A New Enzyme Immunoassay for Prolactin in Serum or Plasma

Reiner Babiel,1 Peter Willnow,1 Martina Beer,1 Michelea van Gent,1 and Volker Ehrhardt1

This enzyme immunoassay (EIA) of human prolactin (hPRL) involves incubation of sample and anti-hPRL antibodies conjugated to horseradish peroxidase (EC 1.11.1.7) in tubes coated with a second antibody to hPRL. The test can be performed within 60 min. No reaction of the antibodies with human placental lactogen and human somatomropin is detectable. The presence of detergent allows assay of both serum and plasma. Precision was improved by including polyethylene glycol in the reaction mixture. To optimize analytical recovery, we added protease inhibitor. Assay of the EIA standards shows good correlation with results for World Health Organization reference preparations. The measurable range is 1 to 400 μg/L. Intra- and interassay CVs are about 5%. Comparisons with two RIAs and two other EIAs show reasonably good correlations. The components of our EIA are stable for 18 months.

Human pituitary prolactin (hPRL) is a single-chain polypeptide of known primary structure similar to the structures of human hPL and hGH; i.e., its peptide sequence is identical to 13% and 16%, respectively, of theirs (1).5 In humans, hPRL plays a crucial role in the promotion of lactogenesis (2) and may be involved in the regulation of normal gonadal function (2-4).

A pathologically increased concentration of hPRL in serum is an important marker of pituitary dysfunction. The major causes of hyperprolactinemia are pituitary adenomas, various drugs (e.g., psychotropics and anxiolytics), hypothyroidism, central nervous system lesions, and stress (5). In women, hyperprolactinemia gives rise to infertility and galactorrhea; in men, it induces decrease of libido as well as impotence. Measurement of hPRL is therefore an essential component for routine evaluation of anovulatory states and male hypogonadism.

Currently, RIA is the method most widely used for measuring hPRL. However, enzyme immunoassay (EIA) represents an obvious alternative to the use of 125I, for example, where lack of gamma counter availability, prolonged reagent storage, or waste disposal all pose problems. We therefore developed, by using two different monoclonal antibodies, a "sandwich"-type EIA for use in routine determinations of hPRL. This one-step assay, based on coated-tube technology, provides a fast and convenient procedure amenable to extensive automation.

Materials and Methods

Materials

Antibodies: Monoclonal antibodies against hPRL were raised in accordance with conventional procedures (6). The characterization of the resulting nine cell lines and the selection of the antibodies used for solid-phase coating and for conjugation with HRP (EC 1.11.1.7) are described elsewhere (7).

Antibody-coated tubes: The capture antibody (1 mg/L) was coated onto polystyrene tubes by the method of Kunst et al. (8).

Antibody-enzyme conjugate: Antibody was conjugated with oxidized HRP as described by Haug et al. (9).

hPRL standards, calibration: "Plasma Diagnostic Base" (Armour, Kankakee, IL) was adjusted to a protein concentration of 6 g/L. Phenylmethylsulfonyl fluoride (Merck, Darmstadt, F.R.G.) in ethanolic solution was added to give a concentration of 0.5 g/L, and the solution was stirred for 1 h at 4 °C. To this matrix we added hPRL (400 μg/L, Brandenberger, Zurich, Switzerland). Standards were prepared by diluting this stock solution with appropriate amounts of standard matrix.

Calibration was accomplished by comparing the EIA standards with the WHO preparations 83/562 and 83/573, dissolved in serum that had been stripped of hPRL by passage through a column containing immobilized antibodies to hPRL. Concentrations of the 10 standards ranged between 80 milli-int. units and 8 int. units of hPRL per liter. The means of triplicate determinations and two runs were used for regression analysis.

Conjugate buffer: The sodium phosphate buffer (40 mmol/L, pH 7.4) contained bovine serum albumin (BSA, 2 g/L; Behringwerke AG, Marburg, F.R.G.), bovine IgG (1 g/L; Boehringer Mannheim GmbH, Mannheim, F.R.G.), polyol detergent (Symperonic F68, 2 g/L; Serva, Heidelberg, F.R.G.), and Polyethylene Glycol 40 000 (1 g/L; Serva).

