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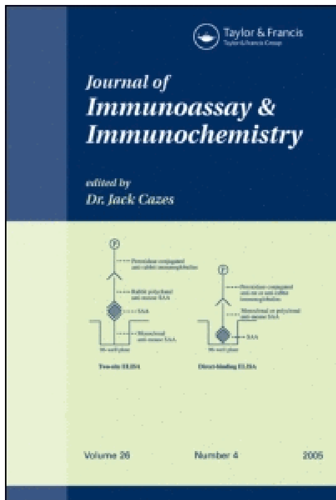

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
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To cite this Article Jose, Sophy M. , Moodbidri, Sudhir B. and Sheth, Anil R.(1992) 'An Enzyme-Linked Immunosorbent Assay for the Detection of Human Seminal Plasma Inhibin', Journal of Immunoassay and Immunochemistry, 13: 1, 127 — 139
To link to this Article: DOI: 10.1080/15321819208019828
URL: http://dx.doi.org/10.1080/15321819208019828

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JOURNAL OF IMMUNOASSAY, 13(1), 127-139 (1992)

AN ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE DETECTION OF HUMAN SEMINAL PLASMA INHIBIN

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(**KEY WORDS** : Inhibin, ELISA, Urine, Seminal plasma)

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ABSTRACT

A simple and sensitive enzyme-linked immunosorbent assay (ELISA) is described for the measurement of inhibin in urine and seminal plasma. Standards used cover a range from 50 ng to 0.05ng/0.1 ml with a detection limit of 0.098 ng/0.1 ml. Coefficients of variation for intra-assay precision and for inter-assay precision were obtained and compared favourably with RIA. Given the ease of application, this technique is an useful alternative to existing radioimmuno-assays for this peptide.

INTRODUCTION

Inhibin belongs to a family of protein hormones that specifically inhibits the release of follicle stimulating hormone from the

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pituitary in mammals (1,2). The determination of inhibin secretion is a helpful parameter in the study of reproductive disorders (3,4) and in various cancers (5-8). A radioimmunoassay for inhibin (isolated from human seminal plasma, HSPI) has been developed in our laboratory (10). However in order to avoid disadvantages inherent in the application of radioisotopes, this ELISA was developed. This is an inhibition assay in which the antigen is coated to polystyrene plate and the antibody is reacted with increasing amounts of soluble antigen in a test solution. Presence of antigen in the test solution is quantified in relation to the amount of inhibition occurring when the pretreated antibody is allowed to react with the coated antigen in the plate.

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MATERIALS AND METHODS

Samples :

Urine samples were collected from healthy laboratory personnel and from patients suffering from benign prostatic hypertrophy. They were stored at -20°C until the time of assay without adding any preservative. Semen samples were collected from patients attending a fertility check clinic. Seminal plasma was separated from sperm cells by centrifugation at 3000 rpm for 10 mins and was stored frozen until use.

Chemicals :

Biotinylated antibodies and Avidin-Biotin Complex (ABC) were obtained from Vector Laboratories Inc., Burlingame, CA.

Bovine serum albumin (BSA) and orthophenylene diamine (OPD) were purchased from Sigma Chemical Company, St. Louis, MO. Tween-20 was purchased from Hi Media, India. All other reagents were of analytical grade.

Buffers and Solutions :

Coating buffer was a sodium carbonate buffer (0.05 M) pH 9.6.

Incubation buffer (PBS) consisted of sodium phosphate buffer (0.1M) pH 7.4 containing 0.14M NaCl and 0.1% BSA.

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As washing solution, isotonic saline with 0.05% Tween-20 was used.

Substrate was dissolved in 0.1M phosphate and 0.05 M citrate buffer, pH 5, containing 0.8 g/l of OPD and 2.5 ml of 30% H_2O_2 W/V per litre.

Microtitre plates (Maxisorp F 16 4 - 69914) with 96 flat bottom wells were purchased from Nunc, Roskilde, Denmark.

Antigen :

Inhibin was isolated from human seminal plasma as reported earlier (9). Same preparation was used for coating the plate and as the standard. Standard concentrations ranged from 50 ng to 0.049 ng per 0.1 ml as a series of double dilutions.

Antisera :

Antibodies to inhibin were raised in rabbits by active immunization by an already established procedure (10). This antiserum is routinely used in our laboratory for RIA and the same antiserum was used for ELISA.

