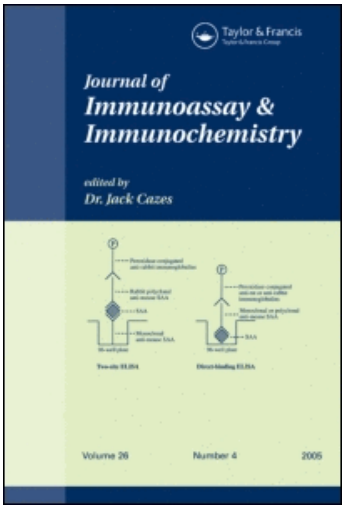

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

A Quantitative Enzyme-Linked Immunosorbent Assay for Rat Insulin

H. L. Bank[a]
[a] The Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, SC

To cite this Article Bank, H. L.(1988) 'A Quantitative Enzyme-Linked Immunosorbent Assay for Rat Insulin', Journal of Immunoassay and Immunochemistry, 9: 2, 135 — 158
To link to this Article: DOI: 10.1080/15321818808057037
URL: http://dx.doi.org/10.1080/15321818808057037

JOURNAL OF IMMUNOASSAY, 9(2), 135-158 (1988)

A QUANTITATIVE ENZYME-LINKED IMMUNOSORBENT ASSAY FOR RAT INSULIN

Bank H.L.

The Department of Pathology and Laboratory Medicine
Medical University of South Carolina
Charleston,SC 29425

Keywords: Diabetes mellitus, Islets of Langerhans, Quantitative Immunoassays, ELISA, RIA, Insulin.

ABSTRACT

A simple, quantitative micro-ELISA (Enzyme-Linked Immunosorbent Assay) has been developed for rat insulin. The micro-ELISA is a solid phase, indirect, competitive immunoassay. The useful range of the micro-ELISA was superior to that of a commercial RIA for rat insulin (e.g. 0.4 to 46.0 ng/ml for the ELISA; 0.2-8.6 ng/ml for the RIA). The ELISA's sensitivity (the lower limit of detection) was 1.0 ng/ml ± 0.13ng/ml, (mean, ± SEm; 9 assays) or 20 ± 2.6 pg/determination, and compared favorably with the sensitivity of a radioimmunoassay (RIA) for rat insulin (0.38 ± 0.10 ng/ml; 4 assays). The ELISA measured pure rat insulin standards accurately. Correlation experiments showed that the results of the ELISA agreed with those of the RIA (r=0.91), when rat insulin was assayed in crude extracts of isolated pancreatic Islets of Langerhans. When the standard curve was plotted as a log of the dose response curve, a sigmoidally shaped curve was obtained which could be transformed into a straight line relationship with a logit-log program. The goodness of fit of the transformed standard curve to a straight line relationship was excellent (r=0.97 to 0.99: n=4 ELISAs). The transform facilitated dose interpolation, tests of parallelism, and quality control. Tests of parallelism showed that the ELISA was specific for rat insulin. The precision of the ELISA was superior to the precision of the rat insulin RIA tested. The intraassay precision of the ELISA was always <10% (CV%), and its interassay precision was always ≤ 15% (CV%). The micro-ELISA is versatile, since it can be used to measure human, porcine, rat, and probably mouse insulin.

Address Correspondence to:

Dr. Harvey L. Bank
Pathology Department
Medical University of South Carolina
171 Ashley Avenue
Charleston, SC 29425

Research supported by NIH grant #DK18115

INTRODUCTION

Since experimental Diabetes research frequently employs isolated rat Islets of Langerhans (1,2), measuring rat insulin quantitatively is important. At present. rat insulin is usually measured with competitive radioimmunoassays (RIAs) (3,4,5). Although RIAs are sensitive, accurate, specific, and precise (6), they have several disadvantages. RIAs employ radioactively labeled molecules whose activity decays continuously. Consequently, the 125-I labeled insulin must be replaced frequently. and each new batch of 125-I insulin must be recharacterized. RIAs are also time-consuming, costly, and potentially hazardous to perform. These disadvantages can be circumvented by using an Enzyme-linked Immunoassay (EIA) which employs enzyme-labelled antibodies or antigens (7,8,9,10,11). The activity of enzymes is less susceptible to change during storage. Although ELISAs have been developed to measure porcine, and human insulin (13,14,15,16,17,18.19), a quantitative ELISA for rat insulin has not been reported.

The development of a quantitative ELISA for rat insulin represented a challenge since rat insulin is only available in limited quantities. To be practical, an ELISA for rat insulin had to use 10-100 times less antigen (rat insulin) than is commonly used in most ELISAs, and yet, still remain quantitative.

The aims of this research were: to develop a simple, solid phase micro-ELISA for rat insulin, to show that this ELISA is quantitative (i.e. precise, accurate, sensitive and specific), to compare the results of the ELISA with those of an RIA for rat insulin, to test the ELISA's ability to measure insulin in experimental samples (extracts of isolated islets), and to demonstrate the feasibility of applying this assay to other insulins including human and porcine insulin.

MATERIALS AND METHODS

Chemicals and Supplies:

Ortho-phenylenediamine dihydrochloride (OPD) and RIA grade bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO USA). Hydrogen peroxide was obtained from Mallinckrodt (Paris, Kentucky USA). Tween-20 was obtained from Fisher Scientific Company (Atlanta, GA USA). Human albumin (#126658) and trasylol were obtained from Calbiochem Behring (San Diego, CA USA). All chemical salts used in the buffers were obtained from Fisher Scientific Company (Atlanta, GA USA) or Sigma Chemical Company (St. Louis, MO USA). In addition, deionized, glass distilled water was used in all buffers and reagents. Type I Immunoplates (Nunc, Denmark) were used throughout this study and were obtained from Gibco Laboratories (Grand Island, NY USA).

