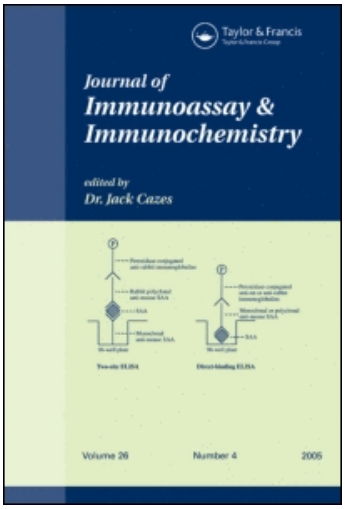

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Development of a Highly Sensitive Enzyme Linked Immunosorbent Assay for Human Serum Progesterone using Penicillinase

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DEVELOPMENT OF A HIGHLY SENSITIVE ENZYME LINKED IMMUNOSORBENT ASSAY FOR HUMAN SERUM PROGESTERONE USING PENICILLINASE

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ABSTRACT

A simple, highly sensitive, heterologous, competitive, penicillinase based tube ELISA for human serum progesterone has been developed. Specific antisera against progesterone was raised in rabbits using 11 α - hydroxyprogesterone hemisuccinate-BSA. Progesterone-3-CMO was coupled to penicillinase by the carbodiimide method. The standard curve covered a wide range from 5 pg/tube to 800 pg/tube. The sensitivity of the assay was 3.8 pg/tube. The intrassay coefficient of variation ranged from 8.6 % to 11.2 % at low concentration while at medium and high concentrations it ranged from 5.3% to 8.9%. The interassay coefficient of variation for the low, medium and high concentrations of progesterone were 9.6%, 10.7% and 6.5% respectively. Parallelism between the standard curve and the sample dilution curve indicated accurary of the assay. Samples measured by RIA and ELISA showed an excellent correlation (r=0.98).

(KEY WORDS: ELISA, Penicillinase, Progesterone)

INTRODUCTION

Progesterone, a sex steroid hormone is elaborated by the corpus luteum, placenta and adrenal cortex. The level of plasma progesterone rises from 1.3 - 6 nmol/l in the follicular phase to a level of 6 - 63 nmol/l in the luteal phase of the human menstrual cycle (1). The level of this post ovulatory rise is taken as a biochemical parameter of ovulation and reflects the adequacy of the luteal phase.

The indications for specific assay of progesterone are to document ovulation and to assess luteal phase insufficiency. Recent papers highlight the importance of single serum progesterone estimation in the diagnosis of abnormal pregnancy (2-4).

Radioimmunoassay (RIA) remains a standard procedure for quantitation of progesterone. However, the disadvantages associated with the use of radioactive materials necessitated the development of immunoassays using alternative label. In fact, progesterone has been estimated by enzymeimmunoassay (5-11), chemiluminescence immunoassay (12-15) and fluoroimmunoassay (16-17). Among the enzymes, β-galactosidase (5), alkaline

phosphatase (11) and horse radish peroxidase (6-10) have been utilized in the development of enzymeimmunoassay for plasma progesterone. However, solid phase enzymeimmunoassay required microtitre plates and ELISA reader which are either expensive or not available everywhere. Current efforts are to opt for an assay system which requires simpler instruments. We have chosen penicillinase as a marker because of its absence in the biological fluids, high turnover number and stability at 40° C for 2 yrs (18) . In addition to its use in ELISAs for some hormones (19-24), the enzyme has also proved to be useful in the serodiagnosis of filariasis (25), toxoplasmosis (26), amoebiasis (27) and in the detection of plant viruses (28). The quantitative ELISA for hormones utilizing penicillinase, however, suffers a major drawback. The method envisages transfer of the products formed from each well of the microtitre plate to a separate tube containing starch iodine solution for the final color development. We report, here, for the first time a simple and highly sensitive enzyme linked immunosorbent assay (ELISA) using penicillinase as the enzyme marker for quantitation of serum progesterone which obviates the necessity of any transfer of solution.

