Simultaneous Assay of α-Fetoprotein and Free β Subunit of Human Chorionic Gonadotropin by Dual-Label Time-Resolved Immunofluorometric Assay

Kim Pettersson,¹,² Henrik Althan,³ Ulf-Håkan Stenman,³ Ursula Turpeinen,³ Mikko Suonpää,¹ Juanita Söderholm,¹ Severin Olesen Larsen,⁴ and Bent Nørgaard-Pedersen⁴

We developed a simple, rapid two-step dual-label assay for the noncompetitive determination of α-fetoprotein (AFP) and β subunit of human chorionic gonadotropin (hCGβ) in serum. Monoclonal antibodies to detect AFP and hCGβ were labeled with europium (Eu) and samarium (Sm), respectively. Highly fluorescent chelates were developed by using the Delfia® enhancement principle. The detection limits for AFP and hCGβ were ~0.02 kIU/L and ~0.2 IU/L, respectively. The within-run precision was <5% over the whole range of AFP (1–500 kIU/L) and hCGβ (1–200 IU/L) concentrations tested. Cross-reaction of intact hCG was <0.03%. The AFP concentrations determined with the dual-label assay correlated well with those obtained by Delfia AFP single-label kit. The concentrations of hCGβ were in good agreement with recently published data. Storing the serum samples for 24 h or 1 week at room temperature increased the hCGβ concentration by 4% and 26%, respectively. At 35°C this dissociation of hCG increased 30–40-fold. Repeated freezing and thawing had no effect on the hCGβ concentration.

Indexing Terms: screening · fetal status · Down syndrome · sample handling

Second-trimester pregnancies at high risk of trisomy 21 (Down syndrome) can be identified by maternal screening. The most frequent analytes used are α-fetoprotein (AFP) (1, 2), AFP in conjunction with human chorionic gonadotropin (hCG) (3–6), and AFP in combination with hCG and unconjugated estriol (7–10). Recently, the use of the β subunit of hCG (hCGβ) has been evaluated as an alternative to total or intact hCG (11–13). The study by Macri et al. (11) suggested that the determination of hCGβ instead of total hCG improves the detection rate of Down syndrome pregnancies. Spencer (12) could not confirm this finding but concluded that measurement of hCGβ provides clinical discrimination at least as well as total hCG. In a subsequent study by Spencer et al. (13), hCGβ was superior to hCG when used in early gestation and in women younger than 30 years of age. Measurement of hCG in second-trimester pregnancies with a noncompetitive immunoassay design requires dilution. This is not needed for hCGβ because its concentration is only ~1% of that of hCG.

Time-resolved detection of fluorescent lanthanide chelates is applicable in multiple-label assays because of the excellent emission wavelength resolution of the method (14, 15). As many as four lanthanides have recently been used for a quadruple-label assay that combined one competitive and three noncompetitive assays in a one-step assay (16). With the commercial Delfia® enhancement principle (17), one can combine europium (Eu) and samarium (Sm) chelates for sensitive dual-label applications. A third lanthanide, terbium (Tb), can also be included by using a simple modification of the Delfia enhancement solution (18).

Here we describe a rapid and sensitive noncompetitive procedure for the simultaneous measurement of AFP and hCGβ using time-resolved detection of Eu and Sm chelates. We obtained reference values of these analytes in sera from pregnancies of 14 through 20 weeks' gestation and compared the results with those of single-label assays of AFP and intact hCG. We also studied the stability of hCGβ in serum.

Materials and Methods

Materials

Immunoreagents and buffers. For the dual-label AFP assay, we used the design and immunoreagents from the Delfia AFP kit (Wallac Oy, Turku, Finland). Monoclonal antibodies (mAbs) of the IgG₁ class were used for capture and detection. In the two-site assay for hCGβ, the capture antibody was an mAb to an epitope present only on the free subunit, whereas for detection we used an mAb recognizing the free subunit as well as intact hCG. Both mAbs were of the IgG₁ class. The assay design is illustrated in Figure 1.

We used Delfia assay buffer, enhancement solution, and wash solution identical to those used in the Delfia AFP and hCG kits from Wallac Oy (for their composition see refs. 17, 19).