ELISA :

ELISA was performed by an Avidin Biotin amplified method as follows. All wells of a 96 well plate except two 'air blanks' were filled with antigen solution (50 ng/100 µl/well) and incubated overnight at 4°C . They were then emptied, tapped onto filter paper and washed three times with washing solution. To saturate any potential binding sites not already occupied by the protein, 2% BSA in PBS was then added to each well and incubated at 37°C for 1 hr. The plates were washed again three times. The concentration of antibody and coating antigen was determined by checker board titration. An antigen concentration below the plateau region was chosen because sensitivity was better at lower concentrations of coating antigen and the antigen was less likely to bring down the antigen antibody complexes in solution in the equilibrium state (11). Measurements were performed in duplicate and a standard ELISA curve was included in each plate to counter any between plate variation. Samples or standards were preincubated with antibody for one hour and were added to the wells. The plate was incubated for 2 hrs. This being an inhibition assay, it was necessary to incubate

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the primary antibody with antigen (to allow the primary reaction to occur) before adding the antigen-antibody mixture to wells coated with antigen. Urine and seminal plasma were tested in duplicate at a 1:20 and 1:40,000 final dilution respectively in PBS containing 0.1% BSA. The plates were washed three times and 1:2000 diluted biotinylated antibody (100 μl, Anti Rabbit IgG) in incubation buffer was added and allowed to incubate for half an hour. Plates were washed again and 100 μl of 1:1000 dilution of pre-formed Avidin-Biotin Complex (1:1, Avidin DH and Biotinylated Horse radish peroxidase-H) was added. Plates were washed and 100μl of substrate solution was added. After 15 min incubation in the dark at room temperature, the reaction was stopped with 25 μl of 4N H_2SO_4. An ELISA reader (Titertek, Multiscan MC, Flow Laboratories Inc., MC Lean, VI, USA) was used to measure the absorbance at 492 nm.

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Precision and Accuracy of the ELISA :

Intra-assay (on the same plate) and inter-assay (on different days) variations were obtained for three samples. The accuracy of the ELISA was determined by adding a known amount of purified inhibin to 1:5 diluted urine followed by measurement of the inhibin concentration in the urine.

RIA of Inhibin :

RIA was performed according to the method reported by Vaze et al (10).

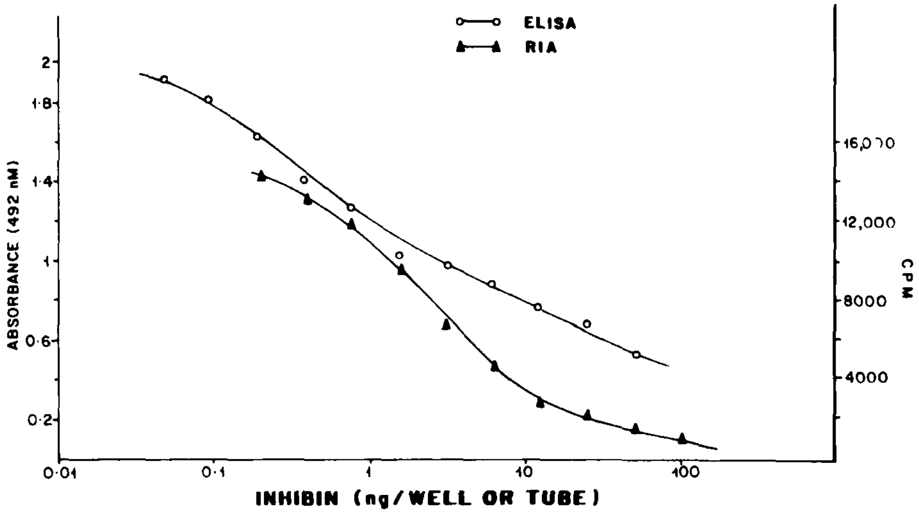

Fig. 1 : Standard curve for inhibin immunoassay (ELISA- absorbance, RIA-cpm is plotted versus inhibin concentration in ng/well or tube on semi log scale paper)

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RESULTS

Standard Curves :

Standard curves for RIA and ELISA obtained from the same dilution series of standard inhibin are shown in fig.1. When the coefficients of variation of the absorbance values at various points on the binding curve were calculated for assays performed on 5 different days, it was found to vary between 3-15%, for ELISA. The coefficient of variation was thus lower at 0.049 ng/0.1 ml as compared to 50 ng/0.1 ml. ELISA was thus more accurate and reproducible at the lower concentration of the standard curve. Non specific binding was 0.124 ± 0.016 absorbance units for 10 assays.

