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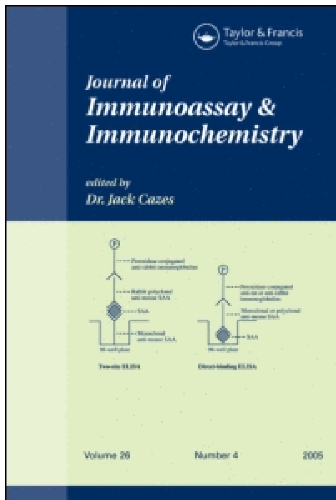

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Development of Isotopic and Non-Isotopic Microwell Based Immunoassays for hCG Using ^{125}I and Biotin Labeled hCG

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Abstract: Isotopic and non-isotopic immunoassays of hCG, based on the principle of competitive inhibition, using micro-well as solid support and ^{125}I and biotin as labels for hCG, have been developed. In both the assays, rabbit polyclonal antibody was immobilized onto micro-wells. In the non-isotopic assay, to the hCG antibody coated micro-wells, 50 μL of standard or samples along with 100 μL of biotinylated hCG were incubated for 1 hour at 37°C. After incubation, wells were washed and 100 μL of streptavidin-HRP conjugate was added to each well and incubated again for a half hour at 37°C. Bound enzyme activity was measured using tertramethyl benzidine/hydrogen peroxide (TMB/H_2O_2) as substrate. In the isotopic assay, to the hCG antibody coated micro-wells, 50 μL of standard or samples along with 100 μL of ^{125}I-hCG were incubated for 1 hour at 37°C. The bound radioactivity was measured using a gamma counter. The sensitivities of the non-isotopic and isotopic assays were 0.12 IU/mL and 0.1 IU/mL, respectively. The intra- and inter-assay CVs for both the assays were less than 12.3%. There was a good correlation between the developed non-isotopic and isotopic immunoassays (r = 0.97, n = 20).

Keywords: hCG, Immunoassay, Micro-well, Isotopic, Non-isotopic, Avidin, Biotin, ELISA, RIA

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INTRODUCTION

Human chorionic gonadotropin (hCG) was first identified and characterized by Aschheim and Zondek.[1] It is a member of the glycoprotein hormone family, composed of two dissimilar subunits, α and β, joined by charge interactions (non-covalently). The molecular weight of intact hCG is 36,700 Daltons, whereas the free α is 14,500 and the free β is 22,200 Daltons.

Normally, hCG is produced by the placenta and is secreted into the blood of both mother and fetus. Secretion of hCG is dynamic and changes throughout pregnancy. It is first detected in maternal serum within 6 to 9 days after conception.[2] The rate of secretion of hCG in serum increases in a logarithmic fashion, reaching maximal levels (~100,000 IU/L) 8 to 10 weeks after the last menstrual period. Thereafter, hCG levels slowly decline, reaching a nadir (~20,000 IU/L) at approximately 18 weeks gestation that persists until delivery.[3] After delivery, hCG remains in the maternal circulation up to 3–10 days of post partum in normal pregnancy.

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The physiological role of hCG in human pregnancy is to maintain the corpus luteum function after implantation of a fertilized egg for continuous production of progesterone[4] and relaxin. It is also responsible for regulating syncytiotrophoblast mass and for production of trophoblast hormones.[5] It inhibits cyclic release of LH hormone. hCG induces physiological changes, such as nausea and vomiting, craving, drowsiness, decreased physical activity, etc.[6]

Human chorionic gonadotropin is primarily pregnancy dependent. It is the potential marker for pregnancy detection and a valuable analytical tool for the diagnosis of abnormal pregnancy, e.g., miscarriage and ectopic pregnancy. Many abnormal pregnancies are associated with 50% lower levels of hCG as compared to the normal gestation of the same duration. Most of these pregnancies result in spontaneous abortion or miscarriage.[7] In the case of ectopic pregnancies, hCG increases at a much slower rate. In normal gestation, the doubling time of hCG is 36–48 hours whereas, in ectopic pregnancy, it takes a longer time. Levels of hCG are elevated in women with multiple fetuses and women with hydatidiform mole or choriocarcinoma.[8] Hence, quantitative estimation of hCG is useful for the diagnosis and management of abnormal pregnancies.