Substrate solution: The phosphate–citrate buffer (100 mmol/L, pH 4.4) contained 500 mg of sodium perborate and 950 mg of ABTS (Boehringer Mannheim GmbH) per liter. Specimens: Serum samples were from healthy individuals, patients with increased concentrations of IgG or rheumatoid factors, and pregnant women.

Other hormones: To assess the specificity of the antibodies, we checked their reactivity with other proteohormones of human origin, all from Boehringer Mannheim GmbH. The concentrations of these hormones were calibrated against the following reference preparations: hGH (1st IRP), hTSH (2nd IRP 80/559), hCG (1st IRP 75/573), hFSH (2nd IRP 78/549), and hLH (1st IRP 68/40). No reference preparation is available for hPL.

Comparison methods: Two RIAs (Riabead® and Riabead® II from Abbott, North Chicago, IL) and two EIAs (Tandem®-E PRL from Hybritech, Liège, Belgium; and PROL-ENZELSA, Compagnie ORIS Industrie S. A., Gif sur Yvette, France) were used for method-comparison studies.

Instruments: Measurements were performed either man-
ually, with the fully automated ES 600 system (10), or with the partly automated ES 22 instrument (11), both instruments from Boehringer Mannheim GmbH.

Procedure

Incubate 50 µL of sample or standard with 1 mL of antibody–enzyme conjugate containing 100 U of horseradish peroxidase per liter at room temperature in antibody-coated tubes for 30 min. Aspirate the fluid, rinse the tube twice with tap water, add 1 mL of substrate solution, and incubate for another 30 min at room temperature. Then measure the absorbance (with the ES 22 at 405 nm; manually or with the ES 600 at 422 nm) and calculate the concentration of hPRL by using a calibration curve.

Assay Validation

Sensitivity: The lower detection limit was calculated as the mean plus 3 SD of 20 measurements of the standard matrix (12).

Linearity and recovery: The linearity and the dilution characteristics were checked with the highest-concentration standard (400 µg of hPRL per liter) and with a human serum having an hPRL concentration of 450 µg/L. Standard matrix or a human serum sample with a low hPRL concentration were used as diluents.

Analytical recovery was tested by blending a low-concentration serum (5 µg/L) or the standard matrix with a pooled specimen of high-concentration serum (450 µg/L) and assaying, and also by mixing equal volumes of conjugate buffer containing increasing amounts of purified hPRL with samples with a low hPRL concentration (3 µg/L) and assaying. Each sample was determined in duplicate, the mean value being used to calculate the recovery.

Imprecision: As recommended by the National Committee for Clinical Laboratory Standards (13), we evaluated the imprecision in two separate runs on 20 days. We measured the hPRL concentration in three human serum pools (low, medium, and high concentration), at two determinations per sample per run.

Method comparison: For this we used freshly collected human sera. The measurements with the different methods were performed in several runs, but the same serum samples were used on the same day.

Interference: Interference by hemolysis, lipemia, and bilirubin was investigated by blending a high-hPRL serum containing no interfering substances with increasing amounts of hemolytic (hemoglobin up to 40 g/L), lipemic (triglycerides up to 12 g and cholesterol up to 4.5 g per liter), or icteric (bilirubin up to 360 mg/L) sera containing low concentrations of hPRL.

Interference by drugs was investigated in vitro by adding solutions of the relevant pharmaceutical to samples of a human serum pool (intermediate hPRL concentration) and measuring the hPRL value in comparison with samples mixed with appropriate volumes of the particular solvent (14).

Stability of EIA components: Lyophilized reagents (antibody–enzyme conjugate, standards), incubation buffer, and coated tubes were stressed by storage for 18 months at 4 °C, and then used in the hPRL EIA. Recovery of hPRL in control sera (Precinorm® IM and Precipath®; Boehringer Mannheim GmbH) and the lower detection limit with the stressed reagent were compared with the data obtained with freshly prepared reagents and freshly coated tubes.

Results

Optimization of Assay Components

The presence of at least 2 g of polyol detergent per liter in the conjugate buffer caused the analytical recovery of hPRL with our EIA in various types of plasmas to be identical to that in serum (Table 1).

The imprecision of our EIA at high hPRL concentrations could be decreased by about five- to ninefold by adding small amounts of PEG. The precision in measuring the zero standard was not significantly influenced by this addition, whereas nonspecific binding was slightly increased (Table 2). Analytical recovery of hPRL in patients' sera and control sera was identical in buffer with and without PEG (data not shown).