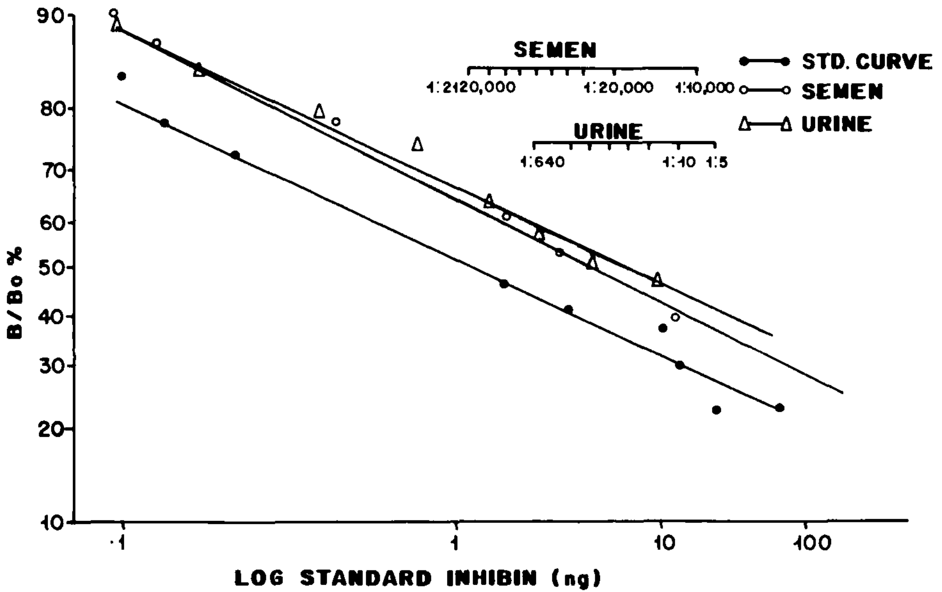

Figure 2 : Parellism between standard curve and sample dilution curve (sample : 1 : 5 urine diluted in a series of double dilutions; semen : 1 :10,000 semen diluted in a series of double dilutions)

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Sensitivity, (lower detection limit) defined as the content of inhibin detected in the 2 SD range of inhibin free sample was found to be 0.098 ng/ 0.1ml.

Precision and Accuracy :

The CV for the intra-assay precision was calculated from 10 replicates of 3 samples and was found to be 13.2%. Inter-assay precision was calculated by coefficient of variation of 3 urine samples on five days and was found to be 9%. In recovery experiments, urine was spiked with 320, 160, 120, 80, 50 and 30

Table 1

Various hormones and peptides checked for cross-reactivity in ELISA of inhibin

Hormone/Peptide	Source	Concentration at which cross-reactivity was checked and found to give no reaction
Oxytocin	synthetic	5 µg - 0.1 µg
Gastrin 1	human (synthetic)	5 µg - 0.1 µg
Vasoactive intestinal peptide	human (Synthetic; fragment 1-12)	5 µg - 0.1 µg
Secretin	porcine (synthetic)	5 µg - 0.1 µg
Luteinising hormone	human	1.5 µg ; 0.5 µg
Follicle Stimulating hormone	human	1.5 µg; 0.5 µg
Prostate specific antigen	human	1 µg; 0.5 µg
Prolactin suppressing factor	human	1 µg; 0.5 µg
Substance P	synthetic	1 µg; 0.5 µg
Fibroblast growth factor	bovine (synthetic; fragment 1-24)	1 µg; 0.5 µg
β-endorphin	human (synthetic; fragment 61-91)	1 µg; 0.5 µg

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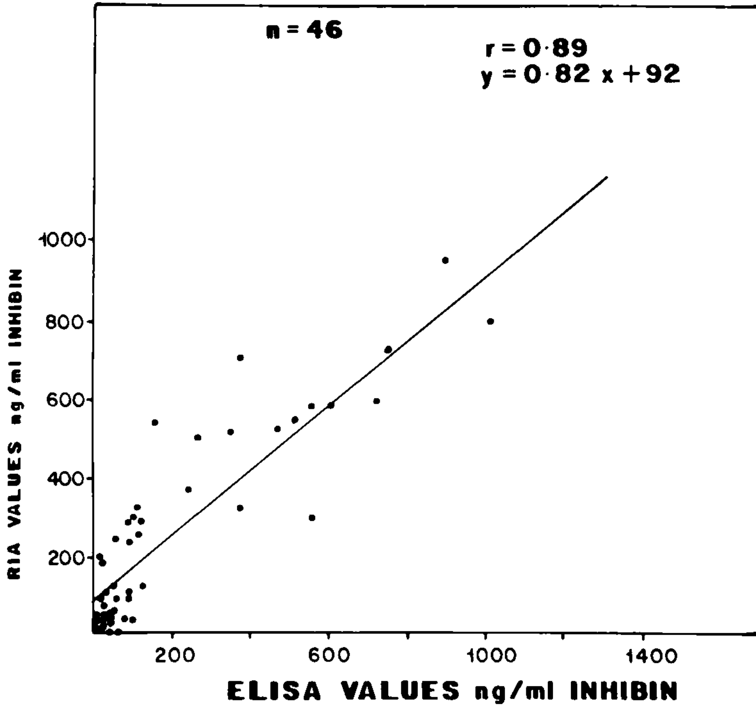

Fig. 3 : Correlation between ELISA and RIA of urine samples

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ng/ml inhibin. Analytical recoveries of the added inhibin ranged from 94.5% to 110%.

