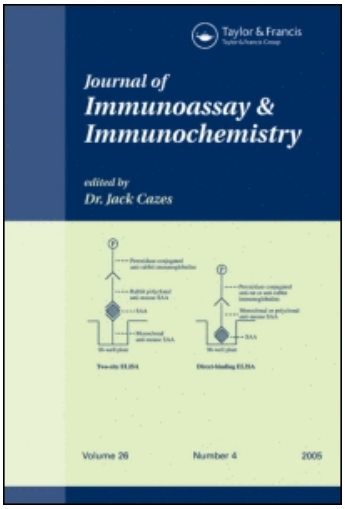

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Importance of Selection of Separation System in the Development of Enzyme Immunoassay: An Experience with Follicle Stimulating Hormone (FSH) Assay

Meena P. Desai[a]; M. Ikram Khatkhatay[a]; Govind M. Sankolli[a]; Usha M. Joshi[ab]
[a] Institute for Research in Reproduction (ICMR), Bombay, INDIA [b] Deputy Director, Institute for Research in Reproduction (ICMR), Bombay, INDIA

To cite this Article Desai, Meena P. , Khatkhatay, M. Ikram , Sankolli, Govind M. and Joshi, Usha M.(1991) 'Importance of Selection of Separation System in the Development of Enzyme Immunoassay: An Experience with Follicle Stimulating Hormone (FSH) Assay', Journal of Immunoassay and Immunochemistry, 12: 1, 83 — 98
To link to this Article: DOI: 10.1080/01971529108055058
URL: http://dx.doi.org/10.1080/01971529108055058

JOURNAL OF IMMUNOASSAY, 12(1), 83-98 (1991)

IMPORTANCE OF SELECTION OF SEPARATION SYSTEM IN THE DEVELOPMENT OF ENZYME IMMUNOASSAY : AN EXPERIENCE WITH FOLLICLE STIMULATING HORMONE (FSH) ASSAY.

Meena P. Desai, M. Ikram Khatkhatay, Govind M. Sankolli and Usha M. Joshi
Institute for Research in Reproduction (ICMR),
Jehangir Merwanji Street, Parel,
Bombay 400 012, INDIA.

ABSTRACT

An antiserum to follicle stimulating hormone (FSH) obtained as a gift from National Institute of Health (NIH), U.S.A. could not be adsorbed on microtitre ELISA plates, although two other FSH antisera raised in authors' laboratory could be adsorbed. A good precision profile for the FSH assay using these three antisera could be achieved with only one separation system viz. solid phase anti rabbit gamma globulin (ARGG), out of the five separation systems tried. The study suggests that a few antisera used for radio-

Address Correspondence to :

Dr.U.M.Joshi,
Deputy Director,
Institute for Research in Reproduction (ICMR),
J.M.Street,
Parel,
Bombay 400 012,
INDIA.

immunoassay (RIA) purposes may not by themselves get adsorbed on plastic plates. However, they could be effectively used for ELISA purposes using solid phase second antibody.

[KEY WORDS : Enzyme Immunoassay (EIA), Follicle Stimulating Hormone (FSH), Separation System, Solid Phase Anti Rabbit Gamma Globulin (ARGG)].

INTRODUCTION

Competitive enzyme linked immunosorbent assay (ELISA) using penicillinase as a marker and microtitre ELISA plate as a solid phase with primary antibody passively adsorbed on it have been described by us for the estimation of various reproductive hormones and their urinary metabolites (1-3). With the availability of antiserum to human follicle stimulating hormone (FSH) from National Institute of Health (NIH), U.S.A. through Indo-U.S. Subcommission Program, we decided to develop a similar ELISA for FSH. However immobilization of this antiserum to ELISA plate either by passive adsorption or through covalent linkage could not be achieved. In this paper, we describe our experience in designing competitive ELISA for FSH with this NIH antiserum using alternate separation system. Further, we demonstrate the influence of different separation systems in the performance of the assay with three different antisera.

