Time-Resolved Fluoroimmunoassay of Human Choriogonadotropin

Kim Petterson,1 Harri Siltari,1 Ilkka Hemmi,1 Erkki Solin,1 Timo Lövgren,1,4 Veil Hänninen,2 Pirjo Tanner,2 and Ulf-Håkan Stenman3

We describe time-resolved fluoroimmunoassay for human choriogonadotropin involving monoclonal antibodies directed against the β- and α-subunits. The latter antibody was labeled with europium, which was measured by counting for 1 s after the immunoreaction was completed. In the solid-phase sandwich assay, both a one-step and two-step procedure were used; the respective measuring ranges were 0.7–135 and 0.7–350 int. units/L, the latter covering a 500-fold dynamic range. The CV within the assay was between 4 and 8%, depending on the dose. Cross reactivity with lutropin in the one- and two-step procedures was 1.6% and 1.0%, respectively.

Additional Keyphrases: monoclonal antibodies • lutropin • peptide hormones • europium label • "sandwich" assay

Human choriogonadotropin (hCG) of placental origin is the primary marker for pregnancy detection (1–3). Immunological pregnancy tests have long suffered from poor sensitivity, mainly because of the inability of anti-hCG antiserum to distinguish between hCG and lutropin (luteinizing hormone). With the development of antisera directed towards hCG-specific sites on the hormone’s β-subunit, one could reliably detect hCG by RIA as early as eight days after ovulation (4). Modifications of hCG RIA (5) are now finding increasing use in the diagnosis of gynecological emergencies; and the quantitative measurement of hCG in serum is important in monitoring therapy of trophoblastic disease and other hCG-producing tumors (1, 3).

Although RIA methods generally have satisfactory sensitivity, many researchers and routine laboratories have begun to search for alternative markers to radioactively labeled substances. Apart from the radiation hazards of the analyte itself to the personnel who handle radioactive substances, the iodination procedure involves conditions that may alter the antigenic properties of the tracer (5–6).

We have developed a highly sensitive and specific two-site "sandwich" fluoroimmunoassay for quantifying hCG in serum. Purified monoclonal antibodies to the hCG β-subunit, immobilized to polystyrene tubes, are used as the solid phase. For a tracer we used purified monoclonal antibodies to the α-subunit of lutropin, to which we covalently attached europium complexed to an EDTA-derivative. By using time-resolved fluorometry (TR-FIA) (7) to detect the europium label, we minimized background fluorescence and obtained maximum sensitivity. With this procedure we could detect hCG at about 1 int. unit/L in a 1-h assay.

Materials and Methods

Materials

Monoclonal mouse antibodies, anti-hCGβ (lot no. 5008) and anti-LHa (lot no. 3031) purified from ascites fluid, were obtained from Medix Laboratories, Helsinki, Finland. According to the manufacturer, the cross reactions of the anti-hCGβ as determined by conventional competitive RIA were as follows: hCG 100%; hCGβ 118%; hCGα 2.2%; lutropin 0.4%; thyrotropin 0.05%. This antibody has been used in a conventional RIA and gives results comparable with those obtained with conventional β-specific antibodies (8).

Highly purified hCG standards were obtained from Organon, The Netherlands [specific activity 10 900 int. units/mg according to the First International Reference Preparation for immunoassays (IRP)], and from Boehringer, Mannheim, F.R.G. [specific activity 13 500 int. units/mg in terms of the 2nd International Standard for bioassays (IS)]. Human lutropin (specific activity 6700 int. units/mg according to IS) as well as purified α- and β-subunits of hCG were obtained from Boehringer.

Diethylstilbestrol was obtained from Sigma Chemical Co., St. Louis, MO 63178. Eu2O3 was obtained from Fluka AG, Buchs, Switzerland. Other chemicals used were of analytical-purity grade.

The chloride salt of Eu was prepared by dissolving Eu2O3 in hydrochloric acid and evaporating the excess acid. Diazophenyl-EDTA was synthesized by a modification of the method of Sundberg et al. (9) by adding Eu before conjugating it to the protein. 2-Naphthoyltrifluoroacetone was synthesized according to the method of Reid and Calvin (10).