With Plasma Diagnostic Base as matrix for the standards, the recovery of hPRL at 20 °C was about 15% higher than at 30 °C. Addition of the protease inhibitors phenylmethylsulfon fluoride or aprotinin led to identical recoveries at both temperatures.

Test Performance

The correlation of the EIA standards (y) with the WHO reference preparations 83/562 and 83/5730 was good. The regression equations (15, 16) were \( y = 14.6x + 11.4 \mu g/L (r = 1.00, n = 10) \) and \( y = 14.5x + 12.2 \mu g/L (r = 1.00, n = 10) \), respectively. One microgram in our standards corresponds

<table>
<thead>
<tr>
<th>Table 1. Effect of Adding Detergent to the Conjugate Buffer on Recovery of hPRL as Measured by EIA in Serum and Various Plasmas</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Detergent concn, g/L</strong></td>
</tr>
<tr>
<td>Sample</td>
</tr>
<tr>
<td>Serum</td>
</tr>
<tr>
<td>Oxalated plasma</td>
</tr>
<tr>
<td>Citrated plasma</td>
</tr>
<tr>
<td>Fluoridated plasma</td>
</tr>
<tr>
<td>EDTA-treated plasma</td>
</tr>
<tr>
<td>Heparin-treated plasma</td>
</tr>
<tr>
<td>Mean recovery, %</td>
</tr>
</tbody>
</table>

Serum and plasmas were obtained at the same time from the same blood donor. Determinations in triplicate. * Recovery of serum hPRL value measured with EIA without detergent (Syneronic) addition in serum.
Table 2. Effect of Adding Polyethylene Glycol (PEG) on Assay Precision

<table>
<thead>
<tr>
<th>Conc added, g/L</th>
<th>Zero standard</th>
<th>hPRL standard, 402 µg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aborbance*</td>
<td>CV, %</td>
</tr>
<tr>
<td>PEG 6000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>0.054 (0.006)</td>
<td>11.0</td>
</tr>
<tr>
<td>2.5</td>
<td>0.056 (0.005)</td>
<td>9.5</td>
</tr>
<tr>
<td>PEG 20000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>0.051 (0.007)</td>
<td>13.0</td>
</tr>
<tr>
<td>2.5</td>
<td>0.052 (0.009)</td>
<td>18.0</td>
</tr>
<tr>
<td>PEG 40000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>0.052 (0.009)</td>
<td>16.0</td>
</tr>
<tr>
<td>2.5</td>
<td>0.052 (0.010)</td>
<td>19.0</td>
</tr>
<tr>
<td>None</td>
<td>0.040 (0.006)</td>
<td>16.0</td>
</tr>
</tbody>
</table>

*Aborances were recalculated for an optical path length of 1 cm from data obtained with 3-mm cuvettes. SDs of 12 determinations are listed in parentheses.

Table 3. Reaction of Various Proteohormones in the hPRL EIA

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Concentration added</th>
<th>Absorbance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>hTSH*</td>
<td>75 mili-int. units/L</td>
<td>0.050 (0.005)</td>
</tr>
<tr>
<td>hGH</td>
<td>2.5 int. units/L</td>
<td>0.053 (0.003)</td>
</tr>
<tr>
<td>hPL</td>
<td>130 mg/L</td>
<td>0.052 (0.004)</td>
</tr>
<tr>
<td>hFSH</td>
<td>38 klo-int. units/L</td>
<td>0.050 (0.005)</td>
</tr>
<tr>
<td>hCG</td>
<td>900 klo-int. units/L</td>
<td>0.052 (0.007)</td>
</tr>
<tr>
<td>hLH</td>
<td>40 klo-int. units/L</td>
<td>0.050 (0.003)</td>
</tr>
<tr>
<td>None*</td>
<td>—</td>
<td>0.054 (0.003)</td>
</tr>
</tbody>
</table>

* Mean of triplicate measurements. SDs are listed in parentheses. * Hormones dissolved in standard matrix. * Standard matrix alone.

