Point-of-Care Time-resolved Immunofluorometric Assay for Human Pregnancy-associated Plasma Protein A: Use in First-Trimester Screening for Down Syndrome

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Background: Screening for Down syndrome in the first trimester by a combination of fetal nuchal translucency thickness and maternal serum pregnancy-associated plasma protein A (PAPP-A) and free β-human chorionic gonadotrophin has been shown to be effective and efficient. We aimed to develop a fast point-of-care assay that could be placed in one-stop clinics for the measurement of PAPP-A.

Methods: We developed a two-site, one-step assay that uses two monoclonal antibodies (mAbs) to PAPP-A, based on a dry-reagent, all-in-one immunoassay concept with a stable fluorescent lanthanide chelate and time-resolved fluorometry. One antibody (mAb 10E1) was biotinylated, and the other (mAb 234-5) was europium-labeled, both via the ε-amine groups of surface lysine residues. The assay was performed on an AIO immunoanalyzer at 36 °C in single, streptavidin-coated microtitration wells that contained the dry reagents. PAPP-A, either in free or complexed form, was detected by the antibodies used.

Results: The assay procedure required 20 min and used 10 μL of sample. The calibration curve was linear from 5 to 10 000 mIU/L. The detection limit was 0.5 mIU/L. Intra- and interassay imprecision (CV) was ≤4.3% and 8.3%, respectively, for whole blood, plasma, or serum samples. Recovery was 93–96% for serum, 95–108% for heparin-derived whole blood, and 98–103% for heparin-derived plasma. Parallelism was observed in all three matrices. Results correlated [slope = 0.85 (confidence interval, 0.82–0.87); intercept = −33 (confidence interval, −58 to −9); y₀ = 85 mIU/L; r = 0.991; n = 100] with those obtained by a Delfia assay. Heparin did not affect the assay, but EDTA markedly reduced PAPP-A values. PAPP-A was stable at 4 °C for at least 18 days in serum and for 8 days in heparin-derived whole blood or plasma.

Conclusions: The present assay appears suited for use in one-stop clinics for screening for Down syndrome in the first trimester, with results available within 1 h.

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leased by cleavage of the binding protein (4–6). In addition to the syncytiotrophoblasts and the trophoblast-derived septal X cells from placenta (7), PAPP-A can also be secreted by osteoblasts (4), marrow stromal cells (5), fibroblasts (4), granulosa cells (8), and vascular smooth muscle cells (9). PAPP-A secreted from all these sources possesses IGFBP proteinase activity.

In uncomplicated pregnancies, PAPP-A concentrations in maternal serum increase with gestational age until term. As a marker, PAPP-A was once suggested to be a risk marker for threatened abortion (10). However, monitoring circulating concentrations of PAPP-A was then shown to be of no clinical value in predicting the outcome of pregnancies threatened by abortion (11). Later, decreased PAPP-A in the first trimester was found to be associated with fetal Down syndrome (DS), and it is now one of the best biochemical markers in early pregnancy, commonly used to screen for DS during the first trimester (12–16).

DS is the most prevalent autosomal chromosome abnormality in live births and the most common chromosomal abnormality causing mental retardation (17). No method is currently available for its treatment, but avoidance of DS birth through prenatal screening followed by invasive prenatal diagnosis and selective therapeutic termination of the affected pregnancies has become an option for pregnant women. Second-trimester serum screening (between 15 and 20 weeks of pregnancy) for DS by maternal age and measurements of α-fetoprotein, human chorionic gonadotropin, and unconjugated estriol, with or without inhibin A can identify 65–75% of affected pregnancies with a false-positive rate of ~5% (18). First-trimester screening (between 10 and 13 weeks of pregnancy) for DS, based on maternal age, PAPP-A and free β-human chorionic gonadotropin concentrations, and measurement of an ultrasound marker, i.e., nuchal translucency, can detect 85–90% of affected pregnancies with a false-positive rate of ~5% (19–22). In addition to the higher detection rate, screening in the first trimester would allow more time to counsel the patients, and more importantly, pregnancy termination would become much easier to perform, leading to less complications occurring thereafter (23). In consequence, there is now increasing evidence to support performing DS screening in the first trimester.

The future trend for first-trimester screening for DS could combine the measurements of PAPP-A, free β-human chorionic gonadotropin, and nuchal translucency in a near-patient environment. The measurements must be performed quickly with adequate analytical quality to provide rapid results to allow doctors to estimate the risks for a pregnant woman at the time of her clinic visit. Thus, considerable savings both in time and economy and better quality of care can be achieved. A recent prospective study has shown promise in this direction (22).

