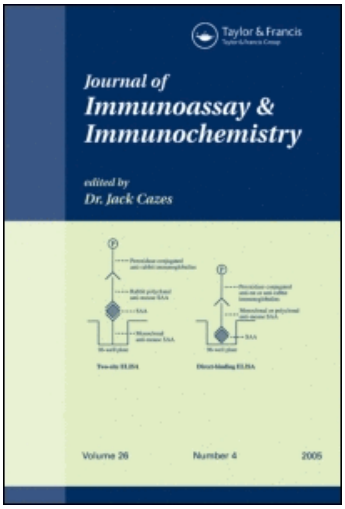

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Immunoradiometric Assay (IRMA) for Human Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) Using Common Avidin Solid Phase

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ABSTRACT

This paper describes the use of avidin-biotin interaction as an affinity system, wherein avidin immobilized magnetizable particles (cellulose) are used as a common separation system in immunoradiometric assay (IRMA) for hormones of the human reproductive system, human follicle stimulating hormone (FSH), and luteinizing hormone (LH). Biotinylated probe was prepared by biotinylation of specific

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monoclonal antibody for respective antigen using the caproyl derivative of biotin *N*-hydroxysuccinimide. The detector antibody for the respective antigen was radiolabelled with ^{125}I by a chloramine-T oxidation method and purified by gel filtration. In the IRMA procedure, standard/sample, respective biotinylated, and radiolabelled antibody as a single reagent, and avidin solid phase were added simultaneously to the assay tubes. After incubation for 3 h with shaking, the bound complex was quantitated for its radioactivity associated with the common avidin solid phase. Results showed that the developed assay protocol is applicable to IRMA of FSH and LH with good precision (intra and inter assay CV less than 8% and 11%, respectively), good assay range (0–200 mIU/mL) and analytical recovery (87–110%). The assay could detect 0.5 mIU/mL and 0.9 mIU/mL of FSH and LH, respectively, and showed good correlation with commercially available kits (FSH $y = 0.98x + 0.21$ and LH $y = 0.99x + 0.18$).

Key Words: Avidin-Biotin interaction; Biotinylation; FSH; LH bicarbonate buffer; pH 8.4.

INTRODUCTION

The avidin-biotin interaction has been used for many years in a variety of different applications[1–3] due to the high affinity constant of biotin with avidin or streptavidin ($K = 10^{15}$ L/M). The use of the avidin-biotin interaction can be exploited in two different ways: as an affinity system or as a detection system.[4] Here, we have demonstrated the use of avidin-biotin interaction as an affinity system.

Immunoassays such as radioimmunoassay (RIA) and immunoradiometric assay (IRMA) are now widely used for assaying a variety of biologically important substances in body fluids.[5,6] An essential step in these procedures is the separation of immune complex from the reaction mixture. The choice of the separation system determines the precision and, thereby, the sensitivity, reproducibility, batch size, and cost of the analysis. In the IRMA system, the capture antibody is linked to an inert solid support. Inert support in the form of polystyrene/polypropylene tubes is, by far, the most preferred. However, success of such systems depends on the availability of uniformly sized and good quality tubes, an efficient method for immobilization, bulk quantity of antibody, and an automation facility for the manufacture of such coated tubes, even on a moderate scale.[7–9] Magnetizable particles (microcrystalline cellulose-coupled iron oxide) thus afford a less expensive and convenient alternative to the plastic tubes.[10,11]

This paper describes various steps in the development of an IRMA for human FSH and LH using an avidin-biotin conjugated system, where avidin coupled magnetizable cellulose serves as a common solid support for FSH and LH IRMA. This assay system depends on the formation of an immune complex, on avidin coupled magnetizable cellulose.

