Specific enzyme immunoassay for human chorionic gonadotrophin

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Abstract. A sandwich-type enzyme immunoassay system specific for human chorionic gonadotrophin (hCG) was prepared with the antibody Fab′-β-D-galactosidase complex and the antibody F(ab′)2-immobilized silicone rubber solid phase by using a purified antibody to β subunit of hCG (hCGβ).
The assay system cross-reacted less than 4% with human luteinizing hormone (hLH) and human follicular stimulating hormone (fFSH), and proved to be highly sensitive with hCG measurable at levels as low as 0.3 mIU per assay tube.
Using 50 μl of serum sample, 6–600 mIU/ml of hCG in serum could be determined specifically with the same degree of precision as in radioimmunoassay but without sample interference with the assay.
The coefficients of variation within-run and between-run were 8.6–8.9%, and 4.9–10.7%, respectively.
Values obtained with the enzyme immunoassay correlated well with those of radioimmunoassay (r = 0.98, slope = 0.94, y-intercept = 10.2 mIU/ml for 75 serum samples).
Results of the immunoassay of hCG levels in serial samples of serum from healthy women and patients with choriocarcinoma show that this method is useful in the clinical diagnosis of trophoblastic disease.

Human chorionic gonadotrophin (hCG), secreted from the trophoblastic cell, appears in the serum and urine of pregnant women and patients with trophoblastic neoplasia. Levels of hCG are a useful guide to treatment in patients with trophoblastic neoplasia after surgery or during chemotherapy.

For this reason a sensitive radioimmunoassay system for hCG has been developed with antibody to the whole hCG molecule (Tomoda & Hreschhyshyn 1968). However, hCG has two distinct subunits, α and β. Theα subunit of hCG (hCGα) is structurally and immunologically very similar to the β subunit of hLH (Morgan & Canfield 1971; Pierce 1971), and antiserum to the whole hCG molecule also reacts with hLH. The immunoassay for hCG with this antiserum cross-reacts with hLH.

Vaitukaitis et al. (1972) described a specific radioimmunoassay for hCG using antibody to the β subunit of hCG (hCGβ). A similar radioimmunoassay system developed by Tomoda et al. (1977) has been an effective aid to clinical diagnosis in our hospital. However, several disadvantages result from the use of radioactive isotope.

We describe a sensitive and specific enzyme immunoassay system for hCG. This is a non-isotopic method, in which purified antibody to hCGβ is used to increase the sensitivity and to avoid cross-reaction with hLH.

Materials and Methods

Anti-hCGβ antiserum

The antiserum was raised in New Zealand white rabbits by injecting hCGβ obtained from Calbiochem-Behring Corp., La Jolla.

The emulsion of hCGβ (100 μg) in 0.5 ml of physiological saline with equal volume of Freund’s complete adjuvant and dried tubercle bacilli (5 mg) was injected intradermally at 30–50 sites into each animal. Similar booster injection was given on the 30th day. The rabbits were bled one week after the booster injections.
**Purification of the anti-hCGβ**

The antibody was purified with immunoaffinity chromatography of the immunoglobulin G (IgG) fractions from the antisera.

About 100 ml of the antisera was fractionated with 50% saturation of (NH₄)₂SO₄. The precipitated IgG fractions were dissolved with 100 ml of 0.02 M sodium phosphate buffer, pH 7.0 containing 0.1% NaN₃, and dialyzed against the same buffer (2 x 2) at room temperature. After centrifugation at 10000 x g for 10 min, the IgG fractions were applied to a column (1 x 10 cm) of hCG-coupled Sepharose 4B equilibrated with 0.02 M sodium phosphate buffer, pH 7.0 at a flow rate of 4 ml/h at room temperature (18-24°C). The column was washed successively with the above buffer (20 ml) and the same buffer containing 1 M NaCl (40 ml). Then the anti hCGβ antibody, each with one combining site, linked by one disulphide bond were separated with a Sephadex G-150 column as described by Kato et al. (1976). This procedure effectively removed small amounts of IgG in the purified antibody preparation, which was contaminated during affinity chromatography.

