Estimation of Urinary Angiotensin II by Radioimmunoassay

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FUKUCHI, S. *Estimation of Urinary Angiotensin II by Radioimmunoassay.* Tohoku J. exp. Med., 1974, 114 (3), 205-213 — Urine samples were collected from fasting subjects after maintaining posture for 2 hr in early morning. Urinary angiotensin II was extracted with SE-Sephadex. The extracts, after being dissolved in phosphate buffer, pH 7.5, were measured by radioimmunoassay. Recovery, sensitivity and accuracy were found to be satisfactory. The normal values obtained from 6 subjects were 52-280 pg/2 hr. The values were almost normal in essential hypertension and in chronic glomerulonephritis. They were high in 3 out of 6 cases with renovascular hypertension and subsequently dropped after surgery. In 6 cases with primary aldosteronism, very low levels were found. These increased after the removal of adrenal adenomas. No positive correlation between simultaneous plasma and urinary angiotensin samples was apparent. Also no positive correlation between urinary angiotensin and urine volume was found. In renovascular hypertension, during glucose infusion, lower values in urine volume and angiotensin excretion were observed on the stenotic side as compared to the intact side. Thus, the angiotensin excretion rate does not appear to be regulated by arterial angiotensin concentration, but rather by the angiotensin perfusion rate. — renin; angiotensin II; hypertension; immunoassay

Angiotensin, an important pressor substance, is a peptide of low molecular weight, about 1000, and is also dialyzable. It may therefore be assumed that circulating angiotensin is filtered through the glomerulus into the tubular fluid, and that at least some of it is excreted in the urine, escaping reabsorption or destruction. Apart from the suggestion put forward by Rappelli and Peart (1968) that very little renin appears in the urine of rats which have been intravenously injected with renin, there has been practically no paper on urinary angiotensin to date.

The following paper describes how a simple radioimmunoassay method was used in an effort to clarify the problem of the urinary excretion of angiotensin. Its importance in certain abnormal clinical states, especially in hypertensive diseases, is also discussed.

METHODS

Urinary angiotensin II was measured by a modification of the method of radioimmunoassay used by Catt et al. (1967) to measure plasma angiotensin II. Intramuscular

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injections of porcine-gamma globulin angiotensin II-amide complex produced by carbodiimide condensation were used to produce antibodies in rabbits. Angiotensin II (Hypertensin, CIBA) was radioiodinated by a modification of the chloramine-T method described by Hunter and Greenwood (1962).

In the experiment, blood and urine were collected from 50 subjects. These included 6 normal subjects, 21 subjects with essential hypertension, 9 with renovascular hypertension, 4 with chronic glomerulonephritis, 6 with primary aldosteronism and 2 with Cushing's syndrome. Apart from the normal subjects they were all studied under metabolic ward conditions on a 250-mEq sodium and 75-mEq potassium diet. Either the subjects had never been treated for hypertension before or, if they had been treated, they were off all medication for at least three weeks before starting the study.

Blood for measuring plasma angiotensin was drawn initially from the femoral artery while subjects were recumbent in the early morning. Urine samples were then collected from the subjects after they had been fasting and in an upright posture for 2 hr. The volume of freshly voided urine was measured, and a 10-ml to 30-ml aliquot was immediately acidified to pH 5.0 with acetic acid and centrifuged. After centrifuging, the supernatant was stored in a deep freezer until the extraction was performed.

The samples were transferred to ice-cooled polyethylene tubes and centrifuged. If the urine contained protein, this was removed by boiling and centrifuging before extraction. The extraction procedure was carried out at 4°C and all glassware used was siliconized. The angiotensin II was extracted from the urine with 400 mg of SE-Sephadex, which was then washed with 20 ml of 80% methanol solution and acidified with acetic acid. The peptide was subsequently eluted from the SE-Sephadex with 20 ml of 80% methanol solution alkalinized with triethylamine, taken to pH 6.0 with acetic acid, and the peptide was then dried in a rotary evaporator.

