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Measurement of plasma renin activity by semi-micro radioimmunoassay of generated angiotensin I

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SYNOPSIS A semi-micro method for determination of plasma renin activity (PRA) by radioimmunoassay of generated angiotensin I (AI) is described. The method permits measurement of PRA on 250 μl of plasma without loss of specificity, sensitivity, accuracy or precision.

The small sample size has considerable application in terms of investigation of infants and young children.

A reference range was established for healthy children on free diets. There was a 15-fold decline in PRA with age from a mean value of 1404 pgAI/ml h^{-1} (ngAI/1. h^{-1}) in infancy to a mean of 85 pgAI/ml h^{-1} (ngAI/l. h^{-1}) in adult life.

Several methods utilizing radioimmunoassay techniques have been reported for the measurement of plasma renin activity (PRA) based on the generation rate of angiotensin I (AI) in plasma (Boyd *et al*, 1969; Haber *et al*, 1969; Vallotton, 1969; Hollemans *et al*, 1969; Cohen *et al*, 1971; Kurtz, 1971; Rössler *et al*, 1971; Menard and Catt, 1972; Katz and Smith, 1972; Sealey *et al*, 1972; Malvano *et al*, 1972; Brech and Franz, 1972; Viol *et al*, 1972).

Many of these techniques required comparatively large quantities of blood and this has hampered the investigation of the renin angiotensin system in young children. This study describes a semi-micro method for measuring PRA and a preliminary reference range which has been determined in children.

Principle

Since it is not possible to measure the level of plasma renin directly, indirect methods have been developed. Plasma renin activity measures the rate of generation of AI as a result of the action of renin on its natural substrate. This reaction takes place under controlled conditions of incubation and in the presence of enzymatic inhibitors. which prevent AI degradation. The generated AI is measured by radioimmunoassay.

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Materials

BUFFER

The buffer was 0·05M barbitone/hydrochloric acid at pH 8·6 containing human serum albumin, 2·5 g/l (Lister Institute of Preventive Medicine, Elstree, Herts), 0·02M calcium chloride, and 0·2 g/l neomycin sulphate.

STANDARD

Synthetic Asp^1 $Ileu^5$ angiotensin I, Code No. 71/328 (National Institute for Biological Standards and Control, London) stored at $-20°C$ as 100 μg/l solution. A picogram/μl (μg/l) solution was prepared in barbitone buffer before use.

LABELLED HORMONE

I^{125} labelled Asp^1 $Ileu^5$ angiotensin I, specific activity 1000 mCi/mg (CEA-IRE-SORIN, Sallugia, Italy). Diluted in barbitone buffer such that 50 μl would give 5000 counts/100 seconds.

ANTISERUM

Angiotensin I antiserum, raised in rabbits using a conjugate of Asp^1 $Ileu^5$ AI with rabbit serum albumin (Dr G. W. Boyd, St Mary's Hospital, London) was stored at $-20°C$ as a 1 in 80 solution in barbitone buffer. This was diluted 1 in 5120 in barbitone buffer before use so that 60-70% binding of labelled AI in the absence of unlabelled hormone occurred.

DEANGIOTENSINIZED PLASMA

Deangiotensinized plasma was prepared by adsorbing AI on to Fuller's Earth (BDH Chemicals Ltd, Poole, Dorset), as described by Boyd *et al* (1969), but the adsorption procedure was performed twice, and 0·5 ml aliquots of plasma were stored at −20°C until used.

CHARCOAL

Dextran-coated charcoal suspension was prepared freshly on day of use. 20 g/l Norit SXI charcoal (Hopkin and Williams Ltd, Chadwell Heath, Essex) containing 1·25 g/l T_{40} Dextran (Pharmacia, Uppsala, Sweden) in barbitone buffer.

OTHER SOLUTIONS

0·5M disodium EDTA (BDH Chemicals Ltd, Poole, Dorset) in deionized water at pH 7·6; 0·2M dimercaptopropanol (BAL) obtained from Sigma Chemical Company, St Louis, USA, and made up freshly on day of use in deionized water; 0·34M 8-hydroxyquinoline sulphate (8HQ) obtained from Schwarz Mann, Orangeburg, USA, and dissolved in deionized water.

APPARATUS

2 ml (47 × 12 mm) polystyrene tubes with tight-fitting caps (Sterilin Ltd, Richmond, Surrey) for incubation; 2 ml (64 × 10 mm) polystyrene tubes (Greyward, supplied by A. J. Seward, London) for radioimmunoassay; syringes and repeating dispensers (Hamilton Micromesure BV, The Hague, Netherlands); Finpipettes (Kemistien oy, Helsinki, Finland); micro-combination glass/reference pH electrode (Russell pH Ltd, Auchtermuchty, Fife), cool tray (Chemlab Instruments Ltd, Ilford, Essex).

