Radioimmunoassay of Human Homologous Prolactin in Serum with Commercially Available Reagents

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A clinically useful and reproducible radioimmunoassay for human homologous prolactin, established with commercially available reagents, was studied and validated. We present detailed conditions for iodination and purification of labeled prolactin and the optimal conditions for the assay. By the method, we found values (μg/liter) as follows for serum prolactin: normal men, 8.9 ± 5.2 (mean ± SD); normal women, 11.8 ± 5.5; normal women taking contraceptive pills, 9.2 ± 5.0; pregnant women in the third trimester, 183 ± 60.9; patients with various disorders other than the hypothalamic-pituitary axis, 9.3 ± 6.3; in some patients with amenorrhea and galactorrhea of diverse origin, 78.2 ± 87.4; and in some patients with surgically proven pituitary tumor, 1414 ± 980. Results under provocative testing are also presented for a patient with normal hypothalamic-pituitary function.

Additional Keyphrases: hypothalamic and pituitary tumors • infertility • acromegaly • prolactin concentrations during pregnancy • tests of hypothalamic-pituitary function • normal values

The development of various homologous and heterologous radioimmunoassays for human prolactin and their impact on clinical endocrinology have recently been reviewed (1). Determination of serum prolactin has proved to be of great utility in the assessment of the endocrine function of the hypothalamic-pituitary axis (2-4). It is all-important in the diagnosis, management, and followup of prolactin-producing pituitary tumors. Hyperprolactinemia is a sensitive indicator of hypothalamic-stalk disease. Provocative testing of hypothalamic-pituitary prolactin axis with chlorpromazine, thyroliberin, or L-dopa has proved advantageous in the understanding of the physiology and pathophysiology of the axis and in delineating the site of any impairment in the axis (5-10). Hyperprolactinemia is thought to be present in about 20% of women with amenorrhea. Suppressing prolactin secretion in these women, for example with bromocriptine (“CB-154”), has resulted in restoration of normal menstrual function, ovulation, and pregnancy in a substantial proportion of these patients (11, 12).

Here we report our experience in establishing and validating a reliable and reproducible homologous human prolactin radioimmunoassay from commercially available antiserum and antigen.

Materials and Methods

Reagents and Solutions

*Phosphate-buffered saline.* Dissolve 10 mmol of Na₂HPO₄·7H₂O, 0.15 mol of NaCl, and 15 mmol of NaN₃ (2.68 g, 8.77 g, and 1.0 g, respectively) in 900 ml of water, adjust the pH to 7.4 with 1.0 mol/liter HCl, and dilute to 1 liter with water.

*Assay buffer.* Dissolve 10 g of bovine serum albumin (Cohn Fraction V; Sigma Chemical Co., St. Louis, Mo. 63178) in 1 liter of phosphate-buffered saline and adjust the pH to 7.4.

*Normal rabbit serum solution, 20 ml/liter.* Dissolve 50 mmol (5.65 g) of disodium ethylenediaminetetraacetate in 1 liter of phosphate-buffered saline, readjust the pH to 7.4. Dilute 2 ml of normal rabbit serum to 100 ml with this solution.

*Purified human prolactin, 2 μg/vial (Calbiochem, La Jolla, Calif. 92037, lot no. 628019),* for iodination.

*Human prolactin antiserum, rabbit, lyophilized* (Calbiochem, lot no. 620021). Reconstitute the lyophilized antiserum with 1.0 ml of water and dilute to 10 ml with the normal rabbit serum solution. After titration according to the assay procedure to be described later, further dilute the solution with the normal rabbit serum solution to a concentration such that 200 μl will bind about half of labeled prolactin.

*Human prolactin standard, 200 ng/bottle (Calbiochem; we used lot no. 628007).* Reconstitute the contents of the bottle with 1.0 ml of water and use this as
stock solution. Dilute the stock solution twofold serially with assay buffer to yield standard solutions of 100, 50, 25, 12.5, 6.25, 3.13, and 1.56 μg/liter for the standard curve; use the assay buffer alone as the 0 μg/liter standard.

