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A sensitive enzyme immunoassay for angiotensin II in serum

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Abstract: A sensitive and specific enzyme immunoassay for measuring angiotensin II (AII) has been developed as a convenient alternative to a radioimmunoassay. An antiserum to AII was prepared using AII conjugated by carbodi-imide to rabbit serum albumin, and coated on to microwell plates. The labelled antigen was prepared from AII and horseradish peroxidase using the periodate method. This enzyme immunoassay was a simple two-step procedure: 0.1 ml of AII-extracted plasma was incubated for 1 h at 37°C; and 1 ml of labelled AII was incubated for 1 h at 37°C. Bound horseradish peroxidase activity was then determined using *o*-phenylenediamine as chromogen by measuring the absorbance at 492 nm. The lower detection limit of the assay was 3.5 pmol l^{-1}. Between- and within-assay RSD values were 8.8–18.3% and 6.9–17%, respectively, for concentrations of 10–40 pmol l^{-1}. The accuracy of the assay, determined by recovery and linearity experiments, was 89–106% for recovery and 91–126% for parallelism. The results obtained by the present ELISA method were well correlated with those obtained by an established radioimmunoassay ($n = 10$, $r = 0.96$, intercept = 0.9 and slope = 1.02). This assay is easy to perform, rapid and does not require radioisotopes; thus it could be widely applied in clinical laboratories.

Keywords: *Enzyme immunoassay; angiotensin II.*

Introduction

There is accumulating evidence for the implication of AII in essential hypertension [1] and its determination can serve as a valuable screening test for patients with hypertension of renal origin [2].

Radioimmunoassay as a measurement method is particularly suitable for large-scale operations but the short shelf-life of the reagents, the rather sophisticated and expensive equipment, the extended counting times for accurate quantitation in some cases, and the strict regulatory controls on the use of isotopes have encouraged the development of alternative techniques.

In 1967 a radioimmunoassay (RIA) for the determination of AII was developed, with good specificity. However, a sample pretreatment procedure with a gel column made the applicability of the analysis difficult [3]. Later other radioimmunoanalysis techniques were developed but owing to the necessity of long incubation times the use of these has been limited [4].

Since the introduction of non-isotopic immunoanalysis as a promising alternative to RIA, the enzyme immunoassay has become an important technology in clinical biochemistry. In this paper the development of a new method for AII is proposed, using a quantitative enzyme immunoassay with 96-well microtitre plates and native AII for the standard curve. The applicability of this assay to measure AII in normal subjects was assessed and the results were compared with those obtained with a commercially available RIA. Since centrifugation and radioisotopes are not used, this assay has the potential for widespread application in clinical and research laboratories.

Materials and Methods

Immunization and collection of rabbit antiserum

Polyclonal antiserum from the female New Zealand rabbit was obtained after its inoculation with hapten-carrier complex (AII-rabbit serum albumin), following the protocol of immunization of Vaitukaitis [5]. The IgG fractions of antiserum to AII were isolated by ion-exchange chromatography [6]. The antibody titre was determined by RIA [7] and the cross-reaction was then studied [8] with homo-

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logous peptides such as angiotensin I (Asp^1-$Ileu^5$) (Boehringer Mannheim, Indianapolis, IN, USA), angiotensin II (Sar^1-$Ileu^8$) (The Sigma Chemical Co., St Louis, MO, USA) and angiotensin III (Sigma). The dissociation constant of the anti-serum was determined by measuring the antibody affinity for AII [9] by the Scatchard method [10], using the program EBDA by G.A. McPherson (1983).

Labelling of AII with peroxidase

Labelling of AII with peroxidase (EC, 1.11.1.7.) was performed at pH 7.4, room temperature and PBS buffer (phosphate buffer saline: NaCl, KCl, Na_2HPO_4, KH_2PO_4) by means of the periodate method [11]; the labelled AII was purified with Sephadex gel g-25 [12]. The stability of this preparation was studied at 4°C and at −20°C.

Coating the microplates

The wells of plastic microtitre plates (Immulon II; Dynatech Laboratories, Alexandria, VA) were coated by incubating them overnight at 37°C with 0.1 ml of anti-serum in PBS (pH 7.4). The wells were then washed twice using an automatic washer (LKB, mod. 1296-024) in a solution of 0.5% Tween 20 in water and were used immediately or stored at −20°C [13].

Polyparametric study of optimization

Five dilutions of antiserum (1/1000, 1/5000, 1/10,000, 1/20,000, 1/50,000), three dilutions of different conjugates (1/100, 1/500, 1/1000), nine different sequential times of incubation (15 min–3 h) and three different temperatures (4°C, room temperature and 37°C), were used for this study.

