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THE APPLICATION OF BRADYKININ RADIOIMMUNOASSAY TO PLASMA KININOGEN DETERMINATION

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Abstract—Bradykinin radioimmunoassay was applied to plasma kininogen determination by measuring the bradykinin content of the trypsin hydrolysate of heat-acid denatured plasma. The bradykinin antibody was produced in rabbits. The immunogen was prepared by coupling bradykinin to human serum albumin with ethyl-carbo-di-imide. In the radioimmunoassay [^{125}I]tyr^{8}-bradykinin was used as tracer. The antibody was suitable for serial determinations of kininogen from 0·2 ml of plasma. The dose-response pattern of bradykinin standard solutions was the same as that of kininogen hydrolysates and the recovery in the hydrolysates was 95–110%. The mean plasma kininogen content of 26 healthy human individuals was 3·36 μg/ml (S.D. 0·49) in bradykinin equivalents which is in good agreement with previous results of kininogen bioassay.

The lack of reliable and simple methods for the determination of the different components of kallikrein–kininogen–kinin system is the main reason for the limited information about the pathophysiological role of this system. Kininogen, the protein precursor of plasma bradykinin, has so far been determined by bioassay of bradykinin liberated from kininogen. The mostly used method has been that described by Diniz *et al.* [1] and Diniz and Carvalho [2], where bradykinin is liberated by trypsin from heat-acid denatured plasma and then assayed biologically on guinea-pig ileum or rat uterus. However, in this method other peptides are liberated which potentiate the smooth muscle contracting activity of bradykinin [3–5]. The trypsin hydrolysates can be purified by ion exchange chromatography, but this is time consuming and losses of bradykinin activity occur during the various steps [5].

In the search for more specific methods for plasma bradykinin determination radioimmunologic procedures have been developed [6–10]. These are complicated by the fact that bradykinin, being not antigenic, must be coupled to a larger carrier molecule. Further, if radioiodine is used as a label, a tyrosine analogue of bradykinin must be used as bradykinin itself does not accept iodine.

We have applied bradykinin radioimmunoassay for plasma kininogen determination in order to get a more specific method suitable for serial determinations and to avoid the interference of tryptic peptides in bradykinin bioassay.

MATERIALS AND METHODS

1. Production of antiserum: Bradykinin immunogen was prepared by coupling bradykinin to human serum albumin using water-soluble carbo-di-imide. 12·5 mg of human serum albumin (Kabi) was dissolved in 0·5 ml of distilled water. 30 mg of bradykinin triacetate (Sigma) was added and dissolved and followed by 450 mg of 1-ethyl-3(3-dimethyl-aminopropyl)-carbo-di-imide HCl (Ott Chemical Company). The solution was mixed, kept in ice-bath for 30 min and dialyzed against distilled water for 48 hr at +4°. 4 ml of distilled water was added and the solution was thoroughly mixed with 4 ml of Freund's complete adjuvant (Difco). The amount of immunogen thus obtained was used to immunize 10 rabbits. The immunogen was injected intramuscularly into all four legs. The immunization was repeated at 2–4 week intervals until the titre of the antibody did not increase. The rabbits were bled from the central artery of the ear 10 days after each booster-dose. Most of the animals did not produce any detectable amounts of antibody. One rabbit out of fifteen produced a usable antiserum; this rabbit was immunized 3 times. The antiserum was used in a dilution of 1 to 100. Using this solution the ratio of the radioactivity bound to antibody in absence of unlabelled bradykinin was about 40%.

2. Radioimmunoassay: The radioimmunoassay procedure was a slight modification of that of Talamo *et al.* [9]. A radio-labelled tracer was prepared according to Greenwood and Hunter [11]. To about 2 mCi [^{125}I] sodium iodide (IMS 30, The Radiochemical Centre, Amersham) 20 μl of 0·5 M phosphate buffer pH 7·0, 50 μl of tyrosine^{8}–bradykinin solution (0·5 mg/ml) (New England Nuclear) and 25 μl of Chloramine-T (5 mg/ml) (Merck) were added. After 45 sec 100 μl of sodium metabisulfite (2·5 mg/ml) (Merck) was added and immediately thereafter 5 μl of potassium iodide solution (20 mg/ml in 2 N acetic acid) (Merck). The solution was purified from unbound iodine by chromatography on a 4 ml Dowex column (Dowex 1 × 8 Cl^- 200–400 mesh) (Fluka) in a disposable plastic pipette. The elution was made by water. The fraction (0·5 ml) containing the top activity of bound iodine was diluted to get a working solution of 2000 counts per min per 5 μl.

