Radioimmunoassay of Angiotensin I, for Estimation of Plasma Renin Activity

Introduction

Renin is a proteolytic enzyme produced by, stored in, and secreted from the juxtaglomerular apparatus of the kidney. Its secretion is controlled by a wide variety of stimuli that generally relate to fluid and volume regulation. Stimuli to renin secretion include upright posture, hemorrhage, dehydration, sodium depletion, and compromise of the renal vasculature. Renin secretion is inhibited during expansion of plasma volume and as a consequence of mineralocorticoid excess. Estimation of renin secretion is important to both physiologists and clinicians, and various methods for doing so have become of prime importance in the diagnosis of functionally significant primary or secondary aldosteronism, stenosis of the renal artery, and in the management of hypertension in subjects with chronic renal failure. More recently, it has been proposed that estimation of renin secretion is a valuable method for determining the prognosis and the most appropriate therapy for subjects with essential hypertension (1). As a proteolytic enzyme, renin acts in the general circulation to cleave a decapeptide from an α2-globulin substrate, angiotensinogen, which is produced in the liver and is abundant in plasma. This decapeptide, angiotensin I, is further cleaved to angiotensin II by converting enzymes that are primarily located in the lung. Angiotensin II has two important biological roles: (a) stimulation of aldosterone production by the adrenal and (b) a direct vasopressor activity.

No direct means of measuring renin is available. In most of the currently popular methods for measuring renin, its activity is measured; that is to say, they are kinetic assays of enzymatic activity. Thus, we term the assay “plasma renin activity.”

Principle

Plasma is incubated at 37 °C for 3 h under conditions designed to inhibit angiotensin I degradation or conversion to angiotensin II. Some controversy exists regarding the completeness of this inhibition. The amount of angiotensin I generated during this 3-h period is determined by radioimmunoassay. The amount of angiotensin I generated will depend on the amount of renin activity in the plasma, because excess substrate (angiotensinogen) is present in plasma, and angiotensin I is generated in direct proportion to the action of renin on substrate (2–4). Angiotensin generated by renin action on endogenous substrate is generally linearly related to time for at least 3 h. This may not always be true, but can be determined by estimating angiotensin generation at several time periods during the incubation. However, it is generally satisfactory to measure angiotensin I at 0 and 3 h of plasma incubation.

The angiotensin I in plasma before and after incubation is determined by a highly sensitive and specific radioimmunoassay. In this assay a standard amount of 125I-labeled angiotensin I and standard amounts of antibody to angiotensin I are incubated at 4 °C in the presence of either various known quantities of unlabeled angiotensin I or plasma containing endogenous angiotensin I to be measured.
The competition of the labeled angiotensin for antibody binding sites with plasma angiotensin I is compared to that of the known quantities of angiotensin I.

Bound antibody is separated from free (non-antibody-bound) angiotensin I with dextran-coated charcoal, which adsorbs the unbound angiotensin I but tends not to adsorb the antibody-bound angiotensin I. Materials used for several modifications of this method, which was originally described by Haber et al. (3), are currently marketed commercially in kit form, and any of these is likely to provide a convenient means of measuring plasma renin activity. We describe here our modification of one such kit method (Schwarz/Mann).

**Materials and Methods**

**Equipment**

- Disposable 12 x 75 mm polystyrene tubes (Cat. No. 2054; Falcon Plastics, Oxnard, Calif. 93030).
- Metabolic-shaking incubator, (“Precision-Dubnoff”); Precision Scientific, Chicago, Ill. 60647).
- Serological pipettes, 1.0 ml (Cat. No. 37035; Kimberly Products Div., Owens-Illinois, Inc., Toledo, Ohio 43601).
- Biotette, 0.2 ml (Cat. No. 0010-19), Biotips (Cat. No. 0010-20), and Biotette Adapter Kit (Cat. No. 0010-18), all from Schwarz/Mann, Orangeburg, N. Y. 10962).
- Gamma-ray scintillation counter (Model 5320; Packard Instrument Co., Downers’ Grove, Ill. 60515).
- Vacutainer tubes (lavender-top tubes, each containing 6 mg of disodium ethylenediaminetetraacetate; Cat. No. 4770; Becton, Dickinson & Co., Rutherford, N. J. 07070).