MATERIALS AND METHODS

Antisera

Antiserum (Code A) : NIADDK-anti-hFSH-6 was obtained from NIH, U.S.A.

Antiserum (Codes B & C) : $^{1}FSH_{1}$ and $^{2}FSH_{1}$, respectively were generated at our Institute using radio-iodination grade human FSH for immunization of rabbits.

Chemicals and Reagents

Pure FSH iodination grade hormone, antiserum to FSH was obtained from NIH, U.S.A. through the Indo-U.S. Sub-Commission Program. First International Reference Preparation (1st IRP, Code 70/45) for urinary FSH and LH was obtained from National Institute for Biological Standards and Control, Holy Hill, Hampstead, London and Pergonal (75 IU FSH and 75 IU of LH) was purchased from Serono Laboratories and was used as a secondary standard. Enzyme penicillinase (E.C.3.5.2.6) specific activity 66,000 Pollock units per mg protein and its substrate Penicillin V were supplied to us by Hindustan Antibiotics Limited, Pune, India. Glutaraldehyde 25% was obtained from E.Merck, Germany; soluble starch from Reanal, Budapest, Hungary and bovine serum albumin and lysine from Sigma Chemicals Co., St.Louis, U.S.A. Skimmed Milk powder was purhcased from National Dairy Development Board (NDDB) Government of Maharashtra, Bombay, India.

Heat killed protein A bearing Staphylococcus aureus were obtained from Microbiology Department of K.E.M. Hospital, Bombay. Polystyrene, Polyvinyl chloride, Immulon-1 and Immulon-2 microtitre ELISA plates were obtained from Dynatech Inc., Japan. Titertek polyvinyl chloride, high activated microtitre ELISA plates were purchased from Flow Laboratories, Finland. Sheep anti-rabbit gamma globulin (ARGG) was raised at our Institute. All other chemicals and salts used in this study were of analytical grade and available locally.

Buffers

Coating buffer 50 mmol/L sodium carbonate-bicarbonate pH 9.5.

Immunoassay buffer 100 mmol/L sodium phosphate buffer, pH 7.2 (PBS) containing 154 mmol/L sodium chloride and 0.1 g/dl BSA.

Washing buffer PBS containing 0.05% Tween-20.

Starch-iodine Penicillin V Substrate Solution (SIP):

To 3.8 mg of Penicillin V, 25 ml of starch iodine solution (prepared by adding 0.2 ml of 18 mmol/L iodine in 3325 mmol/L potassium iodide in distilled water, to 100 ml of 200 mmol/L sodium phosphate buffer pH 7.0 and 30 ml of 20 g/l hydrolysed starch solution) was added.

FSH-Penicillinase Conjugate

FSH-Penicillinase conjugate was prepared by Glutaraldehyde method as described earlier (4).

Assay Protocols

1. Passive adsorption system

200/ul of suitably diluted antisera in coating buffer were dispensed in wells of microtitre ELISA plates. Plates were kept at 4-8°C for 16-18 hrs and washed thrice with washing solution. To duplicate wells of the plate, 100/ul of immunoassay buffer (0 standard), standards or excess standard (NSB) and 100/ul of conjugate was added and plates incubated at 37°C for 2 hrs. Plates were washed and enzyme activity was determined in the bound fraction as described earlier (4).

2. Covalent linkage system

Microtitre polystyrene ELISA plates were pretreated with 0.2% (v/v) glutaraldehyde in 100 mM sodium phosphate buffer, pH 5.0 for 4 hrs at room temperature (± 28°C) (5). The plates were washed and incubated with suitably diluted antisera in 100 mmol/L sodium phosphate buffer pH 8.0 for 3 hrs at 37°C. Later the plates were washed and blocked with 100 mmol/L lysine in sodium phosphate buffer, pH 8.0 for 1 hr at 37°C after which

the plates were wahsed and competitive immunoassay carried out as described above.