MATERIALS AND METHODS

Marterials

Penicillinase (EC 3.5.2.6), 1-ethyl-3(3 dimethyl aminopropyl)carbodiimide hydrochloride, phenoxymethylpenicillinic acid (pencillin V) and progesterone were purchased from Sigma Chemical Company, St. Louis, USA. Progesterone was recrystallized thrice before using as a standard. Sephadex G-25 (super fine) and dextran T-70 were obtained from Pharmacia Fine Chemicals, Uppsala , Sweden. Iodine (cryst, pure, AR for TLC), 1-4 dioxane (for HPLC) and ethyl ether (for HPLC) were procured from Spectrochem Pvt. Ltd., Bombay, India. High density semitransparent polypropylene tubes of 5 ml capacity were obtained from Sarad Biochemicals, Bangalore, India. Radioimmunoassay was performed using reagents supplied by the W.H.O. RIA Matched Reagents Programme.

Reagents

Assay buffer : 1% BSA in 10 mM phosphate buffersaline (PBS), pH-7.0 containing 0.1% sodium azide.

Washing buffer : 0.2 M PBS, pH-7.0

Substrate buffer : 0.2 M sodium phosphate buffer pH -7.2

Substrate solution : 1mM penicillin V in substrate buffer

Iodine reagent : 2% crystalline iodine in 3.2M potassium iodide in distilled water.

Starch Iodine Solution : 1.12 ml iodine reagent in 200 ml starch solution (30ml 2% starch solution mixed with 190 ml distilled water)

Methods

Generation of progesterone antiserum

Specific antisera against progesterone were generated by immunising New Zealand strain albino rabbits against 11 α -hydroxyprogesterone hemisuccinate-BSA. An emulsion of this immunogen was prepared in Complete Freund's Adjuvant. Primary immunisation was done by injecting 500 ug of the emulsified immunogen per rabbit intradermally with the same dose at monthly intervals. A total of sixteen rabbits were immunised at different time intervals. The antibody titre was checked after three booster injections. Seven rabbits responded after a few boosters by producing desired antibody

titre of 30% - 50% binding at 1:10,000 initial dilution. The remaining rabbits even after repeated boosters either yielded low titre antibody or died. The bleeds of one of the rabbits having antibody titre of 41 % - 60 % binding at 1:10,000 dilution were subjected to cross reaction analysis. The results indicated a cross reactivity of only 0.98 % with 17α-hydroxyprogesterone at 50 % displacement. Other related steroids at 50% displacement exhibited less than 0.2 % cross reaction (Fig. 1). This antiserum was pooled, aliquoted and kept at -20°C for the assay.

Preparation of the Progesterone-penicillinase Conjugate

Progesterone-3-CMO was coupled to penicillinase following the carbodiimide method of Mattox et al with some modifications (29). Briefly, 2-3 ug of 1-ethyl-3 (3 dimethyl aiminopropyl) carbodiimide/100 ul of distilled water was mixed to 2 mg of progesterone-3-CMO/100 ul of dioxane and kept at room temperature for 30-40 minutes. The activated steroid was then added to a tube containing 200-250 ug of penicillinase/250 ul of distilled water.The reaction mixture was kept at 4°C for 14-16 hours. The conjugate,thus prepared, was

