Critical Variables in the Radioimmunological Technique for Measuring Immunoreactive Insulin with Use of Immunosorbents

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A commercial kit for determining immunoreactive insulin, the "Phadebas Insulin Test," was methodically evaluated to establish the optimal conditions for this immunoassay. Sensitivity, day-to-day and within-run precision, recovery, accuracy, and specificity are excellent. Results by this method compare favorably with those of the double-antibody technique. This rapid, simple, convenient, and reliable procedure for insulin immunoassay can be easily adapted to the facilities of a clinical laboratory as a standard diagnostic test or for clinical research.

Additional Keyphrases: "Phadebas" test kit • double-antibody technique compared

Radioimmunoassay is based on the competition for antibody between radiolabeled and unlabeled antigen. Antibody-bound and unbound antigen can be separated by paper chromatography (1), alcohol precipitation (2), use of gamma globulin antisera (3), or by adsorption on charcoal (4); one or both of the radioactive fractions is then measured.

Axen and Porath (5) demonstrated that enzymes and hormones can be chemically attached to insoluble polymers and still retain their physiological and chemical properties, thus stimulating a great deal of work in this particular area. The polymers used as immunosorbents were characterized by their high stability, moderate capacity for dispersion, high affinity for the antigen, and especially by their adsorptive specificities.

With this information as a background, and taking into account the several modifications that were applied in order to improve the immunoassays of polypeptides in biological fluids, use of immunosorbents began to be widely accepted among investigators.

Wide and Porath (6) described a radioimmunoassay to measure human chorionic gonadotrophin and luteinizing hormone, in which the antibodies were coupled covalently to a cross-linked dextran, "Sephadex." Free and antibody-bound radiolabeled antigen were subsequently separated by centrifugation. A similar technique was used to determine concentrations of immunoreactive insulin in serum and other biological fluids.

The importance of this new technique is obvious because of its advantages over previous methods: it eliminates the time-consuming steps required by the conventional double-antibody techniques, incubation time is shorter, and antibodies coupled to a solid phase are easily separated from unbound antigen by routine centrifugation.

This technique is the basis for the "Phadebas Insulin Test." We report here an evaluation of the variables in this new procedure for the measurement of immunoreactive insulin in biological fluids, our object being determine the optimal conditions for the assay.

Materials and Methods

Equipment

Vertical rotator ("Heto Rotamix"; Heto, Bregnerodvej 121, Birkerod, Denmark).

Gamma counter system (Model 4224, automatic dual channel; Nuclear Chicago Corp., Des Plaines, Ill. 60018).

Centrifuge, refrigerated (Model PR-2; International Equipment Co., Boston, Mass.).

pH meter (Model 27b "Radiometer," Copenhagen, Denmark).

Plastic tubes or siliconized glass tubes (10 × 75).

Plastic test tubes with screw-on caps (16 × 125 mm), for counting (Nuclear Chicago).

Glass "Lang-Levy" micropipets. These should either be siliconized or rinsed with buffer before using.

Reagents

Phadebas Insulin Test (Pharmacia Fine Chemicals, Inc., Piscataway, N.J. 08854). Each kit contains the following reagents, sufficient for 100 insulin determinations:

Buffer substance, dry power.

Sephadex-anti-insulin-complex, lyophilized.

Insulin standard (320 microunits/ml) after reconstitution, lyophilized.

[125I]Insulin, 8 ng, 3 μCi (at date of manufacture).

Preparation of Reagents

Buffer solution: Dissolve the buffer powder in 200 ml of distilled water. The resulting pH is 7.4.

Sephadex-anti-insulin complex: Transfer quantitatively to a beaker containing a magnetic rod, by use of 100 ml of buffer solution.

[125I]Insulin: Reconstitute the lyophilized material by adding 10 ml of buffer solution.

From the Diabetes Center, Department of Medicine, New York Medical College, New York, N.Y. 10029. Received Aug. 12, 1972; accepted Nov. 29, 1972.
Insulin standard: Reconstitute the lyophilized material by adding 4 ml of redistilled water. From this stock, working standards are prepared with a final concentration of 0, 1.25, 2.5, 5, 10, 20, 40, 80, 160, and 320 microunits/ml. Repeated freezing and thawing of insulin standard and [125I]insulin should be avoided.

Borate buffer: 8.25 g boric acid, 2.70 g sodium hydroxide, 12 molar HCl and water to make 1 liter at pH 8.0. To this, 5 g of bovine serum albumin (Sigma Chemical Co.) was added to each 100 ml of buffer. Additional HCl (0.1 mol/liter) is added if necessary to maintain a pH of 8.0.

Guinea pig anti-insulin serum: From Dr. C. R. Morgan, Department of Anatomy, University of Minnesota, Minneapolis.