ELISA Plate Reader:

A modified, solid state color densitometer (ESECO Speed-master, Model No.T85D; Eseco, Cushing. Oklahoma USA) equipped with a 490 to 510 nm interference filter (Ealing Corporation, Soutn Natick, MA USA) was used to measure the optical density of samples. The maximum optical density was measured with a Biotek ELISA plate reader.

Buffers:

The composition of the phosphate buffered saline (PBS) was 137mM, 1.1mM KH_2PO_4 8.1mM Na_2HPO_4, 2.7mM KCl, pH 7.2-7.4. The wash buffer contained 0.05% Tween-20 in PBS. The sample buffer for samples containing insulin contained 6.1% human (or 1% bovine) serum albumin. 0.05% Tween-20, 0.1% Trasylol, a protease inhibitor, 10mM HEPES buffer and 0.01% merthiolate (vol/vol) in PBS. The competition buffer contained the same components, but lacked merthiolate, HEPES, and Trasylol.

Reagents:

Insulins:

Purified rat insulin (>95% pure), and purified, biosynthetic human insulin (25 uU/ng) was kindly provided to us by Dr. Mary Root and Dr. R.E. Chance of Lilly Research Laboratories (Div. Eli Lilly Co., Indianapolis, Indiana USA). Pro-insulin free rat insulin was purchased from Novo Research Institute (Bagsvaerd, Denmark). In all the experiments reported in this paper, the Novo rat insulin (20μU/ng) was used as the solid phase antigen on the plate and as the liquid phase insulin in the standards. Rat insulin from both sources behaved well in this ELISA. For the purpose of this research the rat insulin standards were prepared so as to be compatible with both RIAs and ELISAs so that the performance of both assays could be compared directly. Crystallized porcine insulin was obtained from Sigma Chemical Company (24uIU/ng; St. Louis. MO USA). Human insulin standards were obtained from the World Health Organization (London, England). The final insulin concentrations of the human insulin standards were confirmed with an independent RIA for human insulin (Cambridge Diagnostics, Cambridge, MA, USA).

Anti-insulin Serum

Guinea pig anti-insulin serum (Lot No. 623) with an insulin binding capacity of 1.3 IU/ml serum) was purchased from the Department of Pharmacology, University of Indiana School of Medicine (Indianapolis, IN, USA). Wright et al. showed that, guinea pig anti-insulin serum crossreacts with rat insulin, and can be used to assay rat insulin (3). This polyclonal pig antiserum (AIS) is specific against porcine insulin and exhibits an extremely low cross-reactivity with porcine glucagon (1/10,000). The antisera was reconstituted, divided into 100μl aliquots, and stored at -20°C. The optimum dilution of the AIS was found to be 1:6400 for the rat insulin ELISA. The second antibody, horseradish peroxidase labeled rabbit anti-Guinea pig

IgG (whole molecule) was purchased from Miles Scientific (Div. Miles Laboratories, Naperville. IL USA; Lot No E056). The enzyme conjugate was stored at -70°C in 20ul aliquots of competition buffer in tubes precoated with 1%BSA/ PBS for 2 h at +37°C. The optimum dilution of this second antibody was 1:2000.

Enzyme Substrate and Stopping Solution:

The photosensitive substrate solution was made just before use and was shielded from light. It consisted of fresh 3% hydrogen peroxide, 25mg ortho-phenylenediamine (OPD) in 50ml of a buffer containing 126mM NaHP04, and 37mM citric acid (pH 6.6). The enzyme substrate, hydrogen peroxide was diluted from a concentrated 30% stock solution stored at 4°C. The stopping solution consisted of 5N H2S04.

Procedure

This micro-ELISA is an indirect, competitive, solid phase immunoassay (20). The final optimized procedure used throughout this report is illustrated in Figure 1. Optimization studies were carried out for each component of the rat insulin micro-ELISA, that is, for the primary and secondary antibodies and for the rat insulin concentration. The optimum values mentioned in this report are the final values for the most sensitive, precise, and economical assay. Since the 72 h incubation gave the most precise results, all of the results reported here were performed at that time interval. Precision was adversely affected by overfilling wells, and by washing the wells too vigorously. Wells were washed with a gentle stream of washing fluid from a plastic wash bottle.

ELISA measurements:

Unless otherwise indicated, each immunoplate contained the following: quadruplicate wells for each insulin-containing sample, quadruplicate wells for each of the non-specific binding controls

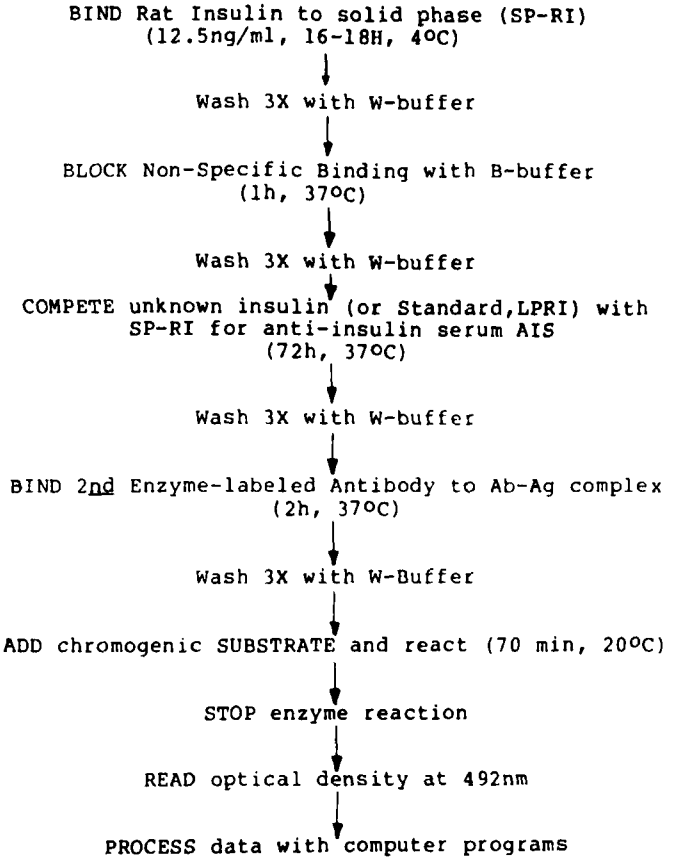

Figure 1. Outline of rat insulin Micro-ELISA.