EXPERIMENTAL

Chemicals and Buffers

Affinity purified monoclonal antibodies for FSH and LH hormones were procured from commercial sources. Magnetizable cellulose was prepared in-house. Standards for FSH and LH of known potency and calibrated against WHO reference preparation were obtained from Immunometrics Ltd. (UK). 1,1′-carbonyldiimidazole (CDI), bovine serum albumin (BSA), purified egg white avidin, biotinamidocaproate *N*-hydroxysuccinimide ester and Tween-20 were procured from Sigma chemical Co. (USA). Sephadex G-100 was from Pharmacia (Sweden). ^{125}I was procured from NEN (USA). Packard automatic gamma counter (USA) was used for measuring the radioactivity. All other chemicals used were of AR grade from local companies in India.

Radiolabelling of the Detector Antibodies

Radiolabelling of detector antibodies was carried out by the conventional chloramine-T oxidation method.[12] 50 μL (50 μg) of monoclonal antibody dissolved in 0.05 M phosphate buffer, pH 7.4 was mixed with 60 μL of 0.5 M phosphate buffer, pH 7.4 and 7–10 μL (~ 750 μCi) of Na ^{125}I followed by the addition of 10 μL (10 μg) chloramine-T dissolved in 0.05 M phosphate buffer, pH 7.4. The reaction was stopped after one minute by the addition of 500 μL of 0.05 M phosphate buffer pH 7.4, 20 μL (20 μg) of sodium metabisulphite and 50 μL (250 μg) of potassium iodide in 0.05 M phosphate buffer, pH 7.4. Purification of radiolabelled product was performed by gel electrophoresis using a Sephadex G-100 column (35 × 1 cm) using 0.05 M phosphate buffer as the eluant. The ^{125}I labelled purified fractions were selected, pooled, appropriately aliquoted, and stored at −20°C. At the time of assay, the aliquot was reconstituted to obtain approximately 1.5×10^5 CPM for 100 μL with 50% counter efficiency.

Standards

The standards for FSH and LH of known potency, obtained from commercial sources, were used. The standard hormone from the stock vial was dissolved in the buffer according to the manufacturer's recommendation. A known amount of this standard was suitably diluted in horse serum and calibrated against the respective WHO reference preparation (83/575 and 80/552 for FSH and LH respectively). Serial dilutions ranging from (0–200) mIU/ml for both analytes were made using the above mentioned calibrated standard, aliquoted and stored frozen at −20°C.

Preparation of Avidin Solid Phase

Avidin was coupled to magnetizable cellulose particles using a 1,1′-carbonyldiimidazole (CDI) activation method with slight modification of the method recommended by SCIPAC.[13] 1 g of the magnetizable cellulose particles were washed thoroughly with DDW and activated for 1 h using 400 mg of CDI dissolved in acetone. The activated cellulose was washed thoroughly with acetone, followed by 0.1 M bicarbonate buffer, pH 8.4 to remove excess of CDI and then particles were suspended in the same buffer. 20 mg of avidin, dissolved in 0.1 M bicarbonate buffer, pH 8.4, was added to the activated cellulose suspension. The coupling reaction, aided by gentle shaking, was allowed to proceed overnight. After completion of the reaction, avidin coupled cellulose (avidin solid phase) was washed thoroughly with 0.1 M acetate buffer, pH 4.0, to remove uncoupled avidin. Free sites of the avidin solid phase were blocked using 2% casein, 0.2 M glycine and 1% BSA in 0.1 M bicarbonate buffer, pH 8.4.[14,15] The saturated avidin solid phase was then thoroughly washed with 0.1 M acetate buffer, pH 4.0, and 0.1 M bicarbonate buffer, pH 8.4, and suspended in a saline phosphate buffer, pH 7.4, containing 0.1% Tween-20, 0.2% BSA, and 1% normal mouse serum at 4°C.

