Enzyme Immunosorbant Assay of Prolactin with Penicillinase as Label

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This competitive, rapid, and sensitive enzyme immunoassay for measuring human prolactin in plasma involves penicillinase (EC 3.5.2.6) as label. Microtiter plate wells coated with goat anti-rabbit gamma globulin are filled with antibody to prolactin and plasma sample or reference prolactin and incubated with penicillinase-labeled prolactin for 1 h at 37 °C. The enzyme activity of the bound complex is then assayed. The assay is as sensitive as radioimmunoassay, the detection limit being 2.5 ng of prolactin per milliliter of plasma. The plasma prolactin values obtained by enzyme immunoassay correlated well with those determined by radioimmunoassay: \( r = 0.98, \) slope = 1.00, intercept = 1.11 ng/mL (\( n = 53 \)).

Additional Keyphrases: hormones • hyperprolactinemia • radioimmunoassay compared

Development of sensitive radioimmunoassays (RIAs) for determining prolactin in plasma or serum (1, 2) has greatly helped in understanding the abnormal physiology related to disturbances of the menstrual cycle, infertility, and hyperprolactinemia in women (3). These RIAs, although the fastest methods currently available, have the disadvantages associated with the use of radioactive labels. The short half-life of \(^{125}\)I-labeled prolactin and the potential health hazards in handling and disposing of gamma ray emitters limit the useful lifetime of a kit and increase its cost, thereby hindering its general application.

To overcome these problems, research to develop nonisotopic immunoassays for some reproductive hormones has been in progress (4). Several enzyme immunoassay (EIAs) involving various enzymes have been developed. An enzymatic assay, in which prolactin labeled with \( \beta \)-galactosidase was used, was described, however, included column-chromatographic separation of the antigen–antibody complex (6).

The use of penicillinase (\( \beta \)-lactamase; EC 3.5.2.6) as a marker enzyme in EIAs has been reported for several steroid and protein hormones (7). So far, however, this enzyme has not been used for measuring the concentrations of estradiol, lutein, folliculin, and prolactin in plasma. We describe here a competitive, fast, and sensitive solid-phase immunoassay for measuring prolactin (hPRL) in human plasma, utilizing penicillinase as a marker enzyme.

Materials and Methods

Materials

The comparison radioimmunoassay kit for hPRL containing "iodination grade" hPRL, anti-hPRL antiserum, and calibration standards (NIADDK-hPRL-RP-1) was supplied by the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases (NIADDK), Bethesda, MD. Penicillinase, glutaraldehyde, Tween 20 (polyoxyethylene (20) sorbitan monolaurate), and DEAE-Septagel were all obtained from Sigma Chemical Co., St. Louis, MO. Polyethyl theraphthalene glycol assay plates with 96 wells were from Costar Europe Ltd., Badhoevedorp, Netherlands. Second antibody against rabbit gamma globulin (goat IgG) was raised in goats by Dr. J. K. Datta of our Institute. All chemicals used were of analytical grade.

Procedures

Preparation of prolactin–penicillinase conjugate. We coupled hPRL to penicillinase by using the one-step glutaraldehyde method of Avramescu (8). We dissolved 50 \( \mu \)g of hPRL (iodination grade) and 150 \( \mu \)g of penicillinase in 0.2 mL of 10 mmol/L phosphate buffer, pH 7.0 (Na\(_2\)HPO\(_4\) - \( 2 \)H\(_2\)O, 895 mg/L, and Na\(_2\)HPO\(_4\) - \( 2 \)H\(_2\)O, 390 mg/L) containing 154 mmol of NaCl per liter (PBS), with added glutaraldehyde, 10 mmol/L. After keeping the reaction mixture at room temperature for 2 h, with intermittent shaking, we diluted it to 0.6 mL and dialyzed against five to eight changes of PBS. We diluted the dialysate to 2 mL and added bovine serum albumin and sodium azide (1 g/L each, final concentration) then stored at −20 °C in 50- or 100-\( \mu \)L portions.

Collection of plasma. Peripheral venous blood was sampled from apparently healthy men and women, selected without conscious bias, as well as from women with disease such as hyperprolactinemia. We collected samples in heparinized vials between 1000 and 1200 hours, separated the plasma after centrifugation, and stored it at −20 °C until analysis.

RIA. We iodinated hPRL by the Chloramine-T method of Hunter and Greenwood (9), then assayed hPRL according to the RIA protocol supplied by NIADDK (Technical Report No. 127, Pituitary Hormones and Antisera Center).

