Enzyme-Linked Immunoassay for Placental Lactogen in Human Serum

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We describe an enzyme-linked immunoassay for measuring human placental lactogen (HPL) in serum. After suitable dilution, sera are pipetted into the wells of polyvinyl microtitre plates previously coated with anti-HPL. After incubation the sera are decanted, and replaced by a conjugate of horseradish peroxidase and anti-HPL. An amount of enzyme proportional to the concentration of HPL in the serum is bound to the wells via an antibody-HPL-antibody bridge. Peroxidase activity is measured by oxidation of o-phenylenediamine, the resulting color being related to the concentration of HPL. Results correlate well with those by radioimmunoassay (r = 0.96). Our between-assay coefficient of variation was 13%; no discernible effect of protein was observed. This 4-h assay can be used to monitor placental function in pregnancy.

Additional Keyphrases: fetal status - trophoblastic tumor - placental function assessment - colorimetry - reference intervals

Measurement of human placental lactogen (HPL) in the serum of the mother is now a well-established test of placental function. Its concentration in serum is related to placental weight (1) and increases during gestation, reaching a peak about the 35th week. An abnormally low concentration of HPL may indicate intrauterine growth retardation (2), fetal danger in preeclamptic toxemia (3), fetal death (4), fetal distress during labor, and asphyxia of the infant at delivery (5). HPL may also be produced by trophoblastic tumors and has been used in investigations of them in patients (6).

To exploit the full usefulness of serum HPL measurements in obstetric medicine, a simple, rapid assay is needed. Previous experience in this laboratory with the enzyme-linked immunoassay of α-fetoprotein (7) and pregnancy-specific β1-glycoprotein (8) led us to apply similar principles to the assay of serum HPL. Here we describe a simple, rapid assay, suitable for estimating HPL in sera from pregnant and nonpregnant women.

Materials and Methods

All reactions were carried out in flexible polyvinyl microtitration plates (M29; Dyratech Laboratories, Sussex, RH14, 8SJ, England).

The absorbance differences were measured with an ABA 100 bichromatic analyzer (Abbott Diagnostic Ltd., Reading, England).

Reagents

**Phosphate-buffered saline.** Dissolve 1.22 g of potassium hydrogen orthophosphate, 0.408 g of potassium dihydrogen orthophosphate, and 8.77 g of sodium chloride in 800 mL of distilled water; adjust the pH to 7.4 with 4 mol/L sodium hydroxide, and dilute to 1 L with distilled water.

**Chicken serum** (Flow Laboratories, Irvine, Ayrshire, Scotland).

**Sodium citrate, 1 mol/L.** Dissolve 294 g of trisodium citrate in 800 mL of distilled water, adjust the pH to 7.4 with dilute HCl, and dilute to 1 L with distilled water.

**Carbonate-bicarbonate buffer.** This buffer is 15 mmol/L sodium carbonate and 35 mmol/L sodium bicarbonate, pH 9.6.

**Wash buffer.** Add 0.1 mL of surfactant (Twee 20) to 1 L of phosphate-buffered saline.

**Antiserum to HPL**, prepared by DAKO-Immunoglobulins Ltd., Copenhagen, was obtained from Mercia Broacades Ltd., Surrey, England. Dilute the antiserum 1200-fold with carbonate-bicarbonate buffer before use.

**Horseradish peroxidase, type VI** (EC 1.11.1.7; Sigma Chemicals, Surrey, England).

**Antibody-enzyme conjugate.** The antibody-enzyme conjugate was prepared by an adaption of the method of Avrameas and Ternynik (9). Dissolve 10 mg of horseradish peroxidase in 0.2 mL of phosphate buffer (0.1 mol/L, pH 6.8), add 5 μL of glutaraldehyde (500 g/L) and allow to stand at room temperature for 18 h. Dialyze the mixture four times against 1 L of distilled water and once against isotonic saline. To the solution containing peroxidase add 0.3 mL of carbonate-bicarbonate buffer and a volume of HPL antiserum containing 10 mg of protein. Store the solution for 24 h at 4 °C, then add 0.1 mL of a 0.2 mol/L solution of lysolecithin and store for another 2 h at 4 °C. Isolate the antibody-enzyme conjugate by passing the solution through a 100 × 2.5 cm column of Sephadex G100 (Pharmacia, Uppala, Sweden). Pool the fractions containing the first protein peak, divide them into 1-mL aliquots, and store at −20 °C. Before use, make a 12-fold dilution in saline/chicken serum (75/25 by volume) and add 0.1 mL of sodium citrate solution.

