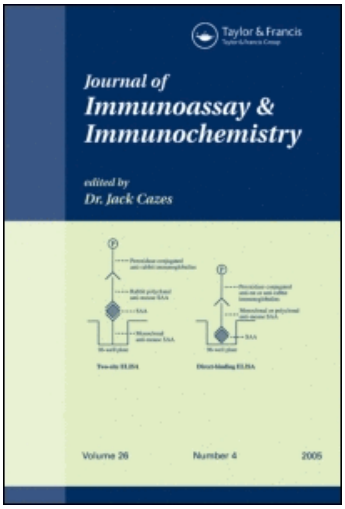

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Development of Colorimetric Enzyme-Linked Immunosorbent Assay for Human Chorionic Gonadotropin

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Abstract: The present study demonstrated the development of a solid phase competitive enzyme linked immunosorbent assay (ELISA) for direct estimation of human chorionic gonadotropin (hCG) in serum and urine. Polyclonal antisera raised against the β- subunit of peak-I hCG was used in the assay. The Peak-IA hCG-penicillinase was used as tracer. The performance of this antiserum and tracer was compared against hCG-β antisera of NIH, USA and penicillinase conjugated to hCG-β obtained from NIH, respectively. Almost parallel standard curves were obtained in both cases, suggesting that these antisera and enzyme label have much potential for developing ELISA system. To the anti-rabbit gamma globulin (ARGG) coated polystyrene tubes, standard or serum or urine samples (50 μL), 100 μL of hCG-β antiserum, 100 μL of peak-I(A) hCG-penicillinase conjugate and 350 μL of assay buffer were incubated at 37°C for 2 hours. Bound enzyme activity was measured using Penicilline V as substrate. In this new strategy, locally available polystyrene tubes were ground from inside and coated with ARGG. The sensitivity of the assay was 17 mIU/mL in urine and 18 mIU/mL in serum. The intra-assay and inter-assay coefficients of variation (CVs) appeared to be within acceptable limits of 10%. The serum and urinary hCG values, obtained by this method, correlated well with those obtained by radioimmunoassay (RIA) $r = 0.98$ ($n = 100$ for serum samples; $n = 250$ for urinary samples).

Keywords: hCG, Immunoassay, Penicillinase, Polystyrene tubes

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INTRODUCTION

Human chorionic gonadotropin and heterogeneous variants of hCG[1–8] are of high clinical importance for the diagnosis and management of pregnancy, monitoring of abnormal and ectopic pregnancies, testing for Down's syndrome, or monitoring therapy of hCG-secreting tumors and trophoblastic diseases. Therefore, clinical measurement of serum or urinary hCG becomes indispensable.

Traditional immunological assays, such as latex agglutination and hemagglutination test have low sensitivity. The sensitivity of these tests allows accurate and reliable detection only after the 42nd day following the last menstrual period.[9] A more sensitive test is needed if pregnancy is to be detected earlier. Moreover, the latex agglutination test cannot be used with serum or urine specimens with high protein contents ($>30\,mg/dL$), as false positive results can be obtained.[10] On the other hand, RIAs have found global use because of their high sensitivity. Nevertheless, its use is not preferred, considering the radiation hazards of radioisotopes used and the capital required. Therefore, in a continuing quest of developing immunoassays devoid of radioisotopes, a variety of non-isotopic immunoassays have been developed, some of which have attained sensitivity near the RIAs.

Solid-phase immunoassays are based on the ability of antibody, coated onto polymers, to specifically bind antigen (in the case of a non-competitive assay) or labeled antigen (in the case of a competitive assay). Sephadex-isothiocyanate[11] and poly-tetrafluoroethylene-γ-isothiocyanatostyrene[12] were the first materials to be used as solid supports for immunoassays. However, solid phase immunoassays became more convenient and popular when Catt et al.[12] demonstrated that plastic tubes could be used as the solid supports. Polystyrene and polypropylene tubes were reported to strongly adsorb antibody molecules that specifically bind antigen molecules. Other methods have also been developed where antigens or antibodies are bound covalently to solid supports, such as cellulose, agarose, nylon, glass, and polystyrene microwells. The ease with which proteins may be immobilized by simple physical adsorption to plastic has made this a very popular method. Binding of antibody to polystyrene increases with antibody concentration and time of incubation, and reaches a certain maximum value.[13,14]