For the quantitative estimation of hCG, different isotopic and non isotopic immunoassays have been developed. Previously, an avidin-biotin system was used by different investigators for the development of sandwich enzyme immunoassays for hCG, where antibody was coupled with biotin.[9,10] Streptavidin-labeled europium chelate was used in a time resolved fluorescence sandwich immunoassay in combination with biotin-conjugated monoclonal antibody.[11] The avidin-biotin system was used to coat monoclonal antibody to the nitrocellulose membrane in the silicon sensor-based filtration immunoassay for hCG.[12] In all of the above methods, the avidin-biotin system helped either in detecting antigen through the biotinylated antibody or in

capturing the biotinylated antibody on the solid phase in sandwich type assays. In this present work, we have used an avidin-biotin system for the development of non-radioactive immunoassay, using biotin labeled antigen (hCG) instead of antibody. Generally, micro-wells are used for non-isotopic immunoassays, e.g., enzyme immunoassay (EIA), fluorescence immunoassay (FIA), and chemiluminescence immunoassay (CIA). Here, in the present study, micro-wells were used for the development of an isotopic immunoassay using ^{125}I labeled hCG. Hence, in the present paper, two types of micro-well based immunoassays have been described: isotopic immunoassay using hCG-^{125}I and non-isotopic immunoassay using biotinylated hCG as labeled antigens.

EXPERIMENTAL

Materials

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Human chorionic gonadotropin (hCG), polyclonal hCG antiserum (against whole hCG), 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide HCL (EDAC-HCL), N-hydroxysuccinimide (NHS), bovine serum albumin (BSA), Freund's complete adjuvant (FCA), diethyl aminoethyl Sephadex-(DEAE Sephadex), and sodium azide were all purchased from Sigma Chemical Company, St. Louis, MO, USA. Streptavidin-HRP and TMB/H_2O_2 were purchased from Bangalore Genei, Bangalore, India. D-Biotin was purchased from Sisco Research Laboratories Pvt. Ltd. India. For radioiodination, Na^{125} I was procured from, MDS Nordion S.A, Zoning Industriel Avenue de l' Espérance, B-6220 Fleurus, Belgium. Breakable strip based micro-well plates were purchased from Thermo Lab System, India. All other chemicals and buffer salts were of analytical grade and were purchased from Sisco Research Laboratory (SRL), Bombay, India.

Primary Antibody (hCG Antibody)

Polyclonal rabbit antibody, against whole hCG, was purchased from Sigma Chemical Company, USA (Cat. No. C 8534, Lot 121 k4891).

Coating of Polystyrene Wells with hCG Antibody

hCG antibody was diluted in 10 mM PBS and coated immunochemically into the micro titer wells according to the method of Shrivastav et al.[13]

Preparation of hCG Standard

Fifty micrograms (50 μg) of lyophilized hCG (from Sigma, USA, Cat. No. C 2047, activity 13,000 IU/mg in terms of IRP 75/537) was dissolved

in 250 μL of horse serum to prepare a stock standard having a concentration 2600 IU/mL. Several working standards (0.15, 0.37, 3.75, 7.5, and 15.0 IU/mL) were prepared by diluting this stock standard with horse serum.

Antibody, coating of micro-wells, and standards were same for the development of non-isotopic and isotopic immunoassays, which are described below.

