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Immunofluorometry of Choriogonadotropin by Time-Resolved Fluorescence Spectroscopy, with a New Europium Chelate as Label

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We describe a new "sandwich"-type non-isotopic immunoassay for human choriogonadotropin (hCG) in serum. In the assay, hCG is captured by a β-subunit-specific monoclonal antibody, which is immobilized in a white microtiter well. The sandwich is completed by adding a second biotinylated monoclonal antibody specific for the whole hCG molecule. The degree of binding of biotinylated antibody, which is proportional to the amount of hCG present in the sample, is quantified by adding streptavidin labeled with the europium chelate 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA), in the presence of excess Eu^{3+}. The fluorescent complex formed on the solid-phase [monoclonal antibody–hCG–monoclonal antibody–biotin–streptavidin–BCPDA–Eu^{3+}] is measured by excitation at 337.1 nm with a nitrogen laser and monitoring the emission at 615 nm in a specially designed gated fluorometer working in a time-resolved mode. A two-step procedure is proposed for routine use to avoid the "high-dose hook effect" of the simpler and faster one-step procedure. The hCG assay described has a dynamic range of 1 to 500 int. units/L, and is precise and accurate. Results agree well with those obtained with a commercially available immunoradiometric and a time-resolved immunofluorometric procedure.

Additional Keyphrases: *non-isotopic immunoassay · solid-phase immunoassay · immunoradiometric, time-resolved immunofluorometric procedures compared · monitoring pregnancy · ectopic pregnancy*

Human choriogonadotropin (hCG) is a glycoprotein hormone, secreted by the trophoblastic cells of the placenta. It is composed of two dissimilar non-covalently linked polypeptides, known as the alpha and beta subunits (*1*).[4] The alpha subunit of hCG is nearly identical to the alpha subunits of the pituitary glycoprotein hormones: lutropin (luteinizing hormone), follitropin (follicle-stimulating hormone), and thyrotropin (thyroid-stimulating hormone) (*2*). The beta subunits also share a substantial homology, but by virtue of differences in amino acid sequence, they confer unique biological and immunological specificities on these hormones (*3*).

The primary use of quantitative or qualitative measurements of hCG is for the early detection and monitoring of pregnancy (*4*) and pregnancy-related disorders, including ectopic pregnancy (*5, 6*) and threatened abortion (*7*). Measurements of hCG have also been found useful in the diagnosis and monitoring of patients with selected malignancies of trophoblastic and non-trophoblastic origin, which are frequently associated with elevated values for hCG (*8–12*).

In recent years, several different analytical techniques have been developed for hCG determination. The initial immunological assays involving sheep erythrocytes or latex particles coated with hCG, the hemagglutination and latex-agglutination methods (*13, 14*), were insensitive (*15*) and were soon replaced with radioreceptor assays (*16*) and radioimmunoassays (*17*). These techniques, although capable of achieving greater sensitivity and earlier detection of pregnancy, significantly cross-reacted with pituitary glycoprotein hormones, particularly lutropin (*18, 19*).

With advances in production of monoclonal antibodies against the beta subunit of hCG, sensitive and specific procedures based on the principle of radioimmunoassay (RIA) and immunoradiometric assay were developed (*20, 21*). This made possible the accurate quantification of very low concentrations of hCG, with negligible cross-reaction with lutropin. However, recent attempts are concentrated towards the development of methodology that eliminates the use of radiolabeled tracers, and thus freedom from handling, exposure to, and disposal of radioactivity, and other associated disadvantages of radioactive labels. The outcome has been the introduction of a new generation of non-isotopic immunoassays with a detection system that incorporates enzymes (*22–24*) or luminescent (*25*) or fluorescent (*26*) probes as labels. Europium complexes have been recently introduced as alternative labels to radioisotopic compounds for development of fluorescence immunoassays. Europium has the advantage of a long fluorescent decay time, with a sharp emission band and a large Stokes shift. Fluorescence from europium complexes can be detected with very high sensitivity and low background signal by time-resolved fluorometry (*27, 28*). The use of europium in time-resolved fluorometry of hCG has been previously reported (*29, 30*), and the system is now commercially available.