**Reagents**

- British anti-lewisite, 0.2 mol/liter (Sigma Chemical Co., St. Louis, Mo. 63178). Dilute a 24.85-g ampule of 2,3-dimercaptop-1-propanol (supplied in peanut oil) with 1 liter of de-ionized water.
- 8-Hydroxyquinoline sulfate, 0.340 mol/liter. Dissolve 0.660 g of 8-hydroxyquinoline sulfate in 10 ml of de-ionized water.
- Buffer, 25 mmol/liter, pH 8.6. Dilute 20.6 g of sodium diethybarbiturate to 1 liter with de-ionized water. Add 500 ml of the sodium diethybarbiturate solution to 60 ml of dilute hydrochloric acid (0.1 mol/liter), and dilute to a final volume of 2000 ml.
- RIA buffer, pH 8.6. Dissolve 2.75 g of bovine serum albumin (Fraction V Powder; Lot No. 176; Miles Labs., Inc., Kankakee, Ill. 60901), 2.29 g of calcium chloride, and 0.2 g of neomycin sulfate in 1 liter of the buffer.
- Norit A untreated charcoal. Dissolve 250 mg of Dextran 40 (Pharmacia, Uppsala, Sweden, or Piscataway, N. J. 08854) in 100 ml of the buffer (pH 8.6), add 4.0 g of Norit A, and mix until homogeneous. This mixture may be stored at 4°C. It should be re-mixed repeatedly during use.

**125I-labeled angiotensin** (Cat. No. 075-53, Schwarz/Mann).
- Anti-angiotensin I antiserum (Cat. No. 0751-03, Schwarz/Mann).

**Standards**

- Stock standard I, Dissolve 1.0 mg of angiotensin I (1-Asp, 5-Ileu; Cat. No. 3380-01, Schwarz/Mann) in 200 ml of the buffer. This is equivalent to a concentration of 5 µg/ml.
- Stock standard II. Dilute 0.05 ml of stock standard I to 50 ml, to give a concentration of 5 ng/ml. Freeze 1-ml aliquots, and store at −60°C.
- Working standard. Dilute 0.5 ml of stock standard II with 1.5 ml of the buffer, to give a concentration of 1.25 ng/ml. Remove 1 ml and add to 1 ml of the buffer. Continue this serial dilution, to produce concentrations of 1.25, 0.625, 0.312, 0.156, and 0.079 ng/ml.

**Radioactivity**

Dilute 125I-labeled angiotensin I with the buffer so that 50 µl contains 5000–10 000 counts per minute. Generally, 0.2 ml diluted to 10 ml is appropriate.

**Sample Preparation**

Collect blood in the Vacutainer tubes and place them on ice. Centrifuge the blood at 4°C for 10 min at 2000 rpm. Transfer aliquots of 0.5 ml to 12 mm x 75 mm plastic tubes for immediate freezing or assaying. For assay controls, use 0.5-ml aliquots of three large plasma pools. One of these pools should have low plasma renin activity, one high plasma renin activity, and the third a moderate plasma renin activity.

**Procedure**

1. Add 5 µl of British anti-lewisite and 10 µl of 8-hydroxyquinoline to each of two 0.5-ml samples of each unknown and to each of the three controls.
2. Incubate a 0.5-ml aliquot of each sample and control pool for 3 h at 37°C (incubated sample). The other aliquot is kept at 4°C (unincubated sample).
3. For each incubated sample, prepare two tubes containing 1.5 ml of the buffer. For each unincubated sample, prepare one tube containing 1.5 ml of the buffer. These tubes are for subsequent dilutions of plasma samples.
4. Set up the radioimmunoassay in duplicate as outlined in steps 6–11, in serially numbered assay tubes. The final volume in each tube will be 0.3 ml.
5. To tubes 1 and 2, add 0.250 ml of buffer. These tubes will not contain antibody and are referred to as “blanks.”
6. To tubes 3 and 4, add 0.200 ml of buffer and 50 µl of antibody. These tubes contain no added unknown or standard, are referred to as “zero’s,” and will show how much of the radioactivity is bound to antibody in the absence of additional angiotensin I.
7. To tubes 5 through 14, add 0.200 ml of the various dilutions of standard angiotensin I (0.079, 0.156,
0.312, 0.625, and 1.25 ng/ml) in duplicate.

(8) Assay each incubated sample at three dilutions, each in duplicate. Assay each unincubated sample at two dilutions, in duplicate. At the end of the incubation, add 2.0 ml of the buffer to each tube, to give a fivefold dilution. Into a 1-ml serological pipette, aspirate 0.7 ml of each fivefold diluted sample, and transfer 0.2 ml to each of the two assay tubes and the remaining 0.3 ml to a tube containing 1.5 ml of buffer. This is the 30-fold dilution. Into a 1-ml serological pipette, aspirate 0.7 ml of the 30-fold dilution and transfer 0.2 ml to each of the two assay tubes and the remaining 0.3 ml to the tube containing 1.5 ml of buffer. This is the 180-fold dilution. Transfer 0.2 ml of this dilution to duplicate assay tubes.

