Two-Site Time-Resolved Immunofluorometric Assay of Human Insulin

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We describe a two-site "sandwich"-type time-resolved immunofluorometric assay for human insulin, based on use of two monoclonal antibodies with different specificities. The first antibody is immobilized on the surface of microtiter plate strip wells, the other is labeled with Eu³⁺. Serum samples can be assayed with one incubation step; two incubation steps are required when plasma samples are assayed. After the immunoreactions are complete, the bound fraction of Eu³⁺-label is quantified by dissociating it in a fluorescence-enhancement solution and measuring its fluorescence with a fluorometer with time-resolution. The sensitivity of the assay is 0.24 micro-int. units/mL. The standard curve is linear from 0.24 to 2400 micro-int. units/mL.

Additional Keyphrases: "sandwich"-type immunoassay · monoclonal antibodies

Since the introduction of radioimmunoassay (RIA) for insulin in 1959 by Berson and Yalow (1), RIA has been the technique used most widely to assay insulin. Because of its high sensitivity, reliability, and easy performance, RIA is well suited for measurement of insulin (2); on the other hand, because of certain disadvantages associated with the use of radioisotopes, the use of alternative methods and labels has been investigated.

In competitive insulin immunoassays, enzyme labels with photometric (3, 4) or fluorometric or luminometric (5) measurements of activity, and fluorescence labels, for use in separation fluoroomunooassay (6) or in homogeneous fluorescence polarization immunooassay (7), have been introduced. However, the competitive assays are not capable of measuring the whole range of clinically encountered insulin concentrations with a single dilution of sample. Use of a "sandwich"-type assay technique allows for a wider working range, and some applications of this technique with enzy-melabeled antibodies have been reported (8-10).

Monoclonal antibodies offer a number of advantages for two-site sandwich immunoassays (11), but until recently, development of monoclonal antibody-based assays has been hampered because suitable monoclonal antibodies were only sparsely available.

We have developed a highly sensitive two-site sandwich time-resolved immunofluorometric assay (TR-IFMA) for human insulin, involving two different monoclonal antibodies with specificities for different epitopes on insulin. The antigen-binding antibody is immobilized on the walls of a microtiter strip well and the second (detecting) antibody is labeled with Eu³⁺ (12). The high sensitivity in the two-site immunoassay is based on using time-resolved fluorometry for Eu³⁺ detection (13).

Materials and Methods

Reagents

Buffers. The assay buffer (DELFIA™ Assay Buffer; LKB-Wallac, Turku, Finland) contained, per liter, 50 mmol of Tris HCl (pH 7.75), 9 g of NaCl, 0.5 g of NaN₃, 5 g of bovine serum albumin (BSA), 0.5 g of bovine globulin, and 0.1 mL of Tween 20 surfactant. We also used phosphate-buffered isotonic saline (PBS, pH 7.4): 2.5 mmol of NaH₂PO₄, 7.5 mmol of Na₂HPO₄, and 145 mmol of NaCl per liter.

Monoclonal antibodies. The monoclonal antibodies OXI-005 (purified from culture supernate) and HUI-018 (purified from ascites fluid and stabilized with aprotinin, 20 μg/mL); human, bovine, and porcine insulines; and human proinsulin were obtained from Novo BioLabs, Novo Industri A/S, Bagsvaerd, Denmark.

The monoclonal antibody HUI-018, of IgG₂κ isotype, was produced against human insulin and cross-reacted with human proinsulin; its Kₐ was 10⁸ L/mol (14). The monoclonal antibody OXI-005, also of IgG₂κ isotype, was produced against bovine insulin. Its affinity for bovine insulin, as estimated from competitive RIA, was 10³ L/mol, 34-fold greater than for human insulin (i.e., 6.4 × 10⁷ L/mol). Its affinity for bovine proinsulin was estimated to be <125-fold lower, and its affinity for human proinsulin to be <823-fold lower, than for bovine insulin. The OXI-006 monoclonal antibody binds isolated insulin B-chains but does not react with Des₂₉-₃₀ or Des₂₆-₃₀ insulin, indicating an epitope near the carboxy-terminal end of the insulin B-chain. OXI-005 bound an epitope including amino acid B₂₀ at the carboxy-terminal end of insulin, proximal to the cleavage site in proinsulin (15, 16). The low binding of proinsulin makes OXI-005 a useful selective tool for use in monitoring insulin concentrations with only negligible interference from proinsulin (16). In preliminary experiments, the HUI-018 and OXI-005 antibodies were shown to bind different epitopes, making this combination a useful one for the development of two-site sandwich-type immunoassays.