**Preparation of antibody F(ab')₂ fragments**

The purified antibody IgG was digested with pepsin (Sigma Chemical Co., St. Louis) and the resulting F(ab')₂ fragments (which comprise a dimer of two identical units, were separated with a Sephadex G-150 column as described by Kato et al. (1976). This procedure effectively removed small amounts of hCG in the purified antibody preparation, which was contaminated during affinity chromatography.

**Anti hCGβ F(ab')₂ immobilized solid phase**

Well washed silicone rubber pieces (3 mm in diameter, from Sanko Plastic Co., Osaka, cut into 4 mm-length) were immersed in a solution of 0.3 ml of 0.1 M sodium phosphate buffer, pH 7.0 containing 0.1% NaN₃ of the antibody F(ab')₂ fragments overnight at 4°C (Kato et al. 1977). The silicone rubber pieces were washed successively with 0.1 M sodium phosphate buffer, pH 7.0 containing 0.1 M NaCl, 1 mM MgCl₂, 0.1% bovine serum albumin, 0.5% gelatin from Difco Laboratories, Detroit, and 0.1% NaN₃. After incubation at 30°C for 3 h with shaking, the reaction medium was discarded by aspiration and each piece was washed twice with 1 ml of buffer A. The piece was then incubated at 4°C overnight with 3 ml of the antibody Fab'-β-D-galactosidase complex in 0.2 ml of buffer A. After reaction, each piece was washed twice with buffer A and transferred to another test tube which contained 0.1 ml of buffer A. The piece was pre-incubated for 5 min at 50°C and then the enzyme reaction was started by adding 50 µl of 3 x 10⁻⁴ M 4-methylumbelliferonyl-β-D-galactoside (Sigma Chemical Co.). After incubation for 20 min with shaking, the reaction was stopped by adding 2.5 ml of 0.1 M glycine-NaOH buffer, pH 10.3.

Fluorescence intensity of 4-methylumbelliferone produced by the enzyme reaction was measured by a spectrofluorometer (MPF-3, Hitachi, Tokyo) at 360 nm for excitation and 450 nm for emission analysis, as described (Kato et al. 1976).

**hCG-coupled Sepharose 4B**

Partially purified hCG preparations (50,000 IU/10 mg, from Mochida Pharmaceutical Co., Tokyo) were coupled with maleimide (Aldrich Chemical Co., Milwaukee) as a bifunctional coupling reagent.

In brief, the F(ab')₂ fragments prepared from the purified antibody IgG were reduced with 15 mM 2-mercaptoethanol, and the resulting mono-valent Fab' fragments, containing sulphhydryl groups, were treated with excess N,N'-o-phenylendimaleimide to introduce maleimide residues. Then the antibody fragments containing maleimide residues were reacted with β-D-galactosidase from Escherichia coli (Boehringer Mannheim, Mannheim), which also contains sulphhydryl groups.

To evaluate the reactivity of the (anti-hCGβ)Fab'-enzyme complex prepared as above, aliquots of the preparation were passed through a small column (3 x 20 mm) of the hCG-coupled Sepharose 4B as described (Kato et al. 1976). Seventy-six per cent of the β-D-galactosidase activity applied was adsorbed in the column, which indicated that about 75% of the enzyme activity in the complex preparation was effectively coupled with the antibody Fab' fragments.

Amounts of the complexes were expressed as units of β-D-galactosidase activity, and one unit of activity is defined as that which hydrolyses 1 µmol of substrate/min under the assay conditions used.