(NSB) (lacking primary anti-insulin antibody, and sample or liquid phase insulin), quadruplicate wells of a reference blank (lacking peroxidase labeled antibodies) and triplicate wells for each of four accuracy standards (1.0, 1.75, 3.0. 10.0 ng/ml of purified rat insulin), and twelve replicate wells for the zero insulin samples (i.e. zero liquid phase insulin sample). Little color was produced in the nonspecific binding wells, i.e. 0.2 optical density units were produced in NSB wells compared to a total 1.3 optical density units in wells with no insulin (i.e. zero sample, liquid phase insulin).

Standard Curve for the ELISA:

To facilitate direct comparisons between the RIA and the ELISA. ELISA data were plotted as a log of the dose response curve (i.e., as a % response versus the log of the insulin concentr- ation). The percent response for each well was determined by obtaining the optical density of each well, correcting it for non-specific binding (NSB), and expressing the optical density as a per cent of maximum binding corrected for nonspecific binding (at the zero insulin concentration). Outliers $> \pm 3$ SD of the mean optical density at the zero insulin concentration were omitted from the calculations.

Statistical Analysis:

A computer program was written to perform the initial data reduction and statistical analysis of both ELISAs and RIAs. The program calculates the mean, standard error of the mean, standard deviation, and variance of each set of replicate measurements. It corrects all values for non-specific binding, and retains an uncorrected mean %B/Bo value for later use in the curve-fitting programs. The results are expressed in optical density units. and as a percent of the maximum bound value %B/Bo. In addition, the program prints the number of replicates (n) for each data point, and the inter-replicate coefficient of variation for each set of

replicates. Using this program, it was possible to define the shape of the ELISA competition curve, which is an important criterion for selecting an appropriate curve fitting program. All data analysis was performed on an Intertec SuperBrain (64K) or an IBM-Personal Computer (512K). A logit-log program, originally written for the analysis of sigmoidally shaped RIA curves (Dr. D. Rodbard, National Institues of Health, USA) was adapted for use with this type of ELISA.

RIA:

A commercially available RIA for rat insulin (Novo, Denmark) 4) was used to determine how well ELISA results correlated with those of an RIA. In the experiments reported here, the RIA was performed according to the manufacturer's recommendations with a few minor exceptions. To facilitate direct comparisons between the RIA and the ELISA, the 125-I radioactivity in the immuno-precipitates was counted (rather than the radioactivity in the supernatants). Like the ELISA results, the RIA results were expressed in terms of %B/Bo (corrected for non-specific binding), and plotted as a log of the dose response curve, like the ELISA results. All radioactive samples were counted with a gamma counter optimized for 125-I (Beckman Instruments, Inc., Fullerton CA USA).

Islets of Langerhans: Isolation and Insulin Extraction :

Rat pancreatic islets were isolated from 150-250g Long Evans Hooded rats according to the method of Lacy and Kostianovsky (1), as modified by Taylor (21). Insulin was extracted from the islets with the following procedure. Groups of 20 similarly sized islets were placed in one ml of the sample buffer, and insulin extraction was carried out by hypotonic lysis of the islet cells (0-4°C) in the presence a protease inhibitor (0.1% Trasylol). To ensure complete disruption of cells, osmotic disruption was followed by 3

cycles of freeze-thawing between 0°C and -28°C. Subsequently, the samples were restored to isotonicity with the addition of cold concentrated (10X) solution of sample buffer, then the samples were centrifuged (5 min, 13,000 g) to remove large cellular debris. The supernatants were stored at -20°C until assayed.

The same ELISA can be used for porcine, and human insulin, if the coating and competing standard insulin are the same. The optimized values for porcine and human insulin ELISAs are given in figure 3A to 3C.

SENSITIVITY

In this report the sensitivity is defined as the lower limit of detection (LLD) of the assay. That is, the sensitivity is that insulin concentration that lies two standard deviations away from the zero insulin concentration on the standard curve.

CORRELATION EXPERIMENTS

In the correlation experiments, rat insulin was measured in crude extracts of isolated rat islets. The rat insulin was extracted from eight groups of isolated islets (20 islets/group; 2 isolations), and the insulin concentration of each stock extract was determined with an RIA. Then, these 8 extracts were diluted further to form a total of 26 experimental samples with a wide range of insulin concentrations. All samples were measured in quadruplicate by each assay.

RESULTS

Useful range

The useful range of the micro-ELISA for rat insulin was greater than that of the RIA tested. For example, the micro-ELISA for rat insulin in Figure 2A had a useful range of 0.4 - 46.0 ng/ml, while the RIA in Figure 2B had a useful range of 0.2-8.6

ng/ml. Typically, the useful range of the RIA was between 0.3-3.0 ng/ml.