We have previously reported the development of monoclonal-based single-label or dual-label time-resolved immunofluorometric assays for PAPP-A in serum (24, 25). The analytical accuracy is well validated by the excellent clinical results obtained from the use of the assays (19, 26–28). However, because of the nature of the antibodies used and the reaction conditions, these assays are time-consuming (3–6 h), and some cannot be used for plasma or whole blood samples.

We describe a rapid point-of-care, time-resolved immunofluorometric assay for PAPP-A developed for one-stop clinics to allow screening for DS in the first trimester. The assay is based on a system that embodies a dry-reagent all-in-one immunoassay concept and utilizes a stable fluorescent lanthanide chelate as well as the time-resolved fluorometry (29). The entire procedure is fully automated and is equally applicable to serum, heparin-derived plasma, and heparin-containing whole blood samples.

**Materials and Methods**

**INSTRUMENTATION**

The 1420 multilabel counter (Victor™) for time-resolved surface measurement of solid-phase fluorescence, the 1230 Arcus fluorometer for time-resolved measurement of DELFIA enhanced fluorescence in tube format, the 1234 DELFIA fluorometer for time-resolved measurement of enhanced fluorescence in plate format, and DELFIA plate-wash were obtained from Perkin-Elmer Life Sciences, Wallac Oy. The AIO immunoanalyzer to perform automated all-in-one assays was from Innotrac Diagnostics Oy. The iEMS Incubator/Shaker from Labsystems Oy was used for plate incubation at 36 °C. The fast-performance liquid chromatography system with a Superdex™ 200 HR 10/30 (a prepacked column) was from Pharmacia Biotech.

**REAGENTS**

ITC-TEKES Eu³⁺ fluorescent chelate of 4-[2-(4-isothiocyanatophenyl)ethyl]imino]-2,6-bis[N,N-bis(carboxymethyl)-amino]methyl]pyridine and biotin isothiocyanate (BITC) were obtained from Perkin-Elmer Life Science, Wallac Oy. Assay buffer and wash solution were prepared as described previously (30). Low-fluorescence I2-well Maxisorp microtiteration strips (ultraviolet-quenched) were purchased from NUNC. Streptavidin-coated single wells and strips were obtained from Innotrac Diagnostics Oy. Bovine serum albumin (BSA) was purchased from Inter- gen. NAP-5™ and NAP-10™ columns were from Pharmacia Biotech. Heparin was purchased from CalBiochem. All other chemicals used were of analytical grade.

**MONOCLONAL ANTIBODIES**

Fourteen monoclonal antibodies (mAbs) against PAPP-A were initially included in this study. Antibodies Hyb234-2, Hyb234-3, Hyb234-4, and Hyb234-5 were obtained from Statens Serum Institut; mAbs 1B2, 1F12, 3B6, 1C3, 4D2, 5H9, 8H1, 10E, 10E2, and 16D4 were obtained from HyTest Oy.
CALIBRATORS
Calibrators were made from a 26-week-pregnancy serum diluted in 50 mmol/L Tris-HCl buffer (pH 7.8) containing 150 mmol/L NaCl and 60 g/L BSA (6% BSA-TSA buffer). The calibrators were calibrated against WHO IRP 78/610 for pregnancy-associated proteins (WHO International Laboratory for Biological Standards, Statens Serum Institut).

ANTIBODY BIOTINYLLATION
Biotinylation of the antibodies, using the BITC reagent, has been described previously (25). Briefly, to 1 mL of antibody solution (1 g/L) in 50 mmol/L sodium carbonate buffer (pH 9.6) we added a 100-fold molar excess of BITC dissolved in dimethylformamide. The mixture was left at room temperature for 3 h. After removal of excess free reagents by gel filtration with a NAP-5 and a NAP-10 column, the biotinylated antibody was stored in 50 mmol/L Tris-HCl (pH 7.8) containing 150 mmol/L NaCl, 0.5 g/L NaN₃, and 1 g/L BSA.

