Micromethod for the Assay of Renin of Seven Species

ERWIN HAAS, PH.D., LAVERA LEWIS, B.S., PAUL SCIPIONE, M.S.,
AND T. JOHN KOSHY, M.COM.

SUMMARY Renin was extracted and purified from human, dog, hog, rat, beef, rabbit and sheep kidneys. The renin "concentration" of these preparations was determined and expressed in international (Goldblatt) units by measuring the pressor effect produced by intravascular injection into normal dogs of a permanent colony. The renin "activity" was determined by bioassay, in the rat, of the angiotensin produced by incubation in vitro with renin substrate prepared from the serum of nephrectomized dogs.

The rate of angiotensin formation was proportional to the concentration of renin, independent of the substrate concentration, and rather similar for renin of all seven species (mean rate = $55 \times 10^4$ ng angiotensin/unit renin/16 hrs).

Due to this uniformity, either of the two international reference preparations of renin (human or hog) from the World Health Organization can serve as an internal standard in the assay of renin of each of the seven species, to express their concentration in terms of the international unit.

Renin substrate from hog plasma was suitable for the assay of human, dog and hog renin (mean rate = $55 \times 10^4$). However, it reacted much more slowly with the renin of rat, beef, rabbit and sheep (mean rate = $9 \times 10^4$) and was, therefore, less suitable for their assay. (Hypertension 1: 112-117, 1979)

KEY WORDS • angiotensinogen • kidney • angiotensin I • concentration of renin • international units

THE concentration of renin in the kidneys of 12 animal species had been determined and compared previously on the basis of the increase of the blood pressure of normal dogs following the intravascular injection of purified renin preparations.

Such test dogs, however, are usually not available in most laboratories and, furthermore, a relatively large dose, such as 1 unit of renin, would be required to raise the blood pressure of such a dog by 30 mm Hg.

The present "indirect" assay procedure makes it possible to determine rather low concentrations; for example, $0.5 \times 10^4$ unit of renin, the amount present in 0.005 mg of rabbit kidney. This procedure is based on the prolonged (16 hours at 38°C) incubation of renin in vitro with its substrate and the subsequent assay of the angiotensin produced.

It has been shown previously for human renin that the rate of angiotensin formation can be affected by a great variety of experimental conditions; an international standard preparation of human renin was introduced, therefore, and employed as reference.

It seemed of interest to extend this concept to the assay of renin preparations from six other species, aside from human, to explore their activity in producing angiotensin, and to express their concentration in terms of an international reference preparation.

Materials and Methods

Definition of Unit

A unit of renin is the quantity which, when injected intravenously (saphenous) or intra-arterially (femoral) into an unanesthetized, trained dog, raises the direct mean femoral artery blood pressure 30 mm Hg in about 2 minutes.

A permanent colony of selected dogs has been maintained in our laboratory, all of which responded uniformly for years to the injection of a standard dose of renin.
Standard Preparations of Human and Hog Renin

Based on an international collaborative study of renin assays, standard preparations of human and of hog renin are now distributed for the World Health Organization (W.H.O.) by the National Institute for Biological Standards and Control (Holly Hill, London, N.W. 3).

The "International Reference Preparation of Human Renin" was established in 1974, and each ampoule (Code No. 68/356) contains, by definition, 0.1 international unit (Goldblatt unit) of human renin. The preparation of hog renin classified as "Research Standard" by the Expert Committee on Biological Standards of the W.H.O. has been available since 1966, and each ampoule (Code No. 65/119) contains 4.7 units of hog renin.

In the present study, either human or hog standard renin was employed, and served equally well as internal standard for the precise assay of renin of any of the seven species.

Standard Angiotensin

The "Angiotensin Research Standard A/65" obtained from the W.H.O. represents the free acid α-L-asp-val'-angiotensin II.

"Hypertensin-Ciba," the pure synthetic Val°-angiotensin II-asp'-β-amide was obtained from Dr. A. J. Plummer, Ciba Research Department, Summit, New Jersey.

The relative pressor activity of Hypertensin-Ciba, as tested by the bioassay in the rat, was 93% of that of Angiotensin A/65.

Extraction and Purification of Renin

The following procedures, summarized in table 1, were used to obtain the standard preparations of renin from the kidneys of seven species.

These renin preparations (table 1) are free of angiotensinase and they have been fractionated to eliminate substances that may cause such undesirable side effects as anaphylaxis, blood pressure depression or hyperthermia.

