Choriogonadotropin Measured with the Tandem-E Immunoenzymetric Assay System

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We evaluated the Hybritech Tandem-E procedure for quantifying choriogonadotropin (hCG) in human serum. In this "sandwich"-type assay, two monoclonal antibodies directed against different regions of the hCG molecule are used, one coated on a plastic bead, the second conjugated to alkaline phosphatase. The assay can detect as little as 1.0 int. unit of the hormone per liter, shows a linear response up to at least 200 int. units/L, and has good precision. By prolonging the incubations for formation of the sandwich and for substrate hydrolysis, one can achieve higher sensitivity at the expense of a narrower linear range. Correlation with a conventional radioimmunoassay for the β subunit of hCG was generally excellent, but in one instance the Tandem-E gave an apparently false positive result.

Additional Keyphrases: monoclonal antibodies • pregnancy • radioimmunoassay compared • β-subunit

Sensitive, highly specific immunoassays for human chorionic gonadotropin (hCG) have gained an important role in clinical practice, particularly for the early diagnosis of pregnancy and the detection of ectopic pregnancy. Until recently, the only widely used assays were radioimmunoassays, with polyclonal antibodies directed toward the β subunit of hCG. Besides requiring radioisotopes, these assays may involve long incubations, a centrifugation step, and multiple-point standardization; they are particularly cumbersome for urgent ("stat") testing of a single specimen.

More recently, immunoenzymometric testing for serum hCG has been introduced (1–4), and several commercial kits are now available. The "Tandem-E hCG" assay (Hybritech, San Diego, CA 92121) is a solid-phase immunoenzymometric assay (EIA) in which two mouse monoclonal antibodies recognize different regions of the hCG molecule. The first antibody is coated on a plastic bead, and the second (soluble) antibody is conjugated to alkaline phosphatase. Because the two antibodies bind to separate molecular sites, they can both be incubated with the serum sample in a single step. The amount of bound enzyme is then determined colorimetrically, with p-nitrophenyl phosphate as substrate.

We present here a detailed evaluation of the performance of the Tandem-E quantitative assay for serum hCG.

Materials and Methods

Reagents: The Tandem-E hCG kit (3) includes the following reagents: plastic beads coated with mouse monoclonal IgG antibody directed against the α-subunit of hCG; a solution, in a "human and animal protein matrix," of mouse monoclonal IgG, directed against the β-subunit of hCG and conjugated with bovine alkaline phosphatase; p-nitrophenyl phosphate substrate in tablet form; buffer for dissolving the substrate; quench reagent containing EDTA for stopping the phosphatase reaction; wash concentrate; and calibrant/control materials based on human serum.

Lyophilized preparations of lutropin (hLH, luteinizing hormone, 20 000 int. units/g, 2nd Int. Ref. Preparation) and hCG were obtained from Sigma Chemical Co., St. Louis, MO 63178. An assay calibrant for follitropin (hFSH, follicle-stimulating hormone, 100 int. units/L, 2nd IRP) was obtained from Serono Diagnostics, Braintree, MA 02184.

Procedure: For routine application we followed the directions in the package insert. In brief, for each standard and sample to be analyzed, put an antibody-coated bead into a 12 × 75 mm plastic test tube. Add 100 μL of alkaline phosphatase-conjugated antibody solution and 100 μL of standard or sample. Incubate on a rotary shaker for 30 min at room temperature. Wash the beads at least three times by forcefully pipetting 1.5 mL of wash solution into each tube, then decanting the wash solution and blotting. Add 200 μL of substrate solution to each tube and incubate, without shaking, for 30 min at room temperature. Stop the reaction by pipetting 1.5 mL of quench reagent into each tube, and measure the absorbances at 405 nm. Construct a linear standard curve from results for the supplied standards containing 0 and 200 int. units of hCG per liter (calibration is based on the WHO Second International Standard, IS

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1.0. For all measurements we used the Hybritech Photon spectrophotometer, in which the original reaction tubes are used as cuvetts and all necessary calculations are performed. All measurements were made in duplicate.

Measurement of hCG by RIA: As a comparison method for the evaluation of this kit we used a double-antibody, polyethylene glycol RIA procedure ("3III"; Serono Diagnostics), which is sensitive to 5 int. units of hCG per liter (2nd BS). Here also, measurements were done in duplicate.

Results

Sensitivity: We assessed sensitivity by replicate measurements of the zero diluent supplied with the kit. The mean final absorbance in 10 measurements was 0.107 A (SD 0.0018). There was no significant difference when we measured distilled water instead of the serum-based diluent. The standard curve indicated 280 int. units/L per 1,000 A. Therefore the sensitivity, defined as that amount of hCG giving a signal exceeding the mean zero signal by two standard deviations, was 1.0 int. units/L.

We found greater sensitivity when we used longer incubations than were specified (see below).