Buffers used in the TR-FIA were: Tris HCl (50 mmol/L, pH 7.7 at 25°C), containing 9 g of NaCl and 0.5 g of NaNO3 per litre (A buffer); A buffer plus bovine serum albumin, 5 g/L (B buffer); B buffer plus 0.5 g of bovine globulin and 1 mL of Tween 20 surfactant per litre (C buffer). Assay tubes were washed with a saline solution containing 9 g of NaCl and 0.5 g of NaNO3 per litre.

Methods

Conjugation of diazophenyl-EDTA-Eu to anti-LHa. Diazophenyl-EDTA-Eu at 20- to 40-fold molar excess was reacted with anti-LHa for 20 h at 0 to 4°C. Passage through a Sephadex G-50 column (1.5 × 20 cm) separated the conjugated protein fraction from excess reagent; each 2-mL fraction was monitored for absorbance at 280 nm and for fluorescence (see below). Incorporation of diazophenyl-EDTA-Eu into the tyrosine and histidine residues of the protein varied from 2.5 to 10 molecules of Eu per molecule of IgG. An incorporation ratio of four or five molecules of Eu per molecule of IgG was found to be optimal and had a negligible effect on affinity. Bovine serum albumin was added to pooled fractions to give a concentration of 5 g/L, and the pooled fractions were stored at 0 to 4°C.
Coating of polystyrene tubes with anti-hCGβ. Anti-hCGβ antibody was immobilized by adsorption to the interior surface of 12 × 50 mm polystyrene tubes (Vapex, Calamandrana, Italy). The tubes were coated with 0.25 mL of purified antibody (10 µg/mL) in 0.25 mL of 0.1 mol/L sodium carbonate buffer, pH 9.5, for 20 h at room temperature. After coating, we added 1 mL of B buffer to each tube and stored them at 0 to +4 °C. Before use, the coated tubes were washed twice with the saline solution.

Time-resolved fluoroimmunoassay. This was done according to two different procedures, a one-step and a two-step procedure.

One-step TR-FIA: To washed, coated tubes we added 100-µL serum samples or hCG-standards in C buffer, followed by 100 µL of C buffer or control serum from males (hCG-free). Then 30–50 ng of Eu-labeled anti-hCGα in 50 µL of C buffer, with 100 and 500 µmol of diethyleneetriaminepentaacetic acid and Ca, respectively, per litre, was added to each tube shortly before use to remove any dissociated free Eu. After gentle vortex-mixing, the tubes were incubated for 1 h at room temperature. We then aspirated the tubes’ contents, and washed the tubes with three 1-mL portions of saline solution. Determinations were done in duplicate if not otherwise stated.

Two-step TR-FIA: To washed, coated tubes we added 100-µL serum samples or hCG standards in C buffer followed by 100 µL of C buffer or male control serum and then an additional 50 µL of C buffer. After gentle vortex-mixing and incubation for 1 h at room temperature, we aspirated the tubes’ contents, and washed the tubes with 1 mL of saline solution. We then added to each tube 30–50 ng of Eu-labeled anti-hCGα in 250 µL of C buffer containing 20 µmol of diethyleneetriaminepentaacetic acid and 100 µmol of Ca per litre.

After vortex-mixing and allowing the tubes to stand for an additional hour at room temperature, we aspirated their contents, then washed the tubes with three 1-mL portions of saline solution.

Measurement of fluorescence. After washing the assay tubes, we measured the Eu bound to the solid phase as a 2-naphthoyltrifluoroacetone chelate by using a single-photon counting time-resolved fluorometer as described by Soini and Kojola (11). (Details of the formation of the 2-naphthoyltrifluoroacetone chelate are to be published elsewhere.) This fluorometer has a xenon 1000-Hz flash lamp. The measuring time was 1 s, the delay time 400 µs, and the counting time 500 µs. The excitation wavelength was 340 nm and the emission wavelength 613 nm. The background fluorescence of 250 cps for noncoated polystyrene tubes was subtracted from all readings.