Development of Non-Isotopic Immunoassay Using Biotin Conjugated hCG

Preparation of hCG-Biotin Conjugate

Human chorionic gonadotropin (hCG) was conjugated to biotin using a carbodiimide mediated N-hydroxysuccinimide reaction. Ten milligrams (10 mg) of D-biotin was dissolved in 100 μL of dimethyl sulfoxide (DMSO) and 300 μL of dimethyl formamide (DMF). To 200 μL of distilled water, 20 mg of N-hydroxysuccinimide and 40 mg of 1-ethyl-3-(3-dimethyl amino-propyl) carbodiimide hydrochloride (EDAC-HCl) were added. An aqueous solution of NHS and EDAC-HCl was added to the organic solution of biotin. The reaction mixture was incubated overnight at 4°C for the activation of the –COOH group of biotin. Fifty micrograms (50 μg) of iodination grade hCG (Sigma, USA, Cat. No. C-2047) was dissolved in 1 mL of 10 mM PBS. Activated biotin solution was added to the aqueous solution of hCG. The reaction mixture was again incubated overnight at 4°C, followed by dialysis against 10 mM PBS to eliminate the unconjugated biotin. The dialysate was centrifuged and the supernatant was collected; to it, 50% of ethylene glycol (v/v), 1% BSA, 9% sucrose, and 10% ammonium sulphate (w/v) were added and the mixture was stored at −30°C. hCG-biotin conjugate was diluted in 50 mM tris-acetate buffer (pH 8.0).

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Preparation of Working Dilution of Streptavidin-HRP Conjugate

Streptavidin-HRP was diluted in 10 mM sodium-acetate buffer to a 1 : 2000 ratio, as per manufacturer's instruction.

Preparation of Substrate Solution

Commercially available combined TMB and H_2O_2 substrate (20 × concentrated), was diluted in a 1 : 20 ratio with distilled water. This was prepared fresh just before its use.

Enzyme Linked Immunosorbent Assay (ELISA) Procedure Using the Avidin-Biotin System

To the hCG antibody coated micro-wells, 50 μL of different concentrations of hCG standards or samples were added, in duplicate, followed by 100 μL of

hCG-biotin conjugate. The wells were incubated for 1 hour at 37°C. After incubation, the contents of the wells were decanted and washed under running tap water. To all the wells, 100 μL of streptavidin-HRP was dispensed and incubated for half an hour at 37°C. The contents of the wells were decanted and washed under running tap water and 100 μL of TMB/H_2O_2 substrate solution (prepared freshly) was added. After 15 minutes of incubation at room temperature, 100 μL of 5 N HCl was added to all the wells to stop the enzyme activity. Absorbance of the developed color was measured with a Tecan-Spectra micro-well plate reader using a 450 nm filter. The hCG concentration of the individual samples was estimated by interpolation from the calibration curve which was prepared as a semi-log graph or with a microcomputer program developed in our laboratory using the logit-log method.

Development of Micro-Well Based Isotopic Immunoassay

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Radio-Iodination of hCG

hCG was radio-iodinated according to the chloramine-T method of Hunter and Greenwood.[14] Fifty microgram (50 μg) of iodination grade hCG (Sigma, Cat. No. C 2047) was dissolved in 250 μL of 0.1 M sodium phosphate buffer. From this stock solution, 25 μL was taken (containing 5 μg of hCG) into a small glass tube. To it, 20 μL of 0.5 M sodium phosphate buffer was added. To this glass tube (kept in ice), 10 μL of $Na^{125}I$ (activity 3700 MBq/mL) was added to react with hCG. Freshly prepared (20 μL) chloramine-T (2 mg/mL), prepared in 0.05 M phosphate buffer, was added to the hCG-iodine mixture to start the iodination reaction. Exactly 45 seconds after the addition of the chloramine-T, freshly prepared 20 μL of sodium meta bisulphate (4 mg/mL), prepared in 0.05 M phosphate buffer, was added to stop the iodination reaction. One hundred micro liters (100 μL) of transfer solution (containing 16% sucrose, 1% potassium iodide, and 0.001% bromophenol blue in double distilled water) was added to the reaction mixture.

The whole reaction mixture was transferred to the Sephadex G-75 column prepared in 0.05 M phosphate buffer. To rinse the glass vial, 100 μL of rinse solution (containing 8% sucrose, 1% potassium iodide, and 0.001% bromophenol blue in distilled water) was added and, after rinsing, it was transferred to the column. The column was eluted with 0.05 M phosphate buffer and 0.5 mL fractions were collected in glass tubes containing 0.5 mL of 2.5% BSA (prepared in 0.05 M phosphate buffer). A total of 25 fractions were collected. The first peak of radioactivity (containing iodinated hCG) was taken and kept for radioimmunoassay of hCG.

