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Radioimmunoassay of polypeptide hormones using immunochemically coated plastic tubes

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Abstract. A method has been developed for immobilisation of antisera on fresh plastic tubes through an immunochemical bridge. This type of immobilisation has been shown to be more consistent than direct adsorption on plastic. Such immunochemically coated antisera on plastic tube has been used in the development of a noncentrifugation radioimmunoassay. This assay system has been found to be technically as sound as the conventional method.

Keywords. Radioimmunoassay; polypeptide hormones; normal rabbit serum; bovine serum; antisera.

Introduction

Radioimmunoassay (RIA), a technique extensively used in all endocrinological investigations, utilizes the double antibody procedure for the separation of bound hormone. This method of separation is time consuming and requires additional reagents. Solid phase RIA's are developed in many laboratories, using antisera (a/s) immobilised on sephadex, cellulose etc. (Leif Wide, 1981; Chakrabarti *et al.*, 1985). Attempts have been made to improve this system using antibody adsorbed on plastic tube, further eliminating a centrifugation step (Parsons, 1981; Murthy and Moudgal, 1986). In the present study, we have immobilised a/s on locally available plastic tube through an immunochemical bridge and report on the advantages of using such a system in RIA.

Materials and methods

Plastic tubes were purchased locally from Sarad Biochemicals, Bangalore. These were moulded of high density polyethylene All hormone a/s used in these investigations were raised by the conventional procedure in rabbit/monkey. Antisera for human chorionic gonadotropin (hCG) and ovine follicle stimulating hormone (oFSH) were raised in rabbit and monkey respectively. AntiIgG (to rabbit or monkey) was raised in goats. hCG was a gift sample from NIH, USA. All other reagents used were of analytical grade. Buffer used for RIA had 0·05 M phosphate and 0·025 M EDTA in physiological saline, pH 7·4 and had 1% bovine serum (BS) (referred as RIA buffer).

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Abbreviations used: RIA, Radioimmunoassay; a/s, antisera; hCG, human chorionic gonadotropin; oFSH, ovine follicle stimulating hormone; BS, bovine serum; PEG, polyethyleneglycol; hLH, human luteinizing hormone.

Preparation of immunochemical bridge

Fresh plastic tubes were coated with 0·5 ml of normal rabbit serum (I) diluted in water (1/1000 diluted), care being taken to see that the solution was pipetted to the bottom of the tube preventing the formation of droplets of the coating solution on the sides of the tube. Following overnight coating, free sites were saturated with in RIA buffer for 1 h. For extension of the bridge the tubes were washed, and 0·6 ml antilgG (A) was added at high concentration (1/300 in RIA buffer, 4°C) to allow saturation of the IgG bound to the plastic surface with A. At this stage we have IgG-antiIgG (-IA) bridge. Further extension of the bridge, when required, was carried out as above using saturating concentration of IgG and antilgG to obtain required length of the immunochemical bridge.

Preparation of the bridge starting from A was carried out in a similar fashion. In all cases immunochemical bridges were made in such a way that the end point of the bridge contains antilgG. These were then stored at 4°C or – 20°C until use. Similarly -IA bridges for monkey system was prepared in an identical fashion using normal monkey serum and antilgG (monkey) in the goat.

Adsorption of a/s on immunochemical bridge

Plastic tubes with immunochemical bridge was washed twice with RIA buffer containing 1% BS, drained on a filter paper for 5–10 min. To these tubes 0·6 ml of a/s (1/10,000 to 1/300,000) diluted in RIA buffer was added. The primary requisite for coating a/s is that the antilgG used should be against the IgG of the species in which the a/s is raised. Binding of a/s was allowed to proceed at 4°C overnight and the tubes were washed twice with 1 ml RIA buffer. These tubes were then stored at 4°C/–20°C until used.

Quantitation of adsorption of a/s

Antisera at 1/100,000 was added to immunochemically coated tube (600 μl). After the adsorption the quantity of a/s remaining in the supernatant was measured with respect to control a/s, by its binding to ^{125}I-hCG. The quantity of a/s adsorbed was then quantitated by the decrease in the binding of ^{125}I-hCG.

Direct adsorption of a/s on plastic

The adsorption was carried out as described by Murthy and Moudgal (1986). Briefly a/s at 1/10,000 dilution in distilled water was added (0·5 ml) to fresh plastic tubes and allowed to adsorb at room temperature overnight. The a/s was discarded, and RIA buffer (0·6 ml) was added to these tubes to saturate the unsaturated binding sites. After 1 h the solution was discarded and tubes were stored at 4°C until further use.