In this report, we present an immunofluorometric assay with a new time-resolved detection system for determination of hCG in serum. The assay is based on the "sandwich" principle, and is performed in microtiter wells coated with a monoclonal antibody to hCG beta subunit. A biotin-labeled monoclonal to intact hCG forms the detection antibody. The degree of binding of the biotinylated antibody to captured hCG molecules is determined by a bridge reaction with a novel europium-saturated chelate, 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA), which has been covalently attached to streptavidin. The fluorescence of the final complex formed (antibody 1–hCG–antibody 2–biotin–streptavidin–BCPDA–Eu^{3+}) is then quantified in the dried solid phase by pulsed fluorescence measurements with the CyberFluor 615 gated fluorometer/analyzer. The BCPDA–Eu^{3+} complex is a highly fluorescent compound, suitable for applications in time-resolved fluorescence immunoassays. The use of the same chelate for the development of sandwich-type and competition assays is

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[4] Nonstandard abbreviations: hCG, human choriogonadotropin; BCPDA, 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid; NHS-LC-Biotin, sulfosuccinimidyl-6-(biotinamido)-hexanoate; BSA, bovine serum albumin; IRP, International Reference Preparation.

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described in a recent review (*31*). With the system described, we were able to combine the high sensitivity and convenience of fluorescence measurements with the specificity of monoclonal antibodies to devise an hCG assay that is suitable for routine measurements. A major advantage of the proposed method in comparison with currently used time-resolved immunofluorometric procedures for hCG is its insensitivity to Eu^{3+} contamination. This advantage is derived from the fact that the label in the present procedure is BCPDA and not Eu^{3+}.

Materials and Methods

Reagents and Buffers

Human thyrotropin (specific activity, 6.6 int. units/mg), human follitropin (specific activity, 6200 int. units/mg), and human lutropin (specific activity, 5000 int. units/mg) were obtained from Sigma Chemical Co., St. Louis, MO 63178, as were bovine serum albumin, bovine globulin, and streptavidin. Sulfosuccinimidyl-6-(biotinamido)hexanoate ("NHS-LC-Biotin") was from Pierce Chemical Co., Rockford, IL 61105. *N,N*-Dimethylformamide was from Fisher Scientific, Ottawa, Ontario, KIG 4A9, Canada. Europium(III) chloride hexahydrate was from Aldrich Chemical Co., Milwaukee, WI 53233. White opaque microtiter plates (96-well) or strips (12-well), Microfluor™, were products of Dynatech Laboratories Inc., Alexandria, VA 22314. All other chemicals were of the highest quality obtainable.

The coating buffer is 0.01 mol/L Tris, pH 8.5, containing 0.1 mol of NaCl per liter. The blocking buffer is 0.1 mol/L carbonate, pH 8.3, containing 1 g of BSA and 0.5 g of sodium azide per liter. The assay buffer is 50 mmol/L Tris, pH 7.8, containing 9 g of NaCl, 0.5 g of sodium azide, 5 g of BSA, 0.5 g of bovine globulin, and 0.1 mL of polyoxyethylenesorbitan monopalmitate (Tween 40) surfactant per liter. The streptavidin–europium buffer is 50 mmol/L Tris, pH 7.8, containing 9 g of NaCl, 10 g of BSA, and 0.5 g of sodium azide per liter. The wash solution is a 9 g/L NaCl solution containing 0.5 mL of polyoxyethylenesorbitan monolaurate (Tween 20) per liter.

Other Materials

hCG standards. Purified hCG (specific activity, 3310 int. units/mg in terms of the first IRP, 75/537, for immunoassay) was obtained from Calbiochem, Behring Diagnostics, La Jolla, CA 92037. The preparation was reconstituted according to the manufacturer's recommendation, and diluted in hCG-free human serum (Chemicon International Inc., El Segundo, CA 90245) to give the desirable standard concentrations.