Micro-Well Based RIA Procedure

To the hCG antibody coated micro-wells, 50 μL of different concentrations of hCG standards or samples, in duplicate, were added. To all the wells, 100 μL

of working dilution of hCG-^{125}I was added. The micro-titer plate was incubated 1 hour at 37°C. After incubation, the contents of the micro-titer plate were emptied into the container for radioactivity waste disposal, washed in water, and dried. The individual wells were broken apart from the strip of the micro-titer plate and bound radioactivity of the wells was counted with a Wallac 1407 Gamma Counter. The hCG concentration of individual samples was estimated by interpolation from the calibration curve prepared as a semi-log graph or with a microcomputer program developed in our laboratory using a logit-log method.

Characteristics of standard curve like slope (m) and intercept (c), affinity of the hCG antibody, and precision of the assay were determined to validate both the assays.

Statistical Analysis and Graphical Presentation of Data

The statistical analysis and graphical presentation of data were performed by statistical analysis software "SPSS," and Microsoft Excel.

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RESULTS

Characteristics of Standard Curves of Non-Isotopic and Isotopic Assays

The standard curves of the non-isotopic and isotopic immunoassays for hCG are presented in Fig. 1. The slope (m) and intercept (c) of the standard curve of the non-isotopic assay were -1.6 and 0.58, respectively, and yielded the following equation: $Y = -1.6\ X + 0.58$ (where Y = Absorbance; and X =

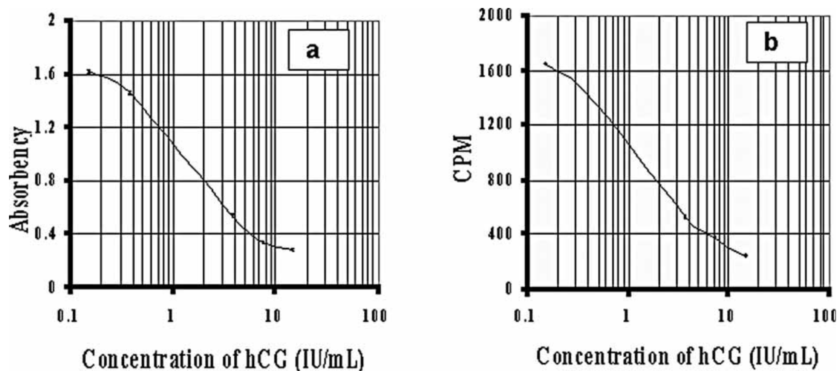

Figure 1. Standard curve of non-isotopic immunoassay (a), and isotopic immunoassay (b) of hCG (X-axis in log scale and Y-axis in linear scale; Plotted by MS Excel).

concentration of hCG in log-dose). The slope (m) and intercept (c) of the standard curve of the hCG RIA were -2.38 and 0.8, respectively, and yielded the following equation: $Y = -2.38\,X + 0.8$ (where Y = radioactivity counts per minute (CPM); X = concentration of hCG in log-dose).

Cross-Reactivity of hCG Antibody

The cross reactivity of the hCG antibody was 11% with hLH, 2% with hFSH, and 5.6% with hTSH.

Affinity of hCG Antibody and Assay Sensitivity of Non-Isotopic and Isotopic Assay

The affinity of the hCG antibody towards hCG, as obtained by non-isotopic immunoassay, was 5.2×10^{8} L/mol, whereas the affinity of hCG antibody towards hCG in the RIA was 7.6×10^{8} L/mol. The Scatchard plots are shown in Fig. 2. The lower detection limit or sensitivity of non-isotopic and isotopic immunoassays were 0.12 IU/mL and 0.1 IU/mL, respectively.