Precision: We estimated precision by repeated measurements of the serum-based reference materials supplied with the kits. Within-run CVs were respectively 8.1% and 4.6% for the 25 and 300 int. units/L materials (n = 15). Between-run CVs were respectively 10.5% and 7.1% (n = 19, all runs performed with a single lot number of reagent kits).

Dose/response curve: By mixing the 200 int. units/L calibrator and zero diluent supplied with the kit, we prepared solutions containing 0, 40, 80, 120, 160, and 200 int. units of hCG per liter. Final absorbance readings of the Tandem-E assay were respectively 0.110, 0.428, 0.720, 1.030, 1.338, and 1.740. This response is highly linear (r = 0.9987, SE = 0.03).

Two patients' specimens were run with serial twofold dilutions. For the first specimen the results were >400, 213, 122, 60.9, 35.7, 17.7, 9.6, and 5.5 int. units/L, for the second, 218, 104, 49.5, 25.3, and 12.1 int. units/L. In both cases a semilogarithmic plot revealed no significant deviation from linearity.

To examine assay response over a wider range of hCG concentration, we dissolved a lyophilized hCG preparation in zero diluent to give an approximate hCG concentration of 5,000,000 int. units/L, and this material was serially diluted. As shown in Figure 1, a "high-dose hook effect" (5) was observed, with maximum reactivity occurring when the hCG concentration was about 5,000 int. units/L. At 5,000,000 int. units/L, response was equivalent to that of serum containing only 43 int. units/L. In the standard assay procedure, hCG values from 400 to about 500,000 int. units/L would generate sufficient signal to be read as ">400," indicating the need for dilution.

Time course, adequacy of washing, and sensitivity to pipetting errors: The effect of varying the duration of the first incubation is illustrated in Figure 2. Evidently "sandwich" formation does not go to completion within the 30-min incubation that is recommended by the supplier.

The recommended incubation times probably achieve a good compromise between good sensitivity and a fairly wide linear range. However, sensitivity of the assay can be improved by prolonging both incubations. Using a 2-h incubation with sample and antibody conjugate, followed by a 4-h incubation with substrate, the assay response was increased to 1.0 A per 10 int. units of hCG per liter and the sensitivity and within-run precision were decreased to 0.2 int. unit/L.

A possible advantage of the Tandem-E type of assay is that, because antibody conjugate is added in excess, small pipetting errors in dispensing it may not be significant (3). However, we observed that assay response was inversely related to volume of antibody conjugate (Figure 3). Binding of antigen and antibody conjugate do not go to completion during the 30-min incubation recommended by the supplier; this may reflect slowed antigen binding due to dilution by the volume of added antibody conjugate. If antibody conju-
conjugate Tandem-E decreased with probably beads, Suspecting These reading obtained used gate were diluted as much as twofold with zero diluent, but used in the specified 100-μL volume, the reading of the 200 int. units/L calibrant was unaffected.

In initial experience with these kits we occasionally obtained discrepant duplicate measurements, with one tube reading 10 to 20 int. units/L and the other reading <5. These samples invariably both read <5 on repeat testing. Suspecting that this was due to inadequate washing of the beads, we tested aliquots of wash solution and observed that substantial phosphatase activity remained after the second wash step. We therefore believe that four wash steps should probably be used to ensure removal of all unbound phosphatase activity. Use of four wash steps in the procedure, each with vigorous mixing and decanting, has substantially decreased this problem of spurious high readings.

Correlation with radioimmunoassay: We compared the Tandem-E IEMA procedure with a standard RIA procedure for the β subunit of hCG, using 187 serum samples submitted to our clinical laboratory for evaluation of pregnancy. Of the 187 samples, 116 gave results of <5 int. units/L by RIA. Of these, 109 were likewise <5 by IEMA, and the remaining seven gave results of 5 to 10 int. units/L. Thirty-five samples were >100 int. units/L by RIA, and all of these were also >100 by IEMA. For the 36 remaining samples having intermediate concentrations of hCG, Pearson correlation analysis (Figure 4) gave IEMA = [1.51 (SD 0.07) × RIA] + [0.64 (SD 2.31)], r = 0.971, and SE = 9.3. The positive slope at least partly reflects a difference in standardization, because the Tandem-E calibrators and controls read about 30% lower by RIA.

Cross reactivity of LH and hFSH: The molecular structures of hFSH and particularly hLH are very similar to that of hCG. To confirm the hCG specificity of the Tandem-E procedure, we tested solutions of hFSH and hLH. Either hormone at a concentration of 100 int. units/L gave an apparent hCG result of <1 int. unit/L. LH at 1000 int. units/L gave a result of 7.6 int. units/L, and at 10 000 int. units/L gave a result of 83.6 int. units/L. (This should represent an upper limit of the LH cross reactivity, because we did not rule out trace contamination of the LH with hCG.)

