Convenient Radioimmunoassay for Urinary Human Choriogonadotropin without Interference by Urinary Human Lutropin

Robert E. Wehmann,1 S. Mitchell Harman,1 Steven Birken,2 Robert E. Canfield,2 and Bruce C. Nisula3

We have devised a radioimmunoassay (RIA) for human choriogonadotropin (hCG) in first morning-voided urine specimens. Concanavalin A, a lectin, is used to extract and concentrate the hCG from urine. A high-affinity antiserum is used, directed to the hCGβ-carboxy-terminal peptide, a unique immunological determinant not shared by the beta subunit of human lutropin. This ensures that urinary human lutropin-related molecules, which interfere with RIAs involving antiserum to the intact hCGβ subunit, will not cross react in this assay. A concentration of hCG as low as 0.4 μg/L can be detected in the first morning-voided urine. The effective sensitivity of this assay for the unequivocal detection of hCG production is somewhat better than that achieved with the serum hCG RIA involving antiserum to the hCGβ subunit. The improved specificity and sensitivity of this assay, and the greater convenience of collecting samples of urine rather than blood, are clinically useful advantages of this approach to assessing hCG production in humans.

Additional Keyphrases: hormones • antiserum directed to the carboxy-terminal peptide of the beta-subunit • cancer • trophoblastic disease

Human choriogonadotropin (hCG)4 is widely measured to assess hCG production in normal pregnancy, trophoblastic disease, and patients with cancer (1), and several assay systems are currently available (2–5). A widely used hCG radioimmunoassay (RIA) involves use of an antiserum generated against the hCGβ subunit (3). Measurements of hCG concentrations in unextracted urine by this and other previously available RIA, radioimmunoassay, or bioassay methods are reliable in those phases of pregnancy or gestational trophoblastic disease when hCG concentrations are so high that the urine can be diluted, which minimizes certain interferences. However, when hCG concentrations are only moderately increased, relatively large volumes of urine must be added in the assay, and the presence of human lutropin (and probably its metabolites) precludes reliable measurement of hCG (6, 7). The recent development of antiserum directed against the unique carboxy-terminal peptide (CTP) region of the hCGβ subunit provides a novel reagent for circumventing the problem of human lutropin cross reactivity in hCG assays (8–12). Concanavalin A bound to a solid-phase matrix is used to partly purify and concentrate glycoproteins such as hCG from biological fluids (13). Using a high-affinity antiserum raised against a sialic acid-containing hCGβ-CTP fragment and concentrating the specimens with concanavalin A, we developed a convenient RIA procedure for measuring hCG in first morning-voided urine specimens.

Materials and Methods

Materials

The antisera used in these studies were SB6, which is directed against conformational determinants of the hCGβ subunit (3), and R529, which is directed against the hCGβ-CTP determinants (11, 12). The method of preparation and the characteristics of the R529 antiserum have been presented elsewhere (11, 12).

Highly purified hCG (CR119) was obtained from the Center for Population Research, National Institute of Child Health and Development, National Institutes of Health, Bethesda, MD 20205; its potency is 13.5 X 10^6 int. units/g in terms of the Second International Standard for hCG in the ventral-prostate-weight bioassay. This material was used both as the reference standard and, when radioiodinated by the Chloramine T method (14), as the radioligand. The specific activity of the radioligand was 50–100 Ci/g; it was purified just before use in the RIA by adsorption chromatography on concanavalin A covalently bound to Sepharose 4B (Con A Sepharose; Pharmacia Fine Chemicals, Piscataway, NJ 08854) as described previously (13). To displace adsorbed glycoproteins from the Con A Sepharose, we used α-methyl-D-mannoside (Grade II; Sigma Chemical Co., St. Louis, MO 63178) (13, 15). The reagents for the RIA of human lutropin and the purified human pituitary lutropin for injection (preparation A3) were obtained from the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases through the National Pituitary Agency, University of Maryland, Baltimore, MD 21201. The commercial hCG used for injection was "Pregnyl" (Organon, West Orange, NJ 07052).

Subjects

Patients or normal volunteers at the National Institutes of Health Clinical Center and the Gerontology Research Center participated in these studies, having given their informed consent. Five men were given injections of Pregnyl, 4000 int. units daily for four days. Blood and urine samples (first morning-voided as well as complete 24-h collections) were obtained before, during, and for five to seven days after the injections of hCG. Five premenopausal, non-pregnant women were given continuous intravenous infusions of purified human pituitary lutropin over 4–6 h, and blood and urine specimens were obtained at intervals. Sera and aliquots of urine were frozen at −20 °C until assayed.