Biotinylation of Capture Antibodies

Biotinylation of capture antibodies was carried out using the caproyl derivative of *N*-hydroxysuccinimide ester.[16] 1 mg of the antibody was dissolved in 1 ml of 0.1 M bicarbonate buffer, pH 8.4. 10 μL of biotin *N*-hydroxysuccinimide ester solution (1 mg/mL in *N*,*N*-dimethylformamide), freshly prepared before use, was added to the antibody

solution. The contents were promptly mixed and allowed to react for 2 h. A second aliquot of ester solution was added to the same vial. The reaction was allowed to proceed for 2 additional hours. The unreacted ester was separated from the biotin-antibody conjugate by extensive dialysis against 3 L of 0.1 M saline phosphate buffer, pH 7.4. The purified biotinylated antibody was diluted in 0.05 M saline phosphate buffer containing 1% normal mouse serum, neomycin (0.1 g/L), chloramphenicol (0.34 g/L), and stored at 4°C.

Characterization of Detector Antibody

Paper electrophoresis was carried out for 40 min in phosphate buffer (0.025 M, pH 7.4) at a potential gradient of 8 V/cm using Whatmann 1 chromatography paper. The sample was applied at 8 cm from the cathode. Following the run, the strips were dried and cut into 1 cm segments and their radioactivities were measured.

Assay Development

A detailed study was carried out for proper selection of the reaction parameters, viz., concentration of reagents, reaction kinetics, sequence of addition of the reagents, and the optimum specific activity of ^{125}I-labelled monoclonal antibody. In order to reduce the number of pipetting steps, two reagents either (biotinylated + radiolabelled antibody) or (biotinylated antibody + avidin solid phase) were mixed in optimum concentration and added as a single reagent in one pipetting step. The effect of these two combinations on the assay performance was studied over the shelf life of the detector antibody.

Briefly, the assay protocol formulated after optimization of reaction parameters consists of adding the reagents in the following sequence, 200 μL of standard/sample + 100 μL of (biotinylated + radiolabelled) antibody mixture as single reagent + 100 μL of avidin solid phase. The reactants were mixed and incubated for 3 h under gentle shaking. At the end of the reaction, 1 mL wash buffer, 0.05 M phosphate buffer, pH 7.4, containing 0.9% NaCl, 0.1% NaN_3, and 0.1% Tween-20 was added to all the tubes (except total), vortexed, and the solid phase was separated using a magnetic rack. The washing step was repeated twice. The radioactivity associated with the avidin solid phase was measured. The same procedure was used for evaluating the performance of the assay. The developed assay was subjected to quality control tests,

e.g., those for precision, sensitivity, specificity, reproducibility, recovery, and parallelism.

To evaluate the effect of avidin-biotin interaction on the developed IRMA procedure, parallel FSH and LH IRMA, using capture antibody coupled to magnetizable cellulose (instead of the biotinylated capture antibodies), and the same detection antibodies were also carried out.

RESULTS

Preparation of Radiolabelled Antibody and Avidin Solid Phase

The radiolabelling method used was simple and reproducible, with iodination efficiency (in terms of % incorporation of iodine) 75–90%, calculated from paper electrophoresis of the reaction mixture. The specific activity, also calculated by paper electrophoresis, was 9–12 μCi/μg with radiochemical purity of the purified fraction $\geq 98\%$ ($n = 28$ and $n = 18$ for FSH and LH, respectively). Figure 1 shows the stability of the radiolabelled antibody in FSH IRMA using freshly prepared and 60-days-old radiolabelled antibody. The radiolabelled antibody was stable for 60 days with no drastic change in the NSB and maximum binding. Quality control serum values were well within the ± 2 SD range.

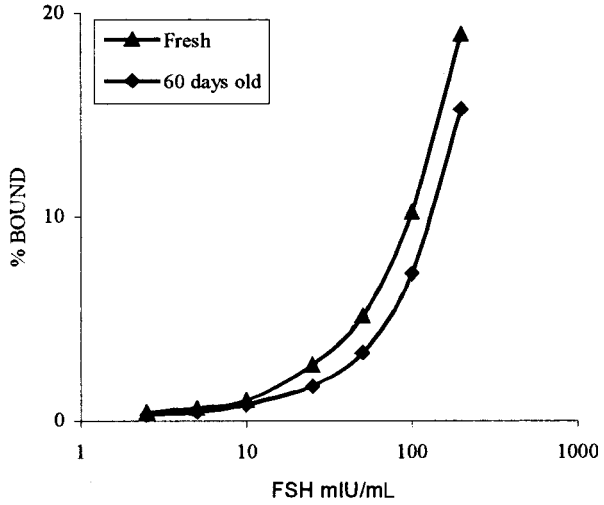

Figure 1. Stability of the radiolabelled antibody in FSH IRMA.