Various enzyme immunoassays for hCG have been developed using different solid supports, viz., polyvinylchloride microtiter ELISA plates,[15] filter paper disc (Whatman no. 541) and bacteria (*Staphylococcus aureus*).[16] Joshi et al.[15] were the first to adopt penicillinase as an enzyme label for measuring hCG, which was later utilized by Talwar et al.,[16] who used different solid supports. In view of the heterogeneous nature of hCG,[1–8] specific assays are still required for measuring hCG or hCG-β subunits.

Therefore, in the present study, we developed a simple, sensitive ELISA of hCG for early detection of pregnancy. We used penicillinase for the

preparation of enzyme-conjugate and locally manufactured polystyrene tubes ground from inside as a solid phase.

EXPERIMENTAL

Chemicals and Reagents

Bovine serum albumin (BSA), diethylaminoethyl (DEAE)-Sephacel, Protein-A-Sepharose, thimerosal (Mercury-[(O-carboxyphenyl)thio] ethyl sodium salt), and polyethylene glycol (PEG) were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. Fetal calf serum was procured from Difco Laboratories, Detroit, Michigan, USA. Penicillinase (β-lactamase, type I from *Bacillus cereus*; E.C. 3.5.2.6) and Penicillin V (Phenoxymethyl penicillinic acid) were obtained from Hindustan Antibiotics Ltd, Pune, India. All other chemicals and solvents used were of analytical grade and were purchased from a local manufacturer.

Buffers

Antibody Buffer

50 mM PBS containing 25 mM EDTA, 0.1% NaN_3, 0.2% BSA, lactose, mannitol, and sorbitol, each at a concentration of 50 mg/dL, (pH 7.4).

Assay Buffer

50 mM PBS containing 0.1% BSA, 0.1% NaN_3, (pH 7.4).

Composition of Buffer for Liquid Phase RIA

50 mM PBS containing 50 mM EDTA, 0.005% thimerosal (i.e., merthiolate), pH 7.2.

METHODS

Preparation of Enzyme Conjugates

Preparation of Peak-I(A) hCG-Penicillinase and hCG-β-Penicillinase Conjugates by Two-Step Glutaraldehyde Method[17,18]

Penicillinase, 2.5 mg (400 μg protein) was dissolved in 0.2 mL of 0.1 M phosphate buffer (pH 6.8) containing 1.25% glutaraldehyde. The reaction mixture was allowed to stand at room temperature ($\sim$25°C) for 18 hrs, and

then passed through a Sephadex G-25 column. The fractions having enzyme activity were pooled and concentrated on PM-10 membrane filter to get final volume 1.0 mL. To this, 100 μg of peak-I(A) hCG (i.e., hCG purified from first trimester pregnancy urine in our laboratory) in 1.0 mL of 0.14 M NaCl and 0.1 mL of 1 M carbonate-bicarbonate buffer, pH 9.5, was added. The reaction mixture was kept at 4°C. After 24 hrs, 0.1 mL of 0.2 M lysine solution was added and allowed to stand for 2 hrs. Subsequently, the reaction mixture was dialysed overnight against 0.01 M PBS and the retentate was stored in small aliquots at −20°C. Similarly, hCG-β (from NIH, USA) was also conjugated with penicillinase.

Coating of Polystyrene Tubes with Secondary Antibody (ARGG)

A solution of ARGG, at a concentration of 20 μg/mL, was prepared in double distilled water. Each polystyrene tube was coated with 12 μg of ARGG.

Checker Board Assay

The hCG-β antiserum was diluted in antibody buffer to give 1 : 500, 1 : 1,000, 1 : 2,000, 1 : 4,000, and 1 : 8,000 titers. Then, checker board assay was performed by following the method of Shrivastav et al.,[19] using second antibody coated polystyrene tubes for separating antibody bound label from free. The titers of antiserum and conjugate were chosen on the basis of wide difference in absorbance observed between specific and non-specific binding in the assay. Similarly, checkerboard assay of hCG-β antiserum supplied by NIH, USA, was also performed.