Study of the incubation time of substrate and cessation of the enzymatic reaction

Using zero-dose control, after the first two times of incubation, 0.1 ml of a working solution of *o*-phenylediamine was added [14] and the absorbance values were read with a BIO-TEK microplate reader (Behring Diagnostic, Hounslow, UK) every 5 min. At 20 min, 0.1 ml of 0.5 M sulphuric acid was added to stop the reaction and the absorbance readings continued until 60 min at room temperature.

Standard curve

For the standard curve the AII standard was diluted in PBS to yield concentrations of 2.4, 4.8, 9.6, 19, 38, 76, 153, 306 pmol l^{-1}.

ELISA protocol for AII

This consisted of six steps:

(1) From each of the calibrators or from the samples (previously extracted with ethanol) 0.1 ml was dispensed into the wells of the plates covered with anti-AII antibodies, and incubated for 1 h at 37°C.

(2) The wells were washed twice in an automatic washer with the washing solution (distilled water containing 0.5% of Tween 20).

(3) Of horseradish peroxidase-conjugated AII, diluted 100-fold in PBS, 0.1 ml was added and both samples were incubated for 1 h at 37°C.

(4) The wells were washed twice with the washing solution.

(5) Of freshly prepared substrate solution (distilled water containing 30 ml of concentrated H_2O_2 and 0.67 g of *o*-phenylenediamine per litre, 0.1 ml were added to all wells for incubation at room temperature (15–30°C) for 15 min.

(6) The reaction was stopped by the addition of 0.1 ml of 0.5 M H_2SO_4 and the absorbance was read at 5 min in a 96-well plate reader at 492 nm when the colour was stabilized.

Validation of ELISA for AII and statistical analysis

The detection limit was determined by substrating twice the standard deviation from the arithmetic mean of the zero-diluent of 10 replicate measurements. The intra- and inter-assay precision was also determined. For intra-assay precision, 10 different samples from healthy blood donors were examined by the same assay six times on each. For inter-assay precision, 10 samples in duplicate were examined on six consecutive days.

Accuracy was also studied by recovery and linearity experiments. The recovery was calculated by adding 0.05 ml of three solutions of 250, 500 and 1000 pmol l^{-1} to samples from four patients. Parallelism was studied by diluting each of the four samples (4/4, 3/4, 2/4, 1/4) with PBS buffer (pH 7.4).

The standard curve of the commercial radioimmunoassay for AII (Buhlmann Laboratories Ltd, Switzerland) was compared with that developed by ELISA and was assessed by correlation analysis and by linear regression.

To determine the levels of significance of differences between sets of experimental data the Wilcoxson rank test was used [15]. Stepwise linear discriminant analysis was applied to determine which combination of analytes provided the best discrimination between the two compared methods; unless stated otherwise, $P < 0.05$ was considered significant. Finally after processing the serum from 10 patients in duplicate results from both methods were correlated by applying linear regression analysis.

A group of 20 subjects (18–40 years) with normal arterial pressure was used as control.

Results

The polyclonal antiserum obtained showed a titre of 1/1113 (Fig. 1) and cross reactions of 0.81% to AI (Asp^1-$Ileu^5$), 0.021% to AII (Sar^1-$Ileu^8$) and 105% to AIII. The dissociation constant for the AII antibody binding was 1.76×10^{-12} M.

In the labelling of AII with peroxidase it was observed that the optimum conditions were PBS buffer (pH 7.4) at room temperature; there was a slight decrease of enzymatic activity at 4°C whereas at −20°C the labelled compound remained stable.

Optimum conditions in the polyparametric study of the developed enzyme immunoassay were: two incubation times for 1 h at 37°C, with a dilution of 1/1000 of the antibody for coating and a working dilution of 1/100 of the conjugate. The concentration range of the calibration solutions was 4.2–67.5 pmol l^{-1} (Fig. 2) and the optimum time of incubation for the substrate was 15 min at room temperature.

The lower detection limit of the method was 3.5 pmol l^{-1}. The precision study showed an intra-assay RSD of 8.8–18.3% while the inter-assay RSD was 6.9–17% for concentrations of 10–40 pmol l^{-1} (Table 1). For lower concentrations (<10 pmol l^{-1}) the intra- and inter-assay RSDs were in the range of 22.2–35% (Table 1). To assess the accuracy of the assay, standard addition and recovery experiments were performed in which known quantities of AII standard were added to normal AII-containing samples, which were then assayed. In this experiment the analytical recovery ranged from 89 to 106% and parallelism ranged from 91 to 126% (Tables 2 and 3). The results of linear regression analysis were not significantly