LABELING OF ANTIBODIES WITH A FLUORESCENT Eu³⁺ CHELATE
The antibodies were labeled overnight at room temperature in 50 mmol/L sodium carbonate buffer (pH 9.6) containing a 100-fold molar excess of ITC-TEKES Eu³⁺ chelate. The labeled antibody was separated from free chelate and aggregated proteins by gel filtration on a Superdex 200 HR 10/30 column equilibrated and eluted with 50 mmol/L Tris-HCl (pH 7.8) containing 0.15 mol/L NaCl and 0.5 g/L NaN₃. BSA and trehalose were added to the labeled antibody solution to final concentrations of 5 and 125 g/L, respectively. Under these conditions, the labeled mAbs contained 7–10 Eu³⁺ molecules per IgG molecule.

ANTIBODY IMMOLIZATION
Biotinylated antibodies were immobilized on the surface of streptavidin-coated microtitration wells at room temperature. To each well we added 50 μL of biotinylated antibody solution. Wells were incubated for 2 h with continuous slow shaking or overnight without shaking. After a washing step, these antibody-immobilized wells were ready for use.

ANTIBODY SELECTION
Fourteen antibodies against PAPP-A were tested in pairs with each used as a capture or a detection antibody. A one-step sandwich assay format was used together with a 5000 mIU/L PAPP-A calibrator and a blank solution. The procedure were as follows: 10 μL of PAPP-A calibrator or blank solution and 400 ng of Eu³⁺-labeled antibody in 20 μL of assay buffer were added, in triplicate, to wells containing 400 ng of immobilized biotinylated antibody. Subsequent incubation was performed at 36 °C for 15 min with shaking. After that, the wells were washed six times with wash solution and dried with a stream of hot dry air for 5 min; the fluorescence was then measured with a 1420 multilabel counter (Victor).

PREPARATION OF AIO DRY-REAGENT WELLS
The procedure used for preparation of dry-reagent wells for PAPP-A was basically the same as that described previously (31). Briefly, to the microtitration wells containing 400 ng of immobilized biotinylated antibody, we added 40 μL of an insulating solution; the wells were then dried overnight. We then dispensed 1 μL of Eu³⁺-labeled antibody solution on top of the dried insulating layer and immediately dried the wells. Dried wells were either packed on top of each other in plastic reagent “pens” or kept within microwell frames, and were thereafter stored in sealed airtight bags with desiccant until use. Dried calibrator wells were prepared similarly to the sample wells. However, in contrast to the sample wells, dried calibrator wells contained a known concentration of PAPP-A in the insulating layer.

ASSAY PROCEDURE
To each AIO dry-reagent well, we added 10 μL of sample (serum, plasma, or whole blood) and 20 μL of assay solution. Subsequent incubation was performed at 36 °C for 20 min with continuous shaking. After six washes, the wells were dried for 35 s under a stream of hot dry air and immediately brought to a time-resolved fluorometer for fluorescence measurement. The concentrations of unknown samples were obtained by calibrating their fluorescence signals against a calibration curve constructed using the calibrator wells. The whole assay procedure was automatically performed by the AIO immunoanalyzer.

SAMPLE MATERIALS
One hundred first-trimester serum samples were collected from a prenatal screening program for severe malformations, infections, and DS at Statens Seruminstiut. The samples were stored at −20 °C before the measurement. Twelve whole blood samples (5 mL/sample) were obtained from healthy volunteers with heparin as the anticoagulant in the blood collection tubes. Each of these 12 whole blood samples was divided into two aliquots. To each aliquot we added a different concentration of PAPP-A, generating a total of 24 whole blood samples containing different amounts of PAPP-A. Subsequently, 24 corresponding plasma samples were prepared by centrifugation at 3000g for 5 min. These samples were stored at 4 °C before the measurement. The hematocrits (Hcts) of these 12 blood samples were determined in Innomet Diagnostics Oy by centrifugation at 14 926g for 3 min in a desk centrifuge (Biofuge Hemo) from Heraeus Instruments. Hct values for the PAPP-A-enriched samples were derived from those of respective original whole blood samples by volume correction.
EFFECTS OF ANTICOAGULANTS
The effect of EDTA was investigated by adding a known amount of PAPP-A to tubes containing 500 μL of the 6% BSA-TSA buffer and different concentrations of EDTA (1–100 mmol/L). These samples were mixed, and their PAPP-A concentrations were measured with two assays, e.g., the present point-of-care assay and a liquid reagent-based, one-step assay configured with a biotinylated mAb (10E1; 400 ng/well), and a Eu³⁺-labeled mAb (234-2; 200 ng/well). For the latter assay, the incubation was conducted at room temperature for 3 h. After the wells were washed six times, 200 μL of enhancement solution was added to each well. After 5 min of slow shaking, the fluorescence in each well was measured.