Preparation of Serum Standards and Buffer Standards of hCG

Both serum and buffer standards were prepared from peak-I(A) hCG,[20] whose immunological potency was estimated to be 10,970 IU/mg using international reference preparation (75/589).

Comparison of hCG-β Antiserum Developed in the Laboratory and Antisera Obtained from NIH, USA by RIA (Double Antibody Precipitation Assay)

Liquid phase RIA, using uncoated glass tubes, were performed for this purpose. All tubes contained 100 μL of hCG standards, 200 μL of hCG-β antiserum (1 : 6,000 dilution used for antiserum from NIH, while 1 : 10,000 dilution for antiserum developed in our laboratory, in the respective tubes) and 200 μL of 1 : 100 diluted normal rabbit serum. The contents were mixed and kept at ~25°C. On the second day, only 100 μL of trace, i.e.,

radioiodinated hCG was added to each tube, mixed, and again kept at ~25°C. On the following day, 200 μL of ARGG solution and 200 μL of 5% polyethylene glycol were added to all tubes, mixed, and kept at 4°C for an hour. Following this, tubes were centrifuged and radioactivity was counted in the precipitate using an Electronic Corporation of India (ECIL) counter.

Comparison Between Two Enzyme Conjugates: hCG-β-Penicillinase and Peak-I(A) hCG-Penicillinase

The activity of both conjugates were compared using ELISA.[20]

Standard Curve

The standard curves for both buffer and serum were established by pipetting 100 μL of hCG standards (55.5 mIU to 6.97 IU/mL or 5.05 ng to 635.3 ng/mL), 100 μL of anti-hCG-β (1 : 2,000), 100 μL of Peak-I(A) hCG-penicillinase (1 : 1,200), and 300 μL of assay buffer in ARGG coated tubes. The assay was performed as described elsewhere.[20]

Affinity

The affinity or association constant (k_a) of hCG-β antiserum developed in the laboratory was estimated by Scatchard analysis.[21]

Analytical Recoveries

Recovery experiments were performed in order to determine the accuracy of the assay. Known amounts of hCG, at concentrations of 500 mIU and 5 IU/mL, were added to non-pregnant serum and non-pregnant urine. The recoveries were determined by ELISA in these spiked serum and urine samples and calculated by expressing the net hCG analyzed as a percentage of added hCG.

Intra-Assay and Inter-Assay Variations

Within-assay and between-assay precision of the method was measured by analyzing the amount of hCG present in three different pools having low, medium, and high concentrations of hCG. These pools were prepared separately using serum and urine from pregnant subjects after measuring hCG in each sample by ELISA.

ELISA Procedure of Urinary and Serum hCG from Pregnant Women

The levels of hCG were determined in urine samples and serum samples by using buffer standard and serum standard, respectively. For urinary hCG quantification, 50 μL of buffer standards or 50 μL of urine samples (1 : 35), 100 μL of hCG-β antiserum (1 : 2,000), 100 μL of peak-I(A) hCG-penicillinase (1 : 1,200), and 350 μL of assay buffer were added to ARGG coated tubes. The assay was performed as described elsewhere.[20]

Radioimmunoassay (RIA) Procedure

The RIA of hCG was performed as per published protocol.[22]

RESULTS

Comparison of Performance of hCG-β Antiserum Developed In-House with NIH Antiserum

Standards prepared for RIA were used to compare the antiserum binding characteristics. The displacement curves run in parallel indicate that hCG-β antiserum prepared in-house was comparable in performance with NIH antiserum (Fig. 1).