Standards, calibration, and cross-reactivity. The AFP standard raw material and the standard diluent were identical to those of the Delfia AFP kit. Highly purified preparations of hCGβ were obtained from Scripps (San Diego, CA) and Boehringer Mannheim (Mannheim, Germany). Recombinant human hCGβ was from Crystal Chem (Chicago, IL). The standards were calibrated against the International Reference Preparation of Chorionic Gonadotropin Beta Subunit, Human, for Im-

¹ Wallac Oy, P.O. Box 10, SF-20101 Turku, Finland. Fax Int. +358-21-678 380.
² Helsinki University Hospital, Department of Obstetrics and Gynecology, Haartmaninkatu 2, SF-00290 Helsinki, Finland.
³ Statens Seruminstitut, Department of Clinical Biochemistry, Arthillerivej 5, DK-2300 Copenhagen S, Denmark.
⁴ Nonstandard abbreviations: AFP, α-fetoprotein; hCG, human chorionic gonadotropin; hCGβ, β subunit of hCG; mAb, monoclonal antibody; and MoM, multiples of the median.
Received February 16, 1993; accepted April 28, 1993.

2084 CLINICAL CHEMISTRY, Vol. 39, No. 10, 1993
The monoclonal antibodies immobilized onto the plastic surface extract the antigens in the serum samples during the first incubation. Eu- and Sm-labeled monoclonal detector antibodies are added in the second incubation to detect AFP and hCGβ, respectively.

Immobilization of mAbs. The capture mAbs were immobilized through physical adsorption onto Maxisorp microtiter strips (Nunc, Roskilde, Denmark). We used 1 μg each of the capture mAbs to AFP and hCGβ in 200 μL of 0.1 mol/L phosphate buffer (pH 5.8) in an overnight incubation at 35°C. After a wash step we added 200 μL of 1 g/L bovine serum albumin in 0.1 mol/L NaH₂PO₄ (pH 4.6) for blocking the coated surface. After overnight incubation at room temperature, the blocking solution was aspirated. The strips were stored at 4°C in sealed plastic bags containing a humidifier.

Conjugation of antibodies with Eu- and Sm-chelates. Basically, the labeling procedure was carried out as described earlier (18). The isothiocyanate derivatives of chelates of Eu and Sm are commercially available from Wallac Oy. Conjugation of Protein A-purified IgG was performed in 100 mmol/L borate buffer (pH 8.6) by overnight incubation at room temperature. The mAb to AFP was labeled to contain 5–8 Eu per molecule of IgG. The labeling yield for the mAb to hCG was 9–12 Sm per IgG molecule.

Assay procedure. We added 25 μL of standards or serum per coated micrtitration strip well, followed by 100 μL of Delfia assay buffer. The plates were incubated with continuous shaking for 60 min at room temperature. After a wash step, we added 100 μL of assay buffer containing 100 ng per well of each of the Eu-labeled mAb to AFP and the Sm-labeled mAb to hCG and incubated the strips for 30 min under continuous shaking at room temperature. After 6 washings and addition of 200 μL of Delfia enhancement solution per well, the strips were shaken for 5 min. We measured Eu and Sm fluorescence at 613 and 643 nm, respectively, for 1 s each with a 1234 Delfia Plate fluorometer (Wallac Oy), using the Eu/Sm dual-label software. The built-in curve-fitting programs of the instrument were used to calculate AFP and hCGβ concentrations of the unknown samples.

The single-label Delfia kits for measuring AFP and hCGβ were from Wallac Oy.

Sample Specimens

We assayed 1059 serum samples from uncomplicated pregnancies obtained from maternal screening programs in Denmark (1985–1991) and Finland (1990–1991). The samples were taken during weeks 14–20 of gestation (see Table 3). The gestational age was estimated as the number of complete weeks from the last menstrual period. Sonographic estimation of gestational age was performed in patients with a positive screening result. After marker determination (AFP and hCG), the serum samples were stored at -20°C. The study was carried out in accordance with Helsinki Declaration II.