Human insulin: A vial containing 0.5 units of crystalline human insulin was generously supplied by Dr. Ronald Chance (Eli Lilly Research Laboratories).


Pork [125I]insulin: Specific activity between 35 and 65 mCi per mg of insulin (Abbott Laboratories).

Procedures

Collection of blood samples: Blood was drawn and allowed to clot for 1 h at room temperature. After centrifugation, the serum was placed in plastic tubes and stored at −20°C until assayed.

The double-antibody technique used in a comparative study was performed according to the procedure of Soeldner and Slone (7).

With the commercial kit, the following standard technique was performed throughout the study, unless otherwise stated.

Procedure: 1. Pipet 0.1 ml of standards or unknown serum.
2. Pipet 0.1 ml of [125I]insulin.
3. Pipet 1.0 ml of Sephadex-anti-insulin complex. Stir the suspension while dispensing.
4. Stopper the tubes and incubate 3 h at room temperature, keeping the particles in suspension by manual rotation of the tubes every 30 min.
5. Centrifuge the tubes at 1500 × g for 5 min and withdraw the supernatant fluid to about 5 mm from the bottom by using a collared suction nozzle coupled to a water aspirator.
6. Add to the tubes 1 ml of saline (9 g of NaCl per liter), and centrifuge 5 min.
7. Withdraw the supernatant fluid and repeat the washings twice more.
8. Discard the supernatant fluid, and determine the radioactivity in the precipitate with a gamma counter.

Calculations of Results

Express the count-rates for each of the standards and unknown samples as a percentage of the mean count-rates of the "zero" samples. Subtract background, if significant.

Plot the values obtained from the insulin standards (expressed as percentages) against the insulin concentration on linear graph paper, and draw a standard curve (Figure 1). Read the concentration of insulin directly from the curve (in microunits/ml) for each of the unknown samples.

Results

Effect of Dilution

The concentration of immunoreactive insulin was determined in the original serum and in serum diluted four-, six-, eight-, and 12-fold. Table 1 shows that dilution has no significant effect on the values for insulin.

Specificity of the Assay

A pooled serum and three different samples were tested for insulin before and after inactivation of the hormone by a modification of the method of Grodsky and Forsham (8): The samples were treated with NaOH (2 mol/liter) until a pH of 11.5 was attained,

![Figure 1. Representative standard curve: bound [125I]insulin vs. concentration of unlabeled insulin](image)

Each point is the mean of 12 determinations

| Table 1. Effect of Dilution on Measurements of Immunoreactive Insulin in Four Specimens of Serum
<table>
<thead>
<tr>
<th>Dilution</th>
<th>Pooled serum</th>
<th>Serum 1</th>
<th>Serum 2</th>
<th>Serum 3</th>
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<tr>
<td>1/2</td>
<td>100</td>
<td>100</td>
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<td>100</td>
</tr>
<tr>
<td>1/4</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>1/6</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
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<td>1/8</td>
<td>50</td>
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<tr>
<td>1/12</td>
<td>40</td>
<td>40</td>
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</tbody>
</table>

*All values are corrected for dilution.
set aside for 72 h at 4°C, and centrifuged. The supernatant fluid was neutralized with HCl (2 mol/liter) and assayed.

After alkaline treatment, no insulin could be detected in samples that had previously contained various amounts of the hormone.

Reproducibility

To investigate the reproducibility (precision) of repeated assays on aliquots of the same pooled serum, we determined the concentrations of immunoreactive insulin, each time against duplicate standards, on 28 different days over a two-month period.

For this particular assay evaluation, the vials with crystalline insulin standards were reconstituted, fractionated in small volumes (0.25 ml) and kept frozen at -20°C in siliconized tubes until used. The [125I]insulin was reconstituted, fractionated, and kept frozen in 2-ml portions until assayed. The values obtained ranged from 28 to 34 microunits/ml (mean: 31; standard deviation: 1.7; standard error of the mean: 0.336; relative standard deviation: 5.7%).

Sensitivity

Twenty determinations of the radioactivity bound to the precipitate (expressed as counts per minute), corresponding to 0 and 2.5 microunits/ml, yielded means of 8080 and 7500 cpm, respectively. Statistically, the means were significantly different ($P < 0.001$). Therefore, the assay is capable of detecting a serum immunoreactive insulin as low as 2.5 microunits per ml.

Variability

To determine the variability of the samples when using the standard procedure, we selected 50 sera in the low, medium, and high ranges, and assayed the immunoreactive insulin, in triplicate.

Reproducibility was excellent; no value for serum deviated more than 3 microunits/ml from the mean value.

Experimental Variables

Washing of precipitate. According to the standard procedure, the precipitates are washed three times. We explored the feasibility of decreasing the number of washings.