**Second-stage precipitation antibody (second antibody), goat anti-rabbit serum** (produced in our laboratory). Titrate each batch to achieve the highest precipitation of bound antigen. The batch used for the present study was found to require 50 μl of undiluted serum for each tube.

Na\textsubscript{125I}, carrier free (New England Nuclear, North Billerica, Mass. 01862), specific activity, 17 kCi/g (mCi/μg).

**Bovine lactoperoxidase** (EC 1.11.1.7; Sigma). Dissolve 1.08 mg in 2 ml of sodium acetate buffer (0.1 mol/liter, pH 5.6).

**Hydrogen peroxide**, 300 g/liter (Mallinckrodt, Inc., St. Louis, Mo. 63160). Dilute 75 000-fold with water.

**Potassium iodide solution.** Dissolve 16 g of sucrose, 1 g of potassium iodide, and 20 mg of sodium azide in 100 ml of water.

**Molecular weight markers:** (a) "Blue Dextran" (av mol wt 2 000 000; Pharmacia, Piscataway, N. J. 08854). Dissolve 0.2 g in 100 ml of water.

**Molecular weight markers:** (b) fluorescein isothiocyanate (FITC) dextran (av mol wt 2900; Pharmacia). Dissolve 2.0 g in 100 ml water.

**Human somatotropin** (growth hormone) is obtained from the National Pituitary Agency (NIH-GH-HS968C).

**Human chorionic somatotropin** (placental lactogen) solutions are prepared from the Pharmacia human placental lactogen test kit standard.

**Iodination of Human Prolactin**

Human prolactin was iodinated with the lactoperoxidase method described by Roth (13), with some modification. Two columns instead of one were applied to purify the labeled prolactin: first, a Sephadex G-25 column, to separate the protein peak from the free iodide peak; then a Sephadex G-100 column, to separate damaged and undamaged labeled prolactin. The modified procedure is described in detail as follows:

Dissolve 2 μg of human prolactin in 20 μl of sodium phosphate buffer (50 mmol/liter, pH 7.4) and add 10 μl of sodium acetate buffer (0.4 mol/liter, pH 5.6).

Add 5 μl of Na\textsubscript{125I} (500 μCi), 5 μl of lactoperoxidase solution, and 5 μl of the freshly diluted hydrogen peroxide. React for 25 s after adding hydrogen peroxide solution with gentle stirring and add 100 μl of potassium iodide solution to stop the reaction. Transfer the reaction mixture to a 0.7 × 15 cm column containing Sephadex G-25, pre-equilibrated with assay buffer. Elute the column with assay buffer and collect 0.5-ml fractions in an ice bath.

Pool the first radioactive peak from the Sephadex G-25 column, add 0.2 ml each of Blue Dextran and FITC dextran solutions, and apply the mixture to a 2.5 × 32 cm column containing Sephadex G-100, pre-equilibrated with assay buffer. Elute at 4 °C with assay buffer and collect 1-ml fractions. Combine the fractions of the third radioactive peak and store in portions. The labeled prolactin is stable for about three weeks if stored frozen. Before assay, dilute each aliquot of the labeled prolactin with assay buffer to a concentration of about 10 000 cpm/100 μl.

**Sample Preparation**

Blood was drawn from nonfasting patients in the morning. After the blood had clotted, it was centrifuged and 2 ml of the serum was stored frozen until assay.

**Radioimmunoassay**

To duplicate 12 × 75 mm disposable polystyrene tubes, add 100 μl of sample or standard solution and 400 μl of assay buffer. Add 200 μl of the human prolactin antiserum that was diluted so as to bind half of the labeled prolactin. To the blank tubes, add 200 μl of the normal rabbit serum solution instead of diluted prolactin antiserum.

Add 100 μl of labeled human prolactin containing 10 000 cpm, mix well, and cover all the tubes and allow the mixture to stand for 72 h at room temperature, then add 50 μl of second-stage antiserum (goat anti-rabbit serum), mix well, cover, and allow to stand for 24 h at room temperature. Centrifuge for 10 min at 3000 × g in a refrigerated centrifuge, aspirate the supernate, and count the radioactivity of the precipitate in a gamma counter for 5 min. Calculate the percentage of binding in standard or sample tubes vs. zero tube (B/B\textsubscript{0}), and plot a standard curve of B/B\textsubscript{0} vs. prolactin, in micrograms per liter, on semilog graph paper. Read the concentration of prolactin in the sample directly from the standard curve.