Methods

SAMPLE COLLECTION

Samples of 0·5 ml blood were collected by direct venepuncture or through an indwelling needle into polystyrene tubes, at 2°C containing 25 μl, 0·3 M disodium EDTA solution. Samples were centrifuged at 2°C, and plasma (250 μl) was transferred to tightly capped polystyrene tubes and stored at −20°C until assayed.

Plasma Incubation

Plasma samples for assay and deangiotensinized plasma thawed at 2°C. 6 μl 0·2M dimercaptopropanol (BAL) solution and 5 μl 0·34M 8-hydroxyquinoline sulphate (8HQ) solution added to each 250 μl sample. Using a micro-combination glass/reference electrode, the pH of each sample was corrected to 7·4 with a few microlitres of 0·1M HCl. From each sample, two 125 μl aliquots were removed. One was incubated at 37°C in a waterbath for 3 hours and the other kept in a cold room at 2°C for the same period.

The aliquots were then diluted 1 in 3 by the addition of 250 μl barbitone buffer at 2°C. A 500 μl sample of deangiotensinized plasma was treated in precisely the same way as the non-incubated unknown samples, that is, appropriate quantities of BAL and 8HQ were added, the pH was corrected to 7·4 followed by dilution 1 in 3 with barbitone buffer.

Radioimmunoassay

The entire radioimmunoassay of the generated AI was carried out at 2°C and in a total volume of 350 μl. A standard curve was prepared by adding 10, 25, 50, 100, 150, and 200 μl of standard AI solution (1 pg/μl) (1 ng/l) to the assay tubes. To each standard curve tube 50 μl deangiotensinized plasma, diluted 1 in 3 in barbitone buffer, was added. 50 μl quantities of the 1 in 3 dilutions of incubated and non-incubated unknown plasma were then added to assay tubes in duplicate. The remaining components of the radioimmunoassay mixture were 50 μl of ^{125}I AI solution and 50 μl of 1/5 120 dilution antiserum. The contents of all tubes were mixed and allowed to equilibrate at 2°C for 18 hours. The separation of free from bound AI was carried out by adsorbing the former on dextran-coated charcoal. 50 μl of well-mixed dextran charcoal suspension at 2°C was added to each tube. After mixing, all tubes were allowed to stand for 15 minutes and were then centrifuged at 2°C for the same period of time at 1900 g. The supernatant solutions were aspirated and the charcoal counted on an automatic gamma counter for 100 seconds.

Quantitation of Renin Activity

A standard curve was obtained by plotting counts (free AI) on the ordinate against pg AI on the abscissa. The amount of AI generated was determined by subtracting the value of the non-incubated sample from that incubated at 37°C. The PRA values were expressed as picograms AI generated per ml plasma per hour of incubation (pg AI/ml h^{-1}) (ng AI/l. h^{-1}). This can be calculated from the formula:

$$\text{PRA (pg AI/ml h}^{-1}\text{) (ng AI/l. h}^{-1}\text{)} = \frac{(\text{pgAI}^{37^{\circ}\text{c}} - \text{pgAI}^{2^{\circ}\text{c}})\ 60}{3}$$

However, slight dilutional effects due to the addition of enzymatic blockers and 0·1M HCl should be taken into account. To correct for the former,

PRA should be factorized by 1·125, although to account for the presence of EDTA in whole blood a standard haematocrit in all the specimens is assumed. To correct for addition of 0·1M HCl a variable factor is necessary: 1·02, 1·04, 1·06, and 1·08 for 5, 10, 15, and 20 μl of 0·1M HCl respectively.

Results

VALIDATION OF THE METHOD

Specificity

The specificity of the assay system for renin was indicated by the consistent failure to detect AI after incubation at 37°C of plasma from adult anephric patients. Displacement of ^{125}I AI by increasing amounts of the immunoreactive product generated during the incubation of plasma was identical with the displacement obtained by increased amounts of synthetic AI standard (fig 1). There was no evidence of cross reactivity with synthetic Asp^1 Ileu^5 angiotensin II (Schwarz Mann, Orangeburg, USA), with the antiserum used. Non-specific effects of other plasma components were excluded since each incubated plasma was compared with its own non-incubated control.

Sensitivity

The assay system for AI was sensitive with a detection limit of 3 pg.

Accuracy

On addition of synthetic AI to anephric plasma, such that the final quantities in the assay tubes varied from 20 to 200 pg, the recovery was 90-100% even after the 3 hour incubation at 37°C.

Precision

Within assay precision as represented by the coefficient of variation of duplicates ranging through the standard curve was $\pm$5%. Between assay reproducibility was 10-15%.