Monoclonal antibodies. Monoclonal antibodies to the beta-subunit of hCG and to the intact hCG molecule are diethylaminoethyl-column chromatographically purified immunoglobulin fractions, purchased from Medix Biotech Inc., Foster City, CA 94404. The percentages of cross reactivity of these antibodies to human-origin hormones, as determined by the manufacturer in a conventional RIA, were: hCG 100%, hCG beta 100%, hCG alpha 0.0%, lutropin 0.3%, and thyrotropin 0.02% for the antibody to the beta-subunit of hCG; and hCG 100%, hCG beta 0.0%, hCG alpha 0.0%, lutropin 90%, thyrotropin 85%, and follitropin 16% for the antibody to the intact hCG molecule. A monoclonal antibody to human lutropin was also obtained from Medix Biotech.

Specimens. Human serum samples containing various concentrations of hCG were kindly provided by Dr. S. Tilak (Hospital Incommon Laboratory, Toronto, Canada). These samples were from pregnant and non-pregnant women, and had been stored at −20 °C. To exclude any possible effect of storage on hCG concentrations, the samples were concurrently tested with the present method and the comparative kits. Specimens exceeding 500 int. units of hCG per liter were diluted with hCG-free serum to bring them within the measurement range of the assay. Lyphochek immunoassay control serum (human) levels I, II, and III were from Bio-Rad Clinical Division, Richmond, CA 94801.

Procedures

Comparison methods. Two commercially available kits were used as comparison methods. The "EchoClonal hCG Assay" (Bio-Rad) is a sandwich-type immunoradiometric procedure. It makes use of solid-phase monoclonal anti-hCG antibodies bound to immunobeads, and iodine-125-labeled monoclonal anti-hCG antibodies as the tracer. This assay is a single-reagent procedure, combining the solid phase and the tracer in a tracer/immunobead reagent. For counting of radioactivity and data reduction we used the LKB-Wallac (Turku, Finland) 1275 Minigamma counter.

The "DELFIA hCG Assay" (LKB-Wallac) is a time-resolved immunofluorometric procedure. As the solid phase it uses a monoclonal antibody to hCG beta-chain immobilized into wells of microtiter strips. The tracer is a monoclonal anti-hCG alpha-subunit labeled with europium. Fluorescence was measured in an LKB 1230 Arcus fluorometer.

Both kits are calibrated against the World Health Organization (WHO) first IRP 75/537 for immunoassay. Procedures recommended by the manufacturers were followed for duplicate measurements of the specimens.

Biotinylation of antibody. The monoclonal antibody against hCG intact molecule (anti-hCG) was biotinylated according to the following procedure. A 0.5-mL aliquot of the antibody solution (1 mg/mL, in 15 mmol/L potassium phosphate buffer, pH 7.2, containing 0.15 mol of NaCl and 1 g of NaN_3 per liter) is mixed with 0.5 mL of a carbonate/bicarbonate buffer (0.5 mol/L, pH 9.0). To the mixture is then added a 500-fold molar excess of NHS-LC-Biotin dissolved in 100 μL of distilled water and incubated at room temperature for 30 min. The unconjugated biotin is then removed by dialysis at 4 °C for 24 h against several changes of 0.1 mol/L sodium bicarbonate (pH 8.3) containing 0.5 g of sodium azide per liter. The biotin–anti-hCG conjugate is then titrated to determine the optimal concentration for the assay, and stored at 4 °C. Before use, the antibody is diluted 100-fold with the assay buffer.

Labeling of streptavidin with BCPDA. The procedure is the same as described in the companion paper (*32*).

Immobilization of anti-hCG beta-subunit. The monoclonal antibody to hCG beta-subunit (anti-hCG-beta) is immobilized by adsorption onto wells of microtiter plates or strips. The coating is prepared by adding, per well, 100 μL of anti-hCG-beta monoclonal antibody (5 μg/mL) in the coating buffer. After overnight incubation at 4 °C, the wells are washed five times with the wash solution, then two times with water. To block the remaining active sites, 200 μL of the blocking buffer is then added and the mixture is allowed to incubate at room temperature for 2 h. The wells are stored at 4 °C and are stable for several weeks. Before use, the wells are washed two times with the wash solution.