3. Liquid phase ARGG system

The WHO protocol for Radioimmunoassay (6) was followed in which enzyme label was added instead of iodine-label. After centrifugation, enzyme activity in the bound fraction was estimated as follows :

To the pellet, 1 ml of SIP was added. After incubation at room temperature for 30 mins, 0.5 ml of 5 M HCl was added and the readings were taken on a spectrophoto -meter at 620 nm.

4. Bacterial separation system : (S.aureus)

To 100 µl of diluted antisera in a tube, 100 µl of buffer or standard and 100 µl of FSH-Penicillinase conjugate was added and incubated for 24 hrs at 4-8°C. Later, 100 µl of bacteria (1:300) was added in all the tubes and incubated for 45 mins at room temperature (28 ± 2°C). The tubes were centrifuged for 30 mins at 800 g after adding 2 ml of cold PBS (20 ± 2°C). Supernatant was decanted off, sides of the tube wiped and 0.6 ml of SIP was added. After incubation at room temperature for 40 mins the content of the tubes were read on spectrophotometer at 620 nm.

5. Solid phase ARGG system

The assay protocol as described earlier was followed (4). Briefly, standard and antiserum were incubated in tube for 1 hr at 37°C followed by addition of enzyme label and further incubation for 1 hr at 37°C. Solid phase ARGG was prepared using PVC plates. Aliquot of immunoassay mixture transferred to ARGG plate and left overnight at 4°C. Enzyme activity on the plate was determined as mentioned before.

RESULTS

The optimum dilutions of antisera and conjugate for various separation systems tried are given in Table I which were selected by checker board titration, using the following criteria (i) Maximum difference between the reading of total binding ("0" standard) and excess standard (NSB) wells. (ii) Minimum O.D. difference between SIP blank and reading corresponding to excess standard (NSB). Antiserum A could not be immobilized by passive adsorption or by covalent linkage to any of the ELISA plates tried. However, the two other antisera could be adsorbed through both the procedures on PVC as well as polystyrene plates.

Figure 1 gives the representative standard curves obtained with the three antisera using various separation systems, the plastic plates referred to in

TABLE - 1

Optimum dilutions of antisera (Ab) and conjugate (Ag*) used

Separation Systems	Ab-A	Ag*	Ab-B	Ag*	Ab-C	Ag*
Passive adsorption	-	-	1 : 10K	1 : 1.5K	1 : 20K	1 : 1.5K
Covalent linkage	-	-	1 : 15K	1 : 1.5K	1 : 25K	1 : 1.5K
Liquid Phase ARGG	1 : 18K	1 : 5K	1 : 10K	1 : 2K	1 : 20K	1 : 2.5K
Bacterial System	1 : 5K	1 : 0.5K	-	-	-	-
Solid Phase ARGG	1 : 20K	1 : 1.5K	1 : 20K	1 : 1K	1 : 40K	1 : 2.5K