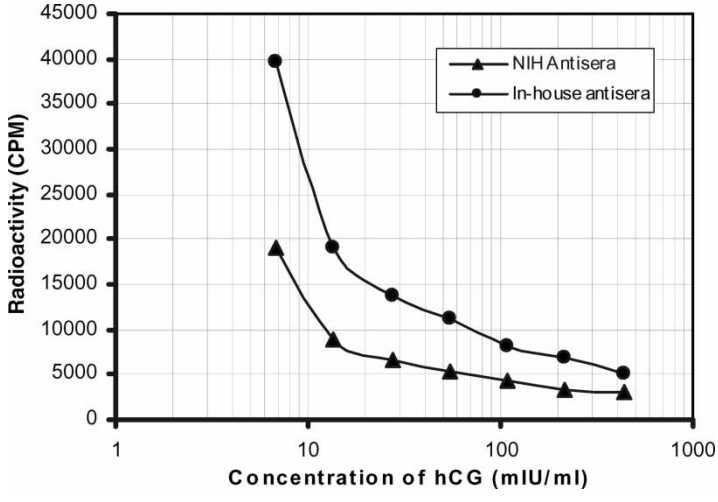

Figure 1. Comparison of standard curves of two different hCG-β antisera by RIA.

Checker Board Assay

The working titer of hCG-β antisera, developed in the present study, was found to be 1 : 2,000 with peak-I(A) hCG-penicillinase showing a titer of 1 : 1,200 and hCG-β penicillinase working at 1 : 600. Using antiserum against hCG-β, obtained from NIH, USA, the dilution of antibody required was 1 : 1,600 with peak-I(A) hCG-penicillinase working at a titre of 1 : 1,200 and hCG-β penicillinase at 1 : 800.

Comparison of Standard Curves of ELISA Using Two Different Conjugates

Peak I(A) hCG and hCG-β subunit (from NIH) were labeled with penicillinase. Using anti-hCG-β antiserum, the conjugates were compared. The range of standard curves varied from 1.0 ng/mL to 880 ng/mL or 10.97 mIU to 9653.6 mIU/mL and both conjugates gave parallel curves (Fig. 2).

Standard Curve

The composite standard curves, plotted on semi log papers, for hCG buffer standard and serum standard are shown in Figs. 3 and 4, respectively. They represent the mean of six assays, conducted separately with duplicate determination of each standard in every assay. The ED_{50} for buffer and serum

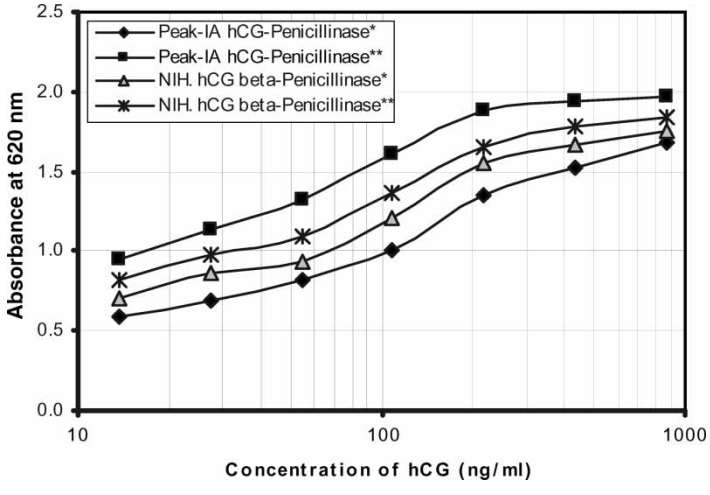

Figure 2. Comparison of standard curves of two different enzyme conjugates or tracers by ELISA. The symbols * and ** indicate that the assays were performed on two different days.

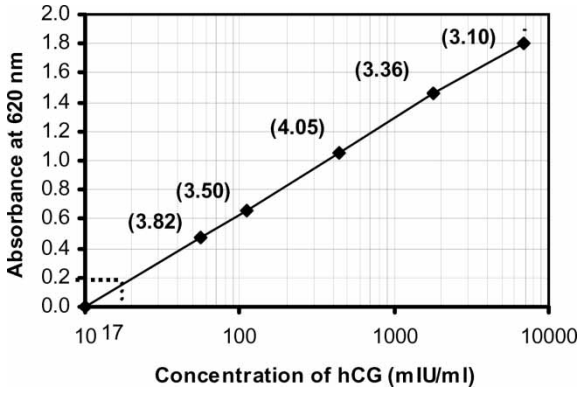

Figure 3. Composite standard curve for hCG buffer standards (from six successive ELISAs of hCG). Figures in the parentheses indicate percent C.V. for absorbance at 620 nm for each value of standard dose. Sensitivity of the assay is 17 mIU/mL, as shown by dashed projection.

standards were found to be 260 mIU/mL and 450 mIU/mL, respectively. The percent coefficient of variation (CV%) for hCG buffer standards ranged from 3.10% to 4.05%, while that of serum standards ranged from 1.15% to 2.76%, indicating that the standard curve obtained over a range of assays remained stable and precise.