Assay procedures. The assay was performed according to a one-step and a two-step procedure. To perform the one-step assay, pipet 50-μL duplicates of hCG standards or serum

samples and 50 μL of biotinylated anti-hCG antibody solution (5 μg/mL in the assay buffer), containing anti-lutropin antibody (1 μg/mL), into microtiter wells. After the wells have been shaken for 3 min in an automatic shaking device, they are allowed to incubate at room temperature for 2 h. The reaction mixture is then removed by decanting, and the wells are washed four times with the wash solution, with use of a 12-well strip-washing device. Binding of biotinylated antibody to hCG is determined by addition of 100 μL per well of the indicator reagent. After 45 min of incubation at room temperature, the wells are washed twice with the washing solution, dried for 5 min by means of an automated cool-air-blowing drying device, and the fluorescence at the bottom of the dried wells is measured in the CyberFluor 615 fluorometer.

To perform the two-step assay, 50-μL duplicates of hCG standards or serum samples and 50 μL of the assay buffer, containing anti-lutropin antibody, 1 μg/mL, are pipetted into microtiter wells. The wells are then shaken for 3 min, and allowed to incubate at room temperature for 2 h. After the wells are washed as described above, 100 μL of the biotinylated antibody solution (2.5 μg per mL, in the assay buffer) is added to each well and allowed to incubate at room temperature for 1 h. The antibody solution is then aspirated and the wells are washed four times with the wash solution. Determination of binding of the biotinylated antibody to captured hCG and the subsequent quantification of the resulting fluorescence by time-resolved fluorometry are done as described for the one-step assay.

Results

Detection Limit and Dynamic Range of the Assay

Figure 1 shows a typical dose–response curve. In both procedures, a near-linear relationship between the response and standard dose exists in the range 1 to 500 int. units of hCG per liter.

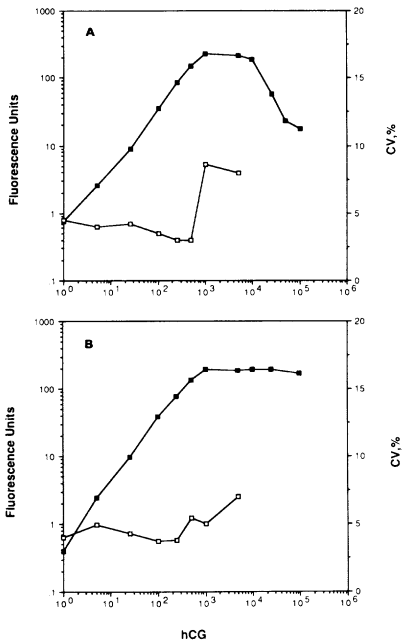

Fig. 1. Dose–response curve (■) and precision profile (□) of the one-step (*A*) and the two-step (*B*) assays

Each point on the standard curves represents the mean of duplicates from which the mean value of zero standard is subtracted. hCG values are in int. units/L, fluorescence in arb. units

Using the value corresponding to the mean plus three standard deviations (SD) of the zero standard for 15 determinations, we determined the detection limits for the one-step and the two-step procedures to be about 1.0 and 1.5 int. units of hCG per liter, respectively. The background fluorescence values of the zero standard are very similar for both assays, averaging about 9.5 ± 0.24 arbitrary units.

The working ranges of the assays were established according to precision profiles derived from 12 replicate measurements of each standard concentration by both methods. Taking a CV of 10% as our upper limit of imprecision, we found the working range for both assays to be 1 to 500 int. units of hCG per liter. However, as the profiles show (Figure 1), in both cases, the CV in the near-linear range of the assay is about 5%.

As shown in Figure 1, the shapes of the standard curves differ for the two assay methods. In the one-step assay, the fluorescence intensity, after reaching a maximum, begins to decline with increasing hCG concentration. This is presumably due to the high-dose "hook" effect, which is often observed with sandwich-type assays (*33*). This effect can be eliminated by performing the assay as a two-step procedure. In this case, the fluorescence intensity reaches a plateau with increasing hCG up to a concentration of at least 100 000 int. units/L.