Sensitivity

The lower limit of detection (sensitivity) of nine separate ELISAs performed over five months was 1.02 ± 0.13ng/ml (± SEM; See Table 1). This corresponds to value of 20 ± 2.6 pg per determination, or 175 ± 23 femtomoles/ml, or 3.5 ± 0.46 femtomoles, or 0.4 ± 0.05 μU/determination. The mean sensitivity of four separate RIAs was 0.38 ± 0.10 ng/ml (mean, ± SEM), which was slightly better than that of the ELISA.

Accuracy and correlation

Table 2 shows that the ELISA measured three highly purified rat insulin standards accurately over a range of insulin concentrations in six separate ELISAs. Subsequent minor modifications to the washing procedure have increased the precision of the assay without comprimising its accuracy.

To determine how well the results of the micro-ELISA for rat insulin agreed with those of the RIA when rat insulin was present in crude extracts of isolated islets, the mean insulin concentrations of 26 samples were determined and plotted on a scattergram. A linear regression gave a correlation equation of Y (ELISA) = 0.8X (RIA) + 0.5ng/ml, and a correlation coefficient of 0.91.

Precision

The precision of the ELISA and RIA were compared directly. The coefficients of intrassay and interassay variation were calculated with the same computation for the same standards using the same number of replicates (Table 3). In general, the rat insulin ELISA was more precise than the RIA tested. The

TABLE 1

ELISA
SENSITIVITY [a]

Assay	Sensitivity (ng/ml)
ELISA 1	1.6
" 2	1.4
" 3	0.7
" 4	1.0
" 5	0.7
" 6	1.2
" 7	1.3
" 8	0.9
" 9	0.4

Mean = 1.02 (± 0.13, SEM) ng/ml
= 175 (± 23.0, SEM) femtomoles/ml [b]
= 3.5 (± 0.46, SEM) femtomoles/well
= 3.5 (± 0.46, SEM) femtomoles/determination

a Lower Limit of Detection of ELISA

b Assumes a M.W. of 5,700 daltons for rat insulin

TABLE 2

ELISA

ACCURACY

No.	Accuracy Standards [Rat Insulin] 1.0	1.75	3.0 (ng/ml)	
1)	1.65	2.39	2.81	(30)
2)	1.42	1.88	3.63	(30)
3)	1.02	3.12	3.18	(30)
4)	1.68	2.80	3.07	(30)
5)	1.11	1.80	3.37	(3)
6)	1.12	1.42	2.24	(3)
Grand Mean =	1.33	2.24	3.05	

coefficients of intrassay variation for the optimized ELISA were >10% (CV%), while the coefficient of intrassay variation were always less than or equal to 15% (CV%). The coefficients of intraassay variation for the RIA were less than or equal to 11.3% (CV%), while the coefficients of interassay variation were less than or equal to 39% (CV%).

To compare the precision between two very different assays, having two different useful ranges, the precision was compared at or near the midpoints (on a logarithmic scale) of the standard curves. This is the region of the standard curve where the

TABLE 3

ELISA PRECISION

Nominal [Insulin] (ng/ml)	No. Assays	No. Replicates/ Assay	CV% Intra-Assay	CV% Inter-Assay
1.75	6	3	8.4%	14.8%
3.0	5	3	5.5%	15.0%
10.0	5	3	5.3%	13.6%

RIA PRECISION

Nominal [Insulin] (ng/ml)	No. Assays	No. Replicates/ Assay	CV% Intra-Assay	CV% Inter-Assay
1.0	4	3	11.1%	31.7%
3.0	4	3	11.3%	39%

precision of both assays will be best. The coefficients of intraassay and interassay variation for the RIA at 1.0ng/ml near the midpoint of 1.25 ± 0.105 ng/ml (mean ±SE; 4 RIAs) were 11.1%, and 39%, respectively. The coefficients of intraassay and interassay variation for the ELISA at 10.0 ng/ml (near the midpoint of 7.6ng/ml ± 0.69 ng/ml on a log scale) were 5.3% and 13.6%, respectively. Thus, the ELISA was more precise than the RIA tested.

Shape of the standard curve:

To analyze the performance of this assay, the standard curve was transformed into a straight line relationship, where the slope, intercepts, and statistical parameters of assay performance could be calculated easily. The choice of an appropriate transform required the knowledge of the true shape of the standard curve. Figure 2a illustrates the shape of the ELISA's standard curve. The error bars in figure 2a show the ± 2 standard deviations of the mean of 30 replicates at each insulin concentration. Thus. the approximate 95% confidence limits for the shape of the standard curve show that the ELISA is sigmoidally shaped. This finding was confirmed with three other equally extensive ELISAs.

Since the ELISA,s standard curve was sigmoidally shaped, it could be transformed into a straight-line with a logit-log transformation. The transformed ELISA standard curves resulted in excellent fits to straight line. Correlation coefficients of the goodness of fit for four separate ELISAs were 0.97, 0.98, 0.99, and 0.99. The goodness of fit of RIA standard curves to straight line relationships were not quite as good as those for the ELISAs (figures 2d; r=0.90). Nonetheless, it is apparent that both the ELISA and the RIA curves are similarly shaped, and when the log of the dose response curves (figures 2a and 2b) are transformed with a logit-log transformation (figures 2c and 2d), the performance of

these two assays are easily compared, thus simplifing the quantitative analysis of specificity. That is, it is much easier to test for parallelism between straight lines than between sigmoidally shaped curves.

Specificity

In addition to using antigens of known purity, and antibodies of known specificities, the specificity of the ELISA was verified by testing the experimental and standard curves for parallelism. To adequately test for parallelism, 12-15 different insulin concentrations between 0.4 and 30 ng/ml (8.0-600 uU/ml) were assayed for each curve. The curves were transformed with an unweighted logit-log analysis and the slopes of the transformed curves were computed. The slopes of the transformed experimental curves were the same as the mean slope of the standard curve, within ± 2 standard errors of the mean. The slopes of the transformed curves were: -0.95 ($\pm$ 0.55 SEM), -0.82 ($\pm$ 0.60 SEM), and -0.77 ($\pm$ 0.13 SEM). Since experimental curves are parallel to standard curves, and the experimental samples behave like the purified, pro-insulin free rat insulin over a wide range of insulin concentrations, the assay is specific.