**Results**

**Preparation of Labeled Prolactin**

Figure 1 shows a typical Sephadex G-25 column chromatogram of the mixture obtained from iodination. The first peak, representing protein (fraction 6 and 7), contained 12% of the total radioactivity. The second
peak, representing free iodide (fraction 11 to 16), contained 69% of the total radioactivity. About 6% of the total radioactivity remained in the iodination tube and transferring pipettes; the remaining 13% of the total radioactivity presumably was still on the Sephadex G-25 column.

The fractions of the first peak from the Sephadex G-25 column were pooled and applied together with the two molecular weight markers to a Sephadex G-100 column. Three radioactive peaks were eluted (Figure 2) between the Blue Dextran and the FITC dextran. The fractions of the third radioactive peak were pooled, diluted, and used as labeled prolactin for the radioimmunoassay. No free-iodide peak was observed after the 2900 molecular weight (FITC dextran) fluorescence marker.

Some Analytical Variables

Standard curve and sensitivity. Figure 3 illustrates a standard curve for human prolactin with values from 0.31 to 20 ng per tube. The displacement of prolactin of 0.31 and 20 ng per tube was 95 and 5%, respectively, indicating the sensitivity of the test to be 0.31 ng/tube, or 3.1 μg/liter of serum, and it may cover a determination range up to 20 ng/tube or 200 μg/liter of serum. The amount of prolactin standard producing about 50% displacement is 2.5 ng/tube.

Cross-reactivity with human somatotropin was tested at 5, 10, 20, and 100 ng/tube, and with human chorionmammotropin at 0.4, 0.8, and 1.6 μg/tube (Figure 3). Human somatotropin up to a concentration of 100 ng/tube (1000 μg/liter of serum) cross-reacted negligibly. Human chorionmammotropin, 800 or 1600 ng/tube (8 or 16 mg/liter of serum), showed a cross-reaction of less than 0.04%. This indicates that the assay can be validly used to determine prolactin concentration in pregnant women, because the highest chorionmammotropin concentration in the third trimester of pregnancy is <8 mg/liter (14), or to determine the serum prolactin concentration in the most severe cases of acromegaly.

Intra- and inter-assay precision and quality control. Five different pooled samples and samples from five individual patients were determined in separate assays as well as on separate days for quality control studies. Results are shown in Table 1. "F" was a pooled serum obtained from women having higher prolactin concentration. "½F" was the F pooled serum diluted twofold with assay buffer. S1 to S5 were samples from individual patients. As shown in Table 2, Q1, Q2, and Q3 were pooled sera containing prolactin concentrations within the top, middle, and bottom portions of the standard curve, respectively. These three pooled sera were used for quality-control studies during development of the test and also served as routine quality control samples. In three months, we determined 13 assays and used three different batches of labeled prolactin. We found that the interassay coefficient of variation of the 13 assays was 24.4% for Q1, 13.3% for Q2, and 10.1% for Q3.

Linearity of results on sample dilution. The result for the undiluted F in Table 1 is 70.2 μg/liter; for the twofold dilution it is 37.5 μg/liter. In addition, a sample with a high prolactin concentration was diluted two-, four-, and eightfold with assay buffer to contain 50, 25, and 12.5 μl of original serum per 100 μl, respectively, and their prolactin concentrations were determined. The results (Figure 4) were 93.4, 47.8, and 22.0 μg/liter, respectively, indicating that the assay is unaffected by serum concentration per se, and that samples that contain more than 200 μg of prolactin per liter can validly be diluted and reassayed.