Binding of ^{125}I-hormones to a/s coated tubes

Antisera coated tubes were washed with RIA buffer, drained and to each tube

0·6 ml of RIA buffer containing 50,000–100,000 cpm of ^{125}I-hormone was added. The tubes were left on the bench overnight. Next morning the solution was poured out, tube washed once with RIA buffer and counted in a LKB gamma counter.

Competitive binding assay with a/s coated tubes

Antisera coated tubes were incubated with sample or hormone (varying amount in each tube) and ^{125}I-hormone, all in RIA buffer in a total volume of 0·6 ml as already described (Murthy and Moudgal, 1986). Incubation was carried out on the bench for 20 h at room temperature, the tubes drained and washed once with 1 ml RIA buffer and counted. Conventional competitive binding assays were carried out by the standard procedure using double antibody-polyethyleneglycol (PEG) for separation of the bound hormone from the free hormone (Murthy and Moudgal, 1987).

Binding assays were analysed by Scatchard method (Scatchard, 1949). In each analysis correction for the damage of hormone during iodination was applied as described (Murthy and Freisen, 1985).

Results

Figure 1 shows the optimisation of the antilgG dilution to be used for the

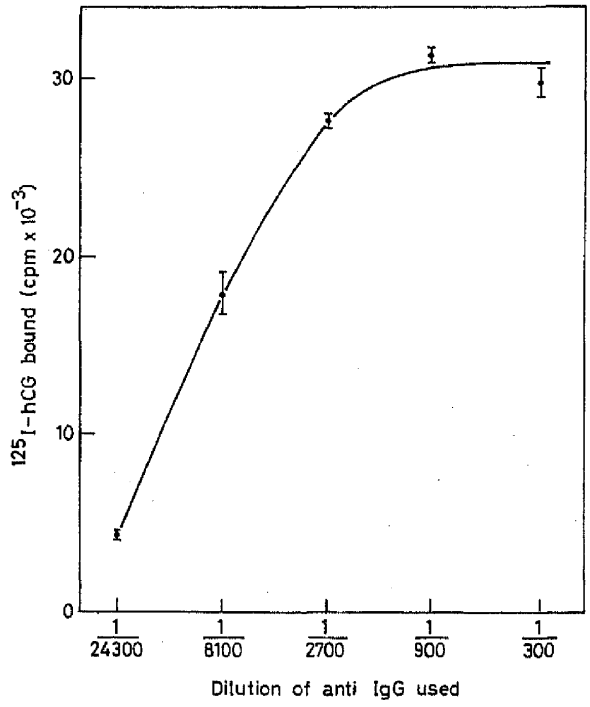

Figure 1. Titration of antilgG concentration required for saturation of IgG adsorbed on the plastic tube. Plastic tubes were first coated with NRS at 1/1000 dilution in water followed by saturation with antilgG at varying concentration.

preparation of the immunochemical bridge. It is seen that a dilution of 1/2000 is required to saturate all the IgG bound to the plastic tube. If the saturation needs to be done in shorter time a higher antilgG concentration (1/100) can be used reducing this period to 2–3 h.

Figure 2 shows the time course of adsorption of a/s by the immunochemical bridge by (i) ^{125}I-hCG binding to the a/s adsorbed tube (curve a) and (ii) by