Results

Assay Performance

Kinetics and dilutions. Standards covering a range of 1 to 500 kIU/L for AFP and 1 to 200 IU/L for hCGβ, pregnancy serum samples, and serum samples from nonpregnant individuals to which assay standards were added were incubated for various times in the first (15, 30, 60, 90, and 120 min) and second (15, 30, and 60 min) incubations. Both the standards and the serum samples reached plateau values in 60 and 30 min, respectively, for the two incubations (data not shown). Serum samples diluted (1:2, 1:4, 1:8, 1:16, and 1:32) into the protein-buffer standard diluent and assayed in the dual-label assay gave results close to linearity for both AFP and hCGβ (Figure 2).

Detection limits and precision. The standard curves for AFP and hCGβ were linear over the ranges determined (Figure 3). The within-run precisions of the standards were between 1.7% and 3.8% for AFP, and between 1.7% and 4.7% for hCGβ. The detection limits (2 SD) were 0.02 kIU/L (0.025 μg/L) for AFP and ≤0.2 IU/L (≤0.2 μg/L) for hCGβ. The signal for AFP was about 50 times that for equimolar concentrations of hCGβ, mainly because of the greater fluorescence intensity of the Eu chelates.

Within- and between-run variations of assays of serum-based controls are summarized in Table 1. The imprecision at clinically relevant concentrations for both AFP and hCGβ was <5%.

Specificity of the hCGβ assay. Given the presence of free β subunits in highly purified preparations of hCG, we could not use these preparations to estimate the cross-reaction of intact hCG in the hCGβ assay. When we used the 1st International Reference Preparation of
hCG (75/537), we found an apparent cross-reactivity of −4% (mol/mol). This was reduced to 0.3% after purification by gel filtration (data not shown). After complete separation of hCG and hCGβ by hydrophobic interaction chromatography of pregnancy serum (20), we estimated the cross-reaction of intact hCG to be <0.03%.

**AFP single-label assay vs dual-label assay.** We assayed on the same day 107 second-trimester specimens (frozen once) with both the Delfia single-label assay and the present dual-label assay. The two assays showed excellent correlation (Figure 4).

### Table 1. Within- (n = 20) and Between-Run (n = 38) Imprecision of Dual-Label Assay of AFP and hCGβ in Serum

<table>
<thead>
<tr>
<th></th>
<th>AFP, kIU/L</th>
<th>hCGβ, IU/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum pool 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within-run</td>
<td>1.34</td>
<td>0.43</td>
</tr>
<tr>
<td>Between-run</td>
<td>1.31</td>
<td>0.42</td>
</tr>
<tr>
<td>Serum pool 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within-run</td>
<td>43.3</td>
<td>28.2</td>
</tr>
<tr>
<td>Between-run</td>
<td>42.8</td>
<td>28.0</td>
</tr>
<tr>
<td>Serum pool 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within-run</td>
<td>159</td>
<td>0.91</td>
</tr>
<tr>
<td>Between-run</td>
<td>161</td>
<td>0.88</td>
</tr>
</tbody>
</table>

**Stability of Standards and Serum Samples**

The stability of three commercial preparations of hCGβ, including a preparation produced by recombinant techniques, was studied at four different temperatures (−20 °C, 4 °C, 22 °C, and 35 °C) at the concentrations used in the standards. After 4 weeks of storage at room temperature, the measured concentrations were 98–100% of those in samples stored at 4 °C or −20 °C. After 2 (and 4) weeks' storage at 35 °C, the recovery rates (%) were 95.5 (92.5) and 95.5 (93) for the two purified natural preparations and 91 (89) for the recombinant preparation.