The effect of heparin was similarly studied. Briefly, a known amount of PAPP-A was added to tubes containing 500 μL of the 6% BSA-TSA buffer and different concentrations of heparin (1–1000 kIU/L). These samples were well mixed, and their PAPP-A concentrations were measured with two assays, e.g., the present point-of-care assay and a liquid reagent-based, one-step assay configured with a biotinylated mAb (234-4; 400 ng/well) and a Eu³⁺-labeled mAb (234-2; 100 ng/well). For the latter assay, the incubation was conducted at room temperature for 3 h. After the wells were washed six times, 200 μL of enhancement solution was added to each well. After 5 min of slow shaking, the fluorescence in each well was measured.

STABILITY
We studied the stability of PAPP-A in different sample matrices, including 6% BSA-TSA buffer, serum, EDTA-based plasma, EDTA-based whole blood, heparin-based plasma, and heparin-based whole blood. These samples were stored at 4°C over certain periods of time during which the PAPP-A concentrations were measured with the point-of-care assay.

BEST ANTIBODY PAIR
Antibody selection was first based on the results of antibody combination studies. Those antibody pairs that gave lower detection limits and linear dose–response signals were preliminarily chosen. Further selection was based on data on assay kinetics and specificity. Only those antibody combinations that enabled rapid measurement of PAPP-A were selected for dry-reagent assay optimization and final characterization. An antibody pair in which 10E1 served as capture and 234-5 as detection antibody fulfilled these requirements and was therefore selected as the best antibody pair.

KINETICS
In the present point-of-care assay, biotinylated 10E1 immobilized on the well surface was kept in close contact with PAPP-A and Eu³⁺-labeled mAb 234-5 by the use of a small reaction volume, higher incubation temperature, and continuous mechanical shaking. Binding of both the low- and high-concentration buffer-based calibrators reached almost 100% in 20 min. Serum samples had virtually the same kinetics as the buffer-based calibrators, whereas plasma and whole blood samples obtained with heparin as the anticoagulant exhibited slightly slower kinetics (91–92% binding). A similar kinetic reaction was observed at concentrations of 5–10 000 mIU/L.

CALIBRATION CURVE AND PRECISION PROFILE
The calibration curve for buffer-based calibrators was linear at concentrations of 5–10 000 mIU/L; the CVs at these concentrations were <10%, as shown in Fig. 1. No hook effect was observed up to at least 50 000 mIU/L, a fivefold higher concentration than the highest calibrator used, because the signal achieved was still increasing.

METHODOLOGIC EVALUATION
Detection limit. The detection limit, estimated as the concentration of PAPP-A giving a fluorescence signal equivalent to that of the mean of 20 replicates of the zero standard + 3 SD, was 0.5 mIU/L.

Intra- and interassay imprecision. Intra- and interassay imprecision (as CVs) was determined with serum samples, heparin-derived plasma samples, and heparin-derived whole blood samples covering low and high PAPP-A concentrations. As shown in Table 1, both intra- and interassay CVs were ≤8.3% irrespective of the sample matrix.

Recovery and parallelism. Analytical recovery of 1000 mIU/L PAPP-A was 93–95% in two serum samples, 98–103% in two heparin plasma samples, and 95–108% in two heparin whole blood samples with low (400–800 mIU/L) and high (3000–5000 mIU/L) PAPP-A.

Parallelism was examined by diluting three samples in different matrices with the 6% BSA-TSA buffer, and these...
diluted samples were measured again as the new samples. Agreement between the expected values and the measured values was good, as shown in Fig. 2.

**Carryover.** The carryover of the AIO instrument was examined by measuring a serum sample with a high PAPP-A concentration (50 000 mIU/L) and the zero standard in sequence. The carryover was 0.0078% in this case, which is below the allowable limit of 0.01% set for third-generation thyroid-stimulating hormone assays (32).

**Effects of anticoagulants.** Heparin at concentrations of 1–1000 kIU/L had no effect on the point-of-care assay, but it did have an effect on the assay that used biotinylated mAb 234-4 and Eu³⁺/H₁₁₀₀₁-labeled mAb 234-2, producing substantially lower signals and, hence, lower measured concentrations of PAPP-A. Noticeably, this effect was dose dependent: the higher the heparin concentration, the lower the fluorescence signal obtained, as shown in Fig. 3.