Results

Sensitivity and Specificity of the Assay

The dose–response curve obtained by TR-FIA is fairly linear, and the fluorescence intensity is nearly directly proportional to the antigen concentration over a large concentration range (Figure 1). The fluorescence intensity of the zero sample (male serum) has not been subtracted in the standard curves shown. If this is done, the standard curves for both assays are linear over 80–90% of the measuring range and the signal is proportional to the dose.

By the one-step procedure, a measuring range of 0.05–10 ng/mL for the Boehringer standard (2nd IS) was obtained. This corresponds to 0.7–135 int. units/L, there being a 200-fold difference between the highest and lowest measurable concentrations. In the two-step procedure, the measuring range was even larger, 0.05–25 ng/mL, or 0.7–350 int. units/L—a 500-fold difference. The minimum detection limit of the one-step procedure was better than 0.7 int. unit/L, but this was the lowest standard dilution used in the assay. If the minimum detection limit is defined as the lowest

![Fig. 1. One-step (A) and two-step TR-FIA (B) results, showing the standard curves with the Boehringer (2nd IS) and the Organon (1st IRP) hCG standards.](image-url)

The cross reactions of anti-hCG antibodies with lutropin (hLH) and free α-hCG subunits are also shown. Each point on the standard curves represent the mean of triplicates; bars indicate 1 SD. Points on the lutropin and α-subunit cross-reaction curves represent the mean of duplicates. The dashed horizontal line denotes the background level plus 3 SD.
The concentration of analyte that produces fluorescence that differs significantly from that produced by the zero sample, then the detection limit is about 0.1 unit/L. This value was defined as the hCG concentration corresponding to the mean fluorescence of 10 aliquots of the zero sample plus 3 SD. For the one-step procedure the background fluorescence was 166 (SD 19) cps, and for the two-step assay it was 428 (SD 58) cps.

A precision profile calculated from 10 replicates of each hCG concentration assayed according to the two-step TR-FIA is shown in Figure 2. When samples are run in duplicate and a counting time of 1 s or 10 s is used, the working range of the assay is from 0.06 ng/mL, or 0.9 int. unit/L, to at least 37 ng/mL, or 500 units/L, respectively, if a CV of 10% is taken as the discrimination limit.

![Precision profile of TR-FIA of hCG](image)

**Fig. 2. Precision profile of TR-FIA of hCG**

Mean of 10 determinations for each hCG concentration (2nd IS, Boehringer) according to the two-step procedure. The CV (in %) for each dose was calculated for counting times of 1 s () and 10 s (). The lowest curve (■) represents the precision profile calculated from duplicate measurements.

The mean background measured for 15 individual serum samples from normal healthy men was 158 (SD 38) cps for the one-step procedure and 470 (SD 65) cps for the two-step procedure. The corresponding sensitivities measured as 3 × SD in these normal samples from men were less than 0.05 ng/mL (2nd IS) for the one-step procedure and 0.06 for the two-step procedure (Table 1). The values obtained with the Organon standard (1st IRP) were about fourfold those obtained with the Boehringer standard (2nd IS) given above, but the shape of the standard curves and the maximum fluorescence observed were practically identical.

The shapes of the standard curves differ for the two assay methods. As expected, the standard curve for the one-step procedure reaches a maximum, after which it decreases with increasing hCG concentration. This reflects the situation in which the tracer increasingly binds to an excess of antigen, which itself cannot bind to the saturated solid-phase antibody. In the two-step assay, the fluorescence intensity reached a plateau that was approximately twice as high as the maximum signal obtained in the one-step procedure. The ratio between the maximum signal and the background was 265 in the one-step assay and 210 in the two-step assay.