**Chromogen.** Dissolve 20 mg of o-phenylenediamine (Sigma Chemicals) in 50 mL of a solution of 22.5 g of disodium hydrogen phosphate and 5.6 g of citric acid per liter of distilled water; the pH should be 6.0. Add 10 μL of hydrogen peroxide (300 g/L) immediately before use.

**HPL standard** was obtained from ICN Pharmaceuticals, Cleveland, Ohio 44128. Dissolve 50 mg in 1 L of chicken serum and store aliquots at −20 °C. Before use, dilute aliquots with citrate-treated human plasma to give a calibration curve over the range 0–50.0 mg/L.

**Sulfuric acid, 4 mol/L.** Carefully add 100 mL of concentrated sulfuric acid to 350 mL of distilled water.

Procedure

Pipet 0.1 mL of diluted anti-HPL into each well of a microtitre plate, cover the plate, and leave at room temperature overnight. Decant the plate and wash it three times with wash buffer.

Dilute the HPL standards and the test sera 50-fold with saline containing 20 mmol of sodium citrate per liter. [The sodium citrate is included to minimize possible interference from serum complement (10).] Pipet 0.1 mL of each standard and test, in duplicate, into the wells of the microtitre plate at timed intervals, cover the plate, and leave at room temperature. Aspirate the contents of each well after 30 min and wash the plate three times with wash buffer. Pipet 0.1 mL of diluted...
antibody–enzyme conjugate into each well and leave at room temperature. Aspirate the contents of each well after 30 min and wash the wells three times with wash buffer.

Pipet 0.1 mL of chromogen into each well and leave at room temperature in the dark. After 20 min add 0.1 mL of diluted sulfuric acid. Measure the change in absorbance of the contents of each well with the bichromatic analyzer, using the 500/600 filter. To construct a dose–response curve, plot on three-cycle semi-logarithmic paper the mean change in absorbance of each standard vs concentration.

Results and Discussion

Optimizing Assay Conditions

Effect of temperature on antibody coating of microtitre plate wells. Diluted antibody (0.1 mL) was pipetted into the wells of three microtitre plates, covered and left overnight (18 h), one plate at 4 °C, one at room temperature (about 20 °C), and the third at 37 °C. After the plates were washed with wash buffer, a series of standards was added to each plate and processed according to the assay procedure. The results are illustrated in Figure 1. Temperature had little influence on the coating of the wells of the microtitre plate with antisera. All three conditions gave an adequate dose–response curve; consequently, for convenience, we let the antisera remain in contact with the plates overnight at room temperature.

Effect of diluting the anti-HPL on coating the microtitre plate wells. Antibody (0.1 mL), diluted 300- to 4800-fold with carbonate–bicarbonate buffer, was dispensed into the wells of five microtitre plates, covered, and left overnight at room temperature. After washing, a series of standards was added to each plate and processed according to the assay procedure. The results are illustrated in Figure 2. We chose an antibody dilution of 1200-fold for routine use because lower dilutions of the antisera, although increasing sensitivity, resulted in a high response to zero dose.

Effect of diluting the antibody–enzyme conjugate. Diluted antibody (0.1 mL) was dispensed into the wells of five microtitre plates, covered, and left overnight at room temperature. Replicate series of standards were added to each plate, all of which were incubated at room temperature for 30 min; after the wells were washed three times with wash buffer, three- to 48-fold dilutions of antibody–enzyme conjugate were added to the wells and processed according to the assay procedure. The results are illustrated in Figure 3 (top). A dilution of less than 12-fold of the conjugate, although increasing the sensitivity of the assay, also increased the response to zero dose.

Effect of incubation temperature on final color development. HPL standards were pipetted into the wells of three microtitre plates and processed according to the standard procedure, except that the first plate was incubated at 37 °C, the second at room temperature, and the third at 4 °C. The results are illustrated in Figure 3 (bottom). The room-temperature incubation produced a calibration curve that covered both the concentration and the photometer ranges and was adequately sensitive without affecting the zero-dose response.

Effect of length of time on the final color reaction. HPL standards pipetted into the wells of four microtitre plates were processed according to the standard assay procedure, except that the final color reaction for the first plate was stopped after 10 min, for the second after 20 min, for the third after 30 min, and for the fourth after 45 min. The results are illustrated in Figure 4. Although prolonging the final color development for more than 20 min increased the sensitivity of the assay, it also increased the zero-dose response.