Applicability to other insulins:

Figures 3 A-C demonstrate the feasibility of using the micro-ELISA to measure other insulins including, porcine (fig. 3B), and human insulin (fig. 3C). Figure 3A shows the log of the dose response curve for a different ELISA for rat insulin than is shown in Figure 2A. Similar results were obtained for both assays. Figure 3B shows the log of the dose response curve for the porcine insulin micro-ELISA. The sensitivity of the porcine insulin ELISA was the greatest of the three ELISAs shown in figure 3 (0.05-0.1ng/ml or 1.3-2.5μU/ml). This corresponds to 0.026 to 0.05μU/ per determination. Figure 3C shows the log of the dose response

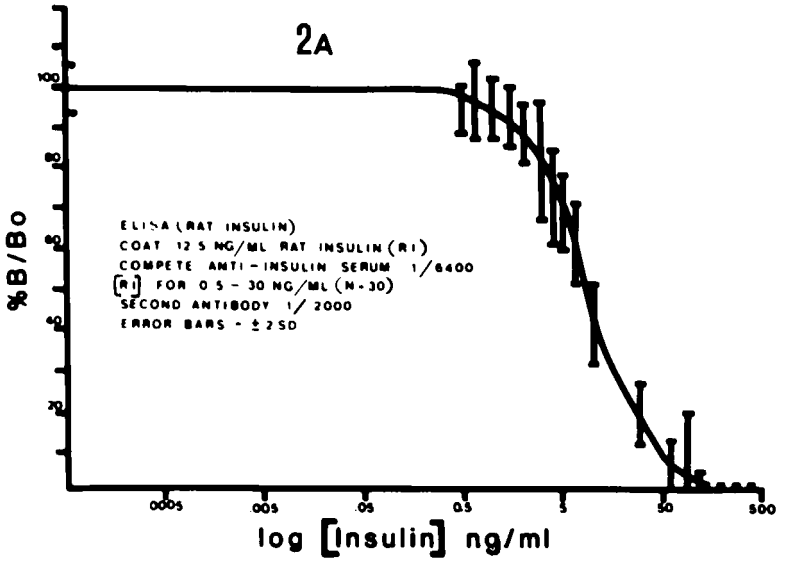

Figure 2A. Log of the dose responsive curve for an ELISA assay (rat insulin). The ELISA curve is sigmoidally shaped, and its useful range (90%-10% B/Bo) is greater than that of a commercial RIA for rat insulin shown in Figure 2C. The error bars for the ELISA curve (Figure 2A) represent the ± 2 SD of 30 replicate determinations for each data point between zero and thirty ng/ml. Thus, the error bars approximate the 95% confidence limits of the standard curve.

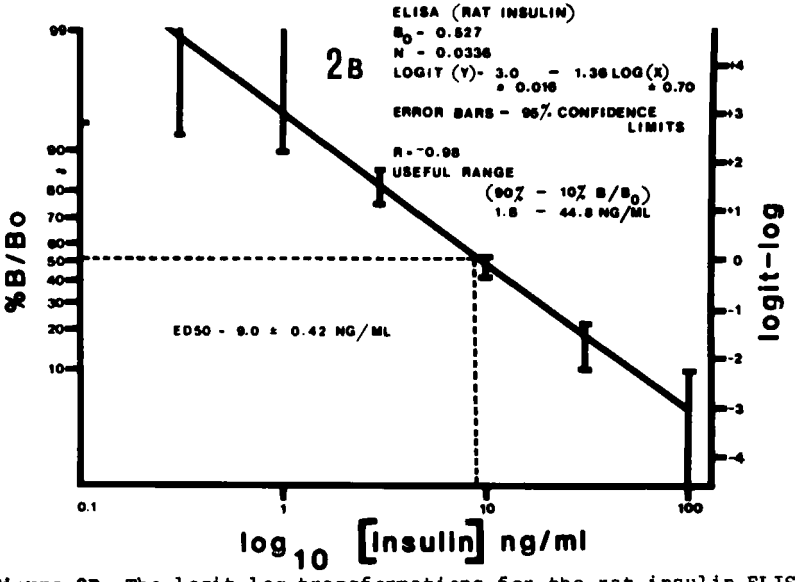

Figure 2B. The logit log transformations for the rat insulin ELISA is illustrated.

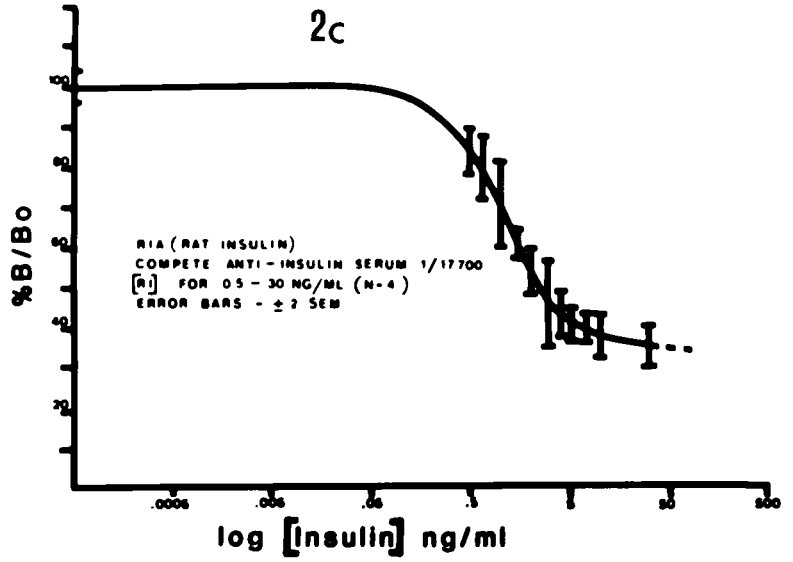

Figure 2C. Log of the dose response for a single RIA (rat insulin) showing that the standard of the RIA for rat insulin is sigmoidally shaped. Each error bar represents ± 2 SEM of four replicates. This RIA has the largest useful range of all the assays tested. Typically its useful range was between 0.03, and 3.0ng/ml.