The cross reaction of anti-hCGα antibody with lutropin was 1.0% for the two-step assay and 1.6% for the one-step assay. These figures represent the maximum cross reaction at hCG concentrations of 1–10 int. units/L. At higher concentrations the cross reactions were lower because of slight non-parallelism between the respective hCG and lutropin dose–response curves. In the one-step assay the curves were parallel up to a lutropin concentration of 100 ng/mL, but the maximum signal obtained with lutropin was only about 10% of that obtained with hCG. In the two-step assay lutropin gave a less steep dose–response curve than hCG. The cross-reaction figures for the Organon standard were about four times higher. The cross reaction with free α-subunit was lower than with lutropin.

**Table 1. Sensitivities of One-Step and Two-Step TR-FIA of hCG with Two Different Standard Preparations**

<table>
<thead>
<tr>
<th>Standard preparation</th>
<th>Sensitivity, µg/L (and int. units/L)*</th>
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<tbody>
<tr>
<td></td>
<td>Control sera (n = 16)</td>
</tr>
<tr>
<td>Boehringer standard</td>
<td></td>
</tr>
<tr>
<td>One-step method</td>
<td>0.05 (0.7)</td>
</tr>
<tr>
<td>Two-step method</td>
<td>0.05 (0.7)</td>
</tr>
<tr>
<td>Organon standard</td>
<td></td>
</tr>
<tr>
<td>One-step method</td>
<td>0.1 (1.1)</td>
</tr>
<tr>
<td>Two-step method</td>
<td>0.2 (2.2)</td>
</tr>
</tbody>
</table>

*Background plus 3 SD for no. of determinations and specimens indicated.

Influence of Free Subunits

The effect of free subunits on the assay was studied by adding a one- to fourfold excess (on a weight basis) of hCGα

![Influence of free α-subunit on the hCG standard curve](image)

**Fig. 3. Influence of free α-subunit on the hCG standard curve**

Different concentrations of a hCG-standard (Boehringer) without (*) or in the presence of one (○), two (□), and four (●) times as much α-subunits as hCG were assayed by the one-step TR-FIA procedure.
to standards containing known amounts of hCG. In the one-step assay a one- to twofold excess of free α-subunits had a very small effect on the lower part of the standard curve (Figure 3). However, the measurement range for high concentrations was significantly decreased. With a fourfold excess of free α-subunits, the measurement range was severely limited and the response curve was displaced to the right (Figure 3). In the two-step assay, no interference was observed even at a fourfold excess of free α-subunits. This corresponds to a 12-fold molar excess.

The effect of free β-subunits was fairly small. The presence of β-subunits in a concentration similar to that of hCG caused a measurable interference only at the uppermost part of the standard curve. The effect was similar in both assays (Table 2). The effect of higher concentrations of β-subunits was not studied because they are not clinically relevant (12).

Table 2. Effect on the One-Step and Two-Step TR-FIA Assays of Increasing the hCGβ Subunits

<table>
<thead>
<tr>
<th>hCGβ, μg/L</th>
<th>One-step</th>
<th>Two-step</th>
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</thead>
<tbody>
<tr>
<td>—</td>
<td>44.5</td>
<td>67.0</td>
</tr>
<tr>
<td>0.5</td>
<td>45.3</td>
<td>65.0</td>
</tr>
<tr>
<td>1.25</td>
<td>43.9</td>
<td>67.5</td>
</tr>
<tr>
<td>5</td>
<td>44.9</td>
<td>64.4</td>
</tr>
<tr>
<td>25</td>
<td>41.4</td>
<td>57.4</td>
</tr>
<tr>
<td>100</td>
<td>33.5</td>
<td>45.4</td>
</tr>
<tr>
<td>250</td>
<td>—</td>
<td>26.4</td>
</tr>
<tr>
<td>hCG concn., 25 μg/L</td>
<td></td>
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</tbody>
</table>

Correlation between the One-Step and Two-Step Assays

Figure 4 shows the correlation between the two immunoassay procedures. In the one-step assay the samples were usually tested in two different dilutions to ascertain that the concentration of hCG in the sample was within the measuring range. Doing this resulted in very good agreement (r = 0.98). Results were slightly lower with the one-step procedure.