Effect of pH on the calibration curve. Stock HPL standard was diluted with citrate-treated plasma to produce samples
with concentrations of 0–50.0 mg/L. Seven calibration curves were prepared by diluting the standards 50-fold with saline containing citrate phosphate buffer at pH 4.5, 5.0, 6.0, 7.0, 7.4, 8.0, and 9.0. The diluted standards (0.1 mL) were dispensed into the wells of a microtitre plate and processed according to the standard assay procedure. The results are illustrated in Figure 5.

The optimum pH for binding HPL with antiserum was
between pH 7.0 and 8.0. Above and below this pH range the binding was significantly decreased. Within this range no difference was observed between using saline containing citrate phosphate buffer and saline containing citrate as the diluting solution for the standards.

**Effect of protein on non-specific antibody–enzyme binding to the microtitre plate.** After a series of HPL standards was pipetted into the wells of three microtitre plates, one plate was processed according to the standard assay procedure (i.e., protein was incorporated into the antibody–enzyme solution). The second plate was washed with a solution containing 5 mL of chicken serum added to 95 mL of phosphate-buffered saline (the antibody–enzyme was dissolved in only the buffered saline). The third plate was washed with the buffered saline only, and the antibody–enzyme was also dissolved in the buffered saline alone. The results are illustrated in Figure 6. Non-specific binding of antibody–enzyme was minimized by incorporating chicken serum in the antibody–enzyme solution.

**Effect of protein on the calibration curve.** Stock HPL standard was diluted with citrate-treated human plasma to prepare standards with a range of concentrations from 0 to 50.0 mg/L. Five calibration curves were prepared by diluting the standards 50-fold with citrate-treated plasma only, or with citrate-treated plasma in saline (50/50, 25/75, or 5/95 by vol), or with saline containing 20 mmol of citrate per liter only. The diluted standards (0.1 mL) were dispensed into the wells of a microtitre plate and processed according to the standard assay procedure. No discernible protein effect was observed.

**Analytical Variables**

**Analytical recovery of HPL.** For our analytical recovery studies we added a man's serum, supplemented with aliquots of HPL standard, to the serum of a clinically normal pregnant woman. The HPL concentrations were measured and compared with the estimated concentrations. Recovery (Table 1) ranged from 90 to 97%.

In another experiment, serum from a normal pregnant woman was diluted in various proportions with serum from a man (contains no HPL); the HPL concentrations were measured and compared with the estimated concentrations. Recovery ranged from 92 to 112% (Table 2).

**Precision.** We assessed assay precision by using six pools of human sera containing various HPL concentrations. The within-assay precision was estimated by 60 replicate analyses of HPL concentrations on three of the pools. The between-assay precision was estimated by performing duplicate measurements on three other pools in 14 consecutive assays on different days. The results are shown in Table 3.

**Correlation between Enzyme-Linked Immunoassay and Radioimmunoassay for HPL**

We compared results of the enzyme-linked immunoassay with those by the radioimmunoassay method of England et al.
Table 4. Cross Reactivity of Somatotropin with HPL Assay

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<th>Hormone, µg/L</th>
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Table 5. Reference Range

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<th>Weeks of gestation</th>
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Characterization of the Antiserum

We tested prolactin and growth hormone (somatotropin) for cross reactivity with the antiserum. Two standard curves were prepared, one set of standards containing prolactin, 0–1000 µg/L, and the other containing somatotropin, 0–1000 µg/L; both sets were processed according to the standard assay procedure.

Prolactin showed no cross reactivity, all the points on the standard curve giving values of less than 1 µg/L. Somatotropin, however, showed some cross reactivity (Table 4).

Reference Range

Blood from clinically normal pregnant women attending hospital for routine prenatal assessment was taken at gestational ages of 29–40 weeks and assayed for HPL. The results (Table 5) must be regarded as provisional until more women have been sequentially monitored until delivery. Although we have data for only the third trimester, the sensitivity of the technique is sufficient for measurements to be made throughout pregnancy, provided a suitable serum dilution is chosen.

The enzyme-linked immunoassay we present here has several advantages over conventional radioimmunoassay. The reagents are easily prepared and are not subject to radioactive decay. Because reactions are carried out in microtiter plates, there are no tubes to be individually labeled and processed. The intensity of the color produced may be measured in any instrument able to accommodate volumes of 0.20–0.25 mL, and in colorimeters fitted with a digital printout, measurements may be made very rapidly. In our experience, one person can assay 100 duplicate samples and have the results available within one-half a working day.

We thank Dr. W. H. Stimson for his continuing interest and helpful advice. We also thank Mrs. M. Sime for typing the manuscript.

References