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RAPID AND HIGHLY SENSITIVE ENZYME IMMUNOASSAY (EIA) FOR HUMAN INSULIN USING POLYSTYRENE BALL OR PLATE AS A SOLID PHASE

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We set up a rapid and highly sensitive sandwich enzyme immunoassay (EIA) of human insulin using anti-porcine insulin guinea pig Fab'-peroxidase conjugate, and polystyrene balls or polystyrene plates coated with anti-porcine insulin IgG. The minimal detectable quantity was 0.15 μU/ml and 0.63 μU/ml using 20 μl sample in polystyrene ball method and polystyrene plate method, respectively. All procedures were completed within three hours. Gel chromatographic analysis of recombinant human insulin showed a single peak of immunoreactive insulin, whereas two peaks, corresponding to insulin and proinsulin, were obtained from plasma obtained from a patient with myotonic dystrophy. The diluted human plasma sample showed linear response parallel to the standard. Plasma immunoreactive insulin levels measured by EIA were well correlated with those of radioimmunoassay. The EIA systems are accurate enough to measure plasma immunoreactive insulin levels within a few hours without any concentration, suggesting a useful tool for evaluating insulin secretion in clinical and basic research.

Key words: enzyme immunoassay / human insulin

INTRODUCTION

Radioimmunoassay (RIA) for immunoreactive insulin was one of the earliest methods established for measurement of circulating peptide hormones (1). Since then, plasma insulin levels have been extensively measured by RIA (2-3). However, there are several disadvantages inherent in RIA. RIA needs radioactive reagents and isolated institution for the handling. Furthermore, radioisotope-labeled reagents are not stable for more than a few months. These disadvantages have been overcome by enzyme immunoassay (EIA) or enzyme-linked immunosorbent assay (ELISA), in which enzyme-labeled reagents are kept stable for at least a year or so. Furthermore, EIA could be routinely performed in small laboratories. Therefore, many RIA systems have been replaced by EIA, but more sensitive and less time/cost-consuming methods have been consistently expected.

In the present study, we established the rapid and sensitive EIA of human insulin using anti-porcine insulin guinea pig antibody and either polystyrene ball or polystyrene plate as a solid phase. Furthermore, gel chromatographic studies were performed to investigate immunological characteristics of the assay.

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MATERIALS AND METHODS

Production of anti-porcine insulin guinea pig serum

Five Hartley strain guinea pigs (Japan SLC Co., Kyoto, Japan) were used throughout the experiment. Porcine pancreas insulin (Sigma, USA) was dissolved in physiological saline, and emulsified in complete Freund's adjuvant. Twenty μg of the conjugate was subcutaneously and multiply injected in each of 5 guinea pigs. Serial injection and blood sampling were performed every week. The highest immunoreactive titer and affinity serum was collected after evaluation by common double antibody RIA.

Preparation of anti-porcine insulin IgG, $F(ab')_2$, Fab', and conjugation with peroxidase

Anti-porcine insulin guinea pig serum was mixed with the same volume of 34% Na_2SO_4 and the precipitate was dissolved in 0.015 M sodium phosphate

buffer, pH 6.5. IgG fraction was separated from the solution by DEAE cellulose chromatography with linear gradient of 0.015 M to 0.2 M sodium phosphate buffer, pH 6.0.

The purified IgG was dialyzed against 0.1 M sodium acetate buffer, pH 4.5, and digested with porcine gastric mucosa pepsin (Sigma) at 37°C for 20h. $F(ab')_2$ fraction was separated from the digested IgG with an Ultrogel AcA 44 (IBF, France) column using 0.1 M sodium borate buffer, pH 8.0. The $F(ab')_2$ was converted to Fab' by 0.1 M 2-mercaptoethylamine at 37°C for 90 min. Fab' fraction was separated by an Ultrogel AcA 44 column using 0.1 M sodium phosphate buffer, pH 6.0, containing 5 mM EDTA.

The Fab' was conjugated with horseradish peroxidase by the maleimide method (4-8). Horseradish peroxidase (Sigma) was incubated with 4-(N-maleimidemethyl) cyclohexanelcarboxylic acid N-succinimidyl ester (Aldrich, Milwaukee, Mich) at 30°C for 30 min. Then, the peroxidase-maleimide conjugate was separated by a Sephadex G-25 column using 0.1 M sodium phosphate buffer, pH 7.0. Fab' was incubated with the peroxidase-maleimide conjugate at 30°C for 60 min. The Fab'-peroxidase conjugate was separated by an Ultrogel AcA 44 column using 0.1 M sodium phosphate buffer, pH 6.5. The conjugate solution was preserved at 4°C with bovine serum albumin (BSA) and thimerosal at the final concentrations of 0.1% and 0.005%, respectively.