Normal Ranges for Healthy Women and Men

To establish the normal range of prolactin, we selected without conscious bias 36 healthy women, ages
Table 1. Quality-Control Study of Prolactin Assay *

<table>
<thead>
<tr>
<th>Samples</th>
<th>Assay 1</th>
<th>Assay 2</th>
<th>Assay 3</th>
<th>Inter-assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>F, pooled</td>
<td>70.9 ± 5.6 (5)</td>
<td>74.0 ± 5.1 (9)</td>
<td>66.0 ± 4.8 (9)</td>
<td>70.2 ± 6.1 (23)</td>
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<tr>
<td>%F</td>
<td>36.9 ± 1.9 (5)</td>
<td>37.6 ± 2.6 (5)</td>
<td>38.0 ± 2.7 (5)</td>
<td>37.5 ± 2.3 (15)</td>
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<tr>
<td>S1</td>
<td>11.5 ± 1.5 (4)</td>
<td>12.7 ± 1.3 (5)</td>
<td>12.5 ± 1.3 (5)</td>
<td>12.3 ± 1.4 (14)</td>
</tr>
<tr>
<td>S2</td>
<td>10.3 ± 1.0 (4)</td>
<td>11.1 ± 3.5 (4)</td>
<td>9.6 ± 2.0 (3)</td>
<td>10.4 ± 2.3 (11)</td>
</tr>
<tr>
<td>S3</td>
<td>48.5 ± 2.8 (5)</td>
<td>49.3 ± 3.5 (5)</td>
<td>46.1 ± 1.7 (5)</td>
<td>48.0 ± 2.9 (15)</td>
</tr>
<tr>
<td>S4</td>
<td>19.3 ± 1.0 (5)</td>
<td>19.6 ± 0.9 (5)</td>
<td>18.7 ± 1.2 (5)</td>
<td>19.2 ± 1.1 (15)</td>
</tr>
<tr>
<td>S5</td>
<td>31.9 ± 1.7 (5)</td>
<td>30.0 ± 1.9 (5)</td>
<td>34.2 ± 2.5 (5)</td>
<td>32.0 ± 2.6 (15)</td>
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</tbody>
</table>

* Values are µg/liter, mean ± SD (no. of determinations); CV = coefficient of variation.

Table 2. Routine Quality-Control Results *

<table>
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<tr>
<th>Samples</th>
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<th>Assay no.</th>
<th>Assay no.</th>
<th>Inter-assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1 pooled</td>
<td>1</td>
<td>6</td>
<td>11</td>
<td>7.3 ± 0.3 (4)</td>
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<tr>
<td></td>
<td>2</td>
<td>7</td>
<td>12</td>
<td>9.4 ± 0.5 (4)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8</td>
<td>13</td>
<td>7.2 ± 0.6 (4)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>9</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>5</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q2 pooled</td>
<td>1</td>
<td>6</td>
<td>11</td>
<td>45.4 ± 0.7 (4)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7</td>
<td>12</td>
<td>48.1 ± 0.8 (4)</td>
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<tr>
<td></td>
<td>3</td>
<td>8</td>
<td>13</td>
<td>42.4 ± 3.1 (4)</td>
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<td></td>
<td>4</td>
<td>9</td>
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</tr>
<tr>
<td></td>
<td>5</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q3 pooled</td>
<td>1</td>
<td>6</td>
<td>11</td>
<td>97.7 ± 1.2 (4)</td>
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<tr>
<td></td>
<td>2</td>
<td>7</td>
<td>12</td>
<td>92.3 ± 4.6 (4)</td>
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<tr>
<td></td>
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<td>8</td>
<td>13</td>
<td>91.3 ± 3.5 (4)</td>
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<tr>
<td></td>
<td>5</td>
<td>10</td>
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</table>

* Values are µg/liter, mean ± SD (no. of determinations); CV = coefficient of variation.

20 to 54 years, and 36 healthy men, ages 23 to 56 years, among clinical laboratory employees. Their blood was sampled and the prolactin concentration determined. For the men the mean prolactin value was 8.9 µg/liter ± 5.2 (SD). For 20 of the women, the mean was 11.8 µg/liter ± 6.8; the other 16 were taking contraceptive pills, and their mean was 9.2 ± 5.0 µg/liter. Because the difference between the latter two subgroups was not statistically significant (P > 0.10), the data were pooled and the combined mean was calculated to serve as the normal value for women: 10.7 ± 6.0 µg/liter.