The extracts from the urine were dissolved in 2 ml of 0.05 M phosphate buffer, pH 7.5, containing 0.5% bovine serum albumin (BSA diluent). After the pH had been adjusted

Fig. 1. Standard curve of angiotensin II.

to 7.5, one milliliter of the angiotensin solution was measured by radioimmunoassay. Radioimmunoassay was performed in small test tubes to which were added 1.0 ml of the angiotensin solution or standard angiotensin II amide, 0.1 ml of ^{125}I tracer angiotensin II amide (about 5000 cpm) and 0.1 ml of antibody diluted 1:10,000 to 1:100,000. This gave a total volume of 1.2 ml in each tube, with a final antiserum concentration of 1:120,000 to 1:1,200,000. The tubes were shaken and kept at 4°C . After 16 hr, 0.2 ml of dextran-coated charcoal was added to each of the tubes, which were then shaken for 20 min and centrifuged for 10 min at 3000 rpm. The supernatant was then discarded, and the tubes were counted in a well-type scintillation counter. The count showed the radioactivity in a fraction of ^{125}I tracer angiotensin which was not bound to antibody. This figure of the percentage of radioactivity in the free fraction divided by the total count of ^{125}I tracer angiotensin added to each tube was used as a quantitative indicator for both the standard and unknown samples of angiotensin (Fig. 1).

In order to determine if the urine extracts had a non-specific influence on the angiotensin II standard curve, a large urine pool was obtained from a patient with primary aldosteronism. The urine was divided into 20 ml aliquots, and extracted and evaporated to dryness. Two ml of angiotensin standard in concentrations of 10, 50, 100, 500, 1000 and 5000 pg/ml were added in duplicate to tubes of dried extracts. Standard curves were prepared using the original angiotensin preparations and the angiotensin added to the dried urine extracts.

The procedure for the recovery of angiotensin was tested over a wide range of concentrations by adding varying amounts of angiotensin standard to 20 ml aliquots of urine obtained from a patient with primary aldosteronism. The samples were extracted and assayed, and the recovery for each dose of added angiotensin was calculated.

A variety of adsorbents were tried for the extraction of angiotensin from urine. These included Kieselgel, silica gel, Fuller's earth, celite, Florisil and SE-Sephadex. SE-Sephadex was found to provide the highest recoveries and was therefore employed for the routine extraction procedure.

The quantity of angiotensin extracted from the urine with SE-Sephadex was investigated. 1200 ml of urine were obtained from a patient with primary aldosteronism. This was adjusted to pH 5.0 with glacial acetic acid, and synthetic angiotensin II was added to achieve a theoretical concentration of 2000 pg/20 ml of urine. The specimen was then divided into 20 ml aliquots and frozen at -20°C . A portion of the original specimen, to which no angiotensin II had been added, was also divided into 20 ml aliquots and frozen. Subsequent assay of aliquots of the original specimen revealed no detectable angiotensin. 20 and 40 ml volumes of the urine specimens with added angiotensin were extracted and assayed and the recovery was calculated in each case. To test the stability of angiotensin in frozen acidified urine, aliquots of the above specimens with and without added angiotensin were thawed after having been frozen for 1, 8, 15, or 22 days and processed on SE-Sephadex as above. All the extracts obtained were assayed at the same time.

Before urine was collected plasma angiotensin was measured. Blood samples were taken from the femoral artery while subjects were recumbent in the early morning, by a syringe containing 0.2 ml of 8% EDTA disodium, and were promptly chilled in ice. The extraction of the angiotensin from plasma was performed by a Dowex 50W-X4 column (H form), and the measurement was done by Fukuchi and Katsushima's method of radioimmunoassay (1971). The plasma pH was adjusted to 2.0 with 2N HCl after the addition of 2 drops of 5% DFP. Then 2 ml of the plasma were heated in boiling water for 10 min, cooled, and centrifuged at 3000 rpm for 10 min. The supernatant was applied to the Dowex 50W-X4 column at room temperature. The column was washed with 2.0 ml of 0.2 M acetate buffer (pH 6.0) and then with 3.0 ml of distilled water. These effluents were discarded. Finally, angiotensin II 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 7.5, of which 0.5 ml were adjusted with the same volume of 1.0% BSA diluent. The eluate was deep frozen until the radioimmunoassay was performed by the method used in urinary angiotensin measurement.

RESULTS

Stability of angiotensin in urine

To test the stability of angiotensin in the urine, synthetic angiotensin II was first added to human urine to a concentration of 1.0 ng/100 ml. The urine was divided into several lots which were stored separately at different pH's and temperatures for various time intervals. In the acidified urine, the angiotensin was quite stable at room temperature for at least 2 days. In non-acidified urine at 37°C, the added angiotensin lost its activity by 20–30% in 60 min and by 30–50% in 120 min. At room temperature it lost its activity by 10–20% in 60 min and 20–40% in 120 min. The level of angiotensin in urine samples dialyzed for 24 hr was negligible.