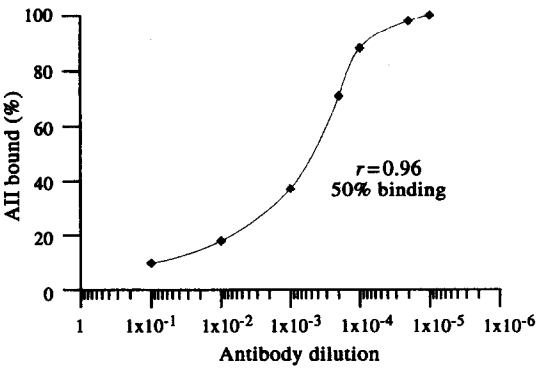

Figure 1
Titre of the antibody by radioimmunoanalysis. The abscissae show the different dilutions of the antibody: 1×10^{-1}, 1×10^{-2}, 1×10^{-3} and 2×10^{-5}, 1×10^{-5}; the ordinates show the percentage of binding.

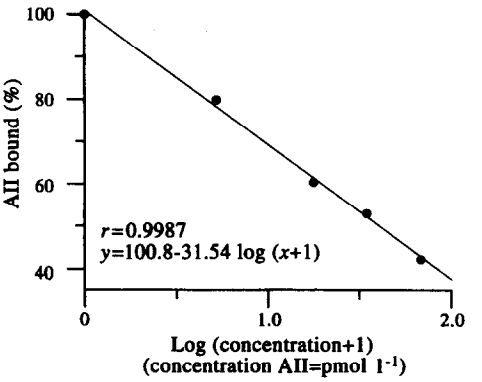

Figure 2
ELISA calibration curve for AII. The incubation time was 1 h at 37°C; the antibody dilution was 1×10^{-3} and the dilution of the conjugate was 1 in 100.

Table 1
Intra- and inter-assay precision* of the ELISA method

Angiotensin II (pmol l^{-1})	Mean	SD	RSD (%)
Intra-assay precision	6.2	1.8	30.2
	9.3	2.6	28.7
	11.8	1.9	16.4
	14.4	2.4	17.0
	15.7	1.7	11.2
	18.3	2.5	14.2
	20.9	1.8	8.8
	26.6	4.8	18.3
	28.9	2.1	7.4
	41.0	3.8	9.3
Inter-assay precision	4.9	1.7	35.4
	8.3	1.8	22.2
	17.3	1.1	6.9
	19.0	2.1	11.4
	26.7	2.7	10.2
	27.9	2.4	8.9
	28.0	1.5	5.6
	31.4	5.3	17.0
	34.8	5.1	14.7
	37.5	4.5	12.1

*n = 10.

Table 2
Analytical recovery* of AII determined by standard addition of AII

Sample	Recovery† solution	Angiotensin II (pmol l^{-1}) Observed values	Expected values	Recovery (%)
1	—	8.9	—	—
	A	22.8	20.4	112
	B	30.6	32.4	94
	C	50.1	56.4	89
2	—	12.1	—	—
	A	24.3	23.6	103
	B	36.4	35.6	102
	C	57.6	59.6	96
3	—	19.0	—	—
	A	29.9	30.0	100
	B	44.3	42.0	105
	C	59.7	66.0	90
4	—	27.1	—	—
	A	40.4	37.8	106
	B	47.1	49.8	95
	C	69.6	73.8	94

*n = 10.
†To 0.95 ml of four samples of serum previously extracted with ethanol were added 0.05 ml of solutions A, B and C (250, 500 and 1000 pmol l^{-1}, respectively).

Table 3
Study* of the parallelism of AII

Sample	Dilutions sample volume/total volume	Angiotensin II (pmol l^{-1}) Observed	Expected	Recovery (%) added AII
1	4/4	30.6	—	—
	3/4	24.7	22.9	108
	2/4	15.5	15.3	102
	1/4	8.2	7.6	108
2	4/4	66.7	—	—
	3/4	48.0	49.8	96
	2/4	32.6	33.3	98
	1/4	17.8	16.6	100
3	4/4	73.1	—	—
	3/4	60.4	54.7	110
	2/4	32.5	36.5	8
	1/4	22.9	18.2	126
4	4/4	24.1	—	—
	3/4	16.5	18.9	91
	2/4	11.6	12.0	96
	1/4	7.1	6.0	117

*n = 10.

different for both methods; correlation between the ELISA method and the commercial RIA method showed a linear regression equation of $y = 0.9133 + 1.0298x$ with a correlation coefficient (r) 0.96 (Table 4).

Table 5 shows the estimated values of the control group as the reference plasma concentration of AII, expressed in pmol l^{-1}.

Discussion

A rapid immunization technique of the animal proposed by Vaitukaitus [5] was chosen; despite obtaining low titres, owing to the use of a homologous carrier (in the case of the rabbit, albumin serum) [16], a highly specific antiserum was obtained.