Measurement of hCG in Urine

Remove any sediment in the urine sample by centrifugation or by allowing to settle. Add volumes of urine up to 3 mL, and the various concentrations of the reference preparation di-

1 Gerontology Research Center, National Institute on Aging, National Institutes of Health, Baltimore City Hospitals, Baltimore, MD 21224.
2 Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, NY 10032.
3 Developmental Endocrinology Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20205.
4 Nonstandard abbreviations: BSA, bovine serum albumin; Con A Sepharose, concanavalin A covalently bound to Sepharose 4B; hCG, human choriogonadotropin; hCGβ, human choriogonadotropin beta subunit; hCGβ-CTP, hCG β-carboxy-terminal peptide; PBS, phosphate-buffered saline (0.01 mol/L PO4, 0.15 mol/L NaCl, pH 7.4); RIA, radioimmunoassay.

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luted in PBS-BSA (phosphate-buffered saline, 0.01 mol/L PO₄, 0.15 mol/L NaCl, pH 7.4, containing 1.0 g of bovine serum albumin (RIA grade; Sigma Chemical Co.) and 1.0 g of Na₂SO₄ per liter) to 12 × 75 mm glass tubes. Adjust the volume to all tubes to 3.0 mL with PBS-BSA. Add 300 μL of a constant-stirred suspension of Con A Sepharose (suspended in an equal volume of PBS); incubate at room temperature for 1 h, and vortex-mix every 10–15 min.

Centrifuge at 1500 × g for 15 min and aspirate the urine from the tubes. Wash the gel with 2.0 mL of PBS-BSA, and recentrifuge the contents of the tubes. Subsequent incubations are at 4 °C. Add 400 μL of a solution of PBS-BSA containing, per liter, 2 mL of antisera (R529), 0.37 mol of α-methyl-D-mannoside and 25 mM of EDTA. Gently vortex-mix. After incubation for 36 h, add 400 μL of PBS-BSA containing 125I-labeled hCG (30 000 cpm) and per liter, 0.25 mol of α-methyl-D-mannoside and 20 mL of normal rabbit serum. Incubate this mixture for 24 h. Add second antibody (200 μL of undiluted sheep anti-β-globulin serum) and incubate for 16 h. Centrifuge the tubes at 1500 × g for 15 min and measure the radioactivity in the sediments. With this procedure, specifically bound radioactivity at zero dose is 20 to 25% of total, after subtraction of nonspecific radioactivity, which is 20 to 30% of total.

Other Procedures

For comparison we assayed some samples by a procedure identical to that described above, but used the hCGβ subunit antisera (SB6) instead of the R529 antisera. In those studies, the final dilution of the SB6 antisera in the assay tube was 50 000-fold. In other studies, the human lutropin content of the extracts was assessed. The Con A Sepharose concentration procedure was identical to that described above for hCG. The human lutropin antisera (National Pituitary Agency anti-hLH, Batch No. 2) was used at a final dilution of 500 000-fold. The human lutropin for iodination was labeled with 125I by the Chloramine T method (14) to a specific activity of 50–70 Ci/g. The reference preparation was LER-907, obtained from the National Pituitary Agency.

The RIA of serum hCG was performed with the hCGβ subunit antisera (SB6) as described previously (3).

Analysis of Data

We analyzed the RIA data by the computerized method of Rodbard and Lewald (16, 17), which provides least-squares linear regression of the log of the standard hormone dose vs the logit of the cpm ratios of tracer bound at each dose (B) to that bound at zero dose (B₀), after correction for nonspecific binding. The regression, weighted for nonhomogeneity of variance, is used to calculate potency estimates for unknown samples. The minimum detectable dose is defined as that amount of ligand displacing enough tracer to give a B/B₀ ratio 2 SD from 1.00; the ED₅₀ is defined as that dose at which B/B₀ = 0.50.

Results

To assess whether first morning-voided urine extracts contain substances that interfere with the RIA of hCG, we prepared serial dilutions of the reference standard in PBS-BSA or first morning-voided urine specimens. We extracted 3-mL aliquots with Con A Sepharose and assayed, using the hCGβ-CTP antisera. The binding-inhibition curves obtained with the extraction of hCG from first morning-voided urine specimens were indistinguishable from that obtained with extraction of hCG from PBS-BSA (Figure 1). The slope of the logit-log plot of the standard was −1.4. The ED₅₀ was 3.6 ng, and the least detectable dose was 1.2 ng in the Con A Sepharose assay; because the potency of hCG in the 3 mL of urine was 92 ± 5% of that added, we designated 0.4 μg/L as the least detectable concentration of hCG in the first morning-voided urine samples. The within-assay CV was 9%, and the between-assay CV was 17%.