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Assay Precision

The intra-assay and inter-assay variations of the non-isotopic assay ranged from 4.6% to 7.0% and 5.3% to 8.7%, respectively (Table 1). Intra-assay

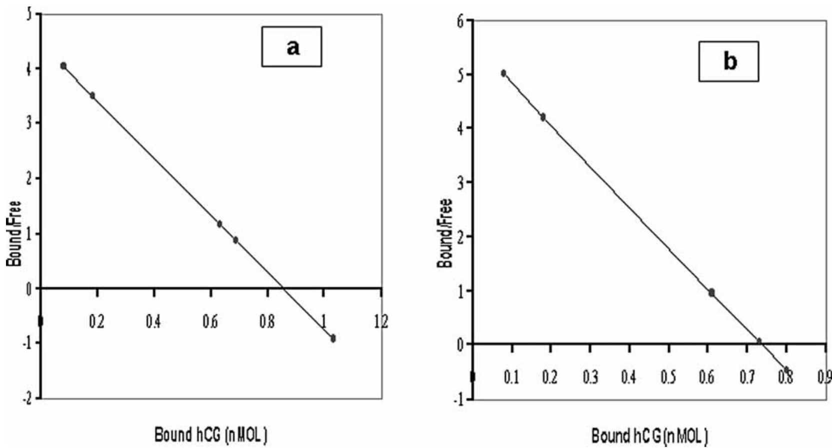

Figure 2. Determination of affinity constant of hCG antibody in non-isotopic immunoassay of hCG (a), and isotopic immunoassay of hCG (b) by Scatchard plot (Plotted by MS Excel).

Table 1. Intra-assay and Inter-assay variation of hCG of four control samples as estimated by developed ELISA

	Pool, A hCG (IU/mL)	Pool, B hCG (IU/mL)	Pool, C hCG (IU/mL)	Pool, D hCG (IU/mL)
Intra-assay variation				
Mean	0.8	2.2	3.85	6.6
SD	0.04	0.17	0.25	0.31
CV (%)	5	7	6	4.6
Inter-assay variation				
Mean	0.81	2.28	3.86	6.59
SD	0.06	0.2	0.25	0.35
CV (%)	7.4	8.7	6.4	5.3

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and inter-assay variations of isotopic assay ranged from 3.8% to 7.9% and 4.7% to 12.3%, respectively (Table 2).

Correlation Between ELISA and RIA

The concentrations of hCG present in different samples (n = 20) were determined separately by the developed non-isotopic immunoassay (ELISA) and by micro-well based RIA. The linear regression equation is as follows:

$$Y\ (\text{ELISA}) = 1.15\ X\ (\text{RIA}) - 1.58$$

The correlation coefficient (r) was 0.97. The regression graph is presented in Fig. 3.

Table 2. Intra-assay and Inter-assay variation of hCG of four control samples as estimated by developed micro-well based RIA

	Pool, A hCG (IU/mL)	Pool, B hCG (IU/mL)	Pool, C hCG (IU/mL)	Pool, D hCG (IU/mL)
Intra-assay variation				
Mean	0.79	2.19	4.15	6.92
SD	0.03	0.15	0.33	0.31
CV (%)	3.8	6.8	7.9	4.4
Inter-assay variation				
Mean	0.83	2.36	4.15	6.9
SD	0.05	0.29	0.26	0.33
CV (%)	6.0	12.3	6.2	4.7