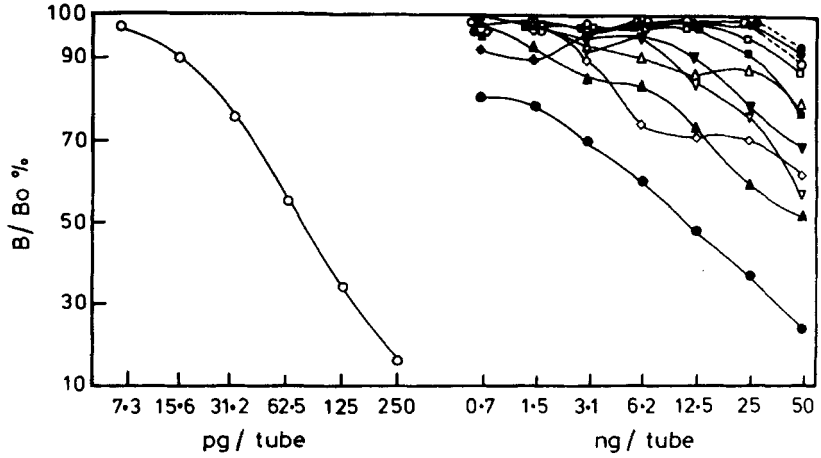

Figure 1
Cross Reaction of the Progesterone Antiserum with Related Steroids. Progesterone (o—o), 17α-hydroxy progesterone (●—●), Cortisol (△—△), Testosterone (▲—▲), Estrone (■—■), Estradiol (□—□), Estriol (▽—▽), 5α-dihydrotestosterone (▼—▼), 4-androstene-dione (◇—◇), 5-androstene 3β,17β diol (◆—◆), 5 androstane 3β,17β diol (o--o), 5 androstane 3β, 17β diol (●--●).

purified by passing through sephadex G-25 column (10 cm x 1.5 cm). The equilibration of the column and the elution of the conjugate were done with 10 mM PBS (pH - 7.0). Fractions containing peak activity were pooled to which sodium azide and BSA were added to a final concentration of 0.1 % and 1% respectively. The conjugate was aliquoted in small volumes and stored at -20°C until use.

Collection of Samples

Blood was collected by venepuncture from otherwise normal healthy males and females of different ages. Serum was separated and stored at -20° C until analysis with sodium azide to a final concentration of 0.1%.

Procedure for ELISA

High density polypropylene tubes used as a solid support were coated with 0.4 ml progesterone antiserum diluted in distilled water and kept overnight at room temperature. The tubes were washed thrice with distilled water. To block the free sites, 0.5 ml of assay buffer was added and the tubes incubated for two hours at 37° C. The tubes meant for the total optical density (TOD) and non specific binding (NSB) were omitted from first coating with the antiserum. To perform the assay, a constant amount of the conjugate in 0.2 ml of assay buffer and 0.2 ml of increasing concentrations of the steroid ranging from 25 pg/ml to 4000 pg/ml or 0.2 ml of serum diluted to 1:10 in assay buffer (subjected to heat denaturation at 60° C for 30 minutes in a separate set of glass tubes) were added to the tubes previously coated with the antiserum. The TOD tubes contained 0.4 ml of assay buffer

while NSB tubes contained 0.2 ml of assay buffer and 0.2 ml of the conjugate. The reaction was allowed to occur at room temperature (R.T.) for 18-20 hours after which the contents were decanted and the tubes washed four times with the washing buffer. The time of incubation with the conjugate and progesterone could be reduced to 2 hours by increasing the temperature to 37°C instead of R.T. . Freshly prepared cold substrate solution in a volume of 0.5 ml was then dispensed rapidly in all the tubes and incubated at 37°C for one hour. Finally, the colour was developed by addition of 1 ml of starch iodine solution to each tube. The reaction was terminated by 1 ml of 5 (N) HCl added to all the tubes. Absorbance was recorded at 570 nm in a Milton Roy spectrophotometer (model 1201).

Radioimmunoassay

All the samples used in ELISA were subjected to radioimmunoassay as per the W.H.O. RIA protocol. The samples were extracted with diethyl ether and the residue was dissolved in RIA buffer prior to RIA.