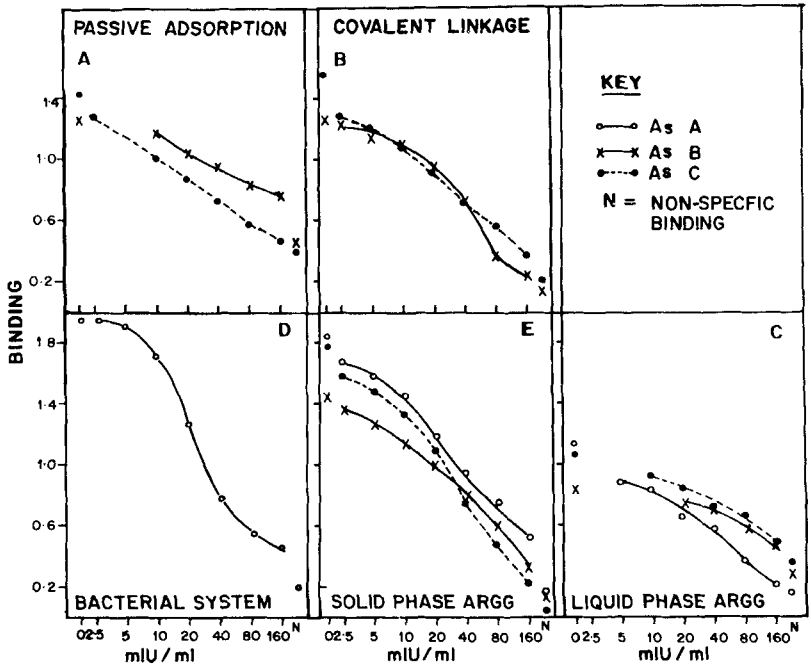

Fig.1. : Representative standard curves with the 3 antisera and 5 separation systems. The binding was calculated as difference beteeen O.D.'s of substrate blank (SIP) and individual standards.

this figure being Dynatech PVC plates. The graph depicts the extent of binding (represented by difference between O.D. of SIP and O.D. of individual standards). In the case of passive adsorption system, non-specific binding of conjugate to the plate was high as shown by difference between SIP blank and reading corresponding

to NSB. This difference ranged between 0.65 - 1.0 for antiserum B and 0.4 - 0.8 for antiserum C in 5 different assays (28-32% of the total binding). The representative standard curves were flat with poor discrimination between the consecutive standard points (Figure 1A). The non-specific adsorption of conjugate could not be reduced by blocking with 3% skimmed milk solution. Hence these assays were considered unsatisfactory for routine use. As compared to the passive adsorption system, NSB in case of covalent linkage method (Figure 1B) was low (15% of the total binding), the standard curve was steeper (-2.3 Vs -1.8, based on logit-log plot of composite standard curves) and assay more sensitive (2.5 mIU - 3.0 mIU/ml Vs 5.5 mIU - 11.0 mIU/ml), sensitivity calculated as described earlier (7).

The liquid phase ARGG system (Figure 1C) was also unsatisfactory as the assay sensitivity with all the 3 antisera was poor (10 - 15 mIU/ml) and the NSB was high (20-25% of total binding). With the bacterial (S.aureus) separation system (Figure 1D), the sensitivity of the assay was good 2 - 2.8 mIU/ml, NSB was low (11% of total binding) with better discrimination between individual standards. However, due to the inconsistent supply of bacteria this system could not be tried with the other two antisera viz. B and C. The assays developed using

solid phase ARGG (Figure 1E) as separation system were found to be the best as sensitivity was high (1.9 mIU/ml - 2.2 mIU/ml) with low NSB (6% of total binding) with all the 3 antisera.

Figure 2 gives the precision profiles of the three antisera with the different separation systems tried. Each point represents the coefficient of variation (C.V.) of triplicates used in the assay. As can be seen the C.V. was $< 15\%$ throughout the standard curve range in only two separation systems viz. solid phase ARGG (for antisera A and C) and bacterial systems. For antiserum B, C.V. was low except for the lowest standard.

DISCUSSION

Most of the current EIA's employ solid phase method for the separation of bound from the free because it eliminates centrifugation and automates the assay. Plastic microtitre plates, tubes and flat surfaces are widely used as solid phase since immunoglobulins (IgG) can be easily adsorbed onto it through hydrophobic interactions between non polar IgG's and non polar matrix (8,9). While developing an ELISA for FSH, our efforts were initially directed towards immobilization of FSH antiserum onto microtitre ELISA plates.

