Measurement of Choriogonadotropin by Chemiluminescence Immunoassay and Immunochemiluminometric Assay: 1. Use of Isoluminol Derivatives

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We have developed immunoassays, monitored by the detection of chemiluminescence, for measuring choriogonadotropin in human urine. These methods involve the use of derivatives of isoluminol and include: (a) a labeled antigen with a second antibody covalently linked to polycarboxylate beads as the solid-phase reagent (i.e., solid-phase chemiluminescence immunoassay); and (b) an excess concentration of a specific antibody passively adsorbed onto the walls of polystyrene tubes and a labeled antibody of different specificity (i.e., two-site immunochemiluminometric assay). After the respective binding reactions, the solutions are aspirated, the antigen-antibody-bound fractions are washed twice with 500 µL of buffer, sodium hydroxide (2 mol/L; 200 µL) is added, and the mixture is incubated for 60 min at 60 °C. After cooling, the label is oxidized with microperoxidase/hydrogen peroxide and the resulting chemiluminescence signal is measured for 10 s. We have evaluated the methods in terms of their sensitivity, precision, and clinical utility, and we compare results with values obtained by radioimmunoassay.

Additional Keyphrases: hormones • urine • radioimmunoassay compared • cutoff value • pregnancy

Human choriogonadotropin (hCG1) is secreted by the trophoblast of the developing embryo and is detectable in the peripheral circulation of the mother shortly after implantation. Renal clearance rates of hCG have been reported, and a discrete immunoreactive degradation product of the β-subunit has been isolated from urine and characterized (1). Early tests for hCG based upon hemagglutination and latex agglutination have been supplemented with radioimmunoassays (RIAs) in which specific antibodies directed against the β-subunit are capable of detecting pregnancy a few days after implantation (2). Recently, several RIA kits have become available commercially and have been evaluated (3). Despite this increasing availability and usefulness of RIA, however, there are still several drawbacks, including: (a) the brief radioactive half-life and radioisolation of the labeled reagent; (b) health hazards associated with the preparation, use, and disposal of radioactive compounds; (c) legislative and emotional biases against the use of radioactivity; (d) the increasing trend towards the development of tests for use outside the laboratory; and (e) the difficulty in establishing RIA procedures in isolated communities. Several proposed alternatives to RIA have been reviewed (4) and various nonisotopic immunoassays for the measurement of hCG in serum, plasma, or urine by use of colorimetry (5, 6) or fluorometry (7) have been described.

As another alternative, chemiluminescent derivatives of isoluminol have been evaluated for their potential as labels in monitoring specific protein-binding reactions (8, 9). We have developed and evaluated several chemiluminescence immunoassays (CIAs) for the measurement of haptens in plasma, urine, or saliva in which the antibodies are labeled with derivatives of isoluminol (10, 11); these methods are as sensitive, precise, and accurate as the corresponding RIAs. In addition, some immunoassays have involved the use of luminol and isoluminol derivatives of proteins (12, 13). Using protein–isoluminol conjugates prepared with a hemisuccinamide derivative of aminobutylyl hexosaminol (ABEI), we have developed a solid-phase CIA and a two-site immunochemiluminometric assay (ICMA) for the measurement of hCG. In these assays we use: (a) a labeled antigen (hCG-ABEI) in a competitive binding assay, and (b) a labeled antibody (rabbit anti-hCG IgG-ABEI) in the immunometric assay. Here, we describe and evaluate the methods, assess their clinical utility for the early detection of pregnancy, and discuss the advantages and limitations of each.

Materials and Methods

Materials

Reagents. A hemisuccinamide derivative of aminobutylyl hexosaminol (ABEI-H; 6-n-(4-aminobutyl)-N-ethylamino-2,3-dihydrophthalazine-1,4-dione) was kindly donated by Dr. T. Lovgren, Wallace Oy, Turku, Finland. The hCG (Canfield CR123) was obtained from Dr. G. Bialy, National Institutes of Health, Bethesda, MD. Rabbit antibodies to the β-subunit of hCG were donated by Amersham International plc, Amersham, Bucks., U.K., and RIA kits for measuring hCG (Amerlex kit; IM 2091) were purchased from the same source. An IgG fraction of the antibody was prepared by affinity chromatography on Protein A–Sepharose CL-4B as already reported (10). Microperoxidase (MP-11), bovine serum albumin (Cohn Fraction V), and Sepharose–Protein A were from Sigma London Chemical Co. Ltd., Poole, Dorset, U.K. Sheep anti-rabbit IgG covalently linked to polycarboxylate beads ("Immuno beads") was from Bio-Rad Laboratories Ltd., Watford, Herts., U.K., and was reconstituted by the addition of 50 mL of phosphate buffer (pH 7.5). All other reagents were obtained from Hopkin and Williams Ltd., Romford, Essex, U.K.