Considering the fact that human lutropin cross reactivity is a problem in most available hCG assays in urine (6, 7, 18), we evaluated the reliability of two antisera that are known to exhibit enhanced specificity for hCG, namely the antisera generated against the hCGβ subunit (SB6), which is widely used to measure serum hCG (3), and an antisera generated against the hCGβ-CTP (R529). Concentrations of hCG immunoreactivity in first morning-voided urines from 21 normal men and 15 premenopausal, non-pregnant women were less than 0.4 μg/L when measured by RIA with the hCGβ-CTP antisera, but when measured by the RIA with the hCGβ antisera, values ranged up to 1.5 μg/L.

We further documented that human lutropin does not cross react in the RIA in which the hCGβ-CTP antisera is used by examining samples from patients with several conditions in which lutropin concentrations are known to be increased.

First, we measured hCG immunoreactivity in the urine of a young woman with apparently normal menstrual cycles. Urine was collected each day through one cycle. The mid-cycle peak of human lutropin in first morning-voided urine specimens was easily demonstrated by RIA with the human lutropin antisera (Figure 2, upper panel) but was not associated with any detectable concentration of hCG determined with use of the hCGβ-CTP antisera (R529) (Figure 2, lower panel). In contrast, the RIA with the hCGβ antisera (SB6) seemed to show human lutropin cross reactivity in most samples, and a pronounced surge coincident with the human lutropin peak (Figure 2, middle panel).

Second, first morning-voided urine specimens were obtained from 25 postmenopausal women and measured in the two assay systems for hCG. The increased lutropin concentrations in urine from these women interfered in the RIA in which the hCGβ antisera was used; there was no interference in the RIA with use of hCGβ-CTP antisera (Figure 3).

Further to verify that human lutropin-related molecules were responsible for some of the immunoreactive material detected in urine by the RIA with the hCGβ antisera (SB6), and that urinary human lutropin does not cross react in the RIA involving hCGβ-CTP antisera, we examined urine obtained from several normal women before and after an infusion of purified human pituitary lutropin. Before the infusion of human lutropin, something in the urine of several
Fig. 2. Concentrations of hCG-immunoreactive material in first morning-voided specimens from a woman presumed to have normal ovulatory cycles. Immunoreactivity in Con A Sepharose concentrates was measured by RIA with the human lutropin (hLH) antiserum (upper panel), hCGβ antiserum (middle panel), and hCGβ-CTP antiserum (lower panel). Shaded areas represent the lower limit of detection in the assays.

of the women cross reacted with the hCGβ antiserum (Figure 4, left panel). Further, during infusion of the highly purified human pituitary lutropin, the concentrations of immunoreactive material increased. However, no immunoreactivity was detected by the RIA with the hCGβ-CTP antiserum either before or during the infusion of human lutropin (Figure 4, right panel).

Studies of the peripheral metabolism of hCG have shown that about 20% of serum hCG is disposed of by renal excretion (15, 19, 20). In fact, with normal urine volumes, the hCG concentration in urine is approximately equal to that in serum. We sought to determine whether values obtained for urine hCG in human subjects by RIA with the hCGβ-CTP antiserum correlated with values obtained for serum hCG by RIA with the hCGβ antiserum. We gave five normal men daily intramuscular injections of 4000 int. units of hCG (Pregnyl) for four days. Urine and serum specimens were obtained during and for several days after the injections. We determined serum hCG concentration by RIA with the hCGβ antiserum and the hCG concentration in first morning-voided urine by RIA with the hCGβ-CTP antiserum. The two were highly correlated ($r = 0.86, p < 0.001$, Figure 5).

To determine how well the first morning-voided urine concentration of hCG reflected the total quantity of hCG in the 24-h urine specimen, we obtained aliquots from the first morning-voided specimens prior to their inclusion in the 24-h urine specimens, and measured hCG in both specimens by

Fig. 3. Concentrations of hCG-immunoreactive material in first morning-voided urine specimens obtained from postmenopausal women as measured by RIA with the hCGβ antiserum or the hCGβ-CTP antiserum. Shaded areas represent the lower limit of detection of the assays.