RESULTS

Standard curve

The composite dose response curve of twelve different experiments for the penicillinase-

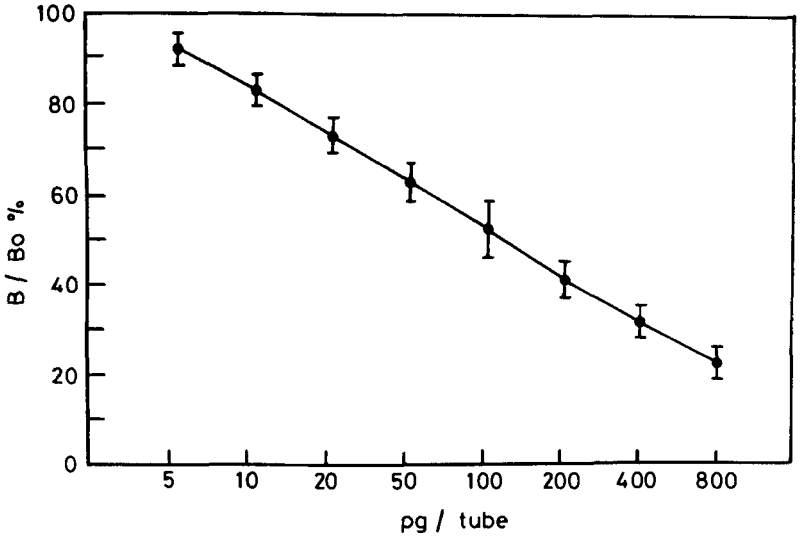

Figure 2
Composite Standard Curve of ELISA for Progesterone (n = 12). B is the amount of labelled progesterone bound to the antibody in the presence of unlabelled progesterone (Bn-Bs)while Bo is the amount of labelled progesterone bound to the antibody in the absence of unlabelled progesterone (Bn-Bz) where Bn, Bs and Bz are the optical density (O.D.) for the nonspecific binding, standard and zero tubes respectively.

-labelled immunosorbent assay for progesterone is shown in Fig.2. The concentration of the hormone ranged from 5 pg/tube (25 pg/ml) to 800 pg/tube (4 ng/ml). The sensitivity of the assay as evaluated by the least amount of the steroid distinguishable from zero concentration at the 95 % confidence limit was 3.8 pg/tube. In contrast, the same antiserum in

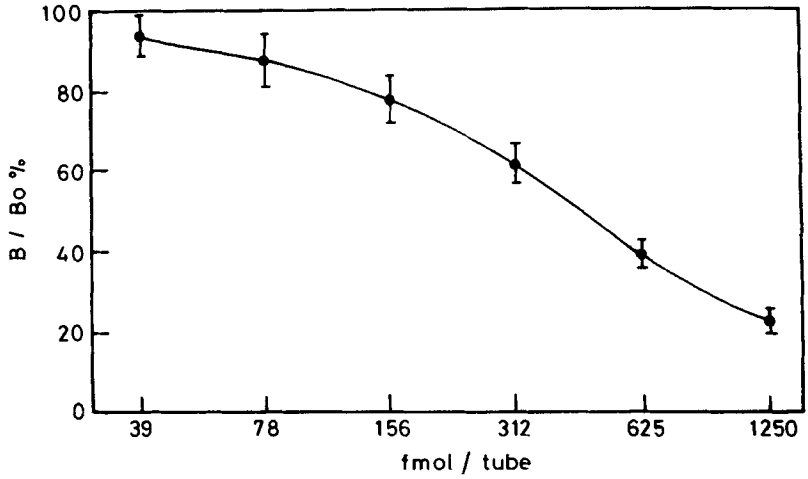

Figure 3
composite Standard Curve of Radioimmunoassay
(n = 8)

radioimmunoassay had a sensitivity of 37.5 fmol/tube (11.8 pg/tube) (Fig.3).

