Enzyme Immunoassay for Pregnancy-Associated Plasma Protein-A

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This enzyme immunoassay procedure for pregnancy-associated plasma protein type A involves a "sandwich"-type system in microtitration plates. One can detect 0.27 mg of the protein per liter of serum, with a between-batch CV of 10.2%, and the antiserum used does not cross react with any of the other placenta derived proteins. A reference interval for the last trimester of pregnancy is presented. The procedure described is suitable for studying the behavior of this protein during pregnancy.

Additional Keyphrases: reference interval • protein concentrations in second and third trimesters of pregnancy • "sandwich"-type immunoassay

Pregnancy-associated plasma protein type A (PAPP-A), a glycoprotein with a molecular mass of 750 000 Da, is produced by the syncytiotrophoblast of the human placenta (1) and secreted in increasing amounts into the maternal bloodstream as pregnancy progresses. Despite considerable study and physico-chemical characterization (2, 3), its physiological function during pregnancy or within the placenta has not yet been identified. Its behavior during pregnancy differs significantly in two ways from that of the other placental proteins. First, it has a substantially longer half-life—51 h—with little day-to-day variation (4). Second, its continuous increase in the serum until term is in contrast both to other placental proteins and to placental weight, which plateau and decrease in the last few weeks before term. These observations, together with those of Halbert and Lin (5) that the concentration of PAPP-A alters in various complications of pregnancy, suggest that further study of this protein might contribute to our knowledge of placental physiology and pathology.

Methods currently available for the measurement of serum PAPP-A in pregnancy are "rocket" immunoelectrophoresis (6), radioimmunoassay (7, 8), and, more recently, an enzyme immunoassay (9). Here we report a simple and reliable enzyme immunoassay for PAPP-A that requires a minimum of laboratory equipment but is sensitive enough to estimate the protein in the second and third trimesters of pregnancy.

Materials and Methods

Instruments

We used flexible polyvinyl microtitration plates (M 29; Dynatech Laboratories, Sussex, RH14 9SJ, U.K.), and measured absorbances with a digital photometer with a drain cell attachment (MSE Scientific Instruments, Manor Royal, Crawley, Sussex, RH10 2QQ, U.K.).

Reagents

Phosphate-buffered saline (PBS). Dissolve 1.22 g of KH₂PO₄ and 8.77 g of NaCl 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, U.K.).


Wash buffer. Add 0.1 mL of Tween 20 polyoxethylene (20) sorbitan monolaurate (I.C.I. Americas, Inc., Wilmington, DE) to 1 L of the PBS.

Chromogen. Dissolve 0.4 g of o-phenylenediamine in 1 L of a solution of 22.5 g of disodium hydrogen phosphate and 5.6 g of citric acid (pH 6.0). Store the solution in 30-mL aliquots at −20 °C. Immediately before use, add 20 μL of saturated (300 g/L) hydrogen peroxide solution.

Sulfuric acid, 4 mol/L.

Anti-PAPP-A. Prepared by Dako-Immunoglobulins Ltd., Copenhagen, and obtained from Mercia-Brocades Ltd., Surrey, KT14 6RA, U.K.

PAPP-A antibody–enzyme conjugate. The horseradish peroxidase–antibody conjugate was prepared by the method of Avrameas and Ternynck (10) as follows. Dissolve 10 mg of horseradish peroxidase, type VI (EC 1.11.1.7; Sigma Chemicals, Surrey, U.K.) in 0.2 mL of phosphate buffer (0.1 mol/L, pH 6.8); add 5 μL of glutaraldehyde (500 g/L) and let stand at room temperature for 18 h. Dialyze the mixture four times against 1 L of distilled water, then once against isotonic saline. To the solution containing peroxidase add 0.3 mL of carbonate–bicarbonate buffer and a volume of PAPP-A 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 lysine and store for a further 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, Uppsala, Sweden). Pool the fractions containing the first protein peak, divide them into 1-mL aliquots, and store at −20 °C.

PAPP-A standard. Because the availability of pure PAPP-A is limited, a secondary PAPP-A standard can be prepared, as follows. Remove the cord and membranes from a fresh human placenta and divide into 50-g portions. Add 200 mL of acetone, previously chilled to −10 °C, to a portion in a blender and chop fine for 5 min. Filter the suspension through a Büchner funnel. Repeat this process for each 50-g portion and allow the combined filter cake to dry at room temperature for 15 min. Suspend the filter cake in 1 L of 1 mol/L potassium iodide solution and stir overnight at 4 °C. Filter the solution through a Büchner funnel, measure the resulting volume, and add solid ammonium sulfate to a concentration of 2 mol/L (2). Harvest the precipitate by centrifugation, redissolve it in 10 mL of PBS, and dialyze versus four changes of PBS. Apply 2.5 mL of the dialysate to a (100 × 2.5 cm) column of G 200 Sephadex, and elute with PBS, collecting the void volume (70 mL); repeat this procedure until all the dialysate has been chromatographed. Concentrate the combined void volumes to 10 mL. A placenta

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Received June 6, 1984; accepted August 28, 1984.
weighing 550 g yields 8 mg of PAPP-A in the final concentrated volume.

Before adding the placental extract to 100 mL of pooled maternal serum, subject both the placental extract and the pooled serum to two-dimensional (crossed) immunoelectrophoresis. The appearance of a single peak in both the placental extract and the maternal serum indicates monospecificity of the antisera used in the assay toward the PAPP-A in both samples. Add the placental extract to the pooled maternal serum and store in 1-mL aliquots at −20 °C. Dilute this preparation in chicken serum/PBS (20/80 by vol) and compare with the WHO reference material 78/160, which contains 45 mg of PAPP-A per liter. Our preparation contained 138.8 mg of PAPP-A per liter; we prepared a standard curve by diluting various amounts of this in chicken serum/PBS to cover the range from 69.4 to 0.27 mg/L.