In contrast to heparin, EDTA had an effect on the point-of-care assay. Assay signals were markedly decreased in the presence of EDTA, as seen in Fig. 4. This reduction was concentration dependent, with more pronounced reductions being observed in the presence of higher concentrations of EDTA. Furthermore, the signal decreased progressively with increasing time, revealing that the decrease was time dependent. However, EDTA had nearly no effect on the assay configured with biotinylated mAb 10E1 and Eu³⁺/labeled mAb 234-2, as shown in Fig. 4, indicating that EDTA affected only the binding of mAb 234-5 to PAPP-A.

**Stability**

The concentration of PAPP-A in the 6% BSA-TSA buffer did not change during 3 months of storage at 4 °C (data not shown). In addition, the PAPP-A concentration in serum did not change when the sample was stored at 4 °C for 18 days. The concentration of PAPP-A in heparin-based plasma was stable over a period of 8 days at 4 °C, as was also the case for heparin-based whole blood. However, the PAPP-A concentration in EDTA-based plasma decreased with time at 4 °C; this observation also applied to EDTA-based whole blood, as shown in Fig. 5.

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**Table 1. Intra- and interassay imprecision with regard to three sample matrices.**

<table>
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<th>Sample</th>
<th>Intraassay</th>
<th>Interassay</th>
</tr>
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<tbody>
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<td>Mean, mIU/L</td>
<td>CV, %</td>
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<td>10</td>
</tr>
<tr>
<td></td>
<td>3090.6</td>
<td>10</td>
</tr>
<tr>
<td>Plasma</td>
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<td>10</td>
</tr>
<tr>
<td></td>
<td>3270.2</td>
<td>10</td>
</tr>
<tr>
<td>Serum</td>
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<td>10</td>
</tr>
<tr>
<td></td>
<td>5138.8</td>
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</tbody>
</table>

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**Fig. 2.** Linearity of results after serial dilution of three samples with different matrices. Each data point is the mean of two determinations.

**Fig. 3.** Effect of increasing concentrations of heparin on PAPP-A concentrations measured by two immunoassays. After the addition of heparin, the samples were left at room temperature for 3 h and then assayed. After background subtraction, each measured binding signal was compared with the binding signal obtained for a sample that did not contain heparin. In this way, the relative binding signal was derived. Each data point is the mean of two determinations.

**Fig. 4.** Effect of increasing EDTA concentration on PAPP-A, as measured by two immunoassays. After the addition of EDTA, the samples were left at 4 °C, and the relevant PAPP-A measurements were performed at different time intervals. Each data point is the mean of two determinations.
The present assay was applied to 24 whole blood samples as well as the corresponding plasma samples. The results from the whole blood samples correlated very well with those from the plasma samples, as shown in Fig. 6A. However, the slope of the curve was ~0.62, indicating that the mean difference between the plasma samples and the whole blood samples was 0.38-fold of the values obtained from the plasma samples. This difference coincided with the mean Hct of the whole blood samples. As shown in Fig. 6B, the slope of the curve reached 0.993 after the whole blood measurements were corrected by the corresponding Hct values.

**Correlation between whole blood samples and plasma samples**

The test results obtained by the point-of-care assay were compared with those obtained by a Delfia assay configured with biotinylated mAb 234-4 and Eu$^{3+}$-labeled mAb 234-2 as described previously (24) for serum samples (n = 100). The correlation was good (r = 0.991) between the results obtained with two methods. The regression analysis yielded the equation: $y = 0.85x - 33$, as shown in Fig. 7A. As can be seen in the difference plot (Fig. 7B), however, the mean percentage of difference between the two methods was ~25% (24.99% ± 17.65%) and was statistically significant ($P < 0.001$, two-tailed t-test). The bias was not concentration dependent over the entire concentration range.

**Discussion**

Screening for DS in the second trimester of pregnancy by measurement of maternal serum biochemical markers is currently widely performed in many countries. However, the trend to move DS screening from the second to the first trimester is strong. This is not only attributable to the fact that first-trimester screening for DS based on fetal nuchal translucency and maternal serum biochemical markers would provide a higher detection rate than biochemical screening in the second trimester of pregnancy, but also to the fact that the implementation of a combined first-trimester screening program and its related diagnostic tests, such as amniocentesis and chorionic villus sampling, is becoming clinically more widespread. It is now generally believed that screening for DS in the first trimester would offer more advantages than screening in the second trimester (33), but this requires the availability of robust and well-characterized assays for the serologic markers, particularly because small increases in assay variation may considerably reduce performance (34).