We did a correlation study between the one-step and the two-step procedure for 30 serum samples. The regression equation found was: y(two-step assay) = 1.659 + 1.088x (one-step assay); r = 0.97. Thus there is close agreement between values observed by the two procedures.

Precision

To evaluate the precision of the assay, we assayed three concentrations of human serum controls for immunoassay (Bio-Rad) and a pooled human serum sample according to the one-step procedure. Within-run precision was determined by analyzing 21 replicates of each sample in the same run; between-run precision, by repeat analysis, in duplicate, of the control samples, in five successive runs. Day-to-day precision was estimated by determining, in duplicate, the hCG concentration of the same samples on 12 different occasions during a 30-day period (Table 1).

Dilution Linearity

Linearity was assessed by serial dilution of different patients' samples with the zero standard. Assaying for hCG, we used the concentration in the undiluted samples to calculate the expected values of the diluted samples. As shown in Table 2, there is a linear relationship between the hCG values expected and the values measured by the one-step (samples 1, 2, and 3) or the two-step (samples 4 and 5) procedures. The correlations between expected and measured values are excellent.

Table 1. Precision of the One-Step Assay

hCG concn, int. units/L								
Within run (n = 21)			Between run (n = 16)			Day-to-day (n = 12)		
Mean	SD	CV, %	Mean	SD	CV, %	Mean	SD	CV, %
14.5	0.6	4.3	14.5	0.9	6.2	13.4	1.3	9.7
52	2.7	5.2	57	3.4	5.9	54	2.8	5.2
210	9.0	4.3	225	18	8.0	212	15	7.2
400	24	6.1	—	—	—	454	31	6.9

Table 2. Linearity: Results for Dilutions of Sera with High hCG Concentration (int. units/L)

	Dilution					
Samples[a]	None	1/2	1/4	1/8	1/16	1/32
Sample 1						
Expected	—	220	110	55	28	14
Measured	440	210	100	49	25	13
Sample 2						
Expected	—	225	112	56	28	14
Measured	450	220	105	52	26	14
Sample 3						
Expected	—	155	78	39	19	10
Measured	310	160	79	40	21	10
Sample 4						
Expected	—	240	120	60	30	15
Measured	480	260	135	72	34	14
Sample 5						
Expected	—	180	90	45	22	11
Measured	360	190	110	50	20	13

[a] Samples 1, 2, and 3 were tested by the one-step assay, samples 4 and 5 by the two-step assay.

Regression analysis of expected (*x*-axis) vs measured (*y*-axis) concentration (sample no., slope, intercept, correlation coefficient): 1, 0.958, −2.44, 0.9997; 2, 0.979, −1.76, 0.9997; 3, 1.028, 0.127, 0.9998; 4, 1.083, 2.29, 0.9993; 5, 1.072, 1.96, 0.996.

Analytical Recovery

To determine the analytical recovery of the assay, we added exogenous hCG at various concentrations to different serum pools. Assays were performed on each sample before and after the addition. Measured and recovered concentrations are shown in Table 3. For the one-step assay (samples 1, 2, and 3) recovery of added hCG ranged from 92 to 105%, averaging 101.5%. Similar results were obtained by the two-step assay (samples 4 and 5): recovery ranged from 97 to 106%, averaging 103%.

Table 3. Analytical Recovery of hCG Added to Five Serum Pools

	Measured	Recovered	
Added[b]	hCG, int. units/L[b]		Recovery, %
Sample 1			
0.0	1.7	—	—
49.5	47	45.3	92
99	105	103.3	104
198	210	203.3	105
Sample 2			
0.0	17	—	—
49.5	67	50	101
99	118	101	102
198	215	198	100
Sample 3			
0.0	98	—	—
49.5	150	52	105
99	200	102	103
198	300	202	102
Sample 4			
0.0	0.0	—	—
49.5	51	51	103
99	105	105	106
Sample 5			
0.0	0.0	—	—
49.5	48	48	97
99	105	105	106

[a] 30-μL aliquots of each standard hCG preparation were added to 3-mL aliquots of each serum pool.

[b] Samples 1, 2, and 3 were tested by the one-step assay, samples 4 and 5 by the two-step assay.