These renin preparations have been employed as renin standards in our studies and have also been furnished for the same purpose to other investigators (table 1), including the W.H.O.

Renin Substrate from Dog Serum

The serum of dogs, 2 days after bilateral nephrectomy, was utilized for the preparation of renin substrate. Blood was collected through a cannula in a carotid artery after sedation of the dog with acepromazine maleate and anesthesia with Nembutal. After 2 hours at room temperature, the serum was separated by centrifugation in the cold at 0–5°C. The serum was either processed directly or it could be stored for more than 2 years in the frozen state at −15°C.

Two types of substrate were prepared from dog serum: 1) By a procedure which included treatment with ethylenediamine tetraacetic acid (EDTA) in alkaline solution for the removal of angiotensinase, fractionation with ammonium sulfate and dialysis. This is essentially the method which we had described previously for the preparation of renin substrate from human serum. 2) By a method involving treatment with acid for the removal of angiotensinase and of converting enzyme.

Specifically, 520 ml serum, obtained from a 15 kg dog, was cooled and all of the following steps were carried out at 0°C. By the drop-wise addition of approximately 18 ml of 2.5 N HC1 and with rapid stirring the serum was acidified to pH 2.5 and it was kept, thereafter, for 2 hours without stirring. The substrate-containing solution was neutralized with 2.5 N NaOH, dialyzed for 20 hours against distilled water, shelf-frozen and lyophilized. The residual dry powder was dissolved in 146 ml of saline to a final volume of 172 ml containing an amount of renin substrate (angiotensinogen) equivalent to 4100 ng angiotensin II/ml. The quantity of substrate was determined by conversion into angiotensin with an excess of dog renin, followed by the bioassay in the rat, using angiotensin II as standard.

Table 1. Procedures for Isolation of Renin from the Kidneys of Seven Species

<table>
<thead>
<tr>
<th>Renin</th>
<th>Specific activity of renin (units/mg protein)</th>
<th>Purification factor</th>
<th>Isolation procedures (reference nos.)</th>
<th>Application by other investigators (reference nos.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td>0.13</td>
<td>75-fold</td>
<td>(6) Procedure C</td>
<td>(10)</td>
</tr>
<tr>
<td>Dog</td>
<td>0.42</td>
<td>21-fold</td>
<td>(7)</td>
<td>(11, 12)</td>
</tr>
<tr>
<td>Human</td>
<td>0.95</td>
<td>470-fold</td>
<td>(8)</td>
<td>(4, 13)</td>
</tr>
<tr>
<td>Beef</td>
<td>1.2</td>
<td>150-fold</td>
<td>(9) Step #4</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>1.3</td>
<td>100-fold</td>
<td>(6) Procedure C</td>
<td>(14, 15)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>2.0</td>
<td>30-fold</td>
<td>(1)</td>
<td>(16)</td>
</tr>
<tr>
<td>Hog</td>
<td>9</td>
<td>850-fold</td>
<td>(9) Step #5</td>
<td>(15, 16, 17)</td>
</tr>
<tr>
<td>Hog</td>
<td>123</td>
<td>8,800-fold</td>
<td>(9) Step #8</td>
<td></td>
</tr>
<tr>
<td>Hog</td>
<td>470</td>
<td>34,000-fold</td>
<td>(9) Step #10</td>
<td></td>
</tr>
</tbody>
</table>
By this procedure, a yield of 700 μg of renin substrate was obtained from the serum of one dog, adequate for the assay of renin in 350 specimens.

The average concentration of renin substrate was 1020 (range 800-1370) ng/ml serum, in 11 batches prepared from the serum of 10 nephrectomized dogs.

With the addition of 0.10 unit of dog renin/ml, all of the substrate was converted within 10 minutes to a maximum amount of angiotensin, and this value remained unchanged, without any loss, in the course of further incubation for 1, 2 and 18 hours at 38°C. Therefore, the substrate preparation and the dog renin were free of angiotensinase.

The angiotensin formed by incubation with dog renin was identified as the decapeptide angiotensin I by the use of small scale counter-current distribution. The substrate, and the renin, therefore, were also free of converting enzyme.

The substrate, even though it had been obtained from the serum 48 hours after bilateral nephrectomy, still contained a low concentration of endogenous renin (0.08-0.17 X 10⁻⁶ unit/ml serum). No attempt was made for its removal. Therefore, a slight correction was applied (as shown in table 3) for the formation of small amounts of angiotensin (6-15 ng/2000 ng substrate) in 16 hours by the endogenous renin.