Procedure

Pipet 20 μL of PAPP-A antiserum into 22 mL of the carbonate buffer, mix, and pipet 200 μL of this solution into each well of a microtitration plate. Cover the plate, and leave it overnight at room temperature. Before use, decant the plate and wash it three times with wash buffer.

Remove complement from standards and test sera, previously diluted 10-fold with the chicken serum/PBS, by heating to 56 °C for 30 min. After cooling, pipet 200 μL of standards and test sample, in duplicate, into the wells of the microtitration plate at timed intervals. Cover the plate and incubate for 60 min at 37 °C. Aspirate the contents of each well at the appropriate time interval and wash the plate three times with wash buffer. Pipet 200 μL of antibody–enzyme solution (containing 13 mL of PBS, 8 mL of heat-inactivated chicken serum, and 1 mL of anti-PAPP-A–peroxidase conjugate) into each well and incubate for 60 min at 37 °C. Aspirate the contents of each well after the appropriate time interval and wash the plate three times with wash buffer. Pipet 200 μL of chromogen into each well and leave the plate at room temperature in the dark. After 30 min add 50 μL of 4 mol/L sulfuric acid, then measure the absorbance of each well in the photometer with the 492-nm filter. Plot a dose–response curve on three-cycle semilog paper; read the concentration of the test sera from the curve, and multiply the results by the dilution factor.

Results

Assay conditions. Various conditions affecting the assay—incubation times and temperature, dilution of antibody–enzyme conjugate—were investigated and optimized. Figure 1 shows a typical dose–response curve.

Sensitivity. We evaluated the sensitivity of the assay (i.e., the least amount distinguishable from zero with 95% confidence) by assaying 12 replicates of a blank and a range of standards between 0.033 and 2.17 mg/L. The sensitivity was 0.27 mg/L.

Precision. In assessing the precision of the assay (Table 1), we used four pools of pregnancy serum. The within-assay precision was assessed from results for 40 replicate analyses on two of the pools, between-assay precision from duplicate measurements on the other two pools in 20 consecutive assays.

Cross reactivity of the antiserum with other placental proteins. We tested our antiserum for any cross reaction with other placental proteins by preparing standard curves for human placental lactogen between 0.04 and 21.5 mg/L, SP1 between 0.09 and 90 mg/L, and choriongonadotropin between 1.9 and 1000 int. units/L, in chicken serum/PBS

![Fig. 1. PAPP-A standard curve read at 492 nm, covering a concentration range from 69.4 to 0.27 mg/L.](image)

Table 1. Precision Data

<table>
<thead>
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<th>PAPP-A, mg/L</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>CV, %</td>
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<td><strong>Within assay</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Pool 1</td>
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<tr>
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<tr>
<td><strong>Between assay</strong></td>
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<td></td>
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</tr>
<tr>
<td>Pool 3</td>
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<td>0.17</td>
<td>9.4</td>
<td></td>
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<tr>
<td>Pool 4</td>
<td>16.6</td>
<td>1.7</td>
<td>10.2</td>
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(20/80 by vol), and assaying these samples for PAPP-A by the method described. All the points on the resulting standard curves gave results less than the detection limit of the assay. We therefore concluded that the antiserum did not cross react with these placental proteins.

Analytical recovery. To investigate analytical recovery of the method, we first diluted serum from a third-trimester pregnancy with various proportions of chicken serum, and measured the PAPP-A concentration as described. Second, we added increasing amounts of a pool of pregnancy serum to chicken serum and measured the PAPP-A concentration. The results are shown in Table 2.

Reference interval. Blood samples from 70 clinically normal women during the last trimester of pregnancy, when they were attending the hospital for routine antenatal assessment, were assayed for PAPP-A. The results, analyzed by use of nonparametric statistics (11), are illustrated in Figure 2.

Discussion

Although most methods for PAPP-A are RIA procedures, our enzyme immunoassay has some distinct advantages. First, pure PAPP-A is not required, which is extremely difficult to obtain for radio-iodination. Our assay is a "sandwich"-type procedure, and the antiserum is available commercially. Also, our labeled antibody preparation is stable at −20 °C for over a year, in contrast to the short shelf-life of the iodinated PAPP-A antigen. The incubation times of our enzyme immunoassay are much shorter than those for the RIA methods, and no radioisotopes are involved. Although this procedure is less sensitive than RIA procedures, which can detect as little as 2 (8) to 10 μg/L (7), it is nevertheless suitable for use in studies throughout pregnancy, the concentration of PAPP-A at 10 weeks' gestation being 3.12 mg/L (7), well within the sensitivity of our procedure. Thus PAPP-A can be measured even earlier in pregnancy than 10
Table 2. Analytical Recovery of PAPP-A after Dilution or Addition to PAPP-A-Free Serum

<table>
<thead>
<tr>
<th>Serum dilution</th>
<th>PAPP-A, mg/L</th>
<th>% recovered</th>
<th>Added</th>
<th>Recovered</th>
<th>% recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Expected</td>
<td>Measured</td>
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<td></td>
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<tr>
<td>1:2</td>
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<td>23</td>
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<td>88</td>
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<tr>
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<td>9.8</td>
<td>98</td>
<td>10.0</td>
<td>100</td>
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</table>

We thank Dr. P. Sizaret (Lyon, France) for his kind gift of the 78/160 reference material, Dr. R. G. Sutcliffe (Glasgow) for his gift of assayed pooled pregnancy serum, and the National Institute of Biological Standards (Holly Hill, London) for providing placental lactogen and chorionic gonadotropin reference material. We also thank Mrs. A. Russell for help in preparing the manuscript.

References