Values measured in males: in a preliminary investigation of the "normal range" of serum hCG as measured by this kit, we collected serum specimens from 43 male hospital patients. Because very low values were anticipated, we extend-

![Fig. 3. Dependence of the Tandem-E assay on volume of antibody conjugate used. Results are for the 200 int. units/L calibrant.](image)

![Fig. 4. Relation between IEMA and RIA results.](image)

ed the incubation times as described above, to improve sensitivity. Of the 43 patients, 34 had unmeasurable concentrations (<0.2 int. unit/L), seven showed 0.2 to 2.0, two showed 2.0 to 5.0, and none had higher concentrations. The two patients with the highest measured hCG values were both elderly males without known malignancies.

An apparent false-positive Tandem-E result: After adopting the Tandem-E assay as our routine laboratory method for serum hCG, we measured 32 int. units/L for a patient thought to have a possible ectopic pregnancy. Confirmatory assays on several subsequent serum specimens also gave results ranging from 32 to 55 int. units/L. The patient then underwent laparoscopy and uterine curettage, neither of which disclosed any fetal tissue. The serum specimens, re-assayed by RIA for the β subunit, gave results of <5 int. units/L. Further investigation showed that these specimens exhibited normal, linear behavior in the Tandem-E assay when diluted with the kit's zero diluent; however, when diluted with mouse serum, the positive reaction was totally abolished. Mouse serum did not abolish the reactivity of several other low-positive patient specimens that we tested.

Discussion

We found the Tandem-E immunoenzymometric assay to be an accurate and convenient method for quantification of hCG in serum. Advantages of the procedure are that incubations are brief, no radioisotopes are involved, no special equipment is needed other than a colorimeter, no centrifugations are necessary, and single-point calibration suffices. A potential drawback is that falsely positive results may result from inadequate washing of the beads, but this problem can be minimized by careful washing (as is emphasized in the package insert) and by duplicate testing. Automation of the washing procedure might be a worthwhile improvement. Another possible pitfall is the "high-dose hook effect" (5), which (e.g.) causes hCG to be measured at <400 when the true concentration is over about 500 000 int. units/L. This presumably reflects overload of the beads' binding capacity, causing some of the antibody conjugate to
be taken up by hCG in solution. This would only be likely to
cause confusion in the case of molar pregnancies secreting
enormous amounts of hCG.

A major difference between the Tandem-E and competi-
tive binding assays is that the former measures only intact
hCG molecules, whereas the latter generally measure either
intact hCG or free $\beta$-subunit. In the case of tumor detection
(6–9) this difference can be important, because some tumors
secrete only free $\beta$ subunit. (Other tumors may secrete free
$\alpha$-subunit, and this would be missed by either type of assay.)
In the case of pregnancy detection, the good correlation we
observed between results of a $\beta$-subunit RIA and the Tand-
em-E assay suggests that the difference is not important.

We found that the sensitivity of the Tandem-E procedure
can be enhanced to allow measurement of as little as 0.2 int.
unit of hCG per liter of serum (equivalent to about 30 ng/L).
Whether such highly sensitive measurements of hCG are
clinically useful is at present unknown. In our study of 43
hospitalized male patients, we found that 20% had measur-
able serum hCG according to the modified Tandem-E pro-
cedure, and in two cases the measured level was >2 int.
units/L. Immunological detection of circulating hCG in
normal, non-pregnant individuals has previously been reported
(4, 10–13). In one of these studies it was shown that the
measured substance is similar to hCG, not only immu-
nologically but also chemically and physiologically. With
the Tandem-E kit, however, we have not ruled out the
possibility of low cross reactivities. If such interferences do
not occur, it should be an especially convenient method for
determining very low concentrations of circulating hCG.

Correlation between results by the Tandem-E procedure
and a standard RIA for hCG was excellent. Measured values
were consistently higher by Tandem-E, but this at least
partly reflected the use of different standards. Taking this
difference into account, there were no clinically significant
discrepancies among the 187 patients’ samples used in our
comparative study. However, we subsequently encountered
a patient whose serum consistently contained 32 to 55 int.
units of hCG per liter by Tandem-E assay, but <5 int.
units/L by RIA. The Tandem-E reactivity was abolished
when her serum was pre-incubated with mouse serum,
suggesting that it resulted from a heterophilic antibody.
Further studies on this false-positive reaction will be report-
ed separately. Heterophilic antibodies have been reported
by others to cause falsely positive reactions in RIAs for the $\beta$
subunit of hCG that involve second-antibody precipitation
(14–16). Other factors may also cause false-positive $\beta$
subunit RIA results (3, 17–19). Because of the considerable
inconvenience and unnecessary surgery that can result from
false-positive pregnancy tests, laboratories probably should
have both an immunometric and a competitive-binding
assay available to check results that appear clinically
questionable (3).

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