Preparation of anti-porcine insulin guinea pig IgG coated polystyrene balls and polystyrene plate

Polystyrene balls (3.2 mm in diameter, Immunochemical Co., Okayama, Japan) as solid phase were coated with anti-porcine insulin guinea pig IgG by physical absorption, as previously described (4, 6, 7). The polystyrene balls were stored with 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1% NaN_3, 0.1% BSA and 0.1 M NaCl. The balls were used after preserved for more than two weeks.

Polystyrene plates (Nunc-Immuno Plate, Denmark) as solid phase were incubated with 0.01 mg/ml of anti-porcine insulin guinea pig IgG in 0.1 M sodium phosphate buffer, pH 7.5 at 4°C for 20 hours. After washing with 0.1 M sodium phosphate buffer, pH 7.2, containing 0.1 M NaCl, the plate was blocked with 0.1 M sodium phosphate buffer, pH 7.2 containing 0.5% BSA at 20°C for 2 hours. After washing with phosphate buffer (0.1 M sodium phosphate buffer, pH 7.2, containing 0.1 M NaCl and 0.05% Triton X-100), the plate was stored at 4°C with 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1% NaN_3, 0.1% BSA and 0.1 M NaCl.

Assay procedure in polystyrene ball method

Anti-porcine insulin guinea pig IgG-coated polystyrene balls were incubated with 20 µl of either human insulin standard, which was calibrated to WHO 1st IRP insulin, or diluted plasma samples and 130 µl assay buffer in duplicate at 37°C for 1 hr with continuous shaking. After removal of the supernatant, the polystyrene balls were washed twice with 2 ml of 150 mM saline and then incubated with anti-porcine insulin guinea pig Fab'-peroxidase conjugate at 20°C for 1 hr. After removal of the supernatant, the polystyrene balls were washed with 2 ml saline. The balls were moved into a new series of assay tube and further incubated with 100 µl of 0.6% 3-(p-hydroxyphenyl) propionic acid as a substrate and 50 µl of 0.015% H_2O_2 at 30°C for 30 min. The reaction was stopped by adding 0.1 M glycine-NaOH buffer, pH 10.3. Fluorescence intensity was measured by spectrofluorophotometer (Shimazu RF5000, Shimazu Co, Kyoto, Japan).

Assay procedure in polystyrene plate method

Anti-porcine insulin guinea pig IgG-coated polystyrene plate was incubated with 20 µl of standard or plasma samples and 80 µl assay buffer in duplicate at 37°C for 1 hr. After removal of the supernatant, the polystyrene plate was washed three times with the washing buffer and then incubated with 100 µl anti-porcine insulin guinea pig Fab'-peroxidase conjugate at 20°C for 1 hr. After removal of the supernatant, the polystyrene plate was washed with phosphate buffer. The plate was incubated with 100 µl of o-phenylene diamine solution at 20°C for 30 min. The reaction was stopped by adding 2 N HCl. The absorbance was measured by a micro plate reader (model 3550, Bio-Rad Laboratories, CA, USA).

Gel filtration study of recombinant human insulin and human plasma of a patient with myotonic dystrophy

In order to clarify the nature of the anti-porcine antibody, gel chromatographic analysis was performed

using EIA of ball method. Five-hundreds μl recombinant human insulin (Humalin R, Shionogi Co, Tokyo, Japan) or plasma sample obtained from a patient with myotonic dystrophy was applied on a Sephadex G-50 column (1.0 x 100 cm), and eluted with 0.01 M phosphate buffer, pH 7.0, containing 0.1 M NaCl, 0.1% BSA and 0.1% NaN_3. The flow rate was adjusted to 10 ml/hr. Fractions of 1 ml were collected. Immunoreactive insulin levels in the effluents were measured by the EIA of ball method.

Dilution effect of human plasma

Human plasma was serially diluted with 0.1 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl and 0.1% BSA. The diluted samples were measured by EIA of ball method.

Correlation between immunoreactive insulin levels measured by EIA and those by RIA

Plasma samples were obtained from 40 diabetic patients before and after 75 g oral glucose administration, and insulin levels were simultaneously measured by EIA of ball method and plate method, and RIA using a commercially available kit (Eiken Chemical Co., Japan).