The uniformity of the tracer was demonstrated by an isoelectric focusing procedure (Fig. 1). The focusing was performed on a 110-ml LKB column (LKB

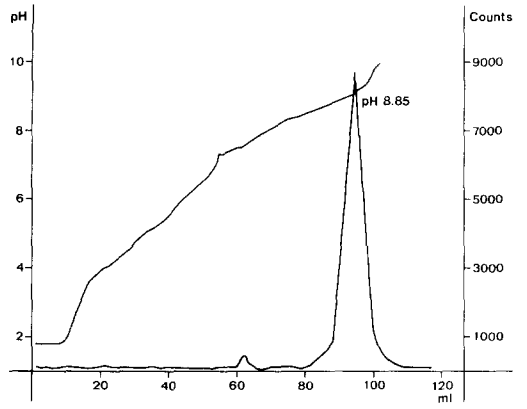

Fig. 1. Isoelectric focusing of the [^{125}I]labelled tyr^{8}-bradykinin used as tracer in the radioimmunoassay.

810) at 1% Ampholine concentration and +10°. The pH gradient was stabilized with a 0–50% sucrose gradient. Focusing time was 2 days, voltage 450 V. Fractions of 1 ml were collected and pH and radioactivity of each fraction recorded at r.t. (We are grateful to Dr U-H Stenman M.D. who performed the isoelectric focusing at The Minerva Institute for Medical Research, Helsinki).

The radioimmunoassay was carried out at r.t. as follows. Throughout the whole procedure all solutions contained 1% of gelatine, which is the best stabilizer of weak bradykinin solutions [5]. Into 1 ml conical plastic centrifuge tubes (Fisher) 20 µl of unknown or standard bradykinin solution, (bradykinin triacetate, Sigma) 5 µl of [^{125}I]tyr^{8} bradykinin working solution and 5 µl of 1 to 100 dilution of the antiserum all in veronal acetate buffered saline, pH 7·4 (veronal 1·47 mg/ml and acetate 9·7 mg/ml in 0·13 M saline) were added in this order. The amount of non-specific binding of bradykinin to rabbit serum was controlled by including a tube of non-immunized rabbit serum to each analysis. This never exceeded 10% of the total binding activity. The incubation was carried out at r.t. for 1 hr which was found to be enough for optimal binding. At the end of the incubation period 400 µl of dextran-coated charcoal suspension was added. This solution was prepared by adding 75 mg of dextran T-70 (Pharmacia) and 750 mg of Norit-A (Sigma) to 100 ml of veronal acetate saline buffer, without gelatine. The mixture was allowed to stand for 10 min, centrifuged and 100 µl of the supernatant was counted for radioactivity. A standard curve was plotted from 6 standard samples each time and the amount of bradykinin in duplicate unknown samples was derived from this curve.

3. Preparation of plasma samples: Blood was drawn from the antecubital vein through a siliconed needle into a heparinized plastic tube and centrifuged at r.t. Bradykinin was liberated from plasma kininogen according to Diniz *et al.*[1, 2] by trypsin incubation of the heat acid denaturated samples. 0·2 ml of plasma was added to 1·8 ml of 0·2% acetic acid in plastic tubes and heated in boiling water bath for 30 min. The pH of the mixture was adjusted to 7·4–7·8 by adding 0·1 N NaOH and buffered to pH 7·8 at +37° (checked by an Astrup pH meter, Radiometer, Copenhagen) by adding 0·5 ml of 0·2 M Tris-buffer. 200 µg of crystallized trypsin in 0·1 ml of saline was added and the mixture was incubated for 30 min at +37° in a water bath. The incubation was stopped by adding 5 ml boiling ethanol and the mixture was further incubated for 10 min at +70°. The mixture was cooled, centrifuged and the supernatant taken to dryness in a siliconed 100-ml round-bottomed flask under reduced pressure (Rotavapor, Buchi). The dried precipitate was stored at −20° if necessary. For kinin analysis it was dissolved in 2 ml of veronal acetate saline buffer with 1% of gelatine.

