Micromethod for the Measurement of Renin Activity

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Abstract

A micromethod for the measurement of renin concentration was described for plasma and renal lymph of cat. It involved the preparation of renin substrate free from renin and from angiotensinase. Renin from 0.1 – 0.2 ml of plasma or renal lymph was made to react with the prepared renin substrate in a incubating medium. Incubation of renin from plasma or renal lymph in the presence of excess substrate was done at pH 7.0 in a polythene tube. To each incubation mixture neomycin sulphate, 2 mg/ml and a kallikrein inhibitor trasylol, 100 units/ml were added and all incubations were done at 37°C for a period of 12 hr with constant rate of agitation. The angiotensin so formed during the period of incubation was absorbed on a column of 1 ml prepared Dowex 50W – X2 (NH_4^+), 100 – 200 mesh and thereafter eluted and freeze-dried. The extracted angiotensin fraction in its freeze-dried form was dissolved in 1 ml of 0.9% NaCl and bioassay was done on the mean systemic arterial blood pressure of ganglion-blocked rats against Val^5-angiotensin II-asp-β-amide. The method measures the angiotensin generating capacity of renin in a controlled environment and can be adapted for the measurement of plasma renin concentration in man.

Introduction

THE PROTEOLYTIC ENZYME RENIN (Munos et al., 1939) is secreted from the juxtaglomerular apparatus of the kidney into the blood stream (Cook & Pickering, 1962) and into the renal lymph (Lever & Peart, 1961; Gan & Lockett, 1973). Renin interacts with its specific alpha-2-globulin substrates (Plentl et al., 1943) from the liver to liberate angiotensin I (Elliot & Peart, 1956) which is then converted to angiotensin II by a converting enzyme (Skeggs Jr. et al., 1956). Conversion of angiotensin I to angiotensin II takes place mainly in the pulmonary vascular bed (Ng & Vane, 1967). Angiotensin II, the active end-product of renin is a very potent vasoconstrictor, evokes aldosterone secretion (Davis, 1962). Renin is known to play a role in certain pathological conditions.

High plasma renin levels are commonly found in association with renal hypertension involving stenosis of the renal artery (Woods & Michekalis, 1968), in pheochromocytoma (Maebashi et al., 1968) and in hepatic cirrhosis with edema (Imai & Sokabe, 1968). Low plasma renin levels are associated with primary aldosteronism (Brown et al., 1968).

In essential hypertension, renin levels can either be high or low (Fasola et al., 1966; Weidmann et al., 1968; Creditor & Loschky, 1968). The β-adrenergic blocking agent is effective in treating essential hypertension with abnormally high renin level. Whereas the aldosterone antagonist spironolactone is particularly good as an anti-hypertensive agent in essential hypertension patient whose renin levels are low (Simpson, 1974). It is apparent that knowledge of exact plasma renin concentration will be useful in studying and treating these pathological conditions.

This paper describes the development of a sensitive micro-method for the measurements of renin concentration from the plasma and renal lymph of cat. With appropriate modifications, it can be adapted for the routine measurements of human plasma renin concentration.

METHODS

Preparation of standard cat renin

Standard cat renin was prepared according to the method of Katz et al., (1966) from 45.5 g kidneys of one animal which had been decapsulated and frozen immediately after removal from the anaesthetized animal. The frozen kidneys were later thawed at room temperature. Freezing and thawing was repeated three times in the same day to increase cell rupture and the availability of renin for extraction (Haas et al., 1954). Thereafter, the rest of the procedure was carried out in a cold room at 5 – 10°C. The kidneys were homogenized using a Waring Blendor in 0.05 M Na_2HPO_4, 3.5 ml per g of tissue. The homogenate was transferred to a sac of Visking tubing (size 24/32 in) and dialyzed for approximately 20 hr against 0.1 M lactic acid buffered to a pH within the range, 3.50 to 3.55 by addition of solid $NaHCO^3$. This process also inactivades angiotensinases (Skinner, 1967). The residual solids removed from the dialysed homogenate by centrifugation for 20 min at 2,000 r.p.m. The supernatant, adjusted to pH 7.0 by the addition of a few drops of 3.5% $NaHCO_3$, was centrifuged for 10 min and the pH of 7.0 was reconfirmed.

This supernatant was put into 130 ampoules each containing 1 ml of standard cat renin. The air in the ampoules was displaced with dry nitrogen, and the ampoules sealed and stored at −20°C.