Sixty-four patients with various diseases other than of the hypothalamic–pituitary axis had a mean prolactin concentration of 9.3 ± 6.3 µg/liter, not significantly (P > 0.10) different from that for the normal population. Figure 5 shows results of these studies and of those in patients with clinically identifiable and surgically proven pituitary tumors, amenorrhea and (or) galactorrhea of diverse origin, and in women in the third trimester of pregnancy.

Response to Chlorpromazine, Thyroliberin, and L-Dopa

To further validate the method, we studied patients' responses to various agents that are known to affect prolactin secretion. Chlorpromazine administration usually leads to increased prolactin secretion, through suppression of prolactostatin (the hypothalamic prolactin inhibitory factor); thyroliberin increases prolactin secretion through a direct stimulating effect on the pituitary; L-dopa decreases prolactin secretion, either by stimulation of prolactostatin or by directly suppressing prolactin release by the anterior pituitary. Figure 6 shows serum prolactin concentrations after the administration of chlorpromazine, thyroliberin, or L-dopa, given singly on successive days to a patient who had normal hypothalamic–pituitary endocrine function.

Discussion

At present, prolactin assay is of great clinical utility...
in the diagnosis and management of disorders of the hypothalamic stalk area and of prolactin-secreting pituitary tumors, and in the assessment of pituitary reserve. For this purpose, an assay of wider range instead of higher sensitivity is required. Therefore, we designed the standard curve of the assay from 3.1 to 200 μg/liter; with the upper range of 200 μg/liter, most of the pathological samples containing high concentration of prolactin still required fivefold, 10-fold, or 20-fold dilutions before assay. If this assay is used for research purposes for which a higher sensitivity is required, the assay should be completely redesigned: lower amounts of antiserum and either higher specific activity or lower counts of the labeled prolactin should be used.

The normal values we found for blood prolactin concentration as determined by the present method compare well with those reported by Frantz (15) and Hwang et al. (16). In Frantz’s studies of 97 healthy, nonhospitalized, ambulatory subjects (54 men and 43 women), the normal mean value for women was 10.27 ± 7.94 μg/liter, and for men, 9.21 ± 5.65 μg/liter (mean ± SD). Hwang et al. (16) used radioimmunoassay to determine prolactin and did not find significant differences between values for men and women and reported a normal range of 0 to 30 μg/liter for both sexes.

The advantage of using two columns instead of one to purify labeled prolactin is that the free iodide can be quickly separated from labeled prolactin with a short Sephadex G-25 column immediately after iodination. Only the radiolabeled prolactin peak, which contained 12% of the initial total radioactivity, was purified in a refrigerated fraction collector and a cold-jacketed Sephadex G-100 column. The elution required 4 to 5 h. Because we could use decreased amounts of radioactivity, the chances of substantially contaminating the equipment were also decreased, and in fact the Sepha-
dex G-100 column could be re-used after extensive washing with buffer.

The supplier (Calbiochem) of the antisera and purified prolactin recommends the use of Chloramine T to iodinate prolactin. We used the lactoperoxidase method in our procedure because Rogol and Rosen (17) demonstrated that for radioimmunoassay the lactoperoxidase method yielded labeled prolactin of much better quality than that obtained by the Chloramine T method. We found that the method gave labeled prolactin of consistently good quality from batch to batch.

For a diagnostic laboratory, we believe that it would be better to develop one's own antisera and assay procedure to ensure a long period of service rather than to purchase test kits. Test kits that include antisera, all the necessary reagents, and even labeled antigen are very convenient to use, but they contain reagent amounts for 100 to 500 tubes and last only a short period. Moreover, antisera vary from animal to animal and sometimes even from bleeding to bleeding in the same animal, and the quality of labeled antigen may also vary according to the shelf life and conditions of shipment. Thus, it is difficult to control the quality of antisera and labeled antigen when they are supplied in kit form. Developing one's own antisera requires relatively pure special antigen and a long immunization period. If the conditions make it impossible to achieve this, we suggest buying antisera in bulk and developing one's own assay method that will give a long period of assured service. In our experience this entails surveying the market, arranging with the prospective supplier to hold a big batch of antisera, purchasing it in a small quantity first, characterizing it carefully, and validating the assay. If all the results are satisfactory, then we purchase the antisera in large quantities that will last for years.

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