RESULTS

Anti-porcine insulin $F(ab')_2$ fraction of 0.658 mg was prepared from IgG fraction of 1.218 mg. Anti-porcine insulin Fab' fraction of 0.496 mg was prepared from the $F(ab')_2$ fraction. Anti-porcine insulin Fab' was conjugated with peroxidase of 0.571 mg. As shown in Fig. 1, anti-porcine insulin Fab'-peroxidase conjugate was eluted as the first major peak. The first peak fraction was used for the following EIA.

Fig. 2 shows a representative standard curve in EIA of polystyrene ball method. The minimum detectable quantity was 0.15 μU/ml using 20 μl sample, which was 3 nU/tube in the polystyrene ball method. The maximum measurable quantity was 640 μU/ml. The intra- and inter-assay coefficiency of variation was 8.9% and 9.8%, respectively. The mean recovery was 95%.

Fig. 3 shows a representative standard curve in EIA of polystyrene plate method. The minimum detectable quantity of was 0.62 μU/ml using 20 μl sample, which was 12.4 nU/tube in the polystyrene plate method. The maximum measurable quantity was 160 μU/ml. The intra- and inter-assay coefficiency of variation was 9.2% and 10.8%, respectively. The mean recovery was 92%.

In order to clarify the immunoreactive characteristics of the assay, gel chromatographic analysis was performed using EIA of ball method. A single peak was obtained as the elution profile of recombinant human insulin was showed in Fig. 4. On the other hand, two major peaks were obtained by the elution profile of plasma sample obtained from a patient with myotonic dystrophy as shown in Fig. 5. The first small peak and the second large peak were considered as proinsulin and insulin, respectively, when evaluated by elution point of cytochrome c with molecular weight of 12.4 KD.

As shown in Fig. 6, diluted human plasma resulted in a linear response ($y = 74.056x + 0.665$, $r = 0.998$) in EIA of ball method, which was parallel to the standard curve.

As shown in Fig. 7, there was a good correlation between plasma insulin levels measured by RIA and those of EIA of ball method (left panel) ($y = 0.983x + 4.058$, $r = 0.968$). Good correlation was also obtained between plasma insulin levels determined by RIA and those of EIA of plate method (Fig. 7, right panel) ($y = 0.738x + 0.972$, $r = 0.968$). The values obtained by EIA were slightly lower than those of RIA.

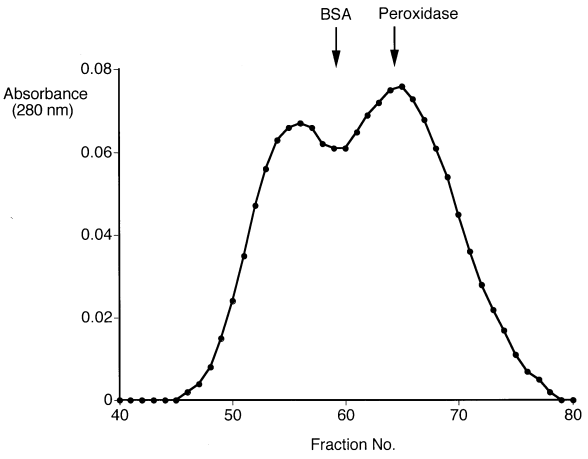

Fig. 1. Gel filtration pattern of anti-porcine insulin guinea pig Fab'-peroxidase conjugate on a Ultrogel AcA 44 column (0.9 x 90 cm) eluted with 0.1 M sodium phosphate buffer, pH 6.5. The volume of each fraction was 0.65 ml. The arrow indicates the elution point of BSA and peroxidase, respectively.

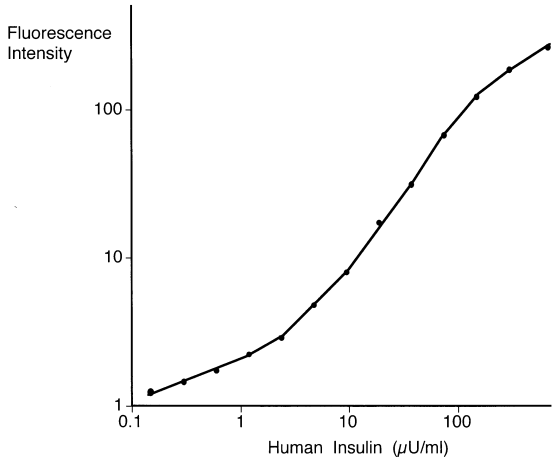

Fig. 2. A representative standard curve of the sandwich enzyme immunoassay of polystyrene ball method for human insulin.