Angiotensin II in acidified urine, frozen for up to 3 weeks before extraction and assay, showed no loss of immunoreactivity. Recovery of the added angiotensin II was complete. The variability present (16.8%) was of the same magnitude as that found in the above determinations of synthetic angiotensin II in a single urine specimen.

Efficiency of adsorbents

Of the several adsorbents tested with ^{131}I -angiotensin II, only Fuller's earth and SE-Sephadex provided ^{131}I -angiotensin recoveries in excess of 70% from 5–50 ml acidified urine. Although Fuller's earth adsorbed as much labeled angiotensin as SE-Sephadex, the labeled hormone could not be eluted conveniently from Fuller's earth. 200 mg of SE-Sephadex and a mixing time of 3 min were sufficient to produce optimal adsorption of ^{131}I -angiotensin from 10–50 ml of acidified urine. Maximal elution of SE-Sephadex occurred with 80% methanol alkalinized to pH 8.0 with triethylamine. The mean recovery of added labeled angiotensin at each step of the extraction procedure from 10 duplicate samples was: SE-Sephadex adsorption step, $93 \pm 3.6\%$. Overall recovery consistently averaged 86% of the added labeled hormone.

Recovery, sensitivity, reproducibility and accuracy

In studies in which varying amounts of angiotensin amide were added to 20 ml of urine, recoveries ranged from 83 to 90%, with a mean value of 86%. The standard error was within 15%, and the lower limit of detection was 10 pg.

When varying amounts of angiotensin II were added to urine extracts from a patient with primary aldosteronism, the immunoassay curve obtained was identical to that resulting from the original angiotensin II preparations. Thus, a nonspecific effect of the urine extract on the angiotensin II standard curve was not apparent. When varying amounts of angiotensin II were added to 20 ml aliquots of urine from a patient with primary aldosteronism and then extracted and assayed, a linear relationship was found between the amount of angiotensin II added and the amount of angiotensin II recovered.

Arrows indicate the change of urinary angiotensin II after the operation

Fig. 2. The levels of urinary angiotensin II excreted for 2 hr in an upright posture in normal subjects and in various hypertensive patients. Arrows show changes of urinary angiotensin II after surgery.

Using SE-Sephadex it was possible to extract angiotensin accurately when either 20 ml or 40 ml aliquots of urine were applied. With either volume, recovery was complete and similar to the value of 87.4% previously obtained using fresh urine. For ease of handling, subsequent extractions were performed using 20 ml aliquots of urine. If the urine volume was below 20 ml, it was set to 20 ml with distilled water.

If the angiotensin solution after extraction was incompletely dried, some substances suppressing the immunoreactivity were found in the residue. It was therefore found necessary to dry the extract completely by washing the tubes several times with methanol.

Urinary angiotensin in various kinds of hypertensive patients

Fig. 2 shows the urinary angiotensin II excreted during 2 hr in the morning both in normal subjects and in various hypertensive patients. The results in 6 normal subjects were 52–280 pg/2 hr. Urinary angiotensin II was estimated in 21 patients with essential hypertension, in 20 of whom the values were within the normal range. The value found for the one remaining patient, was slightly higher: 300 pg/2 hr. Urinary angiotensin II was 420–910 pg/2 hr in 3 patients with unilateral renal artery stenosis, but in the other 6 patients with renovascular hypertension the values were within the normal range. One patient with unilateral renal artery stenosis showed the highest value preoperatively at 910 pg. In this case urinary angiotensin II levels dropped to 510 pg after the operation for renal stenosis. In another 3 patients with bilateral renal artery stenosis normal values were found before operation and the urinary angiotensin II did not change after the operation.

Fig. 3. The relationship between plasma and urinary angiotensin II obtained at the same time. Dotted lines represent the results of urinary and plasma angiotensin II obtained at different times in 2 patients.

In 4 patients with chronic glomerulonephritis, the urinary angiotensin was almost within the normal range. The urinary angiotensin in 6 patients with primary aldosteronism showed the relatively low value of 20–180 pg/2 hr, which increased to 220 pg/2 hr after the removal of the adrenal adenoma in 1 case. In the 2 patients with Cushing's syndrome, urinary angiotensin was in an almost normal range, 100 and 320 pg/2 hr.