Among the most important issues is to design a highly efficient, client-friendly prenatal screening program for DS in the first trimester. In most protocols of published...
first-trimester studies, the biochemical markers and the ultrasound result were examined in different units and the test results were subsequently collected for risk calculation. This procedure is technically very time-consuming. It is also psychologically stressful because a woman must first visit a doctor for pretest counseling and then go to the phlebotomy room where her blood sample is taken and transported to the clinical laboratory for the measurement of biochemical markers. After that, she visits the ultrasound unit where fetal nuchal translucency thickness and crown-rump length are measured. Finally, all the test results are delivered to the doctor for risk assessment. With this procedure, the woman cannot get her risk report until several days or weeks later, when she can schedule posttest counseling with the doctor.

Recently, Spencer et al. (22) proposed a new program in which measurements of biochemical markers and ultrasound markers are quickly performed in a single multidisciplinary clinic. Thus, the risk assessment and related posttest counseling can be completed within 1 h with just a single visit. Women with increased risk are then referred for diagnostic tests for fetal karyotyping. Spencer et al. implemented this program in a fairly large clinical trial involving a total of 4190 singleton pregnancies screened between 10 and 13 weeks of gestation. As a result, they obtained a detection rate of 86% for DS and 95% for all aneuploidies, demonstrating that the new program is feasible and effective. They further showed that integration of ultrasonographic, biochemical, and counseling services into a single clinic allowed more efficient use of total resources. Noticeably, this new procedure was also well accepted by the pregnant women; the percentage of women accepting the screening was high (98%). Furthermore, first-trimester screening is not influenced by mode of conception (35).

The assay developed here is intended for use in such a new screening program. Because it is based on the all-in-one reagent concept, the assay can be automated to yield a precise and sensitive result. Full automation reduces assay imprecision and simplifies the measurement. Consequently, the assay can be conducted by non-laboratory-trained individuals and still give results of adequate analytical quality. Because of the fast assay kinetics, the whole assay procedure is completed in 20 min. Thus, a quantitative test result should be available within 30 min after a blood sample is obtained.

Because >99% of PAPP-A exists as the PAPP-A/proMBP complex in the blood of pregnant women, polyclonal antisera raised against PAPP-A isolated from serum will invariably recognize the proMBP part of the complex as well as the mature eosinophil MBP (2). Unfortunately, serum concentrations of proMBP exceed those of PAPP-A by 4- to 10-fold on a molar basis throughout pregnancy (36). In addition, polyclonal antisera of different preparations have been shown to react with pregnancy-specific glycoprotein 1 and haptoglobin, which are also present in excess of PAPP-A in pregnancy serum (37,38). Therefore, the PAPP-A values measured with the use of polyclonal antibodies may be severely biased unless an extensive adsorption procedure is applied to remove antibody factions reactive with these cross-reactants (7,37). It has been demonstrated that, for DS screening, the performance of polyclonal antibody-based PAPP-A assays in which interfering substances are removed by adsorption methods was similar to that of assays based on the use of mAbs (16,24,37). However,
such treatment is usually time-consuming and may cause considerable damage to the antibodies.

The use of mAbs has made it easier to develop a very specific assay that detects only PAPP-A rather than other blood components. The two mAbs used in the point-of-care assay are reactive only with the PAPP-A subunit of the complex. mAb 234-5, which serves as the detection antibody in the assay, in particular has been extensively characterized (39). No reaction has been observed between this antibody and a large number of human proteins, including proMBP, MBP, hepatoglobin, and pregnancy-specific glycoprotein 1. In addition, mAb 234-5 has been shown to stain the villous trophoblast in immunohistochemical studies of placental tissue. The assay constructed here with these two antibodies is thus very specific for the detection of PAPP-A in either free or complexed form.

