Evaluation of a Solid-Phase Enzyme Immunoassay for Insulin in Human Serum

Kanefusa Kato, Yumiko Umeda, Fujiko Suzuki, Daisaburo Hayashi, and Akira Kosaka

We describe a “sandwich-type” enzyme immunoassay for insulin in serum, in which antibody Fab′–β-D-galactosidase conjugate and an antibody-immobilized silicone rubber solid-phase are used. The interference by serum factors with the solid-phase enzyme immunoassay can now be removed by using a buffer containing gelatin. Serum samples of 50 μL can be analyzed by the enzyme immunoassay, which is as sensitive as radioimmunoassay for human insulin. Our results correlate well with those for radioimmunoassay (r = 0.97, slope = 0.92, y-intercept = 4.6 milli-int. units/L for 181 samples). Between-assay and within-assay coefficients of variation are <15% over the useful ranges of the assay (5–160 milli-int. units/L).

Enzyme immunoassay systems for insulin that use β-D-galactosidase (EC 3.2.1.23) from Escherichia coli as label are as sensitive as radioimmunoassays (1, 2). However, the assay systems are interfered with nonspecifically when serum is present in the sample, especially when separation is by solid-phase techniques. Adding insulin-depleted serum to adjust the standard curve is not reasonable for clinical laboratories, because the interference is different for sera from different individuals (3). Recently, we found that the interference with the “sandwich” enzyme immunoassay systems could be removed by including gelatin, a hydrophobic protein, with a relatively high concentration of salt in the assay mixture (3). We describe here a “sandwich” enzyme immunoassay method for the assay of insulin in serum, based on a technique reported previously (4, 5). A preliminary report of part of the present work has been published (3).

Materials and Methods

Reagents

Buffer A. Sodium phosphate buffer (0.01 mol/L, pH 7.0) containing, per liter, 0.1 mol of NaCl, 1 mmol of MgCl₂, 1 g of bovine serum albumin (fraction V from Armour Pharmaceutical Co., Chicago, IL), and 1 g of NaN₃. The solution is stable for six months at 4 °C.

Buffer G. Sodium phosphate buffer (0.01 mol/L, pH 7.0) containing, per liter, 0.3 mol of NaCl, 1 mmol of MgCl₂, 1 g of bovine serum albumin, 5 g of gelatin (Difco Laboratories, Detroit, MI), and 1 g of NaN₃. The solution is stable for two weeks at room temperature.

Anti-insulin serum. Guinea pig (anti-bovine insulin) serum was obtained from Miles Laboratories, Elkhart, IN (1.2 milli-int. units of insulin bound per microliter of serum). The immunoglobulin G (IgG) fractions were isolated from the antiserum by precipitation with (NH₄)₂SO₄ (50% saturation), dialysis, and chromatography on DEAE-cellulose (5).

Silicone rubber-immobilized anti-insulin. The IgG fractions of anti-insulin serum were immobilized noncovalently on silicone rubber (string, 3 mm in diameter, from Sanko Plastic Co., Osaka, cut into 4-mm lengths) as described previously (5), and stored in buffer A at 4 °C for at least one week before use. The pieces are stable for six months at 4 °C. More than 5000 pieces of the solid-phase medium can be prepared with the IgG fractions from 1 mL of the anti-insulin serum.

(Anti-insulin) Fab′–β-D-galactosidase conjugate. F(ab′)₂ fragments, prepared by digesting the IgG fractions of anti-serum with pepsin (4), were reduced with 2-mercaptoethanol and coupled to β-D-galactosidase from E. coli (Boehringer Mannheim, Mannheim, G.F.R.) by use of N,N′-o-phenylenediamine (Aldrich Chemical Co., Inc., Milwaukee, WI) (4). The amounts of the conjugate are expressed as units of enzyme activity, and one unit of activity is defined as the amount that hydrolyzes 1 μmol of substrate/min under the conditions described. The conjugate prepared from 4 mL of the antiserum (about 16 units) was sufficient for 5000 assays. The conjugate is stable for six months at 4 °C.