The method of coupling avidin to magnetizable cellulose was found to be simple and did not involve any critical steps. Coupling efficiency, monitored by a spectrophotometric protein estimation method, was 75–80%, with high immunoreactivity of the coupled avidin. Coupling efficiency was observed to be more by $8 \pm 2\%$ as compared to conventional IRMA, where the antibody is directly coupled to the solid phase. High binding capacity was expected, due to the four binding sites of avidin and high K value of the avidin-biotin interaction.

Biotinylation of Capture Antibody

We used the caproyl derivative of biotin (biotinamidocaproate *N*-hydroxysuccinimide ester) in order to take advantage of the spacer arm, which reduces the steric hindrance generally associated with binding of four biotinylated protein molecules to one avidin molecule.[17] Labelling of protein with biotin depends only on the reaction stoichiometry and concentration of the protein and biotin ester in the reaction mixture. Higher labelling yield was observed with smaller reaction volume. The reaction mixture should be stirred during the addition of the ester, as well as during the course of the reaction. It was observed that labeling of biotin to protein entirely depends upon the concentration of protein, biotin, and reaction stoichiometry, irrespective of the type of protein being biotinylated. In the case of FSH IRMA, when 1 mg of antibody was used for labelling, the dilution at which it was used in the system was 1:3, i.e., 0.60 μg/tube. In LH IRMA, 2 mg of antibody was used for biotinylation and the final dilution used was 1:6, i.e., 0.66 μg/tube, when all the other biotinylation and dilution conditions were maintained same. The biotinylation procedure did not affect the biological integrity of the protein (antibody molecule) as seen from the high immunoreactivity, up to a certain extent. Hence, we have critically optimized the extent of biotinylation of monoclonal antibodies. Excessive biotinylation ($\geq$1:50) or insufficient biotinylation were detrimental to overall immunoreactivity and sensitivity index. The optimal molar ratio of antibody to biotin was 1:20 and 1:10 for FSH and LH, respectively. The shelf life of the biotinylated antibody was about 24 months at 4°C.

Crossmatching of the Sandwich Partner

For the initial identification of capture and detection antibody to be used in IRMA, both the antibodies in the pair were radiolabelled,

Table 1. Selection of detector (radiolabelled) and capture (biotinylated) antibodies.

		Bound fraction activity Total = 1.3 (10^5 CPM)	
Analyte	Concentration mIU/mL	$SM_1T_2^*$ CPM (A)	$SM_2T_1^*$ CPM (B)
FSH	0	276	162
	2.5	407	714
	200	21628	41476
LH	0	236	118
	2.5	507	661
	200	28430	38505

T_1^*—Radiolabelled Monoclonal Antibody-1; SM_1—Solid phase Monoclonal Antibody-1; T_2^*—Radiolabelled Monoclonal Antibody-2; SM_2—Solid phase Monoclonal Antibody-2.

as well as coupled. The two possible combinations were tested for their response in the assay system by reacting with standard analyte at selected concentrations of 0, 2.5, and 200 mIU/mL for both FSH and LH. The combination 'B', i.e., $SM_2T_1^*$ offering higher maximum binding (at 200 mIU/mL) and high sensitivity index (ratio of response at 0 and 2.5 mIU/mL standard dose) was chosen for the assay development (Table 1).