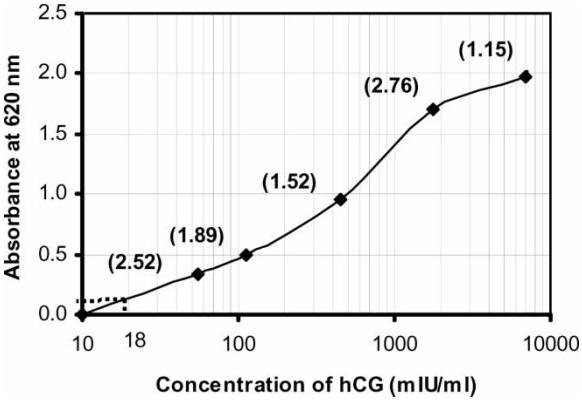

Figure 4. Composite standard curve for hCG serum standards (from six successive ELISAs of hCG). Figures in the parentheses indicate percent C.V. for absorbance at 620 nm for each value of standard dose. Sensitivity of the assay is 18 mIU/mL, as shown by dashed projection.

Affinity

The affinity constant (k_a) of hCG-β antiserum towards hCG was estimated by a Scatchard plot (as shown in Fig. 5) and was found to be 6.074×10^{10} L/mole. The dissociation constant (k_d) was found to be 1.65×10^{-11} moles/liter, suggesting that binding characteristics of the antiserum for an immunoassay are acceptable and provide a sensitive assay.

Sensitivity or Limits of Detection

The detection limit, defined as the concentration equivalent to the reading at zero dose plus two times the standard deviation of the reading at zero dose. The smallest amount of hCG that could be distinguished from '0' dose was 17 mIU/mL in urine and 18 mIU/mL in serum.

Analytical Recoveries

The percentage recoveries of known amounts of hCG added to non-pregnant serum ranged from 90.59–94.28% (for 500 mIU/mL) and 90.59–93.25% (for 5 IU/mL) in solid phase heterogeneous competitive ELISA, while the percentage recoveries of non-pregnant urine ranged from 95.13–95.8% (for 500 mIU/mL) and 98.4–99.3% (for 5 IU/mL) (Table 1).

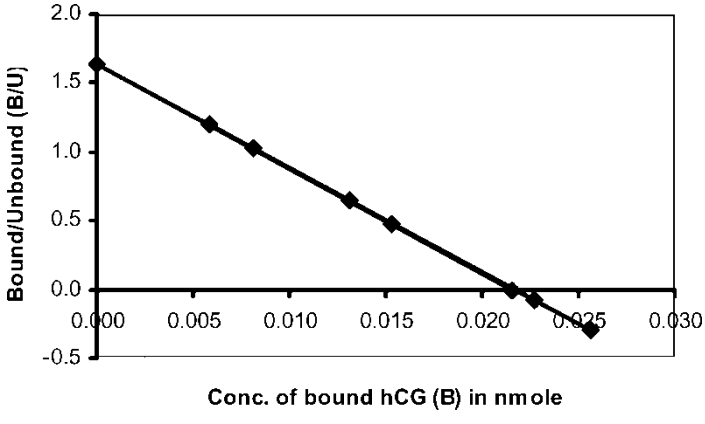

Figure 5. Determination of affinity (equilibrium constant, k_a) of the hCG-β antiserum by radioimmunoassay using Scatchard plot. The k_a is derived from the slope of the line, i.e., k_a = Y-intercept/X-intercept. The concentration of bound hCG (B), in nanomoles per litre, is plotted on the X-axis. The ratio of bound/free (B/U or B/F), using linear regression analysis, is plotted on the Y-axis.