Substrate solution. Ten milligrams of 4-methylumbelliferyl-β-D-galactoside (Sigma Chemical Co., St. Louis, MO) were dissolved in 2 mL of N,N-dimethylformamide at 30 °C. Then, this solution was added dropwise to 98 mL of distilled water at 30 °C (final concentration = 0.3 mmol/L). The solution is stable for one week at 4 °C, and for one month at −20 °C.

Enzyme-reaction-stopping solution. A solution of glycine (0.1 mol/L) was adjusted to pH 10.3 with NaOH. The solution is stable for one month at 4 °C.

Insulin standard. Porcine insulin solution (40,000 units/ml) was used as a standard. Almost identical standard curves were obtained with the porcine insulin, with a standard insulin of the Radioimmunoassay Kit (Insulin RIkit, from Dainabot Radioisotope Laboratories, Tokyo), and with a crystalline bovine insulin (23,000 units/g, from Fluka AG, Buchs, Switzerland). The insulin solution diluted with buffer A is stable for one week at 4 °C.

Procedure

Mix 50 μL of the standard or serum sample with 0.45 mL of buffer G in 10 × 75 mm glass test tubes at 15–25 °C, and place in each tube a piece of the silicone rubber with immobilized antibodies. Incubate the tubes at 30 °C with shaking. After 2 h of incubation, aspirate and discard the reaction medium, and wash the silicone rubber piece successively with 1 mL of buffer G and 1 mL of buffer A in the test tube. Then

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add 0.2 mL of the antibody–enzyme conjugate (3 milliunits, diluted with buffer A) to each tube, and incubate the tubes overnight at 4 °C. Aspirate and discard the reaction medium, and wash the silicone rubber piece twice with 1 mL of buffer A. Transfer the piece to a glass test tube (7.5 × 75 mm) containing 0.1 mL of buffer A. After 5 min of preincubation at 30 °C in a water bath, add 50 µL of the substrate solution to each tube, and incubate the tube for 20 min, with shaking. Add 2.5 mL of the stopping solution to terminate the reaction. Conduct duplicate assays, unless otherwise specified. Set the wavelength of a spectrofluorometer (MPF-3, Hitachi, Tokyo) at 360 nm for excitation, and at 450 nm for emission analysis. Adjust the zero and the full-scale points of the spectrofluorometer with the stopping solution and the 0.1 µmol/L solution of 4-methylumbelliferone, respectively, and then measure the intensity of the fluorescence of the sample. Plot fluorescence intensity as a function of insulin concentration on 3 × 3 cycle log-log paper.

We obtained a linear calibration curve between 5 and 160 milli-int. units of insulin per liter. A representative calibration curve is shown in Figure 1.

**Results**

In experiments for analytical recovery, 10 human sera containing 5–40 milli-int. units of endogenous insulin per liter were used. Each serum sample was assayed in duplicate with and without added porcine insulin (10–120 milli-int. units/L), and the recovery of added insulin was calculated from the calibration curve (Table 1).

The precision of the assay was tested by assaying three frozen sera 10 times in one assay (within-run) or in duplicate in seven consecutive assays (between-run). The precision was best for the value in the mid-range of the calibration curve (Table 2).

To evaluate the accuracy of the present method, we compared the values of 181 serum samples obtained by the enzyme immunoassay with those obtained by a double-antibody radioimmunoassay. Most of the samples tested were sera from diabetic patients, and some of the samples were hemolyzed, lipemic, or icteric. As shown in Figure 2, there was good correlation between the two methods over the entire range studied. The regression equation is \( y = 0.92x + 4.6 \), and the correlation coefficient is 0.97.