Three identical dilutions of insulin standards (A, B, C) were prepared within the range of 2.5 to 320 microunits/ml, and processed according to the standard procedure. After centrifugation the precipitates were washed once for standard A, twice for standard B, and three times for standard C. Percentage of radioactivity bound to the precipitates was plotted against the insulin concentration in order to draw the standard curve.

Inconsistent results were obtained with only one washing; however, Figure 2 shows that differences between two and three washings were insignificant.

Effect of time on precipitation. We investigated the minimum time required to obtain the greatest insulin precipitate. We wished to know at what time the maximum was reached and how long it would remain essentially unchanged. To get triplicate results, we prepared thirty identical tubes of “zero” standards and incubated them while they were being mixed in the Heto Rotamix. At hourly intervals, three tubes were removed and centrifuged, and the precipitates washed and counted for radioactivity during incubation for 9 h, and again after a total incubation time of 24 h. As shown in Figure 3, the maximum was attained by 5 h and remained practically constant for 24 h. We conclude from these data that a 5-h incubation is sufficient.

Influence of specimens-volume on accuracy. It was demonstrated in our laboratory by Oppermann et al. (9) that no significantly different immunoreactive insulin values were determined, when specimens of 25, 50, or 100 μl were used. To investigate this variable with the “kit method,” we processed 100 different sera, each in duplicates of 100 μl and 50 μl. The
Pearson correlation coefficient ($r$) was 0.994 and $P < 0.001$, indicating a statistically significant correlation between the two corresponding values.

Results after adding insulin to serum. Ten different concentrations of crystalline human insulin, dissolved in a solution of bovine serum albumin (50 g/liter), were added to a pooled serum containing a known amount of endogenous insulin. 95% to 102% of the insulin present could be analytically accounted for (Table 2).

Range of variability of standard curves. Twenty identical dilutions of insulin standards were prepared and assayed on 20 different days over a two-month period. The tracer and the standard dilutions were prepared and stored as described in the section on “Reproducibility.” There was no significant difference among any of the standard curves; no sample at any percentage of precipitation deviated more than 3% from the mean value (Table 3).

Phadebas Insulin Test vs. Double-Antibody Technique

To compare the immunoreactive insulin concentrations (microunits/ml) by these two techniques, we processed 200 samples over a two-month period.

The Pearson correlation coefficient ($r$) was 0.99 and $P < 0.001$, indicating a significant correlation of the corresponding values obtained by the two different procedures.

Discussion

In the present study the high quality and usefulness of the Phadebas Insulin Test procedure has been demonstrated. The data confirm the good applicability of the immunological procedures, in which antibodies attached to insoluble polymers are used.

Because this procedure enabled us to incubate all reagents in one step, we obviated the time and effort required in the double-antibody technique for the separation of free from bound insulin.

Because the stability of the reagents, even after reconstitution, is good, they can be used repeatedly if the stock solutions of the insulin standard and the labeled hormone are stored at $-20^\circ$C.

We conclude therefore that this method provides a fast, easy, reproducible, and reliable radioimmunoassay for measuring immunoreactive insulin in serum and other biological fluids.

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Table 2. Immunoreactive Insulin Found in a Pooled Serum Before and After Various Known Quantities of Crystalline (Human) Insulin Were Added

<table>
<thead>
<tr>
<th>Original value</th>
<th>Insulin added</th>
<th>Insulin found</th>
<th>Accounted for, %</th>
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<tbody>
<tr>
<td>34 microunits/ml</td>
<td>5</td>
<td>37</td>
<td>95</td>
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<td>34</td>
<td>12</td>
<td>46</td>
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<td>97</td>
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<tr>
<td>35</td>
<td>200</td>
<td>239</td>
<td>102</td>
</tr>
</tbody>
</table>

Mean: 98
SD: 2.31
SEM: 0.73

Table 3. Variability (in Percent) of Results for Bound $^{125}$I Insulin in 20 Identical Standards of Crystalline Insulin

<table>
<thead>
<tr>
<th>Insulin, microunits/ml</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>80</th>
<th>160</th>
<th>320</th>
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<tr>
<td>Mean</td>
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<td>81</td>
<td>71</td>
<td>57</td>
<td>41</td>
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<td>21</td>
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<tr>
<td>SD</td>
<td>1.14</td>
<td>1.43</td>
<td>1.16</td>
<td>1.37</td>
<td>0.83</td>
<td>1.11</td>
<td>0.96</td>
</tr>
<tr>
<td>SEM</td>
<td>0.25</td>
<td>0.31</td>
<td>0.25</td>
<td>0.30</td>
<td>0.18</td>
<td>0.24</td>
<td>0.21</td>
</tr>
</tbody>
</table>

$n = 20$, at each concn.

References