Relevant Experiments in Development of the Method

CONTROL OF INCUBATION CONDITIONS

Enzymatic Inhibition

Optimal accuracy is obtained when AI is prevented from being degraded and its rate of generation is constant during incubation at 37°C. Effective enzymatic inhibition in incubated plasma was achieved by using a mixture of EDTA, BAL, and 8HQ in concentrations of 20, 4·4, and 6·4 mM respectively. This resulted in almost complete recovery of exogenous AI added to plasma before incubation. When heparinized plasma containing no enzyme inhibitor was incubated no PRA was found. On the other hand, measurement of PRA after incubation of samples of the same plasma containing EDTA, EDTA and BAL, and EDTA, BAL, and 8HQ revealed values of 208, 570, and 923 pgAI/ml h^{-1} (ngAI/l. h^{-1}) respectively.

Angiotensin I Generation Rate and Incubation pH

The validity of this method relies on the assumption that there is a zero order reaction between renin and its substrate during incubation at 37°C. However, the constancy of AI generation with incubation time depends not only on the effectiveness of enzymatic inhibition but on pH variation. The renin reaction is markedly pH dependent with an optimum pH for maximum generation of AI of 5·5-6·0 (fig 2). The pH of plasma samples containing EDTA increased slightly during the first month or two of storage at -20°C, and then remained virtually constant at 8·0 $\pm$ 0·1. Correction of the plasma pH to 7·4 with 0·1M HCl was followed by an increase to 7·7 when unbuffered samples were incubated at 37°C for three hours (fig 3). However, in spite of this pH change, during incubation of high, medium, and low renin-containing plasmas, the AI generation rate remained linear.

Effects of Plasma on Radioimmunoassay

The presence of plasma in the direct assay altered the slope of the standard curve (fig 4). This degree of interference was eliminated by diluting plasma after incubation at 37°C (1 in 3) with barbitone buffer, achieving a plasma dilution in the final radioimmunoassay tube of approximately 1 in 23 (fig 5). However, at times, in the presence of tracer damage, there appeared nonspecific protein effects which were not removed by dilution alone. These effects, on the other hand, were eliminated by ensuring similar protein concentrations in the standard curve as were present in the unknown plasma tubes. This could be achieved by the addition of anephric or deangiotensinized plasma to the standard curve tubes in amounts identical with those present in the unknowns. Since this need for protein in the standard curve could not be anticipated in any particular assay, the addition of deangiotensinized plasma became part of the routine immunoassay procedure.

Separation of Antibody-bound from Free Peptide

An efficient separation of bound from free hormone was achieved using T_{40} dextran-coated charcoal. If addition of dextran charcoal to the assay tubes was achieved in under 3 minutes, a 15-minute time to

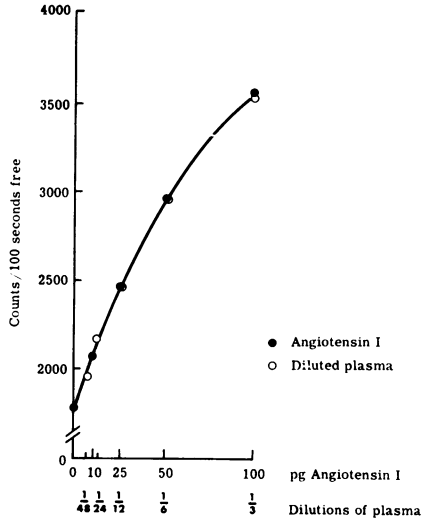

Fig 1 *Comparison of displacement of tracer by synthetic AI and by increasing amounts of immunoreactive product in incubated plasma.*