(9) To each tube except tubes 1 and 2, add 50 µl of the Schwarz/Mann antibody.

(10) To all assay tubes and to an additional four tubes (total count tube) add 50 µl of 125I-labeled angiotensin. Agitate by shaking the tube racks for 2 min.

(11) Incubate the radioimmunoassay at 4 °C for 40 h.

(12) At the end of the incubation period, add 100 µl of dextran-coated charcoal to each tube with the Biopette while the charcoal dextran is being stirred briskly with a magnetic stirrer. Shake the tube rack for 2 min. Perform this step at room temperature.

(13) Centrifuge the tubes at 2500 rpm for 20 min, to cause the charcoal to form a pellet at the bottom of the tube. The charcoal adsorbs the unbound angiotensin I, leaving the antibody-bound angiotensin I in the supernate.

(14) Aspirate the supernate from all tubes by using a source of vacuum or a water-driven aspirator line. (Place several large traps in this line to ensure that all radioactivity is collected).

(15) Count the charcoal pellets, including those from the total count tubes, for 5 min. The counts for each tube are subtracted from the average of the counts from the total-count tubes. This difference equals the counts bound to antibody.

(16) Express the data as (a) the percentage of total counts bound [(bound counts/total counts) × 100], (b) the ratio of bound counts to unbound counts (bound counts/free counts), or (c) simply as bound counts.

(17) Plot the data as expressed above vs. the standard concentrations of angiotensin I on either linear or semilog graph paper (see Figure 1). Determine the amount of angiotensin I in each unknown sample by reading directly from the plot. Multiply this value by the dilution of original plasma to determine the nanograms of angiotensin I per milliliter per sample. If several dilutions of the sample fall within the range of the standard curve, average the values for angiotensin I in the sample.

(18) Plasma renin activity equals (the angiotensin I in the incubated sample minus angiotensin I in the unincubated sample) divided by 3 (hours of incubation). The data are expressed as nanograms of angiotensin I generated per milliliter of plasma per hour of incubation.

**Discussion**

The assay for plasma renin activity described here represents minor modifications of assays described by the various kit suppliers. These changes do not necessarily improve the assay results, but in our experience they work satisfactorily and lead to efficiency. In general, we have adjusted the volume of reagents to eliminate steps in pipetting and have minimized pipetting of small volumes. The sample dilutions permit accurate assays of 0.13 to 75 ng of angiotensin I per milliliter per hour. The capacity to measure such high values is particularly useful if plasma from the renal vein is being assayed. The incubation time of 40 h for the radioimmunoassay has given us the best results in terms of good linearity with sample dilution. Our method eliminates the need to count both the supernate and the charcoal pellets from each tube, and therefore halves counting time.

This assay does not directly measure renin, but measures its activity in terms of its capacity to generate angiotensin I in plasma. The disodium ethylene-diaminetetraacetate, British anti-lewisite, and 8-hydroxyquinoline used in this assay inhibit the destruction of angiotensin I and (or) its conversion to angiotensin II. Preliminary data from our laboratory suggest that hemolysis of erythrocytes in blood collected for determination of plasma renin activity frequently invalidates the assay. However, the time required to process samples between their collection and receipt by the laboratory is not critical for reproducible results.

For our last 25 assays, results for our three control pools have been (in nanograms of angiotensin I per milliliter per hour ± SEM): 1.08 ± 0.03, 2.67 ± 0.08, and 11.78 ± 0.37.

**Normal Values**

The proper interpretation of the activity not only depends on a competent assay but on the clinician's
appreciation of the effects of posture, fluid balance, and drugs on the secretion of renin. For example, in our laboratories, supine normal subjects on a diet containing a liberal amount of sodium have values of from 0.17 to 1.33 ng per milliliter per hour. When the subject has been in the upright posture for 2-4 h, values range from 0.83 to 3.33 ng per milliliter per hour. On a sodium intake of 10 mmol/day, values increase even further. Still further increases may be produced by postural maneuvers and (or) administration of diuretics.

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References

This paper will probably appear as a chapter in the forthcoming AACC volume, Selected Methods of Clinical Chemistry, and criticisms of it are invited from any reader (see Editorial, October 1973). Such criticisms should be addressed to the senior author, with a copy to the Chairman of the Committee on Selected Methods.

No reprints of this paper will be available.