Buffers. We used two assay buffers. The first was a coating buffer (barbital, 70 mmol/L, pH 9.6), prepared by dissolving 14.4 g of sodium barbital in 1 L of doubly distilled water containing 1 g of sodium azide. The other was a phosphate buffer (100 mmol/L, pH 7.5), prepared by dissolving 3.06 g of Na2HPO4 · 2H2O and 11.6 g of Na2HPO4 in 1 L of doubly distilled water containing 5 g of bovine serum albumin, 9 g of NaCl, 1 g of sodium azide, and 1.66 g of EDTA. Microperoxidase was dissolved in phosphate buffer and the stock...
solution (1 mg/mL), stored at 4 °C, was stable for at least three months. The working solution of microperoxidase was prepared by diluting the stock solution 50-fold. The oxidant solution was prepared by adding 100 μL of 300 g/L hydrogen peroxide solution to 10 mL of doubly distilled water.

Preparation of chemiluminescent reagents. We prepared the hCG-ABEI conjugate in two steps. Initially, an N-hydroxysuccinimide ester of ABEI-H was prepared in dry dimethylformamide in the presence of carbodiimide and N-hydroxysuccinimide (Figure 1). Then we added an aliquot (6 μL; 300 nmol) of the activated ester (in dimethylformamide) to a solution of hCG (Canfield 119: 0.6 mg, 15 nmol) dissolved in 1 mL of phosphate buffer (50 mmol/L, pH 8). The reaction mixture was stirred and dialyzed overnight at 4 °C against the pH 8 phosphate buffer. The resulting conjugate was purified by ion-exchange chromatography on diethylaminomethyl-Sephadex prepared in the pH 8 phosphate buffer, and eluted with a gradient of NaCl (from 50 to 500 mmol/L). We checked the protein content of all fractions eluted from the column by using a spectrophotometer and by measuring chemiluminescence activity. Fractions containing the labeled hormone were assessed for immunoreactivity in a double-antibody RIA with 125I-labeled hCG as the competing label. Incorporation of 3 mol of ABEI per mole of hCG did not significantly affect immunoreactivity, whereas 6 mol of ABEI per mole of hCG increased chemiluminescence but decreased immunoreactivity. Consequently, we used the preparation containing 3 mol of label per mole of hCG.

To prepare the labeled antibodies to hCG, we added 10 μL (100 nmol) of the activated ester (in dimethylformamide) to a solution of rabbit polyclonal nonspecific anti-hCG IgG (2 nmol in 1 mL of the pH 8 phosphate buffer). The reaction mixture was stirred and dialyzed against the pH 8 phosphate buffer overnight at 4 °C. We purified the labeled antibody by chromatography on Sephadex G-25 prepared in the pH 8 phosphate buffer. The label was eluted in the void volume (3 mL). We added sodium azide (1 g/L) and bovine serum albumin (10 g/L) to the fractions containing the labeled antibody. The average incorporation of the label was 6 mol per mole of protein.

Antibody-coated tubes. Rabbit anti-β hCG IgG, in excess, was suitably diluted in the coating buffer and 200 μL of this was added to each polystyrene assay tube (PT 0944; Luckham Ltd., Victoria Gardens, Burgess Hill, Sussex, U.K.). After an overnight incubation at 4 °C, the coating buffer was aspirated and discarded and 400 μL of pH 7.5 phosphate buffer was added to each tube. After incubation for 30 min at 22 °C the tubes were stored at 4 °C until required.

