine serum albumin (BSA). Each one

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Fig. 1. Purification of iodinated angiotensin II. The distribution of total radioactivity (●-●) and radioactivity precipitable with excess antibody (open column) in the eluant fraction from the Sephadex column.

Fig. 2. Dose-response curves of angiotensin II, angiotensin I, angiotensin III, arginine vasopressin and bradykinin.

milliliter fraction was collected and measured for radioactivity. Undamaged, labeled angiotensin II was identified by its ability to bind to the antibody.

Radioimmunoassay procedure. Varying amounts of unlabeled angiotensin II (Peptide Institute Protein Research Foundation, Osaka, Japan) or plasma extracts were placed into the assay tubes, and an appropriate amounts of 0.01 M phosphate and 0.14 M sodium chloride, pH 7.0 (Phosphate buffer saline, PBS), including one per cent egg albumin was added to yield a volume of 200 μl. The antiserum was diluted to 1 : 320,000 with 0.05 M EDTA in PBS. One hundred microliters of the diluted antiserum, an amount capable of binding 20 to 25 per cent of $^{125}$I-angiotensin II, was added to each tube. The tubes were incubated at 4°C for 12 hours, and
then 0.8 to 1.0 pg of $^{125}$I-angiotensin II (equivalent to approximately 2,000 cpm) in 100 μl of PBS with one per cent egg albumin was added. The final incubation volume was 400 μl. The incubation was continued for an additional 48 hours at 4°C, then the separation of free from antibody-bound hapten was obtained by the polyethylene glycol (PEG) method. After 1.0 per cent of bovine gamma-globulin and 25 per cent PEG in PBS were added to the tubes and mixed, the tubes were centrifuged at 3,000rpm for 30 minutes at 4°C, and the supernatant was carefully decanted, and the radioactivity in the precipitates was counted by an Packard Autogamma Spectrometer.

The tubes containing the assay buffer were assigned as the control tubes. The counts of the precipitates were expressed as per cent of the control tubes.

**Sampling in normal subjects.** In 13 normal subjects (age; 17 to 47 years), furosemide test in upright position was performed. Basal blood samples were obtained after overnight fasting and recumbency. Forty milligrams of furosemide were then injected intravenously and the subjects were requested to stand for 120 minutes. At 30 and 120 minutes after the injection and standing, blood samples were obtained. Isotonic saline (0.9 per cent) infusion test was carried out in 6 normal subjects (age; 17 to 68 years). After overnight fasting and recumbency, isotonic saline was infused at a constant rate of 500 ml per hour for 3 hours. Blood samples were obtained immediately prior to and 3 hours after the beginning of the injection.

Blood was placed into iced tubes containing EDTA (EDTA-2Na 1 mg per blood 1 ml). The plasma was quickly separated by centrifugation at 4°C and subjected to extraction or stored at −20°C.

**Extraction of angiotensin II from plasma.** Extraction procedure with cold acetone by the method reported previously was employed in this study. Four hundred microliters of plasma were mixed with 800 μl of cold acetone. The mixture was centrifuged at 3,000 rpm for 20
minutes: the supernatant was decanted and thoroughly mixed with 1.5 ml of the assay diluent and subjected to the assay.

In order to investigate the stability of EDTA treated plasma, 4 plasma samples were extracted immediately, and the remainder were extracted after 20 and 40 hours at room temperature to determine the angiotensin II levels. In addition, angiotensin II was measured from two plasma samples which were extracted immediately, and after 40 hours placed at $-20^\circ$C.

Chromatographic study. In order to investigate whether the substance which interferes nonspecifically with this radioimmunoassay system are present in the plasma samples, synthetic angiotensin II, whole plasma, and acetone extracted plasma were applied to 1.0 by 20 cm column chromatography of superfine Sephadex G-25 and eluted with 0.05 M phosphate buffer, pH 7.4. Each one milliliter fraction was collected and angiotensin II concentration was measured using this system.

Plasma renin activity. Plasma renin activity was measured by the radioimmunoassay method of Haber, et al. which is commercially available as a CIS angiotensin I radioimmunoassay kit.

Statistical analysis. Statistical analysis was performed with Student's t-test for unpaired data.

RESULTS

Antiserum. The antiserum was bound to 50 per cent of the added radioactive angiotensin II at a dilution of 320,000. In this assay system, we used the antiserum at a final dilution of 1:1,280,000 in which $^{125}$I-angiotensin II was bound at a rate of 20 to 25 per cent.

Radioiodination. Fig. 1 shows the distribution of total radioactivity and radioactivity which was precipitable with excess antibody in the eluant fractions from the Sephadex column. Two major peaks and one minor peak of radioactivity were observed in the eluant fractions from a short column chromatography of superfine Sephadex G-25. More than 80 per cent of the iodinated hormone was precipitable with a high concentration of the antiserum in the third peak. The radioactivity precipitated in the blank tubes,
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samples in 3 different assays where mean plasma angiotensin II concentration were 3.33 ± 0.24 pg/tube (mean ± S.D.), 5.39 ± 0.18 and 8.87 ± 0.54, respectively. It ranged from 3.3 to 7.2 per cent. Between-assay was evaluated by using the same two plasma samples in 4 separated assays where mean plasma angiotensin II concentrations were 3.59 ± 0.29 pg/tube (mean ± S.D.) and 6.21 ± 0.49. The coefficient of variation was calculated to be 8.0 per cent.