The constancy of the free β subunit concentrations in pregnancy serum was also studied. Aliquots of 20 fresh serum samples were stored for various periods either at −20 °C and 4 °C (controls) or at 22 °C and 35 °C. At higher temperatures the concentrations of hCGβ increased (Table 2). After storage at 35 °C for 24 h the concentrations increased by 2.5 times, and the increase continued over a 7-day period almost linearly. At 22 °C the increase was much slower, but a small (mean 3.9%)
yet significant increase ($P < 0.001$) was apparent after 24 h. A comparison of the concentrations obtained after 1 and 7 days of storage indicated a constant rate of increase over time. The concentrations of hCG$\beta$ after storage at 4 °C for 10 weeks increased on the average by 1% over that of the concentrations measured in aliquots stored at $-20$ °C ($P > 0.05$). Repeated ($5 \times$) freezing and thawing of five serum samples did not cause a significant change in the concentration of hCG$\beta$ (96.6% ± 4.6% of the 4 °C controls).

**Assay of Pregnancy Sera**

Figure 5 shows the concentrations (mean ± SD) obtained by the dual-label assay in comparison with the Delfia AFP and the Delfia hCG single-label assays. The corresponding numerical values are given in Table 3. The 10th and 90th percentiles expressed in multiples of median (MoM) were 0.50 and 2.00 for hCG, and 0.48 and 2.08 for hCG$\beta$ for the whole material. The 2 SD ranges expressed as MoM values were 0.32–3.10 for hCG$\beta$, 0.50–2.01 for dual-label AFP, 0.35–2.88 for Delfia hCG, and 0.49–2.05 for Delfia AFP. The SDs for the four markers showed no dependency on gestational age.

The values obtained initially in the maternal screening program by the Delfia AFP showed excellent agreement with the AFP concentrations obtained by the dual-label assay. The difference (log Delfia AFP − log dual-assay AFP) did not depend on gestational age or on the concentration of AFP, and the SD was only 0.0463. The correlation between log MoM Delfia AFP and log MoM dual-assay AFP was $r = 0.950$ (n = 1059).

**Discussion**

We found that two immunofluorometric measurements using chelates of Eu and Sm as reporter molecules can be combined into a rapid and simple assay without sacrificing the performance characteristics required for second-trimester screening of trisomy 21. The two assays are highly sensitive and have very large dynamic ranges. Because of the high specific activity and low background inherent in time-resolved detection of lanthanide chelates, more than adequate sensitivities for both AFP and hCG$\beta$ are achieved with a low sample volume (25 $\mu$L). Consequently, the antibody-adsorbing capacity of a coated microtitration well can easily accommodate two noncompetitive assays with wide dynamic ranges. We saw no difference between the standard curves of the single-label Delfia AFP kit and the dual-label AFP assay. The correlation between the two assays was excellent.

Because of lower fluorescence intensity and shorter decay times, the samarium chelate used to detect hCG$\beta$ gives a signal ~1% of that given by the Eu chelate. Despite this, the detection limit for hCG$\beta$ was ~0.2 IU/L, corresponding to ~0.25 fmol per well (compared
Table 3. DeltaFis AFP, Dual-Assay AFP, DeltaFis hCG, and Dual-Assay hCGB

<table>
<thead>
<tr>
<th>Pregnancy week</th>
<th>n</th>
<th>AFP, IU/L</th>
<th>hCGB</th>
<th>hCGB dual assay, IU/L</th>
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<tr>
<td>14</td>
<td>31</td>
<td>17.9</td>
<td>18.7</td>
<td>37.3</td>
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<tr>
<td>15</td>
<td>311</td>
<td>(11.9-28.9)</td>
<td>(12.3-28.6)</td>
<td>(20.8-67.1)</td>
</tr>
<tr>
<td>16</td>
<td>180</td>
<td>32.6</td>
<td>31.6</td>
<td>39.4</td>
</tr>
<tr>
<td>17</td>
<td>246</td>
<td>(23.2-45.8)</td>
<td>(22.8-44.3)</td>
<td>(11.1-33.7)</td>
</tr>
<tr>
<td>18</td>
<td>169</td>
<td>37.3</td>
<td>36.3</td>
<td>41.4</td>
</tr>
<tr>
<td>19</td>
<td>83</td>
<td>(29.0-59.0)</td>
<td>(28.4-58.3)</td>
<td>(9.7-24.0)</td>
</tr>
<tr>
<td>20</td>
<td>39</td>
<td>41.4</td>
<td>40.6</td>
<td>44.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(32.7-63.8)</td>
<td>(34.8-61.6)</td>
<td>(8.8-26.8)</td>
</tr>
</tbody>
</table>

with ~0.01 fmol per well for AFP). This sensitivity is more than adequate for measuring the hCGB concentrations encountered in normal pregnancies.