Table 1. Recoveries of hCG added to serum and urine

Concentration of hCG added (IU/mL)	Recovery by ELISA (%)	
	In non-pregnant serum	In non-pregnant urine
500 mIU/mL[a]	92.61 ± 9.7	95.52 ± 2.7
5 IU/mL[a]	92.29 ± 9.1	98.7 ± 4.1

[a]Each concentration assayed 6 times and data are mean ± S.D of 6 replicates.

Intra-Assay and Inter-Assay Variations

Serum and urine from pregnant subjects were pooled separately into three different concentrations of hCG. In ELISA, each pool was analysed six times within the assay and in seven separate assays. The precision profiles appeared to be within acceptable limits of 10% (Tables 2 and 3).

Comparison of ELISA with RIA for hCG values

The values of hCG were measured in 100 serum samples and 250 urine samples by ELISA and by an in-house RIA method. Regression analysis of samples yielded the following equation:

$$Y(\text{ELISA}) = 1.01 \times (\text{RIA}) - 1.3$$

$$r = 0.98(\text{For serum samples})$$

$$r = 0.97(\text{For urine samples})$$

The values obtained by ELISA and RIA were again tested by the Mann-Whitney U test procedure. These two methods were identical ($Z = -1.1$, $p < 0.05$).

DISCUSSION

The heterogeneous competitive ELISA for hCG reported in the present study has good accuracy, sensitivity, specificity, and reproducibility for practical clinical use. It has a sensitivity comparable to RIA procedures available so far. This ELISA procedure does not suffer from disadvantages that other methods for serum and urinary hCG estimation have, e.g., bioassays are expensive and time consuming; radio receptor assays, although rapid, are not as sensitive and cannot distinguish LH from hCG; and radioimmunoassay,

Table 2. Precision profile of ELISA for pooled serum

		POOL-S1		POOL-S2		POOL-S3	
Experiment no.	n	Mean ± SD (IU/mL)	CV (%)	Mean ± SD (IU/mL)	CV (%)	Mean ± SD (IU/mL)	CV (%)
Intra-assay							
I	6	1.99 ± 0.187	9.39	21.49 ± 1.974	9.18	40.37 ± 3.80	9.41
II	6	2.02 ± 0.191	9.45	21.16 ± 1.265	5.98	40.73 ± 3.37	8.27
III	6	2.30 ± 0.194	8.43	20.40 ± 0.474	2.32	38.80 ± 3.50	9.02
IV	6	2.45 ± 0.199	8.12	20.96 ± 1.417	6.76	38.79 ± 3.41	8.79
V	6	2.58 ± 0.189	7.32	21.38 ± 1.954	9.14	39.33 ± 3.06	7.78
VI	6	1.97 ± 0.193	9.79	21.35 ± 2.10	9.83	39.75 ± 2.39	6.02
VII	6	1.99 ± 0.185	9.29	21.22 ± 1.92	9.05	39.71 ± 2.78	7.00
Inter-assay							
Experiments	7[a]	2.18 ± 0.197	9.03	21.14 ± 0.367	1.74	39.64 ± 0.737	1.86

CV = Coefficient of variation.

n = No. of times same sample analysed for intra-assay variation.

[a]No. of times assay carried out for inter-assay variation.

Table 3. Precision profile of ELISA for pooled urine

		POOL-U1		POOL-U2		POOL-U3	
Experiment no.	no	Mean ± SD (IU/mL)	CV (%)	Mean ± SD (IU/mL)	CV (%)	Mean ± SD (IU/mL)	CV (%)
Intra-assay							
I	6	3.95 ± 0.360	9.29	13.80 ± 1.28	9.27	38.68 ± 1.17	3.02
II	6	3.64 ± 0.208	5.71	15.81 ± 1.42	8.98	37.60 ± 0.876	2.33
III	6	3.67 ± 0.307	8.36	15.51 ± 1.37	8.83	37.66 ± 1.824	4.85
IV	6	3.90 ± 0.36	9.23	16.01 ± 1.41	8.81	37.26 ± 2.572	6.90
V	6	3.64 ± 0.284	7.80	15.67 ± 1.082	6.90	37.33 ± 2.229	5.97
VI	6	3.94 ± 0.38	9.64	16.09 ± 1.39	8.64	37.40 ± 3.267	8.74
VII	6	3.63 ± 0.269	7.41	15.68 ± 1.089	6.94	37.61 ± 2.851	7.58
Inter-assay							
Experiments	7*	3.77 ± 0.153	4.06	15.51 ± 0.78	5.03	37.65 ± 0.479	1.27

although very sensitive, requires expensive instrumentation and stringent safety precautions for handling radioactive materials.