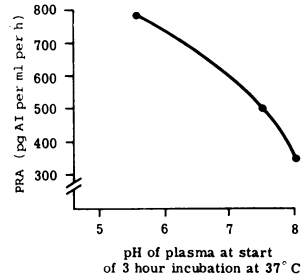

Fig 2 *Effect of plasma pH on renin activity.*

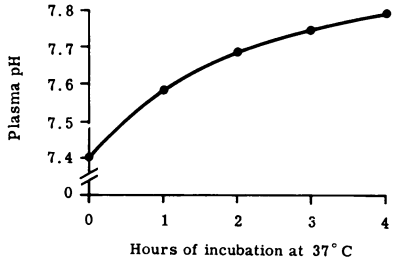

Fig 3 *Variation in plasma pH during incubation at 37°C.*

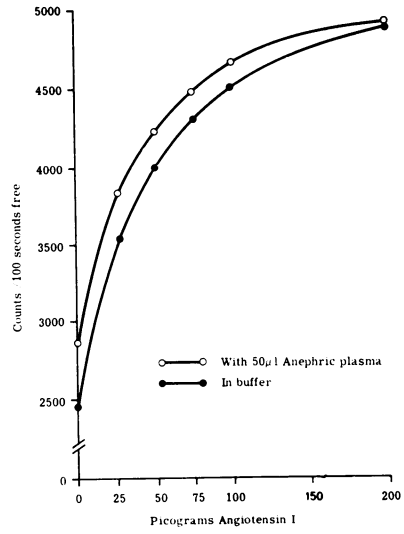

Fig 4 *Alteration of the standard curve by the presence of plasma.*

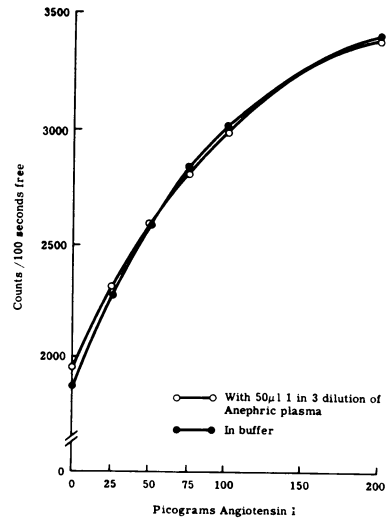

Fig 5 *Elimination of protein interference by diluting plasma 1 in 3 before addition to the assay tube. Final plasma dilution in radioimmunoassay mixture was approximately 1 in 23.*

reach equilibration was necessary before centrifugation.

Reference Ranges

PRA has been determined in children who were in hospital for minor disorders and were free of cardiovascular, renal, acid base or electrolyte disturbance. Venepuncture was performed in the course of other investigations, and the children were supine for 2 hours before the samples were taken.

Age (years)	*n*	*Plasma Renin Activity (pgAI/ml h⁻¹)*	
		Log Mean	*Observed Range*
<1	15	1404	472-3130
1-4	16	824	405-2340
5-9	21	409	55-834
10-15	11	323	175-899
Adult	9	85	22-311

Table *Reference ranges of plasma renin activity (pgAI/ml h⁻¹) (ngAI/l. h⁻¹)*

The results are shown in the table. There was a 15-fold decline in plasma renin activity with age from a mean value of 1404 pgAI/ml h^{-1} (ngAI/l. h^{-1}) in infancy to a mean of 85 pgAI/ml h^{-1} (ngAI/l. h^{-1}) in adult life.

Discussion

The advantage of this method is its small sample size which allows measurement of PRA on 250 μl of plasma without loss of specificity, sensitivity, accuracy or precision. This is particularly critical in neonates or if multiple samples are taken from young children either from peripheral or renal veins in the course of investigation of hypertension.

The control of enzymatic degradation of generated AI has been reported with a variety of chemical agents (Zucchelli *et al*, 1973; Kodish and Katz, 1974). However, effective enzymatic inhibition was achieved in this assay with a mixture of EDTA, BAL, and 8HQ, confirming the work of Haber *et al* (1969), Rössler *et al* (1971), Menard and Catt (1972), and Malvano *et al* (1972). The pH optimum for human renin with renin substrate is known to be between 5·5 and 6·0. At this pH the generation of AI is maximal, and it has been shown that pH changes during incubation are small since the flat portion of the pH optimum curve is utilized (Sealey and Laragh, 1973). However, the addition of comparatively large quantities of acid or dilution with buffer is necessary to achieve this pH range. Neither of these manoeuvres is desirable, and, in particular, dilution of plasma by buffer during the incubation procedure can markedly reduce the rate of generation of AI (Katz and Smith, 1972; Sealey and Laragh, 1973). In view of this the physiological pH of 7·4 was chosen, which allowed constancy of AI generation even though there was some pH instability during the incubation period. Linearity of generation rate within this pH range has been reported by others (Malvano *et al*, 1972) and considered to be due to the minimal effects of pH variation on AI generation within the physiological pH range. In addition, it kept the amount of AI generated within the optimal range for the radioimmunoassay calibration curve (10-200 pg).

Plasma in the assay altered the standard curve. This was eliminated by the addition of deangiotensinized or anephric plasma to the standard curve in precisely the same amounts as unknown plasma was present in the sample tubes, thus confirming the work of Katz and Smith (1972) and Malvano *et al* (1974). Enzymatic inhibitors may also affect the standard curve (Malvano *et al*, 1974), and hence the deangiotensinized plasma used in this assay contained enzymatic inhibitors and underwent pH correction to 7·4 before addition to the standard curve. Non-specific effects caused by other plasma components, including 'angiotensin I-like' proteins (Page *et al*, 1971), were eliminated by subtraction from the incubated plasma of the value measured in the non-incubated control.

The inverse relationship demonstrated in normal children between plasma renin activity and age is of considerable interest and is being studied further. Pathological values in children must be compared with the reference range relevant to their particular age before attempting interpretation of their significance.

Preliminary data on the reference range were presented at the Joint Meeting of the British Paediatric Association and the British Association for Paediatric Nephrology in April 1974 (Dillon and Ryness, 1974).

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