PAPP-A is known to bind to heparin (40), and this property has often been used for the extraction and purification of PAPP-A. Although the amino acid sequence of PAPP-A is known, little is known about how and where the heparin molecules are bound to the PAPP-A complex. It was previously reported that heparin-derived plasma samples gave significantly higher PAPP-A values in an electroimmunoassay than did the corresponding serum samples (41, 42). The reason was attributed to a change in the electrophoretic mobility of PAPP-A caused by the binding of the highly charged heparin (41). In the present study, heparin was not found to have any effect on the point-of-care assay. PAPP-A concentrations determined in the presence of various amounts of heparin were the same as those determined in the absence of heparin. However, we did find that the assay configured with biotinylated mAb 234-4 and Eu$^{3+}$-labeled mAb 234-2 was affected by heparin, leading to a substantially decreased measured PAPP-A value compared with that measured in the absence of heparin. Furthermore, this effect on the measurement of PAPP-A is dependent on the dose of heparin. The reason that heparin has no effect on the point-of-care assay is believed to be that the epitopes recognized by mAbs 10E1 and 234-5 are located at sites distant from the area to which heparin molecules bind on the PAPP-A molecule. Thus, binding of heparin does not affect the two antibodies that react with the respective epitopes. Whereas in the case of the assay constructed with biotinylated mAb 234-4 and Eu$^{3+}$-labeled mAb 234-2, the epitopes recognized by either one or both antibodies are situated in or in close proximity to the heparin-binding area on the PAPP-A molecule. The binding of heparin thus masks the related epitopes, inhibiting binding of the antibodies to the PAPP-A molecule. Furthermore, the number of heparin-bound PAPP-A molecules relative to non-heparin-bound PAPP-A molecules is associated with the heparin concentrations in the samples. The reason that this heparin effect is concentration dependent is therefore understandable.

In studies using rocket immunoelectrophoresis (electroimmunoassays) or RIAs, it was reported that EDTA-derived plasma would yield significantly increased PAPP-A values compared with serum (41, 43). Until now there has been no plausible explanation available for the mechanism of this EDTA effect. In the present study, EDTA was found to affect the point-of-care assay, producing markedly reduced measured PAPP-A values. Furthermore, this effect was EDTA dose dependent. However, EDTA was not found to affect the assay configured with biotinylated mAb 10E1 and Eu$^{3+}$-labeled mAb 234-2. Unlike the heparin effect, the mechanism of the EDTA effect is here thought to involve conformational changes in the PAPP-A molecular structure. As a strong chelator, EDTA can tightly chelate zinc ions. It is known that PAPP-A contains an elongated zinc-binding motif at position 482–492 of its 1547-residue sequence (44). The binding of zinc to this motif is considered important for the structural conformation as well as the proteolytic activity of PAPP-A. This idea is supported by the facts that PAPP-A proteolytic activity can be blocked by EDTA (3) and that substitution of Glu at position 483 with Ala can cause a complete loss of proteolytic activity (45). When EDTA chelates zinc ions from PAPP-A, conformational changes in the PAPP-A molecular structure could occur, which in turn could lead to the disappearance or modification of certain epitopes of the molecule. Consequently, some antibodies would be unable to react with these changed epitopes.

In RIAs or electroimmunoassays, reduced amounts of antigen-antibody immunocomplex are formed because some fractions of the antibodies are incapable of reacting with PAPP-A in the presence of EDTA, which leads to overestimated values derived from the calibration wells in which EDTA is not present. In the present study, the assay that uses biotinylated 10E1 and Eu$^{3+}$-labeled 234-2 was insensitive to EDTA, meaning that the epitopes recognized by mAbs 10E1 and 234-2 were not affected by EDTA. The point-of-care assay based on the same capture antibody (biotinylated 10E1) but a different detection antibody (234-5) was sensitive to the EDTA effect, clearly indicating that the epitope recognized by mAb 234-5 is affected by EDTA. In addition, the fact that the EDTA effect was both dose and time dependent suggests that the interaction of EDTA with PAPP-A is rather slow.

Serum PAPP-A concentrations determined by a polyclonal antibody-based ELISA were previously shown to be stable after storage at 4 or 25°C for 3 days (46). In the present study, PAPP-A in 6% BSA-TSA buffer was found to be stable at least for 3 months. Furthermore, heparin-derived plasma and heparin-derived whole blood samples could be left at 4°C for 8 days with no change in the measured PAPP-A values. This was also true for serum samples but not for EDTA-derived plasma and EDTA-derived whole blood samples. In the latter case, PAPP-A values gradually decreased with time. Although this reduction in PAPP-A concentrations could not be reversed by the addition of calcium (data not shown), it
does not necessarily indicate that PAPP-A molecules are broken down by the action of EDTA. On the basis of these findings, together with those observed in the investigation of anticoagulant effects, it is obvious that serum samples, heparin-derived whole blood samples, and heparin-derived plasma samples are the most appropriate sample materials for the present point-of-care assay for PAPP-A.