**Discussion**

The high sensitivity of the assay for insulin by radioimmunoassay is the result of the highly sensitive detectability of the radioactive isotope. The maximum theoretical specific activity for \(^{125}\)I-moniodinated insulin is \( 2.17 \times 10^6 \text{ Ci/mol} \) (6), given that 1 fmol \((1 \times 10^{-15} \text{ mol})\) of the \(^{125}\)I-labeled insulin displays about 4800 disintegrations per min; consequently 0.1–5 fmol (0.67–33 micro-int. units) of insulin is measurable with competitive-binding methods in which \(^{125}\)I-labeled insulin is the tracer. Replacement of \(^{125}\)I-label by an enzyme that is detectable to the same sensitivity as the isotope should theoretically produce an immunoassay as sensitive as radioimmunoassay. \( \beta \)-D-Galactosidase from *E. coli* in the enzyme immunoassay we report has a turnover number of 53 000

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**Table 1. Analytical Recovery of Insulin Added to Human Serum**

<table>
<thead>
<tr>
<th>No. serum samples</th>
<th>Insulin, milli-int. units/L</th>
<th>Recovery, %</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Added</td>
<td>Recovered</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>10.3 ± 1.6*</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
<td>20.0 ± 2.4</td>
</tr>
<tr>
<td>10</td>
<td>60</td>
<td>58.3 ± 5.9</td>
</tr>
<tr>
<td>10</td>
<td>120</td>
<td>129 ± 22</td>
</tr>
</tbody>
</table>

* Mean ± SD.

**Table 2. Precision of Insulin Assay**

<table>
<thead>
<tr>
<th>No. assays</th>
<th>Insulin, milli-int. units/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Within-run</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample 1</td>
<td>10</td>
<td>7.0 ± 1.1*</td>
</tr>
<tr>
<td>Sample 2</td>
<td>10</td>
<td>47.6 ± 3.5</td>
</tr>
<tr>
<td>Sample 3</td>
<td>10</td>
<td>68.8 ± 5.3</td>
</tr>
<tr>
<td><strong>Between-run</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample 1</td>
<td>7</td>
<td>6.8 ± 1.0</td>
</tr>
<tr>
<td>Sample 2</td>
<td>7</td>
<td>47.2 ± 3.6</td>
</tr>
<tr>
<td>Sample 3</td>
<td>7</td>
<td>69.8 ± 5.4</td>
</tr>
</tbody>
</table>

* Mean ± SD.
The competitive-binding enzyme immunoassay systems for insulin in which β-D-galactosidase was the label, as reported previously (1, 2), were as highly sensitive as radioimmunoassay; however, there was interference by serum factors when serum was present in the sample, which limited the application of the method in clinical chemistry. Interference by serum factors has been also reported with radioimmunoassay (8, 9), especially when solid-phase media are used for separation.

To overcome this interference, we have developed a “sandwich-type” enzyme immunoassay system for insulin. In this method, any serum present in the first immunoreaction is washed out before the addition of the antibody–enzyme conjugate, which prevents a direct effect of serum on the immunoreaction of the conjugate. The interference still observed with the sandwich method is removed effectively by using the gelatin-containing buffer as described previously (3). Here, we report an accurate and sensitive enzyme immunoassay for insulin in serum that is useful in clinical laboratories.

The antibody–enzyme conjugate is stable at least for six months at 4 °C in buffer A, but the enzyme activity of the conjugate is lost if frozen. The fluorescence intensity of the sample is stable for at least 24 h at room temperature when shielded from light. As a solid-phase, commercially available polystyrene balls (Precision Plastic Ball Co., Chicago, IL) were as useful as silicone rubber pieces. One can complete the assay within a day by performing the second immunoreaction at 30 °C for 3 h, instead of letting it set overnight at 4 °C as described in the text.

Although we have not yet tested this variation, it may be possible to conduct the assay colorimetrically by using o-nitrophenyl-β-D-galactoside as the substrate for the enzyme assay and allowing a longer incubation.

The immunoassay of insulin we present avoids the complications and limitations associated with radioisotopes. Standard laboratory equipment is sufficient for the assay. Therefore, the sandwich enzyme immunoassay of insulin in which β-D-galactosidase is the label is useful in current laboratory medicine.

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References