As shown in Fig. 3, no positive correlation between simultaneous plasma and urinary angiotensin samples was apparent. The results of urinary and plasma angiotensin obtained twice in 2 patients showed larger variations of angiotensin II in urine than in plasma, as represented by the dotted lines in Fig. 3. Furthermore, no positive correlation between urinary angiotensin and urine volume was found, as shown in Fig. 4. No correlation was observed between urinary angiotensin excretion and renal function as expressed by the phenolsulfonphthalein test, blood urea nitrogen, creatinine clearance, and *p*-aminohippurate clearance.

In one patient with left renal artery stenosis, urinary angiotensin excretion was 210 pg on the stenotic side and 168 pg on the intact side during glucose infusion. In this case the urine volume was larger from the intact kidney than from the involved kidney. In another case of left renal artery stenosis, no difference was observed in the urinary angiotensin excretion from both sides, despite the marked

Fig. 4. The relationship between urinary angiotensin and volume in hypertensive patients.

differences in urine volume and creatinine concentration. The sodium concentration was higher in the intact kidney than in the involved kidney in the 2 patients with renovascular hypertension.

In cases of unilateral renal disease without a renovascular lesion, urine volume, angiotensin excretion, creatinine concentration, and electrolyte excretion were greater in the intact kidney during glucose infusion. There was no difference in these factors in the urine from either side in the cases of chronic renal failure and essential hypertension.

DISCUSSION

Rappelli and Peart (1968) have reported on the renal excretion of renin infused intravenously in anesthetized rats. Their results show clearly that the relatively large compound, renin, is excreted in the urine. It would be natural to assume that the smaller molecule angiotensin would also be excreted in the urine, escaping reabsorption or destruction. There are, however, no reports confirming urinary angiotensin excretion to date. The reason for this is probably the lack of simple methods of high sensitivity such as radioimmunoassay for the measurement of urinary angiotensin excretion. Our results show clearly that angiotensin is excreted in the urine.

In the present experiment, urine was extracted by a method using SE-Sephadex,

which was shown to provide complete recovery of angiotensin II from the urine. This was coupled with a sensitive and specific radioimmunoassay to estimate angiotensin II excretion. The immunoassay procedure used was sufficiently sensitive to detect the amount of angiotensin II present in the urine of normal subjects. Experiments have shown that, after extraction with SE-Sephadex, normal urinary constituents are not capable of altering the reactivity of the antigen-antibody reaction upon which the assay is based.

It had been observed that angiotensin excretion takes place in large amounts in renovascular hypertension and in smaller amounts in primary aldosteronism. This finding is similar to the results for plasma angiotensin levels measured by the method described in a previous paper (Fukuchi and Katsushima 1971). Urinary angiotensin II increased after the removal of the adrenal adenoma in primary aldosteronism, while it decreased after the improvement of the renal circulation in renovascular hypertension. In general, the hourly variations in urinary angiotensin II were larger than those in plasma angiotensin II. Therefore, it was concluded that the measurement of plasma angiotensin II is more valuable for clinical laboratory tests than that of urinary angiotensin II.

It was previously reported that in renovascular hypertension after water diuresis, urine volume, concentration of sodium and TRF_{Na} decreased on the constricted side (Fukuchi 1970). Creatinine concentration on the constricted side rose in various degrees, but not always markedly (Fukuchi 1970).

Recently larger amounts of renin excretion in urine were detected from the ischemic kidneys of dogs with unilateral renal artery stenosis (Hayduk et al. 1970). In renovascular hypertension it was already been reported that angiotensin in the renal vein was found to be higher on the involved side than on the intact side (Fukuchi 1970). The angiotensin excretion from the stenotic kidney was slightly greater than that from the intact kidney in one case of unilateral renal artery stenosis. In another case of renovascular hypertension, no difference in angiotensin excretion between the kidneys was observed, despite marked differences in urine volume.

In summary, this simple radioimmunoassay procedure for urinary angiotensin II, although less valuable for clinical diagnosis than that for plasma angiotensin II, was shown to be an important means for the diagnosis of various kinds of hypertension, especially primary aldosteronism and renovascular hypertension.

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