Because it uses a lanthanide chelate and time-resolved fluorometry, the point-of-care assay has all the characteristics typical of the time-resolved assays, such as a broad working range and high sensitivity. Compared with conventional time-resolved assays, the point-of-care assay can be performed much faster because of the favorable assay environment for kinetics and the right antibodies. In the point-of-care assay, the lanthanide chelate is fluorescent; its fluorescence is measured in situ on solid well surfaces with a surface measurement mode. This measurement mode eliminates the need for an additional liquid-handling step as is used in dissociation-enhanced lanthanide fluoroimmunoassays (Delfia); at the same time, it avoids potential contamination from external lanthanide ions. Furthermore, this fluorescent chelate is also chemically highly stable; it does not interact with blood cells and any other blood constituents. This allows immunologic reactions to be performed even in the presence of blood cells. We have shown that heparin-derived whole blood is the correct sample material for the point-of-care assay because heparin has no effect on the assay. Importantly, the use of whole blood samples will further shorten the time required for obtaining a test result (i.e., decreased turnaround time) because the need for separation of blood cells from plasma or serum can be obviated. In addition, we have shown that there is an excellent correlation between whole blood samples and the relevant plasma samples; we believe that this is also the case for the relationship between whole blood samples and the corresponding serum samples, as has been demonstrated for other analytes (31, 48). It is noteworthy that after a Hct correction, the results obtained from the whole blood samples become fully comparable to the results obtained for the corresponding plasma samples. Thus, there may be no need to establish a new reference interval for whole blood samples because a conventionally used reference interval might also be valid for the Hct-corrected whole blood PAPP-A values.

Despite excellent correlation with the Delfia assay configured with mAbs 234-4 and 234-2, the point-of-care assay gives concentrations ~25% higher than those obtained with the Delfia assay. This fact is of no consequence for first-trimester screening because it is the multiple of the normal median, rather than the absolute concentration, that is used in screening programs to calculate the individual risk of having a DS-affected pregnancy. When unaffected and affected samples are measured with the point-of-care assay, the multiple of the normal median obtained for each sample would be very close to that obtained with the Delfia assay. Furthermore, we have previously shown that differences in PAPP-A concentrations determined by different mAb combinations did not have a significant effect on discrimination between DS and non-DS pregnancies (24).

The point-of-care assay described here, with a detection limit of 0.5 mIU/L, is among the most sensitive assays developed for PAPP-A to date. Its broad working range obviates the need for preassay dilution for first-trimester samples obtained at different gestational weeks. Because it is automated, the assay has rather low interassay CVs. These features, together with other performance characteristics, such as good recovery and parallelism, are very comparable to those of state-of-the-art laboratory immunoassays.

It is our belief that the present assay is particularly suited for use in one-stop clinics for screening for DS in the first trimester. The rapid and accurate test results obtained directly from whole blood samples combined with the results from other markers produce a risk assessment within a 1-h clinic visit. The decision that the pregnant woman with a positive screening result should have a further diagnostic test can be made before the patient has left the clinic. On the other hand, a screening-negative result will provide the woman with immediate relief from anxiety and stress. Thus, more efficient use of resources and better management of DS screening can be achieved. It is now necessary to test the assay in clinical conditions.

It was recently reported that circulating PAPP-A concentrations were significantly increased in patients with acute coronary syndromes (49). At a cutoff of 10 mIU/L, PAPP-A was shown to detect patients with acute coronary syndromes with a sensitivity of 89.2% and a specificity of 81.3%, suggesting that PAPP-A is a valuable marker for the identification of patients with acute coronary syndromes. The present assay completely covers the concentration range of interest and should thus be well suited for this new application.

In conclusion, we have developed a point-of-care time-resolved fluorometric assay for PAPP-A for use in one-stop clinics for first-trimester DS screening. The assay is sensitive and reproducible. The whole procedure is fully automated and is equally applicable to serum and heparin-derived plasma as well as to heparin-derived whole blood samples. An accurate and quantitative test result should be available within 30 min after a blood sample is obtained. This, together with other marker values, makes it possible to produce a risk assessment within 1 h in a one-stop clinic for screening for DS in the first trimester.

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