The capture mAb of the hCGB assay was highly specific for the free subunit, with barely detectable cross-reactivity with intact hCG purified by hydrophobic interaction chromatography. A difficulty in assessing the true cross-reactivity of intact hCG with mAbs directed against epitopes exposed on the free hCGB subunit is the tendency of hCG to dissociate into its free subunits (20), as is also shown in this study (Table 2). We have also tested a two-site, one-step assay combining the solid-phase mAb for hCGB used in the present assay with another mAb, also directed against an epitope exposed only on the free β subunit. The results obtained with the two methods for serum samples from normal and from trisomy 21 pregnancies gave a very good correlation, yielding almost identical concentrations (data not shown).

A considerable proportion of hCG and hCGB, especially in sera from cancer patients, can be nicked at amino acid positions 47–48 or 44–45 (21, 22). Kardana and Cole have recently shown (23) that mAbs directed against this region either do not measure nicked forms or underestimate them. Lacking a nicked hCGB standard, we have not determined the reactivity of our hCGB assay with nicked hCGB. Comparing the mean ratio of hCGB to intact hCG in this study with the ratios Kardana and Cole reported (23) for three different hCGB-specific mAbs, one of which does not react with the nicked hCGB, suggests that our assay is not influenced by hCGB nicking.

The concentrations of HCGB measured in this study show good agreement with those reported by Spencer (18), who used the commercial kit from CIS, calibrated against the same calibration standard as we used in this study. The measured medians of our study were 3–27% higher than the regressed medians reported by Spencer for pregnancy weeks 16–20. The kit from CIS is based on mAb FBT11 (24), which has been shown to be equally reactive with nicked and intact hCGB (23).

Both the 10th and 90th percentiles and the 2 SD ranges of our study (0.48–2.08 and 0.32–3.10 MoM, respectively) show very good agreement with the values for hCGB reported by Spencer (12), (0.2–2.34 and 0.33–3.00 MoM, respectively). The 2 SD ranges of the DeltaFis hCG (0.35–2.88 MoM) showed a somewhat tighter distribution than for hCGB.

The dual-label assay, for which we used specimens frozen once, was performed in a laboratory separate from the two different laboratories used for the single-label assays. Nonetheless, there was an excellent agreement between the AFP results of the two assays.

Knight and Cole have suggested (25) that artificial increases of hCGB attributable to dissociation of hCG into subunits can occur even after short exposures to room temperature. Our stability studies show that considerable dissociation occurs at 38 °C. At room temperature the dissociation is detectable but quite slow, with a measured increase in hCGB concentration of <4% after exposure to room temperature for 24 h. Serum samples may be exposed to temperatures above the normal room temperature during transportation to the laboratory. However, the stability of purified hCGB itself in standard diluent or a serum matrix is excellent, and repeated freezing and thawing of serum samples is not a problem.

Results from a retrospective study of serum samples from 72 Down syndrome pregnancies by the present dual-label assay have been analyzed and will be presented elsewhere.

Macri et al. (26) recently described a dual-analyte enzyme immunoassay for AFP and hCGB. The present method compares well with the detection limits and dynamic ranges reported for that assay. More important, the direct labeling of the immunoreagents with the lanthanide chelates and lack of overlapping between the Eu and Sm chelates allows a very simple assay protocol that can be easily automated.

In conclusion, this simple and rapid dual-label assay allows the simultaneous immunometric measurement of AFP and hCGB concentrations in serum samples. With the excellent sensitivity provided by the time-resolved detection of Eu and Sm chelates, this assay provides precise and reproducible results over the wide analyte concentration range seen in second-trimester samples from normal pregnancies as well as from pregnancies with Down syndrome or neural tube defects. The increase in the concentration of hCGB caused by dissociation of hCG in serum samples at high temperatures demonstrates the need for proper handling of the samples during transportation.

References