The monoclonal antibodies were purified on Protein A-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). To ensure the binding of these monoclonal antibodies (both IgG₂ isotypes), we adjusted the pH to 8.0. For elution we used citric acid (0.1 mol/L, pH 2.5). The effluent fractions were neutralized immediately with Tris HCl buffer (1 mol/L, pH 8.5). The antibodies were dialyzed against PBS and concentrated to about 5 mg/mL.

Reagents and Apparatus

Eu³⁺-N₂-[p-isothiocyanatobenzyl] diethylenetriamine-N²,N³,N⁴,N⁵,N⁶,tetracetate was synthesized as described previously (17). The Eu³⁺ was quantified from labeled antibod-

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4 Nonstandard abbreviations: BSA, bovine serum albumin; PBS, phosphate-buffered isotonic saline; TR-IFMA, time-resolved immunofluorometric assay.

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ies and in immunoassays by using fluorescence enhancement solution (DELPHA™ Enhancement Solution; LKB-Wallac). The 1230 Arcus Fluorometer with time-resolution (LKB-Wallac) was used in Eu³⁺ measurements.

Labeling of antibodies with Eu³⁺. The purified monoclonal antibody OXI-006 was labeled with Eu³⁺-N³⁺-[p-iodo-4-cyano-2-benzyllamino-1,2,3,4-tetraacetate by incubating it (1 mg/mL) with a 100-fold molar excess of the reagent at pH 9.5 and 4°C overnight. The conjugated antibody was purified from unreacted Eu³⁺-reagent on a 1 x 30 cm column of Trisacyl GF 2000 (LKB-Wallac) by eluting with Tris HCl buffer (0.05 mol/L, pH 7.75). The monomeric IgG fraction was collected and the amount of Eu³⁺ incorporated was measured in fluorescence enhancement solution against diluted Eu³⁺ standard solution (Aldrich Chemical Co., Milwaukee, WI 53201). BSA was added to the labeled IgG fraction as a carrier protein, the final concentration being 2 g/L.

Coating of microtitration strips. The monoclonal antibody clone HUI-018 was immobilized on the surface of microtitration strip wells by physical adsorption. To do so, we incubated 1 µg of antibody in each well with 250 µL of phosphate buffer (0.05 mol/L, pH 8.5) at 20°C overnight. The coated strips were blocked with BSA (5 g/L) and stored in a humid environment at 4°C until use. Just before use, the strips were washed once with wash solution, containing, per liter, 0.9 g of NaCl, 500 mg of NaN₃, and 250 mg of Tween 20.

Immunoassay procedure. Two different procedures were used.

In the “one-step” procedure, 50 µL of standards or serum samples was incubated at room temperature for 2 h in antibody-coated wells with 200 µL of assay buffer containing 100 ng of Eu³⁺-labeled anti-insulin antibody. Subsequently the assay wells were aspirated and washed six times with the washing solution. The bound Eu³⁺ label was then dissociated from the surface with 250 µL of enhancement solution and the resulting fluorescent chelate solution counted (single photon counting) with a time-resolved fluorometer.

In the “two-step assay” 50 µL of standards or samples (serum or plasma) was incubated with 200 µL of assay buffer at 20°C for 2 h. After washing, the diluted Eu³⁺-labeled anti-insulin antibody, 100 ng in 250 µL of assay buffer, was dispensed into the strip wells and incubated for 1 h. After washing, the bound Eu³⁺ label was measured as described above.