Intraassay and Interassay variation

Human serum samples containing low, medium and high concentrations of progesterone as evaluated by RIA were combined separately to form three pools, each of which was further subdivided into six smaller pools. These subpools belonging to low, medium and high concentrations were taken into eight replicates and subjected to quantitative analysis by the ELISA at different times. Table 1 shows the

TABLE 1

Precision Profile

Expt. No.	Low		Medium		High	
Intraassay	Mean ± SD (ng/ml)	CV%	Mean ± SD (ng/ml)	CV%	Mean ± SD (ng/ml)	CV%
1.	0.94 ± 0.1	10.6	5.98 ± 0.5	8.3	21.7 ± 1.7	7.9
2.	1.16 ± 0.1	8.6	5.22 ± 0.4	7.6	19.1 ± 1.0	5.2
3.	1.04 ± 0.1	9.6	5.64 ± 0.5	8.8	20.1 ± 1.8	8.9
4.	1.06 ± 0.1	9.4	5.63 ± 0.3	5.3	19.1 ± 1.7	8.9
5.	1.16 ± 0.1	8.6	6.34 ± 0.5	7.8	21.4 ± 1.8	8.4
6.	0.89 ± 0.1	11.2	4.69 ± 0.4	8.5	18.4 ± 1.4	7.6
Interassay						
	1.04 ± 0.1	9.6	5.58 ± 0.6	10.7	19.9 ± 1.3	6.5

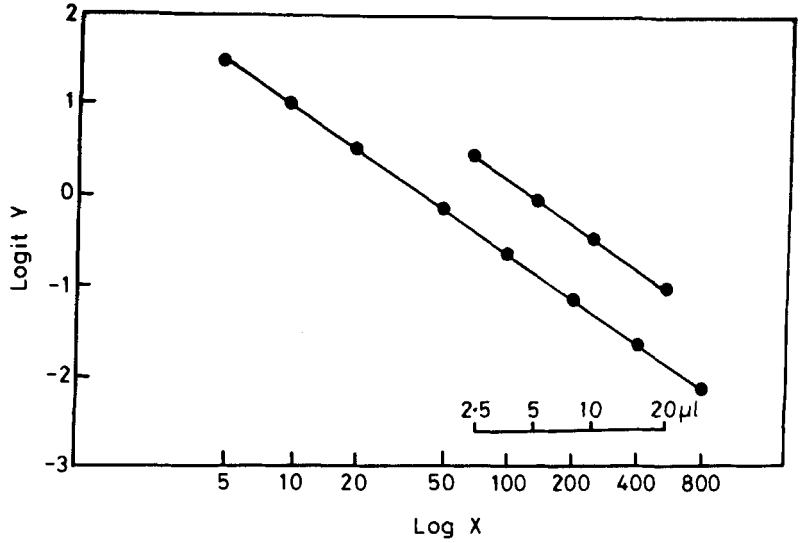

Figure 4
Parallelism between the Standard Curve and Sample Dilution Curve (Logit-Log transformation)

results of these experiments with the intra and inter assay variations. The intraassay coefficient of variation ranged from 5.3 % to 11.2% while the interassay coefficient of variation ranged from 6.5% to 10.7% for the low, medium and high concentrations of progesterone.

Accuracy

Accuracy was checked by the linearity of the data obtained by assaying various dilutions of the sample and the parallelism with the standard curve (Fig.4).