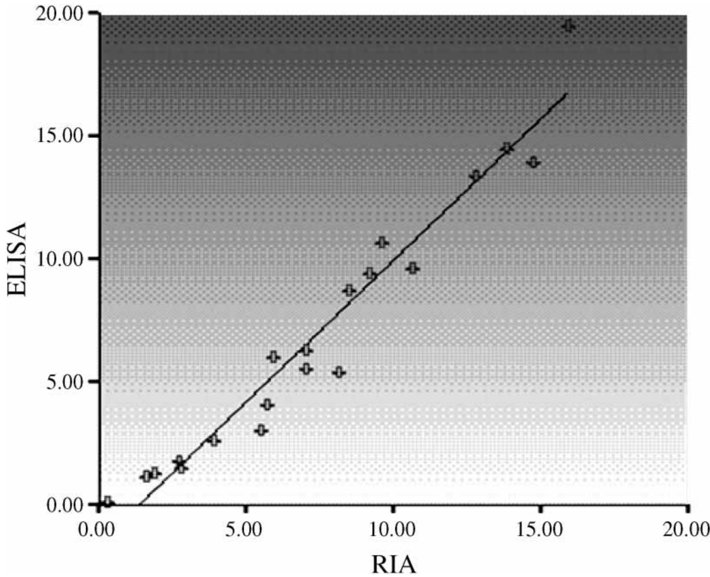

Figure 3. Regression graph of correlation between hCG RIA and hCG ELISA. Values of the RIA plotted in X-axis where as values of ELISA plotted in Y-axis. (Plotted by SPSS software.)

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DISCUSSION

In the present study, micro-well based immunoassays for hCG were developed using biotin conjugated and ^{125}I labeled hCG. The immunoassays were based on the competitive inhibition principle.

In the present assay, rabbit polyclonal antibody was used. Use of polyclonal antibody for the development of an hCG immunoassay has already been reported.[15,16] However, in the light of advancement of hCG immunoassay, the use of polyclonal antibody instead of monoclonal antibody in the present study may appear paradoxical. The advantage of the monoclonal antibody over a polyclonal antibody is in the attainment of higher sensitivity and specificity through a sandwich assay principle. In view of the heterogeneity of hCG (i.e., apart from the regular α and β-subunits, hCG also occurred in different forms like hyperglycosylated hCG, nicked hCG, hCG without β-subunit C terminal peptide, free β-subunit, nicked free β-subunit), the use of monoclonal antibody in hCG assay is questionable and is regarded as one of the main factors responsible for the variations in hCG measurement.[17,18] Because hCG exists in serum or urine in various forms and is unable to recognize all the forms by the particular clone of monoclonal antibody used in the said immunoassay, which causes variation in hCG results.[19] To minimize these variations, generally, in the commercial kits, a mixture of monoclonal antibodies directed against different hCG epitopes

are used. Depending on the degree of mixture of monoclonal antibodies, these assays may measure hCG differently and, hence, is the reason for variation of results among the kits.[17] Keeping in view of the above situation for the use of monoclonal antibody, in the present study, specific polyclonal antibody (against whole hCG) was utilized for hCG assay to ensure that all variants of hCG were measured.

In the present study, an avidin-biotin system was used in the competitive assay with biotinylated hCG. There is an advantage of using biotin, instead of enzyme, for labeling hCG, for the competitive immunoassay. Biotin, being a smaller molecular weight compound attached to hCG, the affinity of the hCG antibodies appeared to be similar to RIA. Because, after biotinylation or iodination of hCG, minor change in the molecular weight and structure of hCG took place, resulting in less of a chance of steric hindrance. But, it may not be the same if the hCG is attached to enzyme for making the enzyme conjugate for competitive immunoassay. It is reported that the lower molecular weight compound attached to the antigen for competitive immunoassay resulted in the enhancement of sensitivity of the assay. In the present study, the sensitivity of the non-isotopic immunoassay is comparable to that of radioimmunoassay.

The sensitivity and specificity of developed assays are very good, whereas analytical variables such as accuracy, precision, and correlation coefficient are in good agreement with the immunoassay standardization protocols. In general practice, recognition of pregnancy becomes possible only after the missed period. The earliest best time for confirmation of pregnancy biochemically is found to be after implantation within the first week of a missed menstrual period.[20] It was reported that, in the 5th week of normal or abnormal pregnancy, the serum hCG level varies from 0.33 IU/mL to 37.3 IU/mL.[21,22] Thus, the developed immunoassays are quite sensitive for detection of the serum levels of hCG in early stages of gestation.

Finally, it is noteworthy to mention here that, for the development of isotopic immunoassay, micro-wells that are generally used for the non isotopic immunoassay were used here. In the present study, suitability of use of micro-wells for the development of the isotopic immunoassay, along with the non-isotopic immunoassay, has been addressed. Hence, from the clue of the present study, it can be concluded that micro-wells can be utilized for the prospective development of simultaneous immunoassay using both isotopic and non-isotopic labels.

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