Dilution tests were carried out for both urine and semen samples. The response curve thus obtained was compared with that for standard inhibin. Both the curves (semen and urine) were parallel to the standard inhibin curve, their slopes being 0.49; 0.51; 0.43 respectively (Fig. 2).

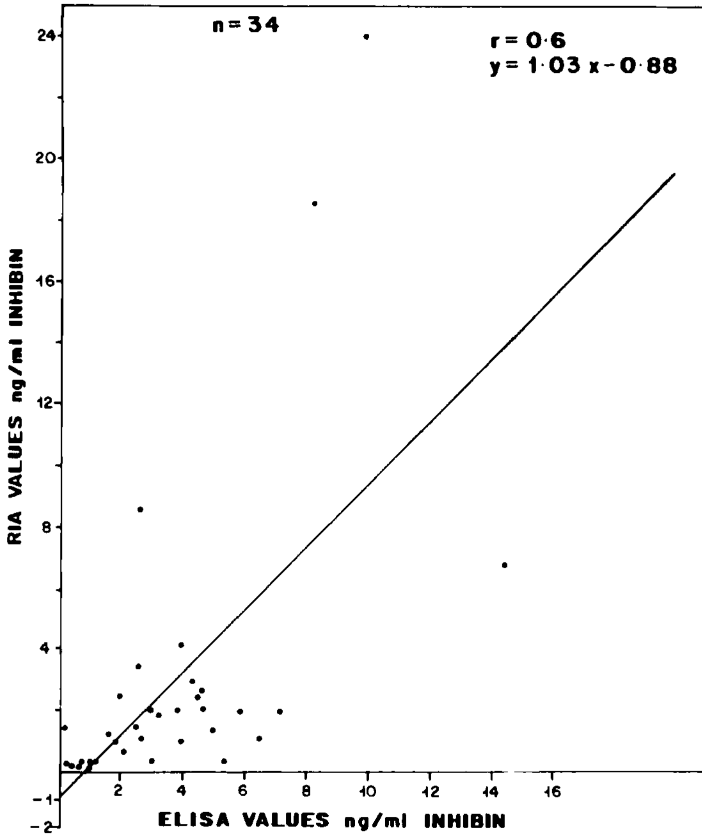

Fig. 4 : Correlation between ELISA and RIA of semen samples

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Specificity :

The cross-reactivity of the antiserum with several proteins was investigated. The assay results were all negative indicating that the antiserum was specific for inhibin (Table 1).

Correlation between ELISA and RIA :

The correlation between ELISA and RIA for 46 urine samples of widely differing inhibin concentrations measured at fixed

dilution is shown in Fig. 3. The coefficient r was 0.89 ($P < 0.001$). 34 semen samples were also compared by both ELISA and RIA. But semen samples showed only moderate correlation ($r=0.6$) though it was significant at $p < 0.001$ (Fig. 4). There were two semen samples that showed elevated levels of inhibin by RIA. These samples were also elevated as estimated by ELISA but were not as high as RIA. This may be due to low precision of ELISA at high concentrations.

DISCUSSION

The results of these studies demonstrate the specificity and sensitivity of the ELISA developed in our laboratory for detection of inhibin in urine and seminal plasma. A highly significant correlation coefficient of $r = 0.89$ ($n = 46$) between values determined by enzyme immunoassay and RIA for urine samples demonstrate the reproducibility of both the techniques. However, there was only moderate correlation for semen samples ($r=0.6$, $p < 0.001$) by the two methods though it was statistically significant. Also, samples containing high concentrations of inhibin could be diluted to fall on the linear portion of the standard curve without loss of precision. ELISA is more convenient to perform because of the stability of the components which are not subject to decay in the same way as a radioactive isotope. Also the hazards of handling radioactive material are eliminated. It is quicker, as reading of a microtitre plate requires less than a minute as compared to RIA which takes a minimum of 2 hours for handling and counting the

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same number of samples. The rapidity and simplicity of the above described ELISA would thus make it a convenient alternative to RIA in elucidating the role of inhibin in reproductive pathophysiology.

ACKNOWLEDGEMENTS

The Senior Research Fellowship awarded to SMJ by Department of Biotechnology is gratefully acknowledged. The authors also wish to thank Mrs. Shyamala Nair for her skilful secretarial assistance.

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