Of the 3 antisera used in the study immunosorbents could be prepared with antisera B and C, but not with

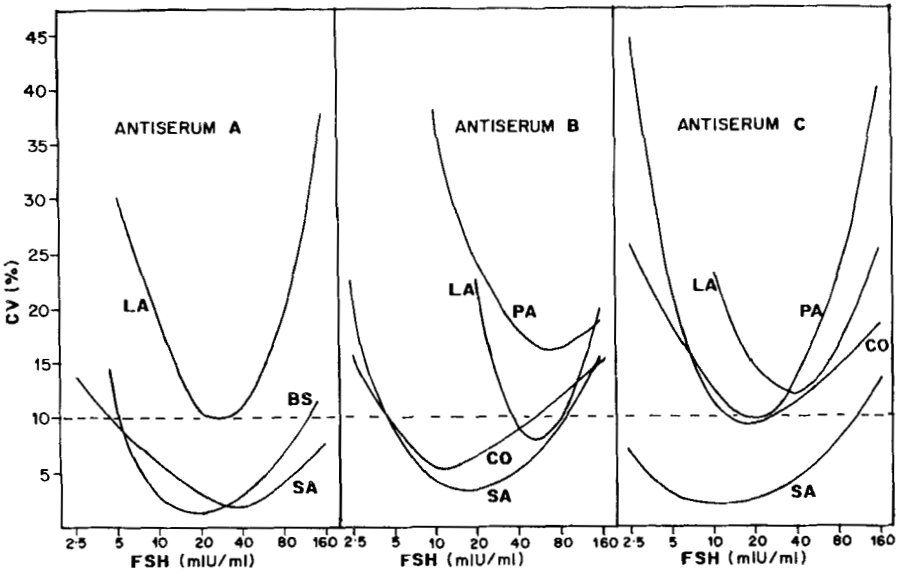

Fig.2. : Precision profile (C.V. V/s. dose) of three antisera and five separation systems. PA : Passive adsorption; CO : Covalent linkage; LA : Liquid phase ARGG; BS : Bacterial system and SA : Solid phase ARGG. C.V. represents variation in estimated concentration of triplicates at each dose. The (----) line represents generally accepted C.V. limit for immunoassays.

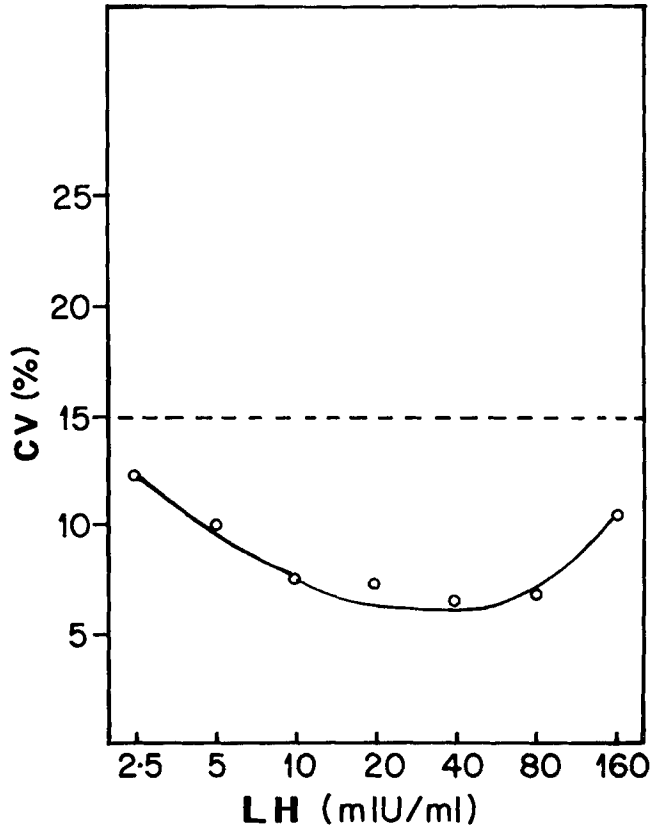

Fig.3. : Precision profile of LH assay, data derived from five assays.

antiserum A. As the antiserum (A) was provided as 1:25 diluted serum in buffer containing 2% NRS, it was thought that NRS components, present in higher proportion than specific IgGs to FSH, were getting preferentially adsorbed. However, it was interesting to find that immunosorbent with LH antiserum provided in the identi-

cal form could be prepared and the resultant assay systems gave excellent precision profile (Figure 3). Since plastics have a limited binding capacity for IgGs ($\cong$ 2 µg/microtitre well), an attempt was made to increase the binding capacity by using coupling agents such as glutaraldehyde. Whereas with 2 antisera (B and C) the effective titre of the antibody increased, immunosorbent could not be prepared with antiserum A. Thus, it can be concluded that antiserum A loses its immunological activity on adsorption, hence preparation of immunosorbent is not possible.