In the study of the cross-reaction the results obtained in comparison with the antiserum antiAII of commercial origin (Bulman Laboratories Ltd, Switzerland) were similar [17] with respect to AII.

The dissociation constant was also similar to that of the commercial antiserum (1.89×10^{-12} pmol l^{-1}). It, therefore, made no difference which antiserum was used in the RIA assay. It is interesting that some authors [18] have confirmed that parallel standard curves

Table 4
Correlation* between RIA and ELISA methods for the determination of AII

Samples	RIA (pmol l^{-1})	ELISA (pmol l^{-1})
1	3.2	4.9
2	12.0	8.3
3	15.6	17.3
4	15.7	19.0
5	20.8	27.9
6	28.4	26.7
7	28.6	28.0
8	29.1	31.4
9	31.5	34.8
10	35.2	37.5

*$n = 10$.

Table 5
Control values, expressed in pmol l^{-1}, of AII by ELISA method

	Subjects	Values
	1	3.5
	2	2.0
	3	1.8
	4	1.7
	5	2.1
	6	2.8
	7	2.9
	8	2.85
	9	2.7
	10	2.5
	11	3.2
	12	1.9
	13	3.3
	14	3.4
	15	3.1
	16	2.0
	17	2.6
	18	2.8
	19	2.6
	20	1.9
Mean ± SD	2.58	0.55

were obtained with RIA and ELISA and that similar cross-reactions were observed using the same antiserum.

The ideal buffer solution for the conjugation of the AII with the peroxidase was PBS (pH 7.4). Others authors used carbonate buffer (pH 9.5) in the conjugation of peroxidase to antibodies [19]. On the other hand, the optimum temperature (room temperature) is the same as that used by other authors in the coupling of the peroxidase to the antibodies [20]. In the stability study of the conjugate, no loss in enzymatic activity was observed in the fractions kept at −20°C; this agreed with published work in which conjugates with peroxidase were stable for 3 years [21]. However, in the fraction kept at 4°C a slight decrease in enzymatic activity of 3.5%, was observed. This decrease can be avoided if the conjugate is lyophilized before storage [22]. The incubation time chosen for the antigen-antibody reaction is in agreement with the times considered by several authors as adequate, as is the use of 37°C as the temperature for reducing the incubation time [13]. The standard curve chosen (Fig. 2) covers normal AII levels known in human serum that vary between 0–30 pmol l^{-1} [23]. The substrate, working solution and solution used to stop the reaction, were chosen in accordance with the study by Sanchez-Vizcaino *et al.* [14] on the use of different substrates for the peroxidase. It is interesting that the optimal incubation time for the substrate found in this study was about one-half that used commercially and by several authors [13]. The lower detection limit for the ELISA method was 3.5 pmol l^{-1} compared with 0.5 pmol l^{-1} which is the detection limit established for commercial RIA (Buhlmann Laboratories, Switzerland). Despite the differences between these two limits, it is possible to consider the detection limit of the ELISA method to be of clinical utility, given the concentration range of AII in human serum.

The high RSD values of the ELISA method are to a large extent due to problems derived from the solid phase. Examples are problems that arise from: variation in the amount of antibodies immobilized at the surface of the wells; variation in the physical structure of the well surface [24, 25]; the type of plastic (propylene, polyvinyl, etc); the selection method for the adsorption of antibodies [26]; and possible changes in temperatures found in the different wells of the plate [27, 28]. These problems may arise, in part, from the use of protein A as a bridging element for the linking of the antibody to the wall of the well [29], or from the use of new separation phases comprising monomers with insoluble polymers [30].

The recovery values found in this study reflect an accuracy that is in concordance with other ELISA data published, which range from 87 to 110% [31]. Similarly the parallelism data show good linearity in comparison with other published methods [32].

Comparison of the variances and the slopes of the standard curves of RIA and ELISA showed no significant differences [15]. Owing to the high variance, the results of both methods are in good agreement [33], with a correlation coefficient of 0.96, especially when compared with other correlations presented in

the literature, which show a correlation coefficient of 0.91 [17], of 0.93 between HPLC and RIA [34], and of 0.87 between FIA and RIA [35].

ELISA techniques in general offer several important advantages in comparison with RIA. There are no radioactive materials to be handled, the shelf-life of reagents is not limited by the half-life of a radioisotope, and only small quantities of antibody are required. However, for RIA the precision is better and the limit of detection is lower. This does not impair the practicability of the ELISA method. Moreover the ELISA method can be improved using a better adsorption technique, as well as a more suitable plastic. It is concluded that ELISA is a useful alternative method to RIA in the measurement of AII. Its main virtues in the present assay are that samples can be measured rapidly and that relatively large numbers of samples may be processed concurrently.

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