Table 4. Precision of the Present Assay

<table>
<thead>
<tr>
<th>Instrument</th>
<th>hPRL concn, µg/L</th>
<th>intra-runa SD, µg/L</th>
<th>CV, %</th>
<th>inter-runb SD, µg/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES 22</td>
<td>9.6</td>
<td>0.6</td>
<td>6.2</td>
<td>0.6</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>76.9</td>
<td>3.1</td>
<td>4.8</td>
<td>4.1</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>256.0</td>
<td>8.2</td>
<td>3.2</td>
<td>11.0</td>
<td>4.3</td>
</tr>
<tr>
<td>ES 600</td>
<td>9.4</td>
<td>0.5</td>
<td>4.9</td>
<td>0.2</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>78.5</td>
<td>4.2</td>
<td>5.4</td>
<td>3.3</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>257.0</td>
<td>16.1</td>
<td>6.3</td>
<td>6.9</td>
<td>2.7</td>
</tr>
</tbody>
</table>

* Twenty duplicate determinations of each sample. b Two separate assay runs (duplicates of each sample) on 20 days.

Fig. 1. Standard Curve for EIA
Determinations represent the mean of four runs and triplicate determinations. Absorances are recalculated for an optical path length of 1 cm from data obtained from 3-mm cuvettes.

Table 4. Precision of the Present Assay

<table>
<thead>
<tr>
<th>Instrument</th>
<th>hPRL concn, µg/L</th>
<th>intra-runa SD, µg/L</th>
<th>CV, %</th>
<th>inter-runb SD, µg/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES 22</td>
<td>9.6</td>
<td>0.6</td>
<td>6.2</td>
<td>0.6</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>76.9</td>
<td>3.1</td>
<td>4.8</td>
<td>4.1</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>256.0</td>
<td>8.2</td>
<td>3.2</td>
<td>11.0</td>
<td>4.3</td>
</tr>
<tr>
<td>ES 600</td>
<td>9.4</td>
<td>0.5</td>
<td>4.9</td>
<td>0.2</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>78.5</td>
<td>4.2</td>
<td>5.4</td>
<td>3.3</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>257.0</td>
<td>16.1</td>
<td>6.3</td>
<td>6.9</td>
<td>2.7</td>
</tr>
</tbody>
</table>

* Twenty duplicate determinations of each sample. b Two separate assay runs (duplicates of each sample) on 20 days.

between 97% and 100% (data not shown).

Correlation of results by this new assay (y) with those for four existing immunoassays for hPRL (two RIAs and two EIAs) for samples within the standard curve range gave the following regression equations (15, 16): y = 1.07x(RIA I) + 2.16 µg/L, r = 0.995, n = 46; y = 1.18x(RIA II) + 0.31 µg/L, r = 0.980, n = 52; y = 0.81x(EIA I) - 1.42 µg/L, r = 0.901, n = 66; and y = 0.81x(EIA II) + 3.73 µg/L, r = 0.990, n = 52.

Hemolytic, lipemic, or icteric samples did not interfere, nor did 37 pharmaceuticals, including bromocriptine.

Storage of the components of our EIA for 18 months at 4 °C did not affect its functioning: The absorbance measured with the highest standard (400 µg/L) was found to be 99% of that measured with freshly prepared reagents; the lower detection limit remained unaltered; and the recovery of hPRL in control sera (Precinorm® IM: 11.8 µg/L vs 12.3 µg/L, Precipath® IM: 72 µg/L vs 74 µg/L), and citrated plasma vs serum (6.1 µg/L vs 6.3 µg/L) did not change significantly.

Discussion

Notwithstanding the high sensitivity of our assay system, the antibodies used in it have no detectable reactivity with the structurally hPRL-related proteohormones hPL and hGH. Therefore, even pathologically high concentrations of these hormones in serum will not affect hPRL determinations.

The addition of the detergent Synpersstonic F68 to the incubation buffer ensures that the hPRL values by our assay are identical for all kinds of plasmas and serum. Other surfactants tested did not reveal this beneficial
Table 5. Analytical Recovery of hPRL Measured by EIA