**Recovery.** When physiologic amounts of synthetic angiotensin II were added to angiotensin II-free plasma and the extraction and assay procedure was carried out, the recoveries were 76.8 ± 5.1 per cent (mean ± S.E.). The recoveries of $^{125}$I-angiotensin II carried through the extraction procedure were 81.5 ± 1.0 per cent.

**Effect of temperature.** Fig. 3 shows the per cent change of plasma angiotensin II after 20 and 40 hours at room temperature, and after 40 hours at −20°C. The values were 293.3 ± 84.9 (mean ± S.E.), 469.8 ± 122.5 and 112.2 ± 0.7 per cent, respectively. Thus, angiotensin II at room temperature increased remarkably, while no significant change was observed at −20°C after 40 hours.

**Chromatographic study.** As shown in Fig. 4, chromatographic studies revealed a single and sharp peak of synthetic angiotensin II (in the upper panel). When the whole plasma was applied to the same column, immunoreactivity showed two fractions (middle panel). The second and major peak, which was coincident with the peak of synthetic angiotensin II, represented endogenous angiotensin II. Plasma extracted with acetone showed a single peak and eliminated the first fraction of the whole plasma.

**Plasma angiotensin II concentration in normal subjects.** The mean angiotensin II concentration in 19 normal subjects was 14.4 ± 1.8 pg/ml (mean ± S.E. ranged from 5.0 to 28.1 pg/ml) in the state of overnight fasting and recumbency. The values were not corrected for the losses during the extraction procedure. The results of furosemide test in an upright position are shown in Fig. 5. The values of plasma angiotensin II concentration before and after 30 and 120 minutes was 14.6 ± 2.2, 56.6 ± 5.7 and 74.3 ± 9.0 pg/ml, respectively, while those of plasma renin activity was 924 ± 139, 4,804 ± 700 and 5,965 ± 820 pg/ml/hr, respectively. Fig. 6 shows the changes of angiotensin II concentration and of plasma renin activity 180 minutes after saline infusion. The concentration of angiotensin II

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i.e. tubes containing no antibody, did not exceed 3 per cent. Thus, the first two peaks represented aggregates and free iodine, and the third, labeled angiotensin II.

Specific activity was 1500 μCi per microgram after purification on Sephadex G-25. When labeled angiotensin II was stored at −20°C, it was stable for as long as 2 months.

**Specificity.** A series of dose-response curves of various peptides are shown in Fig. 2. Angiotensin II exhibited a good dose-response curve, while there was no significant cross-reactivity of angiotensin I and III, bradykinin (Peptide Institute Protein Research Foundation, Osaka, Japan), and arginine vasopressin (Sigma Chemical Company) with the angiotensin II antiserum. The dose-response curve plotted using the extracts of angiotensin II-free plasma (Florisil treated plasma), in which angiotensin II in different amounts was previously added, was in parallel with that of angiotensin II.

**Sensitivity.** At 90 per cent of the buffer control tubes, the assay showed a detection of as little as 0.8 pg of synthetic angiotensin II.

**Reproducibility.** Within-assay coefficient of variation was estimated by 6 replicate plasma

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decreased from 14.1 ± 3.7 to 9.2 ± 1.7 pg/ml (mean ± S.E.), and plasma renin activity from 462 ± 144 to 259 ± 74 pg/ml/hr. There was a significantly positive correlation between plasma angiotensin II levels and plasma renin activities under various conditions (Fig. 7).

DISCUSSION

The foregoing results indicate that the radioimmunoassay described above appears to possess all the requisites for a sensitive and precise measurement of angiotensin II in human plasma. The assay detected as little as 0.8 pg per tube of angiotensin II, i.e., 5 pg per milliliter when extracts from 400 µl of plasma are subjected to radioimmunoassay. In the radioimmunoassay methods previously reported, large sample volumes, approximately 10 ml plasma were required. And the sensitivity described in the present paper was much greater than that hitherto reported. Thus, it was shown that the determination of plasma angiotensin II levels from small amount of plasma samples was possible by using this simplified extraction procedure.

As shown in the chromatographic study (Fig. 4), whole plasma represents two immunoreactive peaks and the second peak reveals endogenous angiotensin II. Therefore, the substance which interferes with the radioimmunoassay non-specifically was recognized in our system. And if we determine angiotensin II concentration in whole plasma by our radioimmunoassay, the angiotensin II concentration might show falsely higher levels. When acetone extracted plasma was applied to the column, the interfering substance was completely eliminated. Although the acetone extraction procedure is a very simplified method and shows good recovery, there has been no report which applied the acetone extraction procedure for radioimmunoassay of angiotensin II.

Our study showed that the converting enzyme activity in EDTA treated plasma was inhibited at −20°C, but not inhibited at room temperature during at least 40 hours (Fig. 3).

The circulating levels of angiotensin II in normal subjects after overnight fasting and recumbency on ordinary diet closely agree with those obtained by others except for one report. After furosemide injection and a standing position, plasma angiotensin II concentrations as well as plasma renin activity significantly increased to high levels. These results coincided with the report of Catt, et al.

Saline infusion reduced both plasma angiotensin II concentrations and plasma renin activity. Furthermore, it was shown that plasma angiotensin II concentrations correlated closely with plasma renin activity under various conditions, i.e., at rest, after furosemide and saline infusion test. It is considered, therefore, that the change in angiotensin II is caused by that of plasma renin activity. These results are consistent with those reported by the other investigators except some.

The sensitive and simplified radioimmunoassay method for measuring plasma angiotensin II presented in this paper may be useful for the investigation of the role of the renin-angiotensin system in the mechanism of various hypertensive diseases.

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