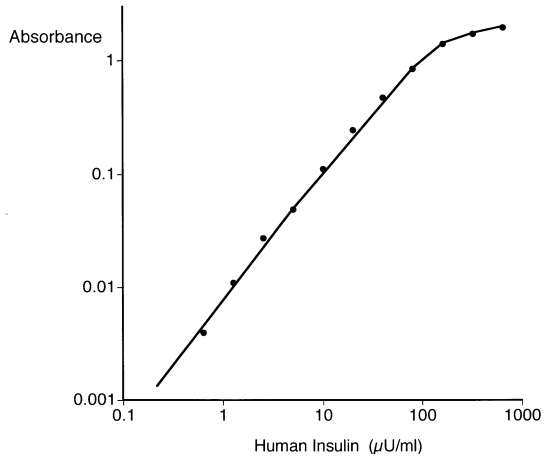

Fig. 3. A representative standard curve of the sandwich enzyme immunoassay of polystyrene plate method for human insulin.

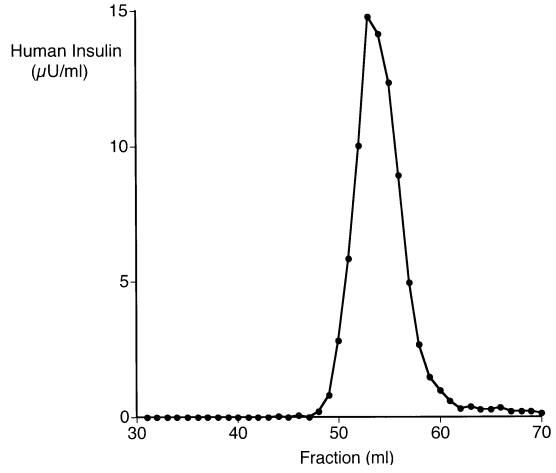

Fig. 4. Gel filtration pattern of recombinant human insulin. Samples of 500 μl were applied on a Sephadex G-50 column (1.0 x 100 cm). The volume of each fraction was 1.0 ml.

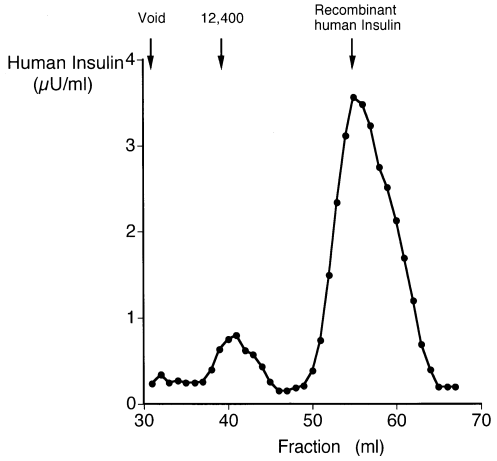

Fig. 5. Gel filtration pattern of plasma from a patient with myotonic dystrophy. Samples of 500 μl were applied on a Sephadex G-50 column (1.0 x 100 cm). The arrow shows the elution point of molecular marker of cytochrome c (12.4 KD). The volume of each fraction was 1.0 ml.

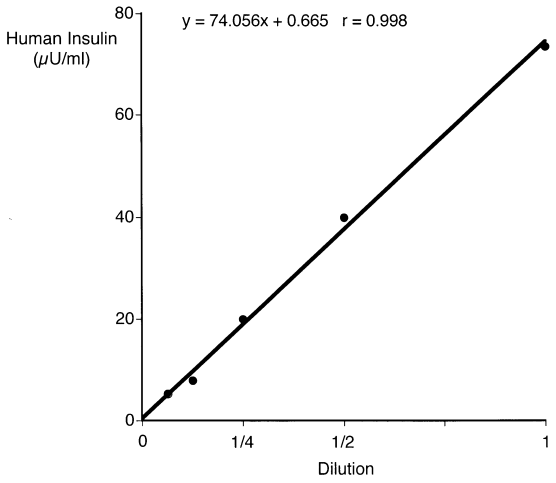

Fig. 6. The dilution effect of human plasma on immunoreactive insulin levels determined by EIA of ball method.