**Immunooassay procedure**

A piece of the silicone rubber immobilized antibody was incubated in duplicate with various amounts of standard hCG or serum sample in 0.5 ml final volume with buffer G (0.01 M sodium phosphate buffer, pH 7.0 containing 0.5 M NaCl, 1 mM MgCl₂, 0.1% bovine serum albumin, 0.5% gelatin from Difco Laboratories, Detroit, and 0.1% NaN₃). After incubation at 30°C for 3 h with shaking, the reaction medium was discarded by aspiration and each piece was washed twice with 1 ml of buffer A. The piece was then incubated at 4°C overnight with 3 ml of the antibody Fab'-β-D-galactosidase complex in 0.2 ml of buffer A. After reaction, each piece was washed twice with buffer A and transferred to another test tube which contained 0.1 ml of buffer A. The piece was pre-incubated for 5 min at 50°C and then the enzyme reaction was started by adding 50 µl of 3 x 10⁻⁴ M 4-methylumbelliferonyl-β-D-galactoside (Sigma Chemical Co.). After incubation for 20 min with shaking, the reaction was stopped by adding 2.5 ml of 0.1 M glycine-NaOH buffer, pH 10.3.

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to 3 g of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala).

**Standard hormones**

The Second International Standard hCG was a gift from the National Institute for Medical Research (Mill Hill, London), and purified hCG (12000 IU/mg) was prepared from the commercial hCG by the method of Van Hell et al. (1968). Purified pituitary hLH (12583 IU/mg) and hFSH (5226 IU/mg) were kindly supplied from the National Pituitary Agency, Baltimore. The hCGα was obtained from Mochida Pharmaceutical Co.

**Serum and urine samples**

The serum and urine samples containing hCG were obtained weekly from 38 patients with trophoblastic neoplasia receiving treatment at Nagoya University Hospital. Daily serum samples were obtained from 2 healthy women with ovulatory menstrual cycles. Serum samples containing high levels of hLH were obtained from 30 post-menopausal women and 18 women who had received LH-releasing hormone (LRH).

**Other methods**

Radioimmunoassay specific for hCG was performed with 'RIA-Quant βhCG Test Kit' (Mallinckrodt, Inc., St. Louis). Radioimmunoassay for hLH was performed with 'LH Kit Daiichi' (Daiichi Radioisotope Lab., Tokyo).

**Results**

A standard curve of the sandwich-type enzyme immunoassay for hCG, referenced by Second International Standard hCG, is shown in Fig. 1A.

Cross-reaction of the assay system with hLH, hFSH, hCGα and hCGβ are shown in Fig. 1B. The hCGβ reaction with the assay was about 140% of hCG on the basis of ng weight. However, hLH, hFSH and hCGα showed low cross-reactions (about 3, 0.1, and 0%, respectively) with the assay.

The effects of serum samples on the immunoassay system were examined by adding various volumes of each sample, and the values of hCG estimated from the standard curve were plotted as a function of sample volume. As shown in Fig. 2, a linear increase in the amount of hCG was observed up to 50 μl with each serum sample. These results
Cross-reaction of the immunoassay with hLH, hFSH, hCGα and hCGβ. Varying amounts (0.01 – 10 ng) of purified hCG, hLH, hFSH, hCGα and hCGβ were incubated with the antibody-immobilized solid phase.

Influence of serum volumes on the immunoassay of hCG. Indicated volumes of each serum sample were subjected to the immunoassay as described in the text.
suggested that the maximal serum volume applicable to the assay system was 50 µl, and indicated that the measurable levels of hCG ranged from 6–600 mIU/ml in serum.

The precision of the assay was tested by assaying three frozen sera 9 times in one day (within-run) or in duplicate in 7 consecutive assays (between-run). As shown in Table 1, the coefficients of variation in the assay were less than 11%.

To confirm the specificity of the present assay, serum samples taken daily from healthy women with ovulatory cycles were measured with the immunoassay. As shown in Fig. 3, the high levels of hLH in serum observed at the ovulatory phase did not cross-react with the immunoassay for hCG under the conditions described. Similarly serum samples containing high levels of hLH (55–290 mIU/ml) taken from post-menopausal women and others did not cross-react with this assay system (data not shown).