Radioimmunoassay. We radioimmunoassayed insulin with a kit for human insulin developed by Novo Research Institute (obtained through Novo BioLabs, Novo Industri A/S, Bagsvaerd, Denmark). The kit contained ²²⁴I-labeled (Tyr A19) porcine insulin (specific activity 100–200 Ci/g), human insulin, and anti-insulin guinea pig serum no. M 8309 (2).

Results

Standardization. We tested the matrix effect of different diluents in insulin standardization by measuring the analytical recovery of insulin from animal sera, using the assay buffer as diluent for standards (Table 1). The tested sera contained cross-reactive insulin giving backgrounds of 0.6 to 15 micro-int. units/mL. Quite comparable recoveries were obtained for human, rabbit, sheep, and horse serum. The standard curves determined with standards in assay buffer and in sheep serum were completely parallel, both with "one-step" and "two-step" assays, with the exception of different backgrounds of zero standards (Figure 1). In subsequent determinations we therefore used the assay buffer as a diluent for insulin standards.

Assay optimization. Varying the amount of immobilized antibodies from 0.5 to 2 µg per well in the coating step had little effect on the characteristics of the standard curves (Figure 2) and 1 µg per well was therefore used in subsequent assays. Similarly, varying the amounts of labeled anti-insulin antibody has only a minor effect on the standard curve, and no "hook" effect was noticed in the "one-step" assay (Figure 1).

Specificity of the assay. The specificity of the assay was tested with porcine and bovine insulins and with human proinsulin. Bovine and porcine insulins gave almost parallel standard curves, though slightly less steep than the curve for human insulin. For bovine insulin the cross-reactivities ranged from 31 to 39%, for porcine insulin from 130 to 240%.

<table>
<thead>
<tr>
<th>Diluent</th>
<th>Endogenous background, micro-int. units/mL</th>
<th>One-step assay Recovery, %</th>
<th>Two-step assay Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human serum</td>
<td>15.0</td>
<td>93</td>
<td>101</td>
</tr>
<tr>
<td>Rabbit serum</td>
<td>0.6</td>
<td>106</td>
<td>128</td>
</tr>
<tr>
<td>Goat serum</td>
<td>2.0</td>
<td>78</td>
<td>96</td>
</tr>
<tr>
<td>Sheep serum</td>
<td>1.5</td>
<td>127</td>
<td>118</td>
</tr>
<tr>
<td>Horse serum</td>
<td>5.8</td>
<td>127</td>
<td>130</td>
</tr>
</tbody>
</table>

Recoveries of insulin, 240 micro-int. units/mL, added to sera shown.

Fig. 1. Standard curves for insulin TR-IFMA with "one-step" (circles) and "two-step" assay (squares) prepared by using insulin standards diluted in assay buffer (solid symbols) or sheep serum (open symbols).
Human proinsulin showed no cross reactivity within its clinical range of 6 to 199 pmol/L.

**Sensitivity and precision.** The dose–response curves for both the "one-step" and the "two-step" assay are presented in Figure 3, A and B. After subtraction of the background signal of the zero sample (900–900 counts/s) the curves were linear from 0.24 micro-int. unit/mL (0.01 ng/mL) to 2400 micro-int. units/mL (100 ng/mL). Figure 4 shows the precision profile obtained from 12 replicates of standards. A CV of <10% was obtained for insulin concentrations ranging from 5 to 1000 micro-int. units/mL.

The sensitivity of the assay, determined as the minimum concentration of insulin in the sample giving a signal of zero +2 SD, was 0.24 micro-int. unit/mL for both the "one-step" and the "two-step" assays.

Interassay precision, tested by measuring three human serum pools during 10 days, had a CV of 5.8 to 10.6% (Table 2).

**Correlation and analytical recoveries.** A correlation study was carried out with human plasma samples previously measured by RIA (2). Because EDTA, when used as an anticoagulant, interferes with Eu-labeled antibodies in the "one-step" assay, the plasma samples were measured by using the "two-step" procedure. For 43 patients' samples the correlation coefficient was 0.977 and the slope 1.008.