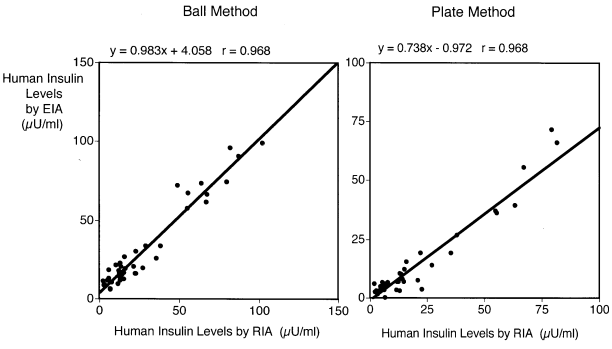

Fig. 7. Correlation between either EIA of ball method (left panel) or EIA of plate method (right panel) and RIA for human insulin. Forty different human plasma samples were simultaneously measured by EIA of ball method, EIA of plate method and RIA.

DISCUSSION

In the present study, we newly set up a rapid and sensitive EIA for human insulin using anti-porcine insulin guinea pig serum. The conjugate prepared with 1.218 mg of antibody IgG was sufficient in amount for performing more than 9,300 assays. The minimum detectable quantity of immunoreactive insulin was 3.0 nU/tube (0.15 μU/ml) in the present assay, which was 5-33 times more sensitive than those (15.1-100 nU/tube) of RIA and EIA previously reported (3, 8-12). The present EIA was more sensitive and accurate compared with other commercially available kits (13).

We could determine plasma insulin levels within three hours including two hours of immune reaction time, while the commercially available RIA kits took an overnight. The shortest immune reaction time has been reported to be 4 hours in EIA for human insulin (8-14). Although the longer incubation time might improve the sensitivity of the present assay, the present sensitivity was sufficient enough for measurement of circulating insulin levels at fasting state.

The ball method has some advantage compared with the plate method. The useful advantage was higher sensitivity and widely measurable range. The minimum detectable quantity was 0.15 μU/ml in the polystyrene ball method, and 0.61 μU/ml in polystyrene plate method, suggesting that polystyrene ball method was more sensitive than the polystyrene plate method. In polystyrene ball method, test tubes were changed twice to new series of test tubes by transferring the balls. These processes decreased non-specific binding of serum and peroxidase labeled antibody to test tubes compared with the plate method. The maximum detectable quantity was 640 μU/ml in ball method, and 160 μU/ml in plate method. The ball method could supply a large amount of stable assay system. The balls of 5,000 pieces could be coated by the diluted antibody at the same time. On the other hand, only five plates could be coated by the same diluted antibody solution in our laboratory. Furthermore, the IgG-coated polystyrene ball could be stored for at least three years. Therefore, it was considered the ball method was more stable, sensitive and accurate assay procedure compared with the plate method. However, the plate method could be available for a computerized system and prevalent in other laboratories if it would be sufficiently supplied.

Immunoreactive insulin levels measured by the plate method were slightly lower than those by the ball method. It is partly explained by serum interference in the sandwich enzyme immunoassay. The ratio of sample volume to total incubation volume in the ball method and the plate method were different and 20 μl to 150 μl and 20 μl to 100 μl, respectively.

The antibody immunized with porcine insulin cross-reacts with insulin of such species as human, bovine, porcine and rat (1). When rat insulin was used as a standard in the present EIA, the minimum detectable quantity was 0.05 ng/ml (data not shown). Furthermore, anti-porcine insulin antibody cross-reacted with proinsulin and insulin at equimolar basis. Plasma proinsulin level was known to be related with insulin resistance (2,15-16) and increased in patients with insulin resistance (17-18). It was reported that plasma immunoreactive insulin levels increased after oral glucose administration in patients with myotonic dystrophy, which consisted of increased proinsulin fraction (15, 19). Hypersecretion of proinsulin and insulin was induced by insulin resistance in peripheral tissue and insulin processing dysfunction in patients with myotonic dystrophy (20-22). Our data of gel chromatography suggests increased proinsulin fraction in the present patient. The present EIA in combination with gel chromatographic analysis was shown to be useful for evaluation of the proinsulin-insulin relationship.

In conclusion, we set up the rapid and sensitive EIA for human insulin using anti-porcine insulin guinea pig serum. These EIA systems proved to be accurate enough to measure circulating insulin levels, providing a useful method for evaluation of insulin secretion in clinical and basic research.

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