RESULTS

Figure 2 shows a typical inhibition curve obtained in our assay. The range of the assay lies between 1 and 20 ng in the sample. Samples of unknown solutions of plasma trypsin hydrolysates are plotted on the same graph. The displacement curves follow the same dose-response pattern as standard bradykinin and the lines run parallel. The standard deviation of the differences from the mean of 12 measurements from the same sample was 2·5% at the level of 63% inhibition and 3·0% at the level of 45% inhibition. The interassay standard deviation was 7% derived from 26 duplicate determinations. When known amounts of bradykinin were added to 8 unknown samples identical dose-response lines were obtained and the recovery was between 95 and 110%.

In order to test the specificity of the assay against physiological split products of bradykinin, synthetic bradykinin in phosphate buffer, pH 7·5, was incubated with human plasma under various conditions. During incubation the measurable activity of bradykinin gradually disappeared totally. The dose-response line was not affected during incubation. Thus it seems that at least the end products of plasma kininase activity do not cross-react with the bradykinin antibody used.

Table 1 shows the obtained kininogen concentrations in bradykinin equivalents from apparently healthy individuals. There is no statistically significant difference between males and females and no correlation to age. The mean kininogen content in plasma

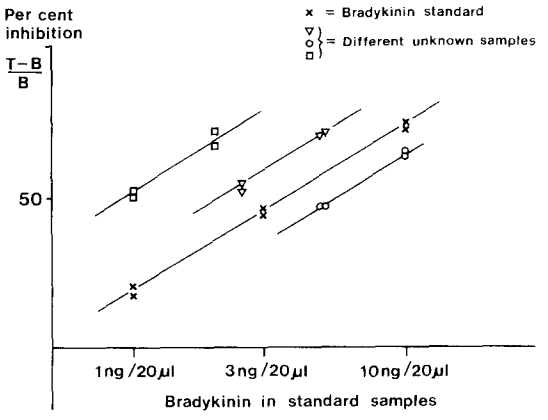

Fig. 2. Standard inhibition curve of synthetic bradykinin samples (×) (semilogarithmic scale). Three different unknown samples (undiluted and diluted 1 to 2) show parallel dose response lines. For the sake of clarity the unknown samples are plotted apart from the standard curve in arbitrary places.

Table 1. Plasma kininogen concentration as bradykinin equivalents in 26 healthy persons

	n	age range	age median	kininogen μg/ml of plasma range	kininogen μg/ml of plasma mean
Females	16	19–58	40	2·2–4·2	3·35
Males	10	21–59	35	2·8–4·0	3·36

expressed in bradykinin equivalents was 3·36 μg/ml (S.D. 0·49).

Plasma samples could be cooled or even frozen for a couple of days without apparent loss of kininogen in contrast to the findings of Periti and Sicuteri [12].

DISCUSSION

It has thus been shown that radioimmunoassay of bradykinin can be applied to the determination of plasma kininogen. As compared to bioassay the radioimmunoassay has the advantage of being more specific and more suitable for serial determinations of biological samples. The range of our radioimmunoassay lies at present between 1 and 20 ng of bradykinin per sample. It is sensitive enough for the determination of the kininogen content of 0·2 ml of plasma, but too insensitive for the determination of blood free bradykinin.

The specificity of the methods depends mainly on the quality of the antibody. The only proofs of the specificity of our antibody lies in the facts that the dose-response lines of standard solutions and different test samples always run parallel and that the physiological split products of bradykinin do not show any activity. Other peptides have not been tested but it is known that 97·5% of trypsin liberated kinin activity in human plasma in bradykinin [13].

As previously pointed out [5] gelatine has been found to be a good stabilizer of weak bradykinin solutions. Moreover, by using 1% gelatine in relevant solutions the assay can be performed at room temperature with no losses of bradykinin activity. The incubation period can be cut down to 1 hr instead of previously used 2 hr [9] or 18 hr [6].

The plasma kininogen concentration of healthy individuals when determined by the radioimmunoassay method (a mean of 3·36 μg/ml of plasma) is in good agreement with that obtained by bioassay after purification of the trypsin hydrolysates and elimination of the potentiating factors [5].

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