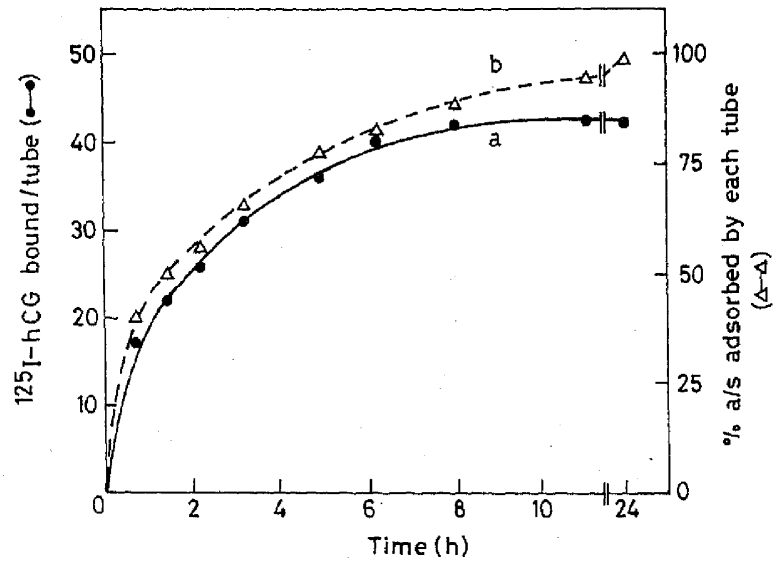

Figure 2. Adsorption of a/s by-IA tubes with time. Curve (a), ^{125}I-hCG bound to a/s coated plastic tube; curve (b), per cent a/s adsorbed by -IA plastic tube. The binding was measured-as described under methods.

decrease in the binding of ^{125}I-hCG to the unadsorbed a/s from the tube in conventional binding assay (curve b). It is seen clearly that increased a/s adsorption as measured by both methods are nearly parallel indicating that binding of a/s on the solid phase through immunochemical bridge results in uniform retention of activity of the a/s.

Table 1 shows a comparison of the consistency of binding of ^{125}I-hormone to a/s adsorbed to plastic tube directly or through -IA, -IAIA and -AIA bridges, along with the specific binding obtained with conventional method (double antibody-PEG). The consistency of binding is about ± 4% for direct binding. In contrast the binding to tubes immunochemically coated with a/s was ±2%. This consistency is almost the same as that obtained for conventional liquid phase assay. Thus it is clear that the. binding of ^{125}I-hormone to immobilised a/s on plastic through immunochemical bridge is highly consistent. It is also seen from the table that this consistency is not only seen for one hormone, but is true for hCG, oFSH and human luteinizing hormone (hLH).

Table 2 shows the stability of the a/s coated tubes (thorough -IA and –IAIA bridge) for storage. It is seen clearly that the binding of ^{125}I-hormone is not significantly decreased on storage as the per cent bound is (last column) nearly same for 45 days (for hCG a/s) and 80 days (for oFSH a/s). Small differences seen in per

Table 1. Comparison of ^{125}I-hormone binding to immobilized a/s through immunochemical bridges*

Type of bridge	n	a/s adsorbed	^{125}I-hormone bound cpm ± SD	± SD (%)
Direct	12	hCG	19444 ± 1750	9·00
adsorption	30	oFSH	27268 ± 1055	3·87
Conventional	20	oFSH	22222 ± 459	2·07
binding	20	hCG	41483 ± 834	2·01
-IA bridge	18	hCG	29572 ± 335	1·13
	10	hLH	26387 ± 473	1·81
-AIA bridge	10	hCG	11537 ± 337	2·92
-IAIA bridge	10	hCG	14303 ± 283	1·98
	5	oFSH	30136 ± 212	0·70
	6	hLH	22645 ± 403	1·78

*Binding of hLH was done for hCG a/s adsorbed on the plastic tube. For hCG and oFSH, plastic tubes coated with corresponding a/s was used.

Table 2 Stability of a/s coated tube.

Days of storage	^{125}I-hormone* added (cpm)	^{125}I-hormone bound (cpm ± SD)	Per cent bound
hCG a/s coated tubes using -IA bridge			
3	54,000	14303 ± 283	26·5
16	48,000	14252 ± 295	29·6
34	52,000	12080 ± 284	23·2
45	60,000	17576 ± 305	29·3
oFSH a/s coated tubes using -IAIA bridge			
3	—	—	—
35	56,000	30136 ± 212	53·8
65	40,000	19798 ± 394	49·5
80	52,000	24520 ± 506	46·0

*Immunologically active

cent bound is not significant considering that the ^{125}I-hormones used are different for each experiment. On storage the consistency of binding (SD) do not change, indicating that the a/s adsorbed on the plastic through immunochemical bridge is infact stable for atleast 90–100 days.

Comparison of the assay using immobilised a/s and normal a/s is shown in figure 3. With regard to sensitivity, binding and consistency the assays are comparable. In the inset of the figure 3 is shown the Scatchard plot of the two assays. The K_a and capacity are infact measurable. The apparent K_a in the solid phase appears to be rather low, compared to that in the liquid phase.