In recovery experiments, various amounts of insulin were added to a human serum pool and measured with the "two-step" TR-IFMA (Table 3). The recovery appeared to be near 100% for all insulin concentrations tested.

**Discussion**

Serum insulin determinations are mostly performed by using competitive radioimmunoassays with working ranges between 5 and 300 micro-int. units/mL and sensitivities of about 2 to 8 micro-int. units/mL. Non-isotopic alternatives, both competitive and sandwich-type assays, which work in about the same concentration ranges, have also been published (3–9). Greater sensitivities (0.2 micro-int. unit/mL) have been reached, e.g., by using horseradish peroxidase-labeled Fab fragments of anti-insulin antibodies raised in the capybara (10).

**Table 2. Interassay Variation of Separate Duplicate Assays of Three Pooled Samples**

<table>
<thead>
<tr>
<th>Mean micro-int. units/mL</th>
<th>SD</th>
<th>Interassay CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>0.8</td>
<td>10.8</td>
</tr>
<tr>
<td>13.8</td>
<td>0.8</td>
<td>5.8</td>
</tr>
<tr>
<td>47.8</td>
<td>4.9</td>
<td>10.3</td>
</tr>
</tbody>
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n = 10 each.

**Table 3. Analytical Recovery of Added Insulin**

<table>
<thead>
<tr>
<th>Insulin conc, micro-int. units/mL</th>
<th>Added</th>
<th>Found</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>7.5 ± 0.7</td>
<td>122 ± 76</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>18.4 ± 0.9</td>
<td>101 ± 9</td>
<td></td>
</tr>
<tr>
<td>100.0</td>
<td>103.8 ± 6.0</td>
<td>96 ± 6</td>
<td></td>
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</tbody>
</table>

*Added to human serum containing 6.24 (SD 0.60) micro-int. units of insulin per milliliter.

*Mean ± SD of n = 12 determinations each.*

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Fig. 2. Effect of amount of "catching" anti-insulin monoclonal antibody (HUI-018) in coating of wells on the standard curve of insulin

Amounts used in coating: 0.5 µg (♀), 1 µg (○), and 2 µg (△) per well.

Fig. 3. Standard curves for insulin TR-IFMA with the "one-step" (♀) and the "two-step" (○) assay procedures.

Fig. 4. Precision profile for the "two-step" insulin TR-IFMA.

The standard deviation (SD) (○) and coefficient of variation (CV, %) (♀) are presented as a function of insulin concentration.
With the present TR-IFMA, one can detect 0.24 micro-int. unit of insulin per milliliter in 50 μL of sample, and the dose–response curve is linear from 0.24 to >2000 micro-int. units/mL. The high sensitivity and wide dynamic range reached in a moderately short incubation time (2 h in the "one-step" assay) is a result of several factors: (a) The use of an easily washable solid-phase separation technique makes non-specific signals low. (b) The availability of two different monoclonal antibodies recognizing different epitopes on the insulin molecule allows use of a two-site assay involving a high excess of labeled antibodies. This results in a high signal, a wide dynamic range, and a low background, even after a brief incubation. (c) The moderately mild coupling reaction in Eu²⁺ labeling allows labeling ratios of over 10 Eu²⁺ per IgG, with no tendency to aggregation of IgG or decreased immunoreactivity. (d) The use of enhancement solution in Eu³⁺ dissociation and fluorescence development after completion of the immunoreaction and the use of time-resolved fluorometry in Eu³⁺ detection results in high detection sensitivity for the label.

The use of monoclonal antibodies against different sites on the insulin facilitated the utilization of the "one-step" assay, and a moderately high excess of labeled antibody produced a broadly linear standard curve. Serum samples can be analyzed rapidly with a one-incubation assay that is as sensitive as one with two incubations. However, a two-incubation assay is required for plasma samples, because of the interference of chelating agents (EDTA or citrate) to the Eu³⁺ chelate. When one is measuring samples from diabetic patients, the effect of possible auto-insulin antibodies evolved during therapy with insulin should be taken into account.

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References