Immunosorbant assay with penicillinase label. We prepared the immunoglobulin (IgG) fraction of the goat antibody in the same way as described elsewhere (10), then coated the 96 wells of the microtiter plate with purified IgG treated with glutaraldehyde as described by Parsons (11). For standards, we dissolved a reference sample of hPRL (2.5–80 ng/mL), obtained from NIADDK (35 kilo-int. units/g), in plasma from which endogenous hPRL had been removed in two ways: (a) Plasma, supplemented with \(^{125}\)I-labeled hPRL, was added to polystyrene tubes coated with antibody to hPRL; after incubation for 2 h at 37 °C, all the labeled hPRL had been stripped from the plasma. (b) Plasma supplemented with \(^{125}\)I-labeled hPRL was incubated first with anti-hPRL antibody, then in polystyrene tubes coated with the goat IgG. This stripped plasma, validated as such by the absence of \(^{125}\)I-labeled hPRL, was used for making standards.

To perform the assay, add 100 \( \mu \)L of standard hPRL or unknown sample, 50 \( \mu \)L of hPRL–penicillinase conjugate, and 100 \( \mu \)L of prolactin antibody in 10 mmol/L PBS solution containing 10 g of bovine serum albumin, 1 g of Na\(_2\)SO\(_4\), and 1 mL of Tween 20 per liter (diluent buffer) to the goat IgG-
costed wells, in duplicate. Incubate the plate for 1 h at 37 °C, then decant the contents of the wells, and wash the plate thoroughly with isotonic saline containing 500 μL of Tween 20 per liter.

To measure the penicillinase activity in each well, add to each well 250 μL of cold, freshly prepared penicillin V reagent, 0.8 mol/L (380 mg/L) in 0.2 mol/L phosphate buffer (Na2HPO4 · 2H2O, 24.28 g/L; NaH2PO4 · 2H2O, 12.17 g/L), pH 7.2, and incubate the plate at 37 °C for 30 min. Then transfer 200 μL from each well to 12 × 75 mm tubes containing 1 mL of starch–iodine reagent.

The starch–iodine reagent is prepared by adding 0.35 mL iodine reagent (18 mmol of iodine in 3.25 mol/L potassium iodide) to 190 mL distilled water and then 30 mL of a 20 g/L solution of hydrolysed starch, with constant stirring.

Stop the reaction after 10 min by adding 0.5 mL of 5 mol/L hydrochloric acid reagent. Measure the resulting blue color at 620 nm. (We used a Pye Unicam SP8-500 spectrophotometer.)

Standardization of the method. To four 1-mL aliquots of the hPRL-stripped human plasma, we added 5, 15, 30, and 50 ng of hPRL. Analytical recoveries were calculated by expressing the net hPRL determined by EIA and RIA as a percentage of added hPRL. Interassay and intra-assay precision of the method was determined by measuring hPRL concentrations in pooled human plasma samples in which the amount of hPRL was measured by RIA.

Clinical studies. In addition to measuring hPRL concentrations in the plasma of apparently normal healthy men and women, we also evaluated patients having a clinical history of amenorrhea and galactorrhea by both EIA and RIA before treatment and, in some, after treatment with bromocriptine. Prolactin was also measured in the plasma of one patient with pituitary adenoma, before and after surgery.

Results

Standard curve. Dose–response curves for the penicillinase-labeled immunosorbant assay and the RIA are shown in Figure 1. The CVs for the A/A0 ratio of each standard ranged from 4.6% to 8.5% for the enzyme immunosorbant assay, and CVs for the B/B0 ratio ranged from 3.9% to 8.0% for the RIA. Thus the standard curves obtained over several assays remained stable and precise.

On logit-log transformation of the curves the equations for the relationships for EIA were y = 2.66x − 2.519 μg/L, and for the RIA, y = −2.55x + 3.88 μg/L. The slopes were thus similar.

Detection limit. The detection limit, defined as the concentration equivalent to the A0 minus 2 SD of A0 and the B0 minus 2 SD of B0, was 2.5 and 3.5 μg/L for the EIA and the RIA, respectively.

Analytical recoveries. Recoveries of known amounts of hPRL added to four pools of stripped plasma ranged from 101% to 115% by EIA and 94% to 120% by RIA.

Intra-assay and interassay variations. Plasma specimens containing approximately the same concentrations of hPRL were combined to form four pools with different concentrations. We analyzed each pool 15 times within assay and also in 10 separate assays by both EIA and RIA. As shown in Table 1, the CV was marginally high at low concentrations of hPRL, both by EIA and RIA, the results being comparable and well within a CV of 15%.

Clinical studies. The mean concentrations of hPRL in plasma of a group of normal women (29 samples) were 13.58 (SD 5.06) μg/L by RIA and 14.99 (SD 4.58) μg/L by EIA. For normal men (29 samples) the corresponding values were 9.90 (SD 3.90) and 11.98 (SD 3.21) μg/L, respectively. In another 53 plasma samples, hPRL was measured both by EIA and RIA and the correlation coefficient was 0.98.