In the present study, locally manufactured polystyrene tubes (ground from the inside) were coated with polyclonal antiserum against hCG-β, developed within the laboratory, through an immunochemical bridge using ARGG-anti-hCG-β. The performance of antibody developed within the laboratory was compared with hCG-β antibody obtained from NIH, USA, in an RIA system. The displacement curves obtained for hCG with both antibodies are comparable.

Most of the hCG enzyme immunoassays reported to date used β-D-galactosidase,[23] alkaline phosphatase,[24] and horse-radish peroxidase[25] as the marker-enzymes. The reasons for selecting penicillinase in this ELISA system were its very high turnover number (1,200,000 as against 6,600 for HRP and 3,500 for ALP), easy availability of the enzyme and substrate (produced in India by M/s Hindustan Antibiotics Ltd, Pimpri, Pune, India), stability at temperatures prevalent in the tropics (in dry condition, stable at 40°C for up to 2 years); simplicity of determining its activity (disappearance of blue colour of starch iodine complex in presence of substrate and enzyme); and absence in biological fluids. An added advantage is its low molecular weight (28,000 Da), so that relatively small amounts need to be used to achieve a necessary molar ratio during the process of labeling the analyte. Therefore, an ELISA for hCG was developed using this enzyme as marker.

Since the development of enzyme immunoassay,[26] only three groups of researchers[15,16,27] have reported the development of an hCG ELISA using penicillinase as the enzyme label. Being a pioneer, Joshi et al.[15] achieved a sensitivity of 500 mIU/mL, while Talwar et al.[16] have reported a sensitivity of 5 ng/mL. Joshi et al.[15] used hCG antiserum and Talwar's group[16] utilised a monoclonal antibody specific for hCG. Using a chromogenic redox substrate system for β-lactamase, Bieniarz et al.[27] reported a sensitivity of 5 mIU/mL ($\sim$25 fmol/mL) utilizing two monoclonal antibodies in a sandwich immunoassay. The sensitivity of 17.0 mIU/mL (with buffer standards) and 18.0 mIU/mL (with serum standards) obtained in the present method used peak-I(A) hCG, as standard, which was standardised against WHO, International reference preparation 75/589. The assay also utilized hCG-β antiserum. This could be further improved if a sandwich assay with monoclonal antibodies is developed. Almost parallel standard curves were obtained using peak-IA hCG-penicillinase and hCG-β penicillinase as the enzyme labels, suggesting that both enzyme labels are equipotent in the system.

In the present study, peak-I(A) hCG-penicillinase conjugates have been prepared using a two-step glutaraldehyde method. It is accepted that hCG levels in normal pregnant women, beyond detection limits of the present assay system, i.e., 17.0 mIU/mL (with buffer standards) and 18.0 mIU/mL (with serum standards) can be accurately measured by this method.

The ELISA developed in the present study may accurately detect only normal hCG or hCG-β present in normal pregnancy serum or urine. But, its ability to detect an hCG molecule with some alterations in the carbohydrate moieties at hCG-β C-terminus, which are found in urine of patients with choriocarcinoma,[28] is not ascertained. Its ability to detect an hCG molecule with polypeptide nicks needs to be investigated.

A minimum of 17 mIU of hCG/mL of urine and 18 mIU/mL of serum can be detected easily and the test can be completed within 3 hours with good precision. Accurate negative results are obtained in urine samples of normally ovulatory women, postmenopausal women, and adult males, suggesting that the physiologic concentrations of leutinizing hormone do not influence the assay results.

In summary, data obtained in the present study demonstrated that this newly developed hCG ELISA is a sensitive, rapid, and reliable method for detecting urinary hCG and diagnosing early pregnancy. By using a more specific hCG antiserum, the sensitivity and specificity can be further improved.

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