Preparation of cat renin substrates

Renin substrates were prepared by the method of Haas et al., (1966) from arterial blood of cat which was nephrectomised under chloralose anaesthesia and bled 10 hr later whilst still under anaesthesia. The blood was collected into a chilled heparinised flask and the plasma was separated at once by centrifugation at 2,000 r.p.m. and 4°C, for 20 min. Solid ethylene-diamine tetraacetic acid (EDTA) 1.88 g/100 ml, was added to the plasma and dissolved at room temperature. The solution was stirred and brought to pH 8.0 by the cautious dropwise addition of 5 N NaOH. After cooling to approximately 5°C the pH was adjusted to 5.3 by the slow addition of 2.5 N H_2SO_4 while stirring continuously. Renin substrate was then salted out by the addition of solid $(NH_4)_2SO_4$, 30.4 g/100 ml plasma, and stirred for 10 min, then capped stood overnight at 4°C.

After 14 hr, the renin substrate was obtained as a centrifugate of the mixture. The substrate was extracted twice with distilled water (2 × 12 ml per 100 ml plasma). The combined extracts were then centrifuged. The supernatant was dialyzed in sacs of Visking tubing against 6 changes of distil water in 24 hr in the cold room at 5 – 10°C. After centrifugation, the residue was discarded and the solution of substrate was freeze-dried and stored at −20°C.

Measurement of renin activity

A micro-method, modified from the method of Boucher et al. (1967) was developed for the measurement of renin concentration in plasma.

Plasma or standard renin, 0.1 to 0.2 ml, was added to 3 ml isotonic saline (0.9% NaCl at pH 7.0, unless otherwise stated) containing neomycin sulphate (Andrew Laboratories) 2 mg/ml, and a kallikrein inhibitor, trasylol 100 units/ml (Skinner, 1967) in a polythene tube. All tubes were immediately capped and incubated, with uninterrupted vigorous and standardized agitation, in a water bath at 37°C for 12 hr before cooling rapidly to 4°C. The tubes were stored at 4°C after incubation before the isolation of angiotensin.

The isolation and elution of angiotensin so formed was carried out in approximately 1 ml prepared Dowex 50W – X2 (NH_4^+) resin, 100 – 200 mesh (Bio-Rad Laboratories), contained in a glass column 14 cm long with an internal diameter of 1.25 cm. Each Dowex column was prepared by washing with 10 ml 0.2 M ammonium acetate at pH 6.0, then with 20 ml 10% (V/V) aqueous acetic acid and finally with 30 ml distilled water before the cold incubate was passed through the column. The angiotensin was adsorbed on the column and was then eluted by passage of 7 ml 0.1 N diethylamine followed by 7 ml 0.2 N ammonia in a Erlenmeyer flask and was freeze-dried. The dry residue was dissolved in 1 ml 0.9% NaCl immediately before assay against Val^5-angiotensin II-asp-β-amide (Hypertensin, Ciba).

Bioassay

Female rats, 150 – 200 g, of an inbred Wistar strain, were anaesthetized by intraperitoneal (i.p.) injection of pentobarbitone sodium, 40 mg/Kg (Nembutal, Abbott Laboratories). The trachea, an external jugular vein and a common carotid artery were cannulated with polythene tubes. Mean arterial blood pressure was recorded from the carotid cannula through an E & M pressure transducer coupled to a Nesco Recorder. Injections were made through the cannula in the external jugular vein in volumes not exceeding 0.15 ml. Each injection was washed with 0.1 ml 0.9% NaCl. Ganglionic blockade was induced in all rats before the assay commenced by injection of pentolinium (Ansolysen, May & Baker), 0.5 mg intravenously followed by 2.0 mg subcutaneously to give a more stable basal blood pressure (Peart, 1955).

All assays were of 4 × 4 Latin square design. The two doses of standard Val[5]-angiotensin II-asp-β-amide most commonly used were 0.5 and 2.5 ng. The two doses of extract were selected to give comparable responses to those elicited by the two standards. The assay were designed and analyzed by the method as described for posterior pituitary extract by Holton (1948). Typical tracings from assays of renin activity in both the renal lymph and arterial plasma are shown in Figure 1.

Collection of blood and renal lymph

For the measurement of renin activity, blood is collected from the carotid artery via a polythere cannula. Collection of renal lymph from cat was previously described (Gan & Lockett, 1973).