Concentrations of hPRL in plasma of 17 patients with clinical symptoms of amenorrhea and galactorrhea (Table 2) showed significantly high values. Nine of these patients, on treatment with bromocriptine, again had low concentrations of hPRL in plasma. In one patient showing a pituitary macro-adenoma and having high values for plasma hPRL, surgical removal of the adenoma returned the circulating concentrations of hPRL to within the normal range.

Discussion

The advantages of using penicillinase as an enzyme marker in the development of EIAs for some steroid and protein hormones have been discussed (7). These include its absence from biological fluids; its greater stability, even at room temperature; and its high turnover rate. The present assay has certain distinctive features that have not been reported earlier, either when penicillinase was used as an enzyme label for other hormones or for measuring hPRL in plasma. Coating of microtiter plates with the purified goat IgG provides a universal solid phase for measuring proteins, steroids, and steroid metabolites, and helps minimize the use of costly primary antibody for assay purposes. The assay

![Graph](image-url)
Table 1. Intra-Assay and Interassay CVs for Measurements of Prolactin in Pooled Human Plasma

<table>
<thead>
<tr>
<th></th>
<th>EIA Mean (SE), µg/L</th>
<th>EIA CV, %</th>
<th>RIA Mean (SE), µg/L</th>
<th>RIA CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within assay (n = 15 each)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.68 (0.30)</td>
<td>13.51</td>
<td>8.02 (0.28)</td>
<td>13.35</td>
<td></td>
</tr>
<tr>
<td>21.29 (0.44)</td>
<td>8.12</td>
<td>21.80 (0.45)</td>
<td>7.90</td>
<td></td>
</tr>
<tr>
<td>48.65 (1.09)</td>
<td>8.75</td>
<td>46.50 (0.44)</td>
<td>3.63</td>
<td></td>
</tr>
<tr>
<td>72.27 (1.02)</td>
<td>5.46</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Between assay (n = 10 each)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.14 (0.30)</td>
<td>13.02</td>
<td>10.20 (0.45)</td>
<td>13.67</td>
<td></td>
</tr>
<tr>
<td>21.02 (0.60)</td>
<td>11.79</td>
<td>24.23 (0.80)</td>
<td>10.30</td>
<td></td>
</tr>
<tr>
<td>42.67 (1.20)</td>
<td>8.6</td>
<td>46.30 (1.17)</td>
<td>7.8</td>
<td></td>
</tr>
<tr>
<td>66.69 (2.05)</td>
<td>9.11</td>
<td>—</td>
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Table 2. Prolactin Concentrations in Some Patients

<table>
<thead>
<tr>
<th>Condition</th>
<th>EIA n</th>
<th>Mean (SD), µg/L</th>
<th>RIA n</th>
<th>Mean (SD), µg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amenorrhea, galactorrhea</td>
<td>17</td>
<td>56.99 (17.92)</td>
<td>17</td>
<td>55.82 (18.09)</td>
</tr>
<tr>
<td>Before treatment</td>
<td>9</td>
<td>16.86 (3.25)</td>
<td>9</td>
<td>15.98 (4.25)</td>
</tr>
<tr>
<td>Pituitary macroadenoma</td>
<td>260.00</td>
<td>265.90</td>
<td>260.00</td>
<td>265.90</td>
</tr>
<tr>
<td>After surgery</td>
<td>1</td>
<td>10.50</td>
<td>1</td>
<td>11.22</td>
</tr>
</tbody>
</table>

The design is such that the first reaction—i.e., that between prolactin antibody, prolactin–penicillinase conjugate, and standard or unknown hPRL—occurs in solution, which ensures rapid reaction rates without restricting diffusion. The stability of the starch–iodine reagent is extended by one year by preparing it in water instead of buffer (7, 10). Actual assay and color reaction can be completed within 2 h, thus making this assay the first available rapid and sensitive EIA for hPRL. The detection limit, 2.5 µg of hPRL per liter by EIA, is comparable with that of the RIA method of our laboratory.

Hyperprolactinemia can occur in association with pituitary tumors, hypothyroidism, and, in some instances, drug therapy. In infertility, with disturbed reproductive patterns, increased hPRL concentrations have been found to be responsible for causing amenorrhea and galactorrhea. Here, we measured hPRL in plasma from some of these cases, before and after treatment with bromocriptine. In one established case of pituitary adenoma, very high values for hPRL in plasma declined to normal after surgery. Thus this is a simple, sensitive, and fast procedure for measuring hPRL to monitor infertility due to hyperprolactinemia and other reproductive disorders.

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References