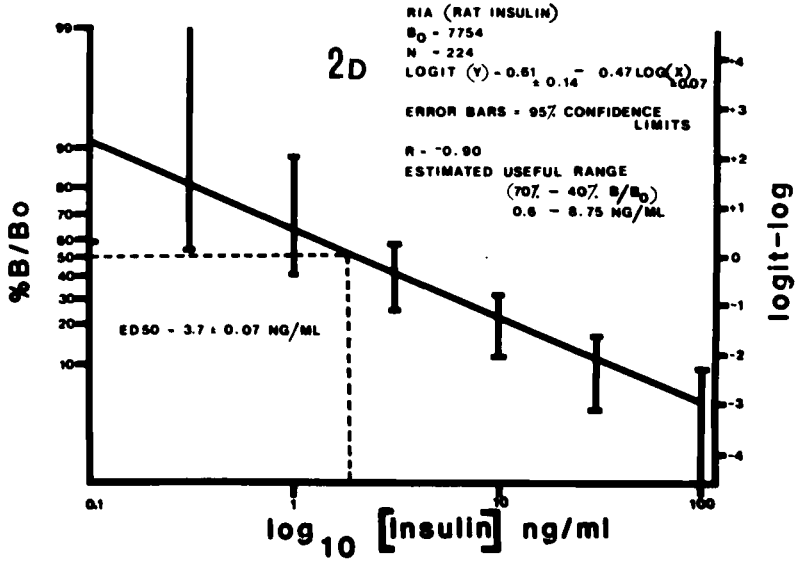

Figure 2D. The logit log transformations for the rat insulin RIA (fig.2C) is illustrated. All 4 curves (figures 2A-2D) were corrected for non-specific binding, and were plotted on similar axes to permit direct quantitative comparisons between the performance of the assays.

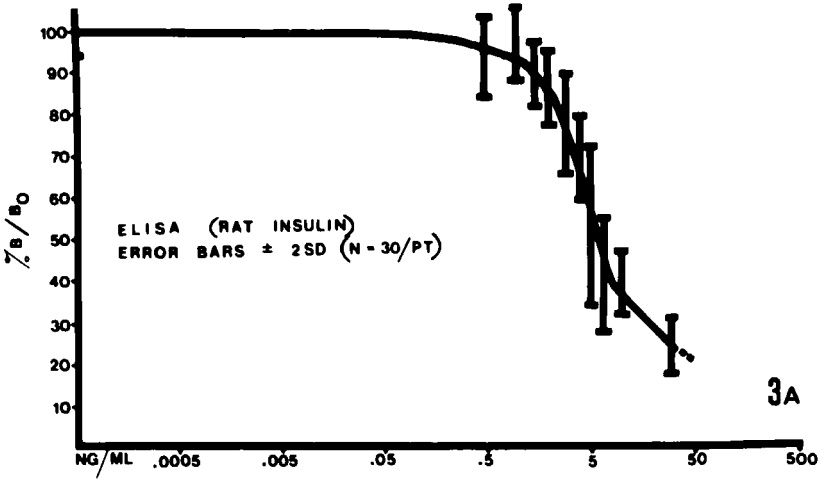

Figure 3. Versatility of the micro-ELISA. Figures 3A through 3C show that the same ELISA can be used to measure other insulins with only minor changes in procedure.

Figure 3A. The figure shows the log of the dose response curve for a rat insulin ELISA. The data are from a different ELISA than that shown in Figure 3A, however the results are similar.

curve for the human insulin micro-ELISA. The sensitivity of the human insulin ELISA was 0.4 ng/ml. This micro-ELISA accurately measured three human insulin standards (10, 50, 150μU/ml) obtained from the World Health Organization (London). The final concentration of each human insulin standard was verified independently with an RIA for human insulin. Since standard curves of each micro-ELISA from Figures 3A to 3C are sigmoidally shaped, the same computation methods could be used.

DISCUSSION

A simple, quantitative micro-ELISA for measuring rat insulin has been developed. The assay is accurate, specific, sensitive, and precise. The results of the micro-ELISA correlate well with those of a comparable RIA, and it measures rat insulin in purified, standards and in extracts of islets. The micro-ELISA is

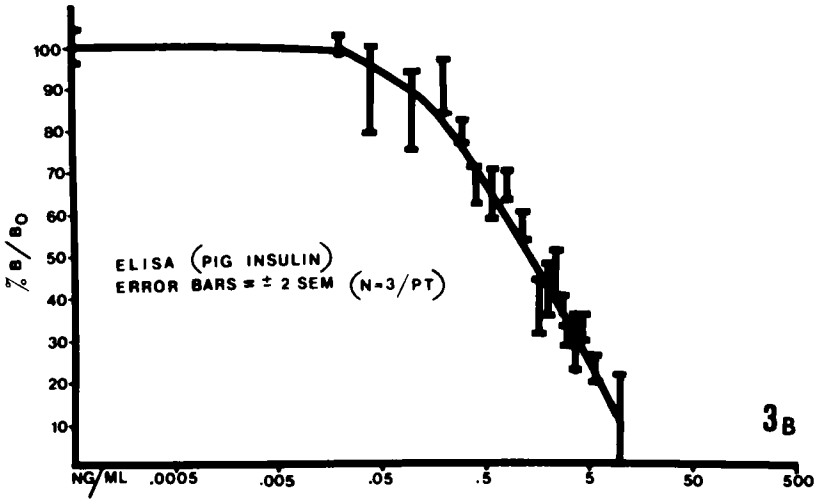

Figure 3B. The figure shows the log of the dose response curve for a Porcine insulin ELISA. In this assay, porcine insulin was used both as the solid phase (coating) and liquid phase (competing) antigen.