Storage of the substrate preparation for 10 weeks in the frozen state, at -15°C, had no adverse effect; i.e., the rate of angiotensin formation with hog renin was unchanged.

Renin Substrate from Hog Plasma

Drs. K. E. Lentz and L. T. Skeggs, Veterans Administration Hospital, Cleveland, Ohio generously supplied four substrate preparations from hog plasma. These had been purified up to 480-fold by the procedure of Skeggs et al. and the highest specific activity was 2100 ng angiotensin II/mg of protein.

Results

The details for the microassay of human renin are specified in table 2; the identical procedure was employed also for the assay of renin of each of the seven species.

The method for the assay of minute quantities of renin (table 2) involves the production of large amounts of angiotensin by incubation of the renin for 16 hours at 38°C, with an excess of renin substrate from dog serum.

This assay can be performed with a rather high precision, because: 1) the amount of angiotensin (60 ng) produced by 1 × 10⁻⁶ unit renin is adequate for 20 bioassays in the rat; 2) the pressor effect of the "unknown" angiotensin is monitored continuously against that of a standard angiotensin to compensate for any change in the responsiveness of the test rat; and 3) standard angiotensin preparations of different or even of an unknown specific activity can be employed, because the results of the assays are reported in terms of the concentration of renin, by comparison with a standard renin preparation and not as the activity of renin, i.e., the amount of angiotensin produced.

The validity of the test procedure (table 2) was investigated under various experimental conditions. For example:

1. The concentration of dog substrate was 2000 ng/2 ml, and under these conditions, the rate of angiotensin formation was 95% of the maximum as shown in figure 1.

2. The rate of angiotensin formation was increased in direct proportion to the concentration of dog renin between 1 × 10⁻⁴ to 6 × 10⁻⁴ unit/2 ml.

3. Angiotensin was produced at identical rates with six batches of substrate from nephrectomized dogs which had been prepared by various procedures such as EDTA-treatment, acid-treatment and fractionation with ammonium sulfate.

4. Angiotensin increased linearly with the time of incubation (2, 4 and 16 hours); i.e., there was no inactivation of renin or of angiotensin with prolonged incubation.

5. The rate of angiotensin formation was also proportional to the concentration of hog renin, and it was identical for hog renin preparations with a specific activity of 9, 123 and 470 units/mg protein, respectively, i.e. after 650-, 8800- and 34,000-fold purification.

6. The validity of the assay procedure (table 2) was further established by the addition of the international reference preparation of human renin. For this pur-
TABLE 2. Test Procedure for the Microassay of Renin

<table>
<thead>
<tr>
<th>Exp. 1</th>
<th>Exp. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50 M phosphate buffer (pH 6.3)</td>
<td>0.20 ml</td>
</tr>
<tr>
<td>0.001 M phenylmercuric acetate</td>
<td>0.10 ml</td>
</tr>
<tr>
<td>0.27 M diisopropyl fluorophosphate</td>
<td>0.01 ml</td>
</tr>
<tr>
<td>Saline</td>
<td>1.19 ml</td>
</tr>
<tr>
<td>Renin substrate of dog serum</td>
<td>0.50 ml = 2000 ng</td>
</tr>
</tbody>
</table>

Total volume 2.0 ml

Human renin added unknown
Standard human renin added unknown
Concentration of added renin (unit) X₁ = unknown
Angiotensin produced† (ng) Y₁ = 220

As in exp. 1

The rate of angiotensin formation by added standard human renin:

\[
\text{Rate} = 61 \times 10^4 \frac{\text{ng angiotensin}}{\text{unit renin} \times 16 \text{ hrs}}
\]

and the concentration of the unknown renin X₁ is:

\[
X₁ = \frac{Y₁}{Yᵢ} = \frac{220}{61 \times 10^4} = 3.6 \times 10^4 \text{ unit.}
\]

The rate obtained in the experiments of table 2 with added standard human renin (61 × 10⁴ ng angiotensin/unit renin/16 hrs) is similar, within the experimental errors, to the mean rate of 55 × 10⁴, which was calculated (table 4) for the activity of renin from the seven species.