Preparation of isotonic buffered incubation salines

Terminology

The Terms renin activity and renin concentration are treated as synonymous. Renin activity is computed as ng angiotensin formed per hr. when 1 ml plasma/lymph or standard renin is incubated with an excess of substrate under standard conditions for 12 hr.

RESULTS

Effects of changes in renin substrate concentration

Different concentrations of renin substrate were incubated, each with 0.1 ml standard cat renin. Figure 2 shows that a maximum yield of angiotensin

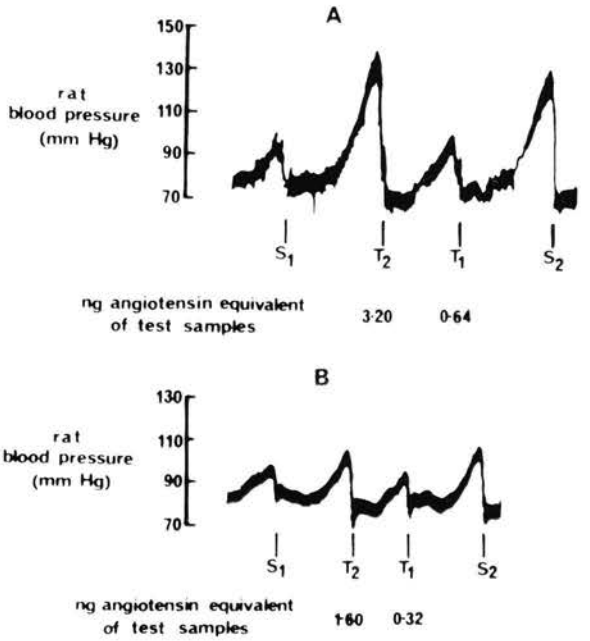

Figure 1

Tracings from two assays of renin activity on the blood pressure of pentolinium-treated rats

A: Assay of renin activity from renal lymph
S = standard angiotensin, S_1 = 0.5 ng, S_2 = 2.5 ng
B: Assay of plasma renin activity
S = standard angiotensin, S_1 = 0.4 ng, S_2 = 2.0 ng
Values indicated beneath T_1 and T_2 show the ng angiotensin found in the injected volumes of the test samples. The calibrated pressure (mm Hg) is shown on the left of each tracing.

Table 1

The composition of isotonic buffered incubation salines

pH	Solution A (ml)	Solution B (ml)	NaCl added (g × 100 ml^{-1})
	0.055M citric acid	**0.055M Na_2HPO_4**	
3.9	50	50	0.48
	0.0016M citric acid	**0.00346M sod. citrate**	
5.0	67.9	32.1	0.54
	0.067M NaH_2PO_4	**0.067M Na_2HPO_4**	
5.9	90	10	0.52
6.5	70	30	0.50
7.0	40	60	0.46
8.0	5	95	0.42

was obtained by the use of approximately 80 mg renin substrate per incubating medium for 12 hr of incubation. To ensure that the substrate presence is in excess, 90 mg of substrate was employed for each measurement of renin activity in plasma and in lymph under the standardized conditions.

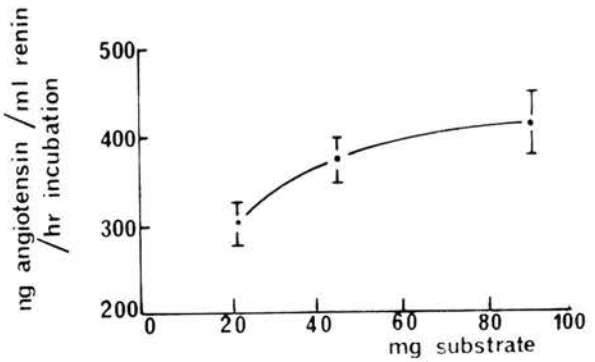

Figure 2

Effect of varied concentrations of renin substrate on the generation of angiotensin by standard cat renin

The vertical bars show the fiducial limits of assays, P = 0.05.

Effects of changes in renin concentration

Figure 3 shows the rate of interaction of standard cat renin with renin substrate with varying concentrations of cat renin. The relationship between ng angiotensin generated /ml renin/hr incubation with different dilutions of renin was linear for up to 12 hr incubation period.