Analytical Variables

The assay procedure was optimized for the estimation of FSH and LH after detailed study of various parameters/factors, viz., concentration of the reagents, reaction kinetics, incubation conditions, and sample volume. We have also critically optimized the concentration and molar ratio of biotinylated antibody, concentration of avidin solid phase, and also the specific activity of the radiolabelled antibody. The concentrations at which the standard curve characteristics, viz., detection limit, slope, and assay range, were optimally selected, are shown in Table 2. The nonspecific binding (NSB) of the radiolabelled antibody in the absence of the standard analyte was less than 0.1% of the maximum binding (Bo) value for both the assays. The effect of washing the immune complex bound to avidin solid phase was also studied. A minimum of three washings of 1 mL each were essential to attain the low NSB and high

Table 2. Optimization of assay parameters/factors for development of IRMA.

Factor/parameter	FSH IRMA	LH IRMA
Specific activity of radiolabelled antibody	9–12 μCi/μg	9–12 μCi/μg
Concentration of biotinylated antibody	0.60 μg	0.66 μg
Molar ratio for antibody to biotin	1:20	1:10
Concentration of avidin	1.5–2.0 μg	1.5–2.0 μg
Reaction condition	3 h, RT	3 h, RT
Sample volume	200 μL	200 μL

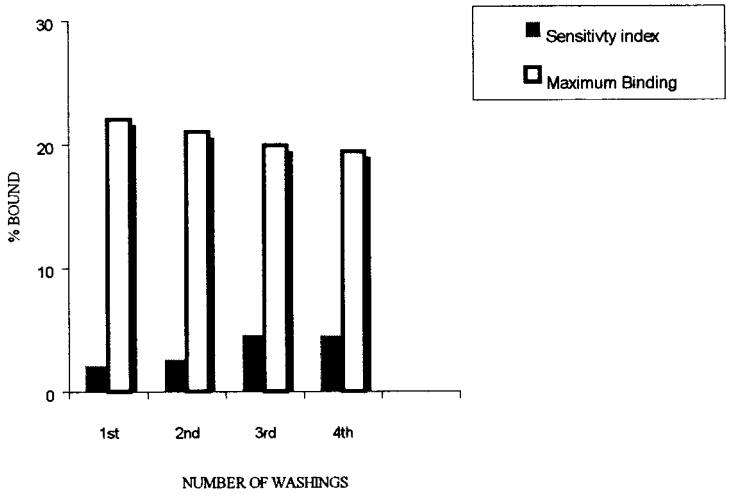

Figure 2. Effect of washing on nonspecific binding and sensitivity index of LH IRMA.

sensitivity index as shown in Fig. 2. A significant increase in sensitivity was seen with washings. However, there was marginal reduction in the maximum binding (at 200 mIU/mL). We have also investigated the effect of incubation time (1 h, 3 h, and overnight) on the immune reaction and we selected 3 h duration. By 3 h itself, almost all the analyte has been converted to the immune complex (Table 3) because of the high affinity constant of the avidin-biotin interaction. It is known that the avidin-biotin system offers faster reaction kinetics as compared to the conventional IRMA procedure, which required incubation for almost 16 h to have a comparable sensitivity using the same pair of monoclonal antibodies (results not shown).

Table 3. Effect of incubation time on LH IRMA.

LH concentration mIU/mL	1 h %B/T	3 h %B/T	Overnight %B/T
0	0.08	0.08	0.09
2.5	0.25	0.46	0.41
200	10.2	19.8	20.7