Discussion

Our results demonstrate that very sensitive and rapid immunoassays can be developed with time-resolved fluorescence as the detection method. The combination of speed, sensitivity, and specificity obtained in this hCG TR-FIA is probably unequaled at present. The properties of this fluorimunoassay method are the result of several factors. The newly developed TR-FIA technology (11) allows very sensitive detection of the tracer. By using purified monoclonal antibodies for both coating the solid phase and for the tracer, very high concentrations of antibody can be added to the sample tubes. This means that sufficient antibody–antigen binding is quickly attained. This explains why we obtained a very high sensitivity for a 1- or 2-h assay.

The observed specificity of the present hCG TR-FIA, mainly determined by the specificity of the anti-hCGβ antibody used, is very similar to that obtained by using the same anti-hCGβ antibody in a conventional RIA (8). Although the cross reaction with LH is only 1.0–1.6%, this has to be considered if the maximum sensitivity of the assay is utilized, e.g., even 10 int. units of lutropin per litre would be detected in the one-step assay and therefore it would be an advantage to dilute the samples five- or 10-fold in routine assays. Diluting would also extend the assay range up to 1350 int. units/L while retaining a sensitivity of about 1 int. unit/L in the one-step assay.

Theoretically, an immunoassay of the present type should not measure free subunits, but a clear dose–response was observed with hCGα. This response was less than 1% of that observed with hCG and was possibly caused by the contamination of the hCGα preparation with hCG.

Addition of free hCG subunits in concentrations occurring in serum samples (12–14) had a negligible effect on the results. The two-step method was especially insensitive to subunit excess.

The two hCG standards that we used differed in potency by a factor of four—not surprising, because they had been calibrated against different standards. The 1st IRP has been recommended for use in immunoassay but, in practice, the 2nd IS is much more widely used. Sensitivity and specificity calculations based on the 2nd IS are therefore comparable with those generally used in clinical practice.

A prominent feature of the hCG TR-FIA is the very wide measurement range. This is a result of the sensitive fluorescence detection method and the high concentrations of antibody used. In a two-site sandwich assay of the type used here, the upper part of the measurement range is mainly limited by the amounts of antibodies added to the sample tubes. However, the present assay does not differ in principle from other sandwich assays (15) except for the type of label used. This suggests that the high sensitivity at least partly results from the use of time-resolved fluorescence. Preliminary results obtained by a similar assay, in which radiolabeled instead of Eu-labeled anti-hCGα was used, seem to confirm this conclusion.

The choice between the one-step and two-step assay methods depends on their application. The one-step method is more rapid, but because of the shape of the standard curve it is necessary to assay at least two dilutions of the sample, and therefore the two-step method seems to have practical advantages. This is especially true for hCG, the concentration of which varies over such a wide range. For many other hormones, such as lutropin, the measurement range of the assay would cover the physiological concentration range.

Engvall et al. (16) recently described a very sensitive enzyme-labeled immunosorbent sandwich assay for alphafetoprotein involving two monoclonal antibodies reacting with different sites on the antigen. This assay is basically
similar to the present TR-FIA method, the main difference being the use of an enzyme instead of Eu for labeling the second antibody. Many of the advantages observed in the fluoroimmunoassay described here were also achieved in the enzyme immunoassay: a sensitivity better than 1 µg/L in a 2-h assay and a linear dose response over a 100-fold concentration range. The TR-FIA method has certain advantages when compared with this and other enzyme immunoassays. The dynamic range of the detection method is wider in TR-FIA; in this study the signal/noise ratio was >200. This can probably not be achieved by routine photometric methods. Even when compared with radioisotopic methods, the measurement range obtained by TR-FIA seems to be superior. In TR-FIA very good counting precision is obtained, even with a counting time of only 1 s.

The main advantages of enzyme immunoassays are shared by our TR-FIA method. The reagents are non-hazardous and stable and the use of the assay is not limited by radiation regulations. This is especially important for emergency applications such as early detection of pregnancy (5, 8). These advantages, in combination with better sensitivity and shorter assay time, make time-resolved fluoroimmunoassay a very competitive immunoassay method.

References