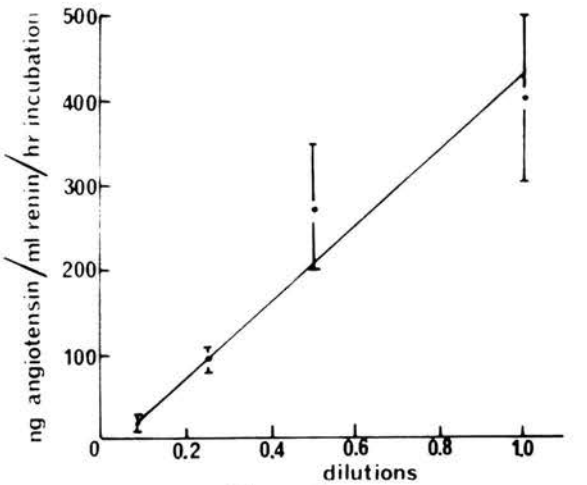

Figure 3

Rate of interaction of standard cat renin with renin substrate

In each case 90 mg of substrate was used. Vertical bars as in figure 2.

Relationship between the quantity of angiotensin generated and the duration of incubation

Standard cat renin was incubated under standard conditions with excess renin substrate. Angiotensin was formed at a constant rate throughout the first 12 hr of incubation as shown in figure 4.

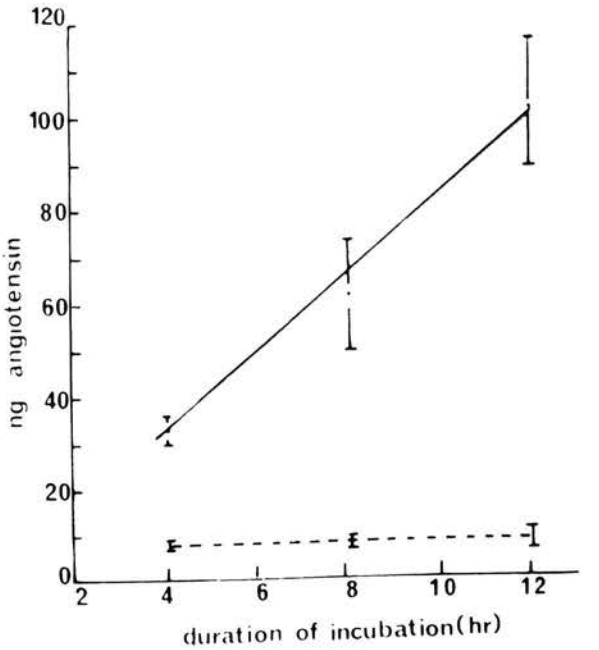

Figure 4

Relationship between the total angiotensin formed and the duration of incubation

The continuous line represents the cummulative total of ng angiotensin generated per ml cat renin incubated with excess substrate and the broken line shows the rate of formation of angiotensin in ng per ml cat renin per hr of incubation. Vertical bars as in Figure 2.

Effects of pH on angiotensin generation

Isotonic buffered incubation salines for various pH values are prepared as shown in table 1. The curves relating pH to ng angiotensin formed per hr in the presence of excess renin substrate was determined for standard cat renin as shown in figure 5. The optimum pH was 7.0. A small hump seen in the curve at pH 3.9 was attributed to the presence of pseudo-renin (Skeggs et al., 1969). Since these authors report that pseudo-renin is almost inactive at pH 7.0 all incubation for the determination of true renin activity should be carried out at pH 7.0.

Recovery studies

Duplicate samples of known concentration of synthetic angiotensin when subjected to the same

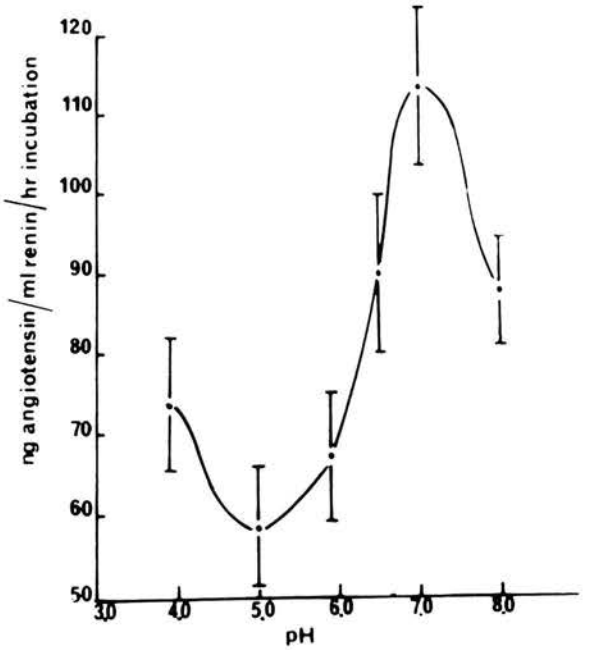

Figure 5

pH curve for the interaction of standard renin with renin substrate

Figure shows the curve relating the rate of formation of angiotensin by renin in 0.1 ml of cat plasma incubated with excess substrate (as ordinate) to pH (as abscissae). In each case 90 mg of substrate was used. Vertical bars as in Figure 2.