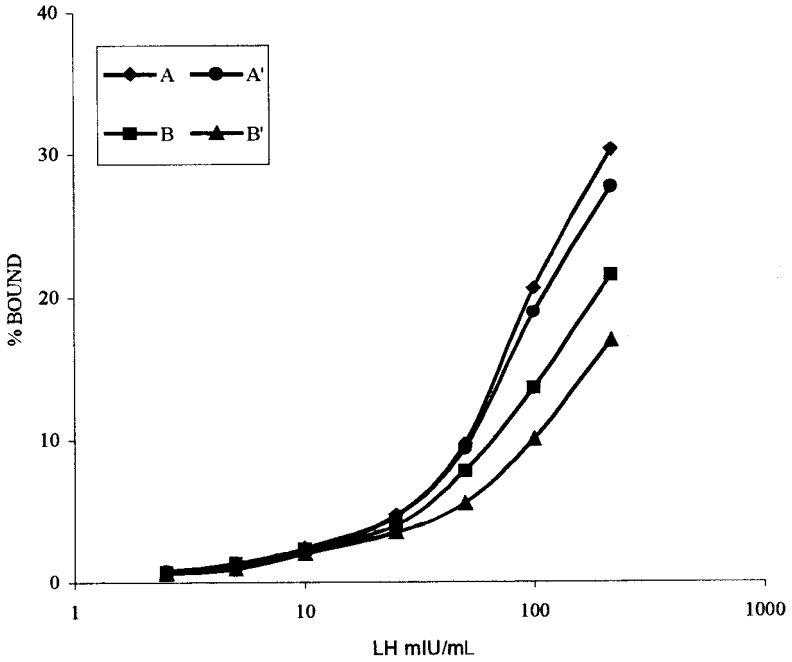

Figure 3. Stability of mixture added as a single reagent in IRMA of LH IRMA. A—Mixture-I (Biotinylated antibody + Radiolabelled antibody), freshly mixed; A′—Mixture-II (Biotinylated antibody + radiolabelled antibody), after 60 days; B—Mixture-I (Biotinylated antibody + Avidin solid phase), freshly mixed; B′—Mixture-II (Biotinylated antibody + Avidin solid phase), after 60 days.

Standard curves for LH assay, using a mixture-I (biotinylated antibody + radiolabelled antibody) and mixture-II (biotinylated antibody + avidin solid phase) are given in Fig. 3. The standard curve A represents freshly prepared and curve A′ for a 60-days-old mixture-I.

Table 4. Typical standard curve data.

Concentration (mIU/mL)	FSH IRMA %B/T	LH IRMA %B/T
0	0.08 ± 0.02	0.08 ± 0.02
2.5	0.30 ± 0.15	0.42 ± 0.2
5.0	0.60 ± 0.25	0.80 ± 0.3
10	1.10 ± 0.4	1.30 ± 0.5
25	2.20 ± 0.3	3.20 ± 0.9
50	4.90 ± 0.8	6.10 ± 1.2
100	10.2 ± 2.2	12.0 ± 2.8
200	21.0 ± 3.0	20.2 ± 4.0

For $n = 21$ assays, using different batches of reagents over a period of 32 months.

Curve B represents the stability of mixture-II and curve B′ represents 60-days-old mixture II. Quality control serum samples which were evaluated using both the mixtures, gave values well within the expected range of ± 2 SD. However, in the case of mixture-II, reduction in maximum binding at 200 mIU/mL was observed as compared to mixture-I. This is due to preferentially occupying the available biotin binding sites on the avidin solid phase by premixed biotinylated antibody. Hence, for further assay development, we have selected mixture-I, the mixture of biotinylated antibody and radiolabelled antibody, in the required concentration (as given in Table 2), as a single reagent.

Typical standard curve data for the assay with biotinylated antibody and radiolabelled antibody as a single reagent for FSH and LH IRMA is shown in Table 4. Sensitivity of the assay, estimated as the minimum dose which can be measured with an error less than 10%, was observed to be 0.5 mIU/mL and 0.9 mIU/mL for FSH and LH, respectively. The intra-assay precision was determined by replicate analysis of the three control serum samples in a single assay and inter-assay precision was estimated by duplicate measurement of the same control serum samples in 12 different runs. We have also evaluated dilution linearity of the assay by assaying samples serially diluted with hormone free serum. A good agreement between the measured and expected values was observed. The analytical recovery of the known concentrations for the exogenous human FSH/LH added to portions of the five serum samples lies within acceptable limits. All the validation results are tabulated in Table 5.

Table 5. Characterization of avidin-biotin based IRMA for FSH and LH.