Sample Collection

Ten healthy female volunteers with regular menstrual cycles (25 to 33 days) were recruited from the infertility clinic at King’s College Hospital. Each woman was requested to record day 1 of her cycle and her daily basal body temperature (to determine the nadir and shift), to collect an early morning sample of urine for each day throughout the cycle, and to undergo pelvic ultrasonography at appropriate intervals to determine the time of follicular rupture. The

![Fig. 1. Proposed structure (tentative) of an activated ester of ABEI-H](image)

The samples were stored at −20 °C until pregnancy was confirmed. For the clinical evaluation of these methods, we measured hCG in samples from each cycle, from the day of the luteinizing peak until a routine pregnancy test gave a positive result (arbitrarily set at > 50 int. units of hCG per liter) on three consecutive days.

Procedures

CIA. Add 100 μL of sample or standard (range, 8 to 328 int. units/L of urine from males) in duplicate to the assay tubes, then add 100 μL of 2000-fold diluted anti-β-hCG antibody, 100 μL of hCG-ABEI (10 ng, producing chemiluminescence of about 100 mV/100 μL of phosphate buffer), and 200 μL of ImmunoBead reagent. After incubating the mixture at 22 °C for 2 h, add 1 mL of phosphate buffer to each tube and vortex-mix. Centrifuge for 10 min at 2000 × g and remove the supernates by aspiration. Repeat the washing step, discarding the supernates, then add 200 μL of 2 mol/L sodium hydroxide to each tube and incubate at 60 °C for 60 min. Cool the tubes to room temperature and add 100 μL of the microperoxidase solution to each assay tube, then place it in the luminometer. Initiate the chemiluminescence reaction by rapidly injecting 100 μL of diluted hydrogen peroxide with an automatic dispenser (we used a "Microspenser" from Hook and Tucker Instruments Ltd., New Addington, Surrey) and measure the light emitted (we used an LKB Luminometer Model 1250 and display, kindly provided by LKB Instruments Ltd., South Croydon, Surrey, U.K.) for 10 s, sufficient to measure 65% of total light emission. Calculate results by comparison with a calibration curve.

ICMA. Add 100 μL of sample or standard (range 0 to 320 int. units/L of urine from males) in duplicate to the antibody-coated tubes, then add 100 μL of phosphate buffer, incubate at 22 °C overnight, and aspirate the liquid. Add 200 μL of suitably diluted (excess) nonspecific anti-hCG IgG-ABEI conjugate and incubate the mixture at 37 °C for 2 h. Again, aspirate the liquid, add 500 μL of phosphate buffer to each tube, aspirate the liquid, and discard. After repeating this washing step twice more, add 200 μL of 2 mol/L sodium hydroxide to each tube and incubate at 60 °C for 60 min. Cool to room temperature, then add 100 μL of the microperoxidase solution to the assay tube and place it in the luminometer. Initiate the chemiluminescence reaction by rapidly injecting 100 μL of diluted hydrogen peroxide, measure the signal for 10 s as described previously, and calculate the results.

Results

CIA

Figure 2 shows a typical calibration curve (mean ± SD; six replicates) for hCG as determined by CIA. The minimum concentration of hCG that could be significantly distinguished from zero (mean ± 2 SD), calculated from three calibration curves, was 2 ± 0.1 int. units/L. Within-batch precision was estimated by the replicate analysis (n = 20) of a sample of urine with an hCG at about the arbitrary cutoff value for the pregnancy test (50 int. units/L). The mean was 52.3 (SD 6.2) int. units/L, the CV 11.9%. The between-batch CV for four assays of the same quality-control sample was 14.1%. The concentrations of hCG in selected samples of early-morning urine collected throughout the 10 cycles, determined by CIA (γ) and by an RIA with an iodinated tracer (χ), correlated well: r = 0.98, n = 60; y = 1.14x − 6.78.

ICMA

Figure 3 shows calibration curves prepared with the use of various incubation times (range 2 h to seven days). The
minimum concentration of hCG that could be significantly distinguished from zero (mean – 2 SD) ranged from 1.8 ± 0.4 int. units/L (2-h incubation) to 0.25 ± 0.05 int. units/L (seven-day incubation). Within-batch precision for hCG in urine (overnight incubation) was estimated as for the CIA. The results were: mean 51.9 (SD 12.9) int. units/L and CV 18.1%. The between-batch CV (four assays) of the same quality-control sample was 18.7%. The concentration of hCG, determined in selected samples of urine collected throughout the 10 cycles by icMA (y) and RIA (x), also correlated well: \( r = 0.97, n = 64; y = 0.978x + 0.63 \).