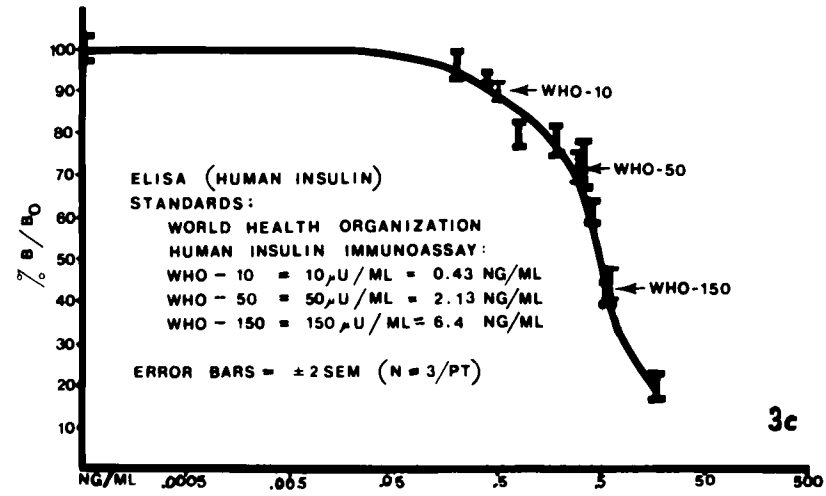

Figure 3C. The figure shows the log of the dose response curve for Human insulin ELISA. In this assay, porcine insulin was used as the solid phase (coating) antigen, and biosynthetic human insulin (Eli Lilly Co.) was used as the competing antigen. The three human insulin immunoassay standards (courtesy of the World Health Organization, London, England) indicate that human insulin was measured accurately by the assay.

versatile since it measures human and porcine insulin. Furthermore, the ELISA can be used to measure mouse insulin, since the amino acid sequence of mouse insulins I and II are identical to those of rat insulins I and II (2).

The ELISA's sensitivity is only slightly less than that of a commercial RIA for rat insulin. The sensitivity of the micro-ELISA for rat insulin is sufficient to measure the insulin content of a single islet in 5.0ml of buffer or to measure the amount of insulin secreted by a single islet into 1.0 ml buffer (assuming that 20% of the insulin in an isolated islet is secreted during a 60 minute static challenge with 11mM glucose).

Since this is the first report of the development of an ELISA for rat insulin, the performance of this ELISA cannot be compared directly with another rat insulin ELISA. However, other enzyme immunoassays (EIAs) have been developed for human and porcine insulins and comparisons can be made with these ELISAs. In general, the lower limit of detection (3.5 femtomoles) for rat insulin compares favorably with the lower limits of detection of other ELISAs for human and porcine insulin. e.g. 2.1 (14), 3.5 (15), and 5.0 femtomoles (16). When compared with colorimetric EIAs for human insulin, the colorimetric rat insulin micro-ELISA is more sensitive than two assays (13,16), and slightly less sensitive than another (17). The rat insulin ELISA is somewhat less sensitive than fluorimetric ELISAs for human insulin (18,19). The sensitivity of the rat insulin micro-ELISA (0.4uU/well) is equal that of one fluorescent EIA for porcine insulin (14), and slightly less sensitive (2X less) than another (16).

The sensitivity of the porcine insulin micro-ELISA shown in figure 3B is more sensitive than two fluorescent EIAs for porcine insulin (19,21), while the human insulin ELISA in figure 3C is

less sensitive than the most sensitive fluorescent ELISA for human insulin (19).

Three observations support the conclusion that this micro-ELISA measures rat insulin accurately. First, the ELISA accurately measured highly purified (>95%) rat insulin standards accurately. Second, the ELISA measured rat insulin obtained from extracts of islets accurately and its results correlated well with those of an RIA. Third, the ELISA measured the amount of insulin/rat islet accurately since the insulin/islet values fell within the range of values reported in the literature (22,23,24).

The useful range of the ELISA was consistantly superior to that of the RIA tested. One possible explanations for the smaller useful range of the rat insulin RIA may lie in the fact that the RIA uses 125-I labeled porcine insulin as a tracer antigen, and rat insulin may not displace all of the labeled porcine insulin. Since the ELISA uses rat insulin for both the bound (solid phase insulin) and free phase (liquid phase), the rat insulin should be able to displace all of the anti-insulin antibody bound to the solid phase rat insulin. One of the practical advantages of the greater useful range of the ELISA is that fewer dilutions and remeasurements of samples are needed.