Cross Reactivity

We tested for interference from other hormones by two experiments. In the first one, we measured the response of the assay to increasing concentrations of thyrotropin, follitropin, and lutropin in the absence of hCG. Of the concentrations of thyrotropin (50–200 milli-int. units/L) and follitropin (50–200 int. units/L) tested, none produced a response significantly different from the two SD range of the hCG negative sample. The hCG response of the sample was significantly increased with lutropin at concentrations >100 int. units/L, with a maximum cross reactivity of about 18% in the one-step assay and 9% in the two-step assay (Table 4).

In the second experiment, the same concentrations of these hormones were added to serum samples containing a fixed concentration of hCG (100 int. units/L). The main purpose of this experiment was to evaluate the possibility that in the one-step assay these hormones at high concentrations might compete with hCG for binding to the biotinylated anti-hCG monoclonal antibody, thus producing a false negative effect. With high concentrations of thyrotropin and follitropin present, the hCG response did not change by more than two SD of the expected value (i.e., 100 int. units/L). Similarly, lutropin did not appear to have any false-negative effect on hCG detection; rather, it generated a false-positive interference. The degree of cross reaction was the same as that noted in the previous experiment, cross reacting at a maximum of about 17% and 8% in the one-step and the two-step assays, respectively.

Because of the significant contribution of lutropin to the assay response at a range that might correspond to physiological as well as pathological concentrations (about 200–400 int. units of lutropin per liter), the possibility of including a scavenger anti-hLH antibody to the system was investigated. A monoclonal antibody against the beta-subunit of lutropin appeared most effective when added to the system during the sample-addition step. This antibody at a concentration of 1 μg/mL (i.e., 50 ng per well) was capable of practically eliminating the cross reaction with lutropin (Table 4). Only about 2% cross reactivity remained at 500 int. units/L, indicating that lutropin, even at pathological concentrations, would not lead to any false-positive or -negative results in the hCG assay performed by the one-step or the two-step methodology.

Correlation with Immunoradiometric and Immunofluorometric Assays

hCG concentrations in serum samples from pregnant and non-pregnant females were assayed, in duplicate, by the present one-step method (FIA) and by the comparative immunoradiometric (Bio-Rad) and immunofluorometric (LKB) procedures. There was a good agreement between the values obtained with the test method and the comparative kits. The regression equations were: y(FIA) = 3.52 + 0.919x (Bio-Rad), r = 0.97 (n = 64) and y(FIA) = 1.87 + 1.014x (LKB), r = 0.99 (n = 74).

Discussion

Immunoassays based on time-resolved fluorescence measurements are relatively new and offer a number of inherent advantages. They incorporate speed and sensitivity of fluorescence detection, with specificity of monoclonal antibodies, and do not have the well-known disadvantages of the radiolabeled reagents. One of the contributing factors is the ability to significantly reduce background signals, by selec-

Table 4. Cross Reactivity with hLH

Assay	Sample 1 (0.0 int. units of hCG/L)		Sample 2 (100 int. units of hCG/L)	
	hCG equivalent	% cross reaction	hCG equivalent	% cross reaction
LH added[a,b]				
100	12	12	15	15
200	30	15	32	16
500	90	18	86	17
LH added[a,c]				
100	1.0	1.0	0.0	0.0
200	1.7	0.8	0.0	0.0
500	14	2.8	5.0	1.0
LH added[b,d]				
100	7.0	7.0	7.0	7.0
200	14	7.0	16	8.0
500	45	9.0	40	8.0
LH added[c,d]				
100	1.5	1.5	1.0	1.0
200	4.0	2.0	3.0	1.5
500	11	2.2	8.0	1.6

[a] hCG one-step assay. [b] assay performed in the absence of anti-hLH. [c] assay performed in the presence of anti-hLH. [d] hCG two-step assay.

tive detection of the fluorescence of europium, which has an exceptionally long decay time. This allows quantifications to be performed with very high sensitivity and low background by time-resolved fluorometry (*27–28, 31*).