isolation process carried out in 1 ml prepared Dowex 50 W – X2 (NH_4^+) renin, 100 – 200 mesh and thereafter freeze dried and assayed give not less than 80% recovery. Results of duplicate samples assayed was in agreement within the limit of ± 5%.

DISCUSSION

The method presented measures the angiotensin generating capacity of renin in a suitably controlled environment. The initial requirements in the development of the method is to prepare renin free from angiotensinase and from substrate, and then to prepare substrate free from renin and from angiotensinase. Proof of the absence of angiotensinase in renin and in substrate was obtained by continued incubation of standard renin with limited amounts of substrate for several hours after the maximum yield had been obtained. The constancy of the maximum yield for several extra hours on incubation demonstrated the absence of angiotensinase activity. Proof of the absence of substrate in renin preparations was obtained by demonstration of the absence of angiotensin formation on incubation of renin without addition of substrate. Proof of the absence of renin from preparations of substrate was made by demonstration of the absence of angiotensin formation when the substrate was incubated without the addition of renin.

The interactions of renin with renin substrate and the subsequent measurements of angiotensin generating capacity of renin assumes that renin substrate is always present in excess. Thus each batch of substrate prepared was interacted with standard renin to ensure that the amount of substrate used was in excess. The simplest way to calibrate substrate was to measure the maximum amount of angiotensin that could be formed from it by a fixed amount of standard renin, and adjustment made if necessary so that the substrate used each time is of equal concentration to the 90 mg shown in figure 2.

The incubation of renin with its substrate was carried out in the presence of a kallikrein inhibitor, trasylol, 100 units/ml and neomycin sulphate, 2 mg/ml in 0.9% NaCl. Both trasylol and neomycin sulphate in the concentrations used had no effect on the reaction rate between renin and its substrate, nor had they any effect on rat blood pressure (Skinner, 1967). Neomycin sulphate was employed to prevent bacterial contamination of the incubation medium because it had been shown previously that bacterial contamination could lead to destruction of angiotensin II (Lever, et al., 1967).

Interaction of standard renin or of plasma/lymph with excess renin substrate under standardized conditions was followed by absorption of the angiotensin so formed on a Dowex column and thereafter eluting and freeze drying. Since injections of freshly prepared solutions of these residues produced clean pressor responses of the mean systemic arterial blood pressure of ganglion-blocked rats, and duplicate incubates yield residues of similar activity, the extraction procedure was also deemed reliable. Moreover, the pressor response elicited in the rat by the injection of extracted angiotensin fractions from both lymph and plasma was of a similar shape and indistinguishable from the pressor responses induced by synthetic angiotensin II (figure 1).

The method allowed detection of renin activity in relatively small volumes of plasma or lymph. Coupled with its relative rapidity and reproducibility, the method was particularly suited to measure small volume of test sample. With appropriate modifications the method can be used to measure renin concentration in human plasma.

SUMMARY

A micro-method was developed for the measurement of renin concentration in the plasma and renal lymph of cat. The method involved the interaction of renin from plasma or from renal lymph with excess renin substrate in a controlled environment in the presence of neomycin sulphate and trasylol for a period of 12 hr. The angiotensin so formed was absorbed onto 1 ml of prepared Dowex 50W – X2 (NH_4^+), 100 – 200 mesh and elution of angiotensin was done by using 7 ml 0.1 N diethylamine followed by 7 ml 0.2 N ammonia in a Erlenmeyer flask and freeze dried. The freeze dried residue was dissolved in 0.1 ml 0.9% NaCl and bioassayed against Val^5-angiotensin II-asp-β-amide on the mean systemic arterial blood pressure of ganglion-blocked rat. The optimum pH of incubation was found to be 7.0 and hence this pH was recommended for incubation purposes. Recovery studies by using synthetic angiotensin was found to be extremely good. The method is reproducible and sensitive enough to detect the renin concentration in 0.1 ml of cat plasma/lymph. The method can be easily adapted to measure the plasma renin concentration in man.

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