The precision of the micro-ELISA was superior to that of the rat insulin RIA tested, but equivalent to that of RIAs known to be precise (17). To enable comparison of precision both RIA and ELISA standards were drawn from the same batch of rat insulin, the same number of replicates were used, the same computational methods were used and both curves were corrected for non-specific binding. The precision of the Elisa was superior to that of the RIA. The ELISA is versatile, since it can be used to measure rat, porcine, and probably mouse insulins. Since the ELISA is a

micromethod, it will be useful in applications where large numbers of precise, quantitative determinations are needed, e.g. in perifusion studies of the kinetics of insulin release, and in studies of the molecular biology, biosynthesis, secretion, and metabolism of rat and mouse insulin. Since World Health Organization human insulin standards are measured accurately with the same type of micro-ELISA as the one for rat insulin, in the future, both clinical and experimental samples may be assayed with essentially the same procedure.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. Michael Taylor (Cambridge U., Cambridge, England) for sharing his islet isolation procedure with us, for his helpful discussions and encouragement. Dr. Patrick Moore and Renita Didiyk for technical assistance, Dr. Philip Rust, and Fu Shing (Medical University of South Carolina, Charleston SC USA) for help with statistical methods, Karen Lattie for her excellent typing assistance.

REFERENCES

1. Lacy, P.E. and Kostianovsky M Method for the isolation of intact islets of Langerhans from the rat pancreas. Diabetes 1967; 16: 35-39

2. Permutt, M.A. Biosynthesis of insulin.In: Cooperstein SJ, Watkins D (eds) The Islets of Langerhans: biochemistry, physiology, and pathology. New York, Academic Press, 1981: pp 75-95

3. Wright, P.H., Malaisse, W.J. and Reynolds, I.J. Assay of partially neutralized guinea pig anti-insulin serum. Endocrinology 1967; 81: 226-234

4. Heding, L.G. Determination of total serum insulin (IRI) in insulin-treated diabetic patients. Diabetologia 1972; 8: 260-266

5. Hales, C.N. and Randle, P.J. Immunoassay of insulin with insulin-antibody precipitate. Biochem J 1963; 88: 137-146

6. Yalow, R.S. and Berson SA (1973) Radioimmunoassay.Insulin measurements. In: Berson SA, Yalow RS (eds) Peptide Hormones. Vol. 2B, New York, Elsevier Publishing Co., Inc., 1973: pp 864-870

7. Avrameas, S. Coupling of enzymes to proteins with glutaraldehyde. Use of conjugates for the detection of antigens and antibodies. Immunochemistry 1969; 6: 43-52

8. van Weemen BK, Bosch AMG. Dawson EC, van Hell H, Schuurs AHWA Enzyme immunoassay of hormones. Scand J Immunol 8 1978; (Suppl 7): 73-82

9. Engvall E, Perlman P Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 1971; 8: 871-874

10. Engvall E, Jonsson K, Perlman P Enzyme-linked immuno sorbent assay. II. Quantitatiave assay of protein antigen, immunoglobulin G by means of enzyme labeled antigen and antibody-coated tubes. Biochim Biophys Acta 1971; 251: 427-434

11. van Weemen BK, Schuurs AHWM Immunoassay using antigen-enzyme conjugates. FEBS Letters 1971; 15: 232-236

12. Bosch, A.M.G., van Hell, H. Brands, J. and Schuurs, A.H.W.M. Specificity, sensitivity and reproducibility of enzyme immunoassays. In: Pal SB (ed) Enzyme Labelled Immunoassay of Hormones and Drugs. Walter De Gruyter, New York. 1978: pp 175-187

13. Miedema, K. Boelhouwer, J. and Otten, J.W. Determinations of proteins and hormones in serum by an immunoassay using antigen enzyme conjugates. Clin Chem Acta 1972; 40: 187-192

14. Kato, K. Yamaguchi, Y. Fukui, H. and Ishikawa, E. Enzyme-linked immunoassay. I. Novel method for synthesis of the insulin-Beta-D galactosidase conjugate and its applicability for insulin assay. J Biochem 1975; 78: 235-237

15. Kitagawa, T. and Aikawa, A. Enzyme coupled immunoassay of insulin using a novel coupling reagent. J Biochem 1976; 79: 233-236

16. Ishikawa, E. Enzyme immunoassay of insulin by fluorimetry of insulin-glucoamylase complex. J Biochem 1973; 73: 1319-1321

17. Yoshioka, M. Taniguchi, H. Kawaguchi, et al. Evaluation of a commercial enzyme immunoassay for insulin in human serum, and its clinical application. Clin Chem 1979; 25: 353-383.

18. Kato, K. Umeda, Y. Suzuki, F. Hayashi, D. and Kosaka, A. Evaluation of a solid-phase enzyme immunoassay for insulin in human serum. Clin Chem 1979; 25: 1306-1308

19. Imagawa, M. Hashida, S. Ishikawa, et al. A highly sensitive sandwich enzyme immunoassay for insulin in human serum developed using capybara anti-insulin FAB1 horseradish peroxidase conjugate. Anal. Letters 1983; 16: 1509-1523

20. Voller, A. Bartlett, A. and Bidwell, D.E. Enzyme immunoassays with special reference to ELISA techniques. J Clin Pathol 1978 31: 507-520

21. Taylor, M.J. Duffy, T.J. Hunt, C.J. Morgan, S.R.A. and Davisson, P.J. (1983) Transplantation and in vitro perifusion of rat islets of Langerhans after slow cooling and warming in the presence of either glycerol or dimethyl sulfoxide. Cryobiology 20: 185-204

22. Andersson, A. Isolated mouse pancreatic islets in culture: Effects of serum and different culture media on the insulin production of the islets. Diabetologia 1978; 14: 397-404

23. Reaven, E.P. Gold, G. Reaven, G.M. Effect of age on glucose stimulated insulin release by the B-cell of the rat. J Clin Invest 1979; 64: 591-599

24. Schatz, H. Gunnerman, B. Schafer, G. et al. Regulation of islet function in cultivated islets of rat pancreas. In: Islet-Pancreas-Transplantation and Artificial Pancreas. Georg Thieme Verlag, Thieme Stratton, Inc. New York, N.Y. 1982; p59-64