The present assay integrates a new time-resolved fluorescence detection method with a "sandwich"-type immunoassay, using the biotin–streptavidin system as the interface. The biotin–streptavidin system was chosen because it takes advantage of a gentle method of covalently binding multiple biotin residues to each antibody molecule, thus generating a highly stable reagent. Each of these biotin residues is capable of binding to a streptavidin molecule, thus amplifying the sensitivity of the system (*34*). In our assay, this amplification is further enhanced by the fact that each streptavidin molecule is covalently linked to several BCPDA residues, each capable of forming a 1:1 complex with europium cations, which are present in large excess. Other advantages of the biotin–streptavidin system is that they are a universal detection system which can be used in both sandwich and competition assays (*31*), and that BCPDA-labeled steptavidin is also a very stable reagent.

As shown in the previous section, the assay demonstrates a detection limit of about 1 to 1.5 int. units of hCG per liter and a near-linear dose–response covering a 500-fold concentration range. The high sensitivity of the assay is due, partly, to the amplification achieved by the use of the biotin–streptavidin system, and partly from the use of time-resolved fluorescence measurements. There is no interference from follitropin and thyrotropin, and cross reactivity with lutropin has been minimized to about 2% at lutropin concentrations ≤500 int. units/L. The observed specificity of the assay is determined by a combined specificity of the coating antibody and of the scavenger anti-lutropin antibody. In the two-step assay, the anti-lutropin antibody is included in the biotinylated antibody solution, and in the two-step assay it is included in the assay buffer. Results with the present assay compare well with those by a commercial immunoradiometric kit (Bio-Rad's EchoClonal) and a time-resolved immunofluorometric assay (LKB's DELFIA), and its performance characteristics are similar to or better than those of RIAs (*20*) and enzyme immunoassays (*23, 25*).

The hook effect, which is mainly associated with sandwich-type assays (*32*), was investigated by examining samples with hCG concentrations as high as 100 000 int. units/L. In the one-step assay, we found that the fluorescence intensity gradually declined with increasing hCG concentration. This indicates the requirement to assay at least two dilutions of the sample to ensure accuracy of the results. The effect occurs at hCG concentrations >1000 int. units/L, mainly because the concentration of the biotinylated monoclonal antibody is finite. With high concentration of hCG present, the excess hCG molecules increasingly saturate both the tracer and the solid phase, thus preventing them from forming the sandwich. However, the one-step assay is more rapid, and it might be preferred when hCG concentrations are expected to be ≤500 int. units/L. The two-step assay is free from the hook effect and is suitable for general routine use.

A sandwich-type time-resolved immunofluorometric assay involving monoclonal antibodies to the beta- and alpha-subunits of hCG has been recently described (*29, 30*), and it is commercially available (LKB-Wallac's DELFIA hCG kit). This assay is reported to achieve a detection limit of 0.5 int. unit/L and a linearity range of up to 1000 int. units of hCG per liter, which are comparable to those of the method described here. Results by the present procedure correlated well with those by the LKB kit in an assay of 76 clinical samples. Although the two procedures are similar in principle, a major difference between them is that the previous method makes use of a detection monoclonal antibody that is directly labeled with europium (through an EDTA derivative). Fluorescence readings are taken in a liquid phase after an enhancement solution is added to dissociate and complex europium cations from the labeled antibody. Because of this principle, the previous method is highly sensitive to background fluorescence resulting from europium contamination present in dust and water, as well as on skin surfaces. To minimize the contaminating effect, a great deal of care is required in the storage and handling of the enhancement solution, and during the performance of the assay. The present procedure, on the other hand, works under conditions of large europium excess (10^{-5} mol/L) and so it is insensitive to contamination by Eu^{3+}.

In summary, this assay is sensitive and specific, with good performance characteristics and a protocol easy to perform. It is a contamination-free system, requiring only 100 μL of serum for duplicate assays, and two or three incubation steps. The label is highly stable. Measurement time per

well, only 1 s, compares favorably with the time-consuming procedures for radioisotope quantification. These advantages, combined with a satisfactory accuracy and reproducibility, may represent a challenge to the procedures now used for hCG quantification in routine clinical chemistry laboratories.

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