If the rate obtained by this procedure is found to be considerably lower than this mean rate of 55 × 10⁴, it would be an indication of an incomplete recovery of angiotensin. Several possible causes for such a loss of angiotensin have been previously outlined. ²

The assay procedure (table 2) was employed also to determine the rate of angiotensin formation by seven renin preparations: 1) with dog substrate prepared by the EDTA procedure (table 3); 2) with a number of different substrate preparations from dog serum (table 4); and 3) with substrate prepared from hog plasma and from dog serum (table 5).
The "direct" in vivo standardization in the test dog, and the "indirect" in vitro assay of the seven renin preparations (table 2) are both based on the enzymatic reaction of renin with the renin substrate of dog serum. It was, therefore, perhaps predictable that under the assay conditions of table 2, a rather uniform rate of angiotensin formation would be obtained, per unit of renin, for the seven renin preparations. This is confirmed by the results of tables 3 and 4.

In contrast, with the substrate from hog plasma (table 5), the rates of angiotensin formation were not uniform, but varied widely for renin of some of the species.

Hog substrate was not suitable, because of its rather low reactivity, for the assay of renin from rat, beef, rabbit and sheep (table 5). However, hog substrate has been used with good results for the assay of renin from human,\(^1\) dog\(^2\) and hog (table 5). In 83 experiments, the mean rate for the reaction of renin of these three species with hog substrate was \(55 \times 10^4\) ng angiotensin/unit renin/16 hrs, the coefficient of variation \(\pm 11.9\%\).

**Discussion**

The assay procedure, as designed in the present study, offers various advantages.

1. The preparation of renin substrate from hog plasma\(^*\) involves rather complex procedures and requires considerable amounts of time, labor and expense. In contrast, in the present study, two simple procedures served to obtain relatively large amounts of a suitable renin substrate from the serum of nephrectomized dogs.

2. The renin preparations of seven animal species produced angiotensin at rather similar rates by reaction with the substrate obtained from dog serum (tables 3 and 4). In contrast, with the substrate from hog plasma, the rates varied widely for renin of certain species. For example, the rate for human and dog renin was 12-times higher than for rat renin (table 5).

3. The renin preparations of rat, beef, rabbit and sheep reacted only at low rates with hog substrate (table 5) which was, therefore, less suitable for the assay of renin of these four species. The substrate from dog serum, however, reacts rapidly with the renin of all seven species investigated and was thus well qualified for their assay (tables 3 and 4).

4. Uniform rates of angiotensin formation were obtained (table 4) from six batches of renin substrate that had been prepared by three methods from the serum of six nephrectomized dogs.

5. The estimates in terms of the "renin activity" published from various laboratories are unsuitable for comparison, because of the great variety of experimental conditions used by different investigators.\(^*\)\(^*\) For example, the values reported for the "renin activity" were widely different when two different standards of angiotensin were used (one commercially available, the other from the W.H.O.). However, when these rates of angiotensin formation were calibrated against the Renin Standard and expressed as renin concentration in international units, the results agreed remarkably well.\(^4\)

6. Angiotensin was produced from dog substrate by each of the seven standard renin preparations with a mean rate of \(55 \times 10^4\) ng angiotensin/unit renin/16 hrs (table 4). Therefore, this mean rate can be used to calculate the concentration of renin in specimens from any of the seven species in terms of the international unit as follows:

\[
\text{concentration of renin} = \frac{\text{ng angiotensin produced in 16 hrs}}{55 \times 10^4} \quad \text{(units),}
\]

\[
\text{concentration of renin} = \frac{\text{ng angiotensin produced/hr}}{3.4 \times 10^4} \quad \text{(units).}
\]

7. In previous studies, it had been suggested\(^*\)\(^*\) that one add a known amount of an international standard preparation of human renin as an internal control in the course of the assay of an unknown sample of human renin. However, because of its low concentration in normal human kidneys (0.10 unit/g kidney),\(^1\) it is difficult to obtain human renin in appreciable amounts and in a satisfactory state of purity.\(^9\) The supply of standard human renin available to the W.H.O. is, therefore, rather limited.

In contrast, hog renin, with a concentration of \(4.3\) units/g kidney,\(^1\) is available in large amounts and at a much higher purity.\(^2\) The present study indicates that such hog renin is suitable to serve uniformly as an internal control and international standard in the assay of renin from each of the seven species.
By reference to such international standard preparations of renin, either human or hog, it will be possible to compare the results of renin assays in various laboratories and to express these as the "concentration of renin" in terms of the international (or Goldblatt) unit of renin not only for human, but also for various animal species.

Acknowledgments

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