Parameter	FSH IRMA	LH IRMA
NSB	< 0.1%	< 0.1%
Sensitivity	0.5 mIU/mL	0.9 mIU/mL
Assay range	0–200 mIU/mL	0–200 mIU/mL
Recovery	90–110%	87–105%
Dilution test	90–110%	90–105%
Intra-assay variation	3–8%	3–6%
Inter-assay variation	7–11%	3–9%
Hook effect	No effect up to 400 mIU/mL	No effect up to 400 mIU/mL
Cross-reactivity	LH < 0.2% hCG < 0.1% TSH < 0.1%	FSH < 0.2% hCG < 0.1% TSH < 0.1%

Table 6. Linear regression correlation with results from comparison assay.

Comparative assay	Slope	Intercept	γ	n
Coated tube FSH IRMA*	0.985	0.21	0.99	70
Coated tube LH IRMA*	0.995	0.18	0.98	60

*Of a commercial source (ICN, USA).

CORRELATION WITH OTHER ASSAY

Comparison of levels of FSH/LH in different serum samples, obtained by the present method, and by using commercially available IRMA coated tubes (ICN, USA) method, exhibited good agreement. The regression analysis showed good correlation with commercially available kits ($y = 0.98x + 0.21$ and $y = 0.99x + 0.18$) for FSH and LH. The correlation details are tabulated in Table 6.

DISCUSSION

Several RIA or IRMA systems, using various solid phases, have been reported in the literature for estimating the levels of antigen circulating in

the body fluids But, the use remains limited because of the constraints of specificity, sensitivity, cost effectiveness, automation facilities, and also the feasibility of the developed assay at production levels. In the conventional IRMA system, analyte specific antibody coated to separate solid phase is mandatory.

The aim of this work was to develop a single, common, and economical solid phase for use in a variety of immunoradiometric assays, which can be easily scaled up to the production level. In the past couple of years, the emphasis on the use of avidin-biotin complex in immunoassay has shifted from detection systems to the improvement of capture systems. The use of avidin in this capacity for immobilizing a biotinylated antibody offered several advantages.

The method of coupling avidin to magnetizable cellulose is found to be technically easy as compared to coupling of antibody to magnetizable cellulose. This is because the tetramer, avidin, is very stable and its biotin binding capacity normally remains intact, even after various chemical modifications during immobilization. This is very unlikely in conventional IRMA procedures where, very often, coupling of the antibody to solid phase causes a deformation of the structure which could affect the specificity or overall sensitivity of the assay. The procedure for coupling avidin to magnetizable cellulose is reproducible and can be carried out in a moderately equipped laboratory without any automation. This helps to reduce the overall cost of the assay, unlike coated tubes, where costly automation and a large quantity of coating reagent is required to ensure uniformity of the coating.

The molar ratio of the biotin to protein has to be critically evaluated in order to satisfy the assay requirements (detection limit, dynamic range, and maximum binding). At very high molar ratio ($>1{:}50$), the biological integrity may be affected. This has been directly reflected in the observed maximum binding values. Also, when compared with conventional IRMA, consumption of capture antibody per tube is less, due to the incorporation of the avidin-biotin conjugated system (Table 2). This might be due to minimal loss of antibody during the biotinylation procedure and, also, more precise and finer control on the use of immunochemically active biotinylated antibody molecules applied to avidin coupled magnetizable particles. Thus, avidin coupled magnetizable cellulose offers a cost effective common solid phase for a variety of antigens. Nonspecific binding is largely dependent on the quality of the radiolabelled antibody. The nonspecific binding in both the assays has been $< 0.1\%$. Generally, such low NSB is very unlikely to be observed with avidin-biotin based assays. Use of nonfat dry milk powder, saline buffer, normal mouse serum, and efficient washing

of immune complex helped to keep the nonspecific binding at such a low level.

In conclusion, the avidin-biotin based system is a simple and economical approach for the manufacturer towards development of variety of IRMAs using a single common avidin solid support. We propose that the present design is well suited for the development of variety of IRMAs using common avidin solid support, in the form of magnetizable particles, tubes, or beads.

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