Clinical Comparison Studies

We used 50 int. units/L as the cutoff value of hCG for diagnosis of pregnancy by RIA, CIA, and icMA, expressing the results for the measurement of hCG as positive or negative according to whether they were above or below this. Contingency tables established to determine the clinical utility of the alternative immunoassays are summarized in Table 1.

Discussion

Recently, CIAs have been developed for the measurement of various hapten, including plasma steroids and their urinary metabolites (10), serum thyroxin (14), thromboxane \( \mathbf{B}_2 \) (15), and salivary progesterone (16). In addition, several attempts have been made to develop CIAs with use of proteins (antigens or antibodies) labeled with derivatives of luminol and isoluminol (12, 13). However, luminol associated with peptides or proteins has a significantly lower quantum yield. More recent studies (17, 18) show that protein–isoluminol conjugates prepared with ABEI–H have acceptable quantum yields upon oxidation (17, 18). Using this derivative, we have prepared hCG-ABEI and a nonspecific anti-hCG IgG-ABEI for use in the development of the CIA and icMA. The stability of each labeled protein is satisfactory, no significant reduction in activity being discerned over 12 months (18). We have substituted the labeled hCG in various RIA kits in place of the iodinated derivative without any significant alteration in sensitivity, specificity, or antibody dilution. Moreover, the labeled antigen procedure combines an acceptable sensitivity with good economy of antibody reagent.

In 1968, Miles and Hales (19) introduced an important new type of immunoassay, immunoradiometric assay (IRMA) based on the use of isotopically labeled antibodies (19). Since then, two-site IRMAs have been developed for compounds with more than one antigenic determinant (epitope) (20). Nonisotopic immunometric assays include immunenzymometric assay (21), immunofluorometric assay (22), and immunochemiluminometric assay (23, 24). The advantages of immunometric procedures include greater sensitivity, broader working range of analyte, improved precision, and shorter incubation times because high concentrations of reagent can be used. The icMA we describe here has the first two characteristics but assay sensitivity is dependent on incubation time, perhaps because of the low concentration of specific antibody bound to the solid-phase. This aspect might be improved by the introduction of well-matched, highly specific monoclonal antibodies.

We have found that the chemiluminescence techniques developed for the measurement of hCG are at least as sensitive and specific as conventional RIA. Both of our assays still need improvement, however. Their relatively poor precision may be due in part to the fact that end-point measurement is preceded by an incubation at high temperature and alkaline pH. Perhaps the discovery of alternative

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<th>Table 1. Clinical Utility of CIA, icMA, and RIA for Pregnancy Tests on Daily Samples of Early-Morning Urine from 10 Cycles</th>
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* Samples of early-morning urine collected on the following day gave a positive result (hCG > 50 int. units/L) with each test.
derivatives of isoluminol with which to label proteins will increase quantum yields of chemiluminescence on oxidation without prolonged incubation under these conditions. At the present, however, for high sensitivity, the addition of sodium hydroxide before oxidation is obligatory to raise the pH, dissociate the antibody-bound complex, and cause the physicochemical nature of the label to change. The conditions for each assay must be optimized in terms of temperature and time, and the concentration of sodium hydroxide. Alternative techniques to enhance sensitivity may be preferable. At present we add the last reagent (i.e., hydrogen peroxide) by rapid injection into the assay tube, which is situated in front of the photo-detector, because the rate of the chemiluminescent reaction is too fast for the reagent to be added outside of the instrument. Alternative oxidation systems that lead to constant, stable light emissions (cf. bioluminescent reactions) might be preferable.

In 1981, Simpson et al. (13) reported the use of acridinium esters as labels in immunoassays. The use of these compounds may increase quantum efficiency, avoid serious quenching when labels are associated with proteins or haphtens, and allow simpler oxidation systems involving alkaline peroxide alone. In the reports of assays with protein–acridinium conjugates (24, 25), the most highly active conjugates of protein and acridinium ester have yielded greater specific activities than those obtained from the equivalent 125I-labeled proteins, and they are stable for at least 11 months when stored at −20 °C. Currently we have developed and are evaluating a CIA and an ICMA for measurement of hCG with protein–acridinium derivatives (26).

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References