The criteria for an ideal assay methodology includes high specificity, a wide standard curve range with a good discrimination (O.D. reading) between two consecutive standards, steeper slope, desired sensitivity, precision and reproducibility of results obtained. It should also have minimum non-specific binding (10,11). Of the 3 different antisera and various separation systems tried, it is obvious that sensitive and precise assays could be developed with solid phase ARGG system (Figure 2). However, the assay developed using this system and antiserum C, was found to be the best.

We therefore suggest that while developing in-house ELISAs with commercially available reagents, it should be noted that it may not be possible to prepare

the immunosorbent with every antiserum. When immunosorbent cannot be prepared this way, alternative separation systems should be tried. Further, we recommend solid phase ARGG system as precision profile obtained with such system is the best amongst other commonly used systems described in the study.

ACKNOWLEDGEMENTS

We thank NIH, U.S.A. for the supply of the FSH antiserum (Code A) and Iodination Grade Hormone FSH through the Indo-U.S. Subcommision Programme. We also thank Dr.V.Raghavan for anti-rabbit gamma globulin, Mr.D.K.Pardhe for technical assistance and Mrs.Annette Fonseca for typing the manuscript.

REFERENCES

1. Joshi, U.M., Shah, H.P., Sankolli, G.M., Khatkhatay, M.I. Development and application of penicillinase linked EIA in reproductive medicine. In : Eng, A.S. Garcia, P. (eds.). Clinical Biochemistry - Principles and Practice. Organizing Committee, Second Asian Pacific Congress of Clinical Biochemistry, Singapore, Pgs.257-262, 1983.

2. Shah, H.P. and Joshi, U.M. A simple, rapid and reliable enzyme linked immunosorbent assay for measuring estrone-3-glucuronide in urine. J. Steroid. Biochem. 1982; 16 : 283-286.

3. Khatkhatay, M.I., Sankolli, G.M., Meherji, P.K., Gogate, J., Choudhary, V., Joshi, U.M. Application of penicillinase linked ELISA of pregnanediol glucuronide for detection of ovulation and assessment of corpus luteum function. Endocrinol. Japonica 1987; 34 : 465-472.

4. Desai, M.P., Khatkhatay, M.I., Sankolli, G.M., Meherji, P.K., Joshi, U.M. Enzyme labelled immunoassay for urinary gonadotropins using penicillinase. Clinica Chimica Acta 1989; 184 : 315-322.

5. Tijssen, P. Practice and theory of Enzyme Immunoassays. In : Laboratory Techniques in Biochemistry and Molecular Biology. Publisher Elsevier, 1985.

6. Sufi, S.B., Donaldson, A., Jeffcoate, S.L. Method Manual - Programme for the provision of matched assay reagents for the radiommunoassay of hormones in reproductive physiology. WHO Special Programme of Research, Development and Research Training in Human Reproduction, Geneva 1986.

7. Ekins, R.P., Newman, G.B., O'Riordan, J.L.H. Saturation assays. In : McArthur, J.W., Colton, J. (eds.). Statistics in Endocrinology, M.T.P.Press, Cambridge, Massachusetts, 1970; 345-378.

8. Parson, G.H., Jr. Antibody coated plastic tubes in radioimmunoassay. In : Langane, J.L., Vunakis, H.V. (eds.). Methods in Enzymology, New York, Academic Press 1981; 73 : 224-239.

9. Catt, K., Tregear, G. Solid-phase radioimmunoassay in antibody coated tubes. Science 1967; 158 : 1570.