RIA, using the hCGβ-CTP antiserum. Again, the values were highly correlated ($r = 0.96, p < 0.001$, Figure 6). Expressing the results shown in Figure 6 as nanograms of hCG per gram of creatinine improved the correlation coefficient only slightly, to $r = 0.98$.

Discussion

Our radioimmunoassay method for hCG has several clinically useful advantages over currently available methods.

First, to circumvent the problem of human lutropin cross reactivity, our RIA for the hCG in urine extracts involves the use of a high-affinity hCGβ-CTP antiserum directed toward determinants on the hCGβ-CTP, a structural region of hCGβ not common to human lutropin (21–23). We have confirmed that urine contains material(s) that cross react in the RIA in

Fig. 4. Concentrations of hCG-immunoreactive material in urine before and after intravenous infusion of highly purified human lutropin into normal young women, as measured by RIA with the hCGβ antiserum (left) or the hCGβ-CTP antiserum (right). Shaded areas represent the limit of lower detection of the assays.

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which the hCGβ subunit antiserum is used (6, 7). The observation that in the urine of post-menopausal women, the concentration of cross reacting material correlates with the concentration of human lutropin immunoreactivity (6), in conjunction with our observation that infusion of purified human lutropin markedly increases the concentrations of cross reactivity, strongly indicates that the various forms of immunoreactive pituitary lutropin account for most of the cross reactivity. Regardless of its exact nature, this cross reacting material hampers the usefulness of the hCGβ antiserum for the measurement of low concentrations of urinary hCG. In contrast, in the RIA involving use of the R529 antiserum directed toward the hCGβ-CTP immunological determinants, there was no cross reactivity of urine specimens collected from postmenopausal women, women during the ovulatory gonadotropin spike, or subjects who received an infusion of human lutropin. Thus, our hCG RIA can measure urinary hCG reliably in the presence of urinary human lutropin.

Second, as a result of the decreased human lutropin cross reactivity, hCG production can be detected reliably at lower rates than with the conventional hCGβ RIA in serum. Experience with the hCGβ RIA system for serum hCG has shown that it provides a more sensitive indication of low rates of hCG production than do the urinary agglutination assays, the radioreceptor assay, or the bioassay of hCG (d). In the report by Vaitukaitis et al. (3), 1 μg of immunoreactive hCG per liter of serum was suggested as a satisfactory lower limit for unequivocal detection of hCG. However, in an extensive survey, Blackman et al. (24) found that normal postmenopausal women had concentrations in serum ranging up to 4.1 μg/L when measured with the hCGβ antiserum (SB6); thus, immunoreactivity up to this concentration cannot be ascribed to hCG with confidence. Normal renal clearance of this amount of hCG would result in an equivalent concentration of 4.1 μg/L in urine, while the limit of unequivocal detection of authentic hCG in urine with our RIA system is 0.4 μg/L. Thus, RIA of urine with use of the hCGβ-CTP antiserum is effectively 10 times more sensitive for the reliable detection of hCG production in postmenopausal women than is the RIA for hCG in serum with the hCGβ subunit antiserum.

Third, the use of urine samples has obvious advantages over blood sampling. Moreover, it is more convenient to use first morning-voided urine specimens than to attempt to collect complete 24-h urine specimens, and values for first morning-voided urine are highly correlated with hCG concentrations in both serum and 24-h urine. The patient can easily collect first morning-voided specimens as frequently as desired by the physician, store them at home at ~20 °C, and deliver batches of specimens to the laboratory at convenient intervals. It is possible to express hCG excretion in terms of creatinine excretion to take into account day-to-day variations in urine volume (25).

Fourth, our extraction procedure with use of Con A Sepharose is simple, and requires only a little more technical time than the routine RIA for serum hCG. Extraction procedures such as the kaolin–acetone method followed by adsorption on Con A Sepharose (26) offer much better sensitivity of hCG detection than does Con A Sepharose extraction alone, but they are more laborious and inconvenient for use with large numbers of samples.

It should be kept in mind that certain molecular forms other than hCG itself will cross react in this RIA. These include the free hCGβ subunit and any other form that contains the hCGβ-CTP immunologic determinant and interacts with concanavalin A.

Our observations suggest the following approach to the use of urinary hCG measurements to monitor gestational trophoblastic disease. Production of hCG can be assessed initially by measuring the hCG concentration in first morning-voided specimens of urine by the method described in this communication. If the hCG concentration is near the limit of detection of this method, procedures to concentrate the hCG from larger volumes of urine (for example, kaolin–acetone concentrates of 24-h collections) may be applied to further improve the capability of detecting low but clinically significant rates of hCG production (27).

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References