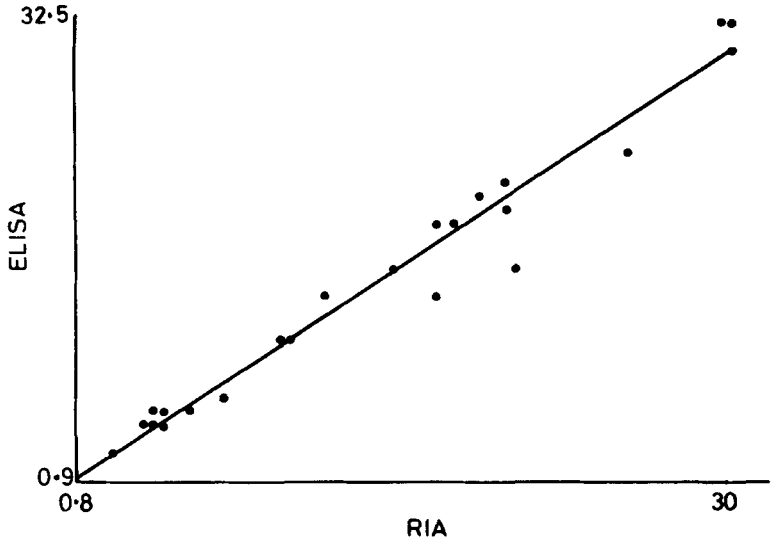

Figure 5
Correlation between RIA and ELISA
Y=1.019x+0.052 ; r=0.985

Correlation coefficient

The validity of the assay was further investigated by comparing the values of unknown samples obtained by the conventional RIA with the ELISA. The results showed a very high degree of correlation (r = 0.98) between the two methods as shown in Fig.5.

DISCUSSION

Radioimmunoassay is used as a sensitive and standard procedure for the measurement of

circulating hormones. However, current efforts are being directed towards the development of simpler and cheaper methods of detection eliminating the hazards of handling radioisotopes. We have used penicillinase as the enzyme marker for the detection of progesterone because of its attractive advantages over that of the other enzymes.

The assay is very sensitive and measures progesterone directly from the serum samples without any prior extraction of the steroid. The heterologous combination of the antiserum and the penicillinase labelled conjugate as well as the choice of the enzyme itself may have contributed to the increased sensitivity of the assay. Sauer et al have recently reported that out of the four enzymes used in a heterologous system, consistent improvement in the assay sensitivity was observed in the order of penicillinase> peroxidase> alkaline phosphatase>β-galactosidase and with penicillinase, the sensitivity generally exceeded that of RIA (30,31). Moreover, the present method is free from two major disadvantages of using HRP as a label (i) the inhibition of the enzyme by sodium azide, a common sample preservative and (ii) the substrate

Table 2

Results of one Enzyme Linked Immunosorbent Assay

No.		O.D.	Average	B/Bo %
1.		0.698		
2.	Bo	0.735	0.731	
3.		0.760		
4.		0.823		
5.	5 pg/tube	0.951	0.889	91.43%
6.		0.895		
7.		1.053		
8.	10 pg/tube	1.002	1.004	85.19%
9.		0.958		
10.		1.115		
11.	20 pg/tube	1.132	1.128	78.47%
12.		1.138		
13.		1.383		
14.	50 pg/tube	1.334	1.337	67.13%
15.		1.296		
16.		1.515		
17.	100 pg/tube	1.555	1.539	56.18%
18.		1.547		
19.		1.794		
20.	200 pg/tube	1.743	1.77	43.65%
21.		1.773		
22.		1.980		
23.	400 pg/tube	2.011	1.977	32.42%
24.		1.940		
25.		2.133		
26.	800 pg/tube	2.108	2.123	24.81%
27.		2.129		
28.		2.571		
29.	NSB	2.579	2.575	
30.		2.673		
31.	TOD	2.689	2.681	

ortho- phenylene diamine being photosensitive and mutagenic.

The consistency of the present tube ELISA is quite satisfactory as evident by the consolidated displacement analysis of twelve assays (Fig.2) and the intra & inter assay coefficient of variation being almost within permissible limits (32). The accuracy of the assay is further elaborated by the parallelism obtained between the sample dilution curve and the standard curve (Fig. 4).

Finally, the values of human samples collected randomly from males and females at different phases of the menstrual cycle and evaluated by RIA as well as ELISA showed an excellent correlation indicating validity of the present assay (Fig. 5). Also, the difference in absorbance between Bo and NSB is 1.84 making the assay workable over a wide range (Table 2).

The added attraction of the assay is that progesterone can be measured directly from the serum without any prior extraction.This ELISA obviates the necessity of the use of an ELISA reader as the readings are taken in a spectrophotometer. It is an important feature of this assay as the method could be exploited widely.

On the basis of these results, we believe that a simple and highly sensitive tube ELISA for quantitation of serum progesterone may be an alternative to RIA.

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