To evaluate the accuracy of the present method, we compared the values of 75 serum samples obtained by the enzyme immunoassay with those estimated by the radioimmunoassay specific for hCG. As shown in Fig. 4, there was a good correlation between the enzyme immunoassay and the radioimmunoassay of serum samples with the regression equation $y = 0.94x + 10.2$, and the correlation coefficient $r = 0.98$.

### Table 1.

<table>
<thead>
<tr>
<th></th>
<th>No. of assays</th>
<th>hCG mIU/ml</th>
<th>CV %</th>
</tr>
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<tbody>
<tr>
<td><strong>Within-run</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample 1</td>
<td>9</td>
<td>57.0 ± 5.1*</td>
<td>8.9</td>
</tr>
<tr>
<td>Sample 2</td>
<td>9</td>
<td>158.1 ± 14.1</td>
<td>8.9</td>
</tr>
<tr>
<td>Sample 3</td>
<td>9</td>
<td>406.2 ± 35.0</td>
<td>8.6</td>
</tr>
<tr>
<td><strong>Between-run</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample 1</td>
<td>7</td>
<td>59.6 ± 2.9</td>
<td>4.9</td>
</tr>
<tr>
<td>Sample 2</td>
<td>7</td>
<td>151.6 ± 9.2</td>
<td>6.1</td>
</tr>
<tr>
<td>Sample 3</td>
<td>7</td>
<td>359.6 ± 42.4</td>
<td>10.7</td>
</tr>
</tbody>
</table>

* Mean ± sd.
Fig. 5 shows serial determinations of hCG levels in serum with the present immunoassay, and those in urine with a haemagglutination assay, in patients with choriocarcinoma receiving chemotherapy. A parallel shift of hCG levels in serum and urine was seen in both cases. Case 1 responded well to the chemotherapy, and the hCG levels in serum decreased concomitantly. An apparent increase in hCG levels in serum and urine on the 11th week is probably due to increased hLH levels in serum, which cross-react with the haemagglutination assay, because the antibody to whole hCG molecule was used in this assay system.

Discussion

The enzyme immunoassay for hCG we describe is as sensitive and accurate as the radioimmunoassay, and is able to measure a low level of hCG (6 mIU/ml) in serum with little cross-reaction with hLH and hFSH.

Kikutani et al. (1978) reported a competitive enzyme immunoassay for hCG using the β-D-galactosidase for labelled hCG. Their assay was as sensitive as the present method, but not specific for hCG, since antiserum to the whole hCG molecule was used for the assay. Furthermore, there was interference from serum factors when serum was present in the sample, which limited the application of the method in clinical chemistry.

In the sandwich immunoassay system we describe, interference by serum with the assay was prevented by using gelatin containing buffer for the immunoreaction mixture (Kato et al. 1979). The sensitivity and specificity of the assay were increased by use of a purified antibody to hCGβ. A slight cross-reaction with hLH (about 3% on the basis of IU) observed in the present method was probably due to the fact that the β subunits of hCG and hLH contain a portion of the same sequence of amino acids (Morgan et al. 1975), and the antibody produced by hCGβ may react partially with hLH. Specific immunoreactions at two sites on hCG molecules (with the antibody-solid phase, and with the antibody-enzyme complex) in the sandwich immunoassay might bring a relatively low cross-reaction of the assay with hLH.

It may seem complicated to label the antibody Fab' with β-D-galactosidase, but the antibody-β-D-
galactosidase complex prepared from 10 mg of the purified antibody is sufficient for 16,000 assays, and is stable at least for 6 months at 4°C in buffer A. The antibody-immobilized silicone rubber solid phase is stable for one month at 4°C in buffer A.

More than 100 samples can easily be assayed in a day.

The immunoassay of hCG we present avoids the problems and limitations associated with radioisotopes, is useful in current laboratory medicine, and needs no special equipment.

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References


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