<table>
<thead>
<tr>
<th>Measured µg/L</th>
<th>Calculated µg/L</th>
<th>Recovery, %</th>
<th>Measured µg/L</th>
<th>Calculated µg/L</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>---</td>
<td>---</td>
<td>0.0</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>44.7</td>
<td>48.6</td>
<td>92</td>
<td>40.6</td>
<td>44.1</td>
<td>92</td>
</tr>
<tr>
<td>91.2</td>
<td>93.2</td>
<td>98</td>
<td>87.9</td>
<td>88.2</td>
<td>100</td>
</tr>
<tr>
<td>138.6</td>
<td>135.8</td>
<td>101</td>
<td>132.3</td>
<td>132.3</td>
<td>100</td>
</tr>
<tr>
<td>174.2</td>
<td>179.4</td>
<td>97</td>
<td>181.1</td>
<td>178.4</td>
<td>103</td>
</tr>
<tr>
<td>218.2</td>
<td>223.0</td>
<td>98</td>
<td>222.5</td>
<td>220.5</td>
<td>101</td>
</tr>
<tr>
<td>255.6</td>
<td>266.6</td>
<td>96</td>
<td>262.5</td>
<td>264.6</td>
<td>99</td>
</tr>
<tr>
<td>301.1</td>
<td>310.2</td>
<td>97</td>
<td>289.3</td>
<td>308.7</td>
<td>94</td>
</tr>
<tr>
<td>333.9</td>
<td>353.8</td>
<td>94</td>
<td>370.8</td>
<td>352.8</td>
<td>105</td>
</tr>
<tr>
<td>390.2</td>
<td>397.4</td>
<td>98</td>
<td>401.7</td>
<td>396.9</td>
<td>101</td>
</tr>
<tr>
<td>441.7</td>
<td>441.0</td>
<td>100</td>
<td>449.0</td>
<td>441.0</td>
<td>102</td>
</tr>
</tbody>
</table>

Mean recovery, %

97

100

* Serum with low hPRL concentration mixed with serum having a high hPRL concentration. 
* Standard matrix mixed with serum having a high concentration of hPRL. Determinations in duplicate.

The antibody/antigen reaction is far from equilibrium (only attained after about 8 h) after the 30-min incubation used in our test, so we suspected that the diffusion of the reactants in the test tube might be a limiting factor for the test's imprecision. Indeed, with the addition of small amounts of polyethylene glycol, which is known to enhance diffusion rates, we improved the precision, particularly so at high hPRL concentrations. To avoid a high-dose hook effect at undesirably low hPRL concentrations and to leave the absorbance with the zero calibrator unaffected, we prefer to add 1 g of polyethylene glycol per liter.

Initial experiments showed that analytical recovery with our EIA is lower at 30 °C than at 20 °C (ambient temperatures that may occur in the laboratory). This temperature effect was abolished by the addition of the serine protease inhibitors phenylmethylsulfonyl fluoride and aprotinin to the standards, suggesting the presence of proteases in the bovine serum used for standard matrix preparation that are activated at 30 °C, leading to the degradation of hPRL in the reaction mixture.

Because physiological hPRL values are distributed over a wide range, the need for very precise and reproducible test results appears not to be so great, and an intra-assay imprecision of <10% (CV) in the range of 30 to 60 µg of hPRL per liter is considered adequate (17). With CVs ranging between 2% and 6.5%, our EIA fulfills this requirement very well.

The dynamic range of our assay is 1 to 400 µg of hPRL per liter, as assessed by measurements of a series of concentrations prepared by mixing the highest standard or a serum having an hPRL concentration, 450 µg/L, with serum having a low hPRL concentration or with standard matrix. Our assay's working range is adequate for clinical purposes, because hypoprolactinemia is a seldom seen phenomenon, and the rarely encountered samples with hPRL concentrations exceeding 400 µg/L can be reassayed after dilution either with standard matrix or with human serum with low hPRL concentration.

The comparison with four existing immunoassays for hPRL shows good correlations. The systematically differing recoveries with the different methods are presumably attributable to different calibration procedures.

Our assay is calibrated to the WHO reference preparations 83/562 and 83/573, and the dilution characteristics of our standard hPRL and the hPRL used in the reference preparations are very similar.

Owing to the convenience of the procedure and the short incubation times, test results with our EIA can be reported after 1 h. The coated-tube technology enables it to be performed in semiautomated (11) and fully automated (10) systems. The components utilized possess excellent stability, indicating a good overall assay performance. Especially with the fully automated ES 600 system, reasonable precision and reliability of testing with a remarkable cost-saving may be achieved by running the hPRL EIA in singletones (18) and with one-point recalibration (19).

A preliminary report published quite recently (20) also demonstrates our EIA's good analytical performance, particularly its high precision, also under routine conditions in a clinical laboratory.

References