Competitive binding of ^{125}I-oFSH to immunochemically immobilised a/s on plastic tube (curve b) and normal a/s (curve a) is shown in figure 4. The profiles are similar and the duplicates are comparable to that of the conventional double antibody assay. Table 3 shows the consistency and parallelism obtained for the measurement of a control sample of oFSH in 5 determinations using the a/s coated plastic tube. Figure 5 provides a consolidated displacement profiles of all these

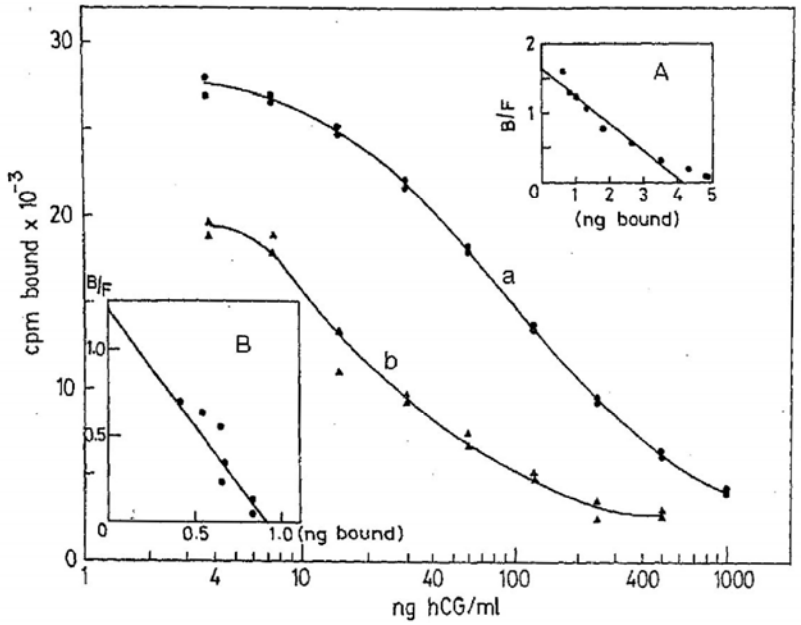

Figure 3. Displacement profile in RIA using immobilised antibody on plastic tube (curve a) and conventional method (curve b) for hCG. Tubes were coated with 1/200,000 a/s (0·6 ml) on -IA bridge. Liquid phase assay was done using 100 μl of 1/100,000 a/s in a total volume of 0·6 ml. ^{125}I-hCG added was 60,000 cpm in both cases. Insets A and B show the Scatchard plots for curves (a) and (b) respectively.

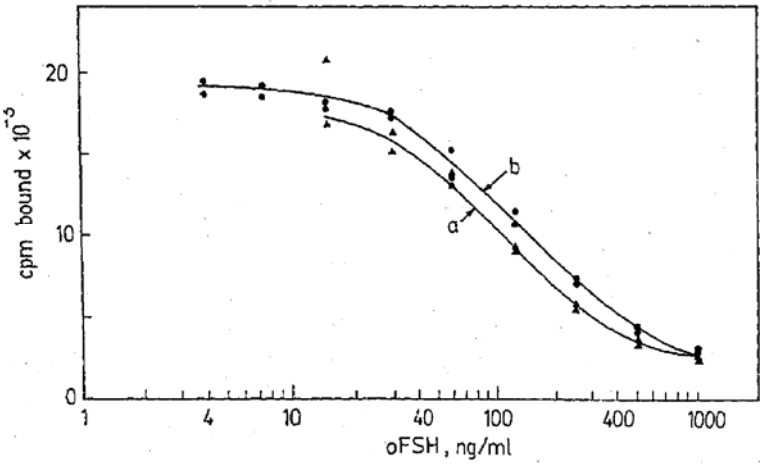

Figure 4. Displacement data of RIA using conventional method (curve a) and noncentrifugation method using immobilised a/s on plastic tube (curve b) for oFSH. Plastic tubes were coated with 1/100,000 a/s (0·6 ml) on -IAIA bridge. Liquid phase assay was done using 1/25,000 (100 μl) a/s. ^{125}I-oFSH added was 50,000 cpm per tube (-IAIA bridge).

Table 3. Analysis of tube RIA using immunochemically coated a/s on plastic tubes through -IAIA bridge.

Volume of control sample used	oFSH ng/ml read in assay nos 1	2	3	4	5	Mean ± SD
25 µl	44	55	50	50	55	51 ± 4
50 µl	100	100	120	120	110	110 ± 10
100 µl	240	230	250	230	250	240 ± 10
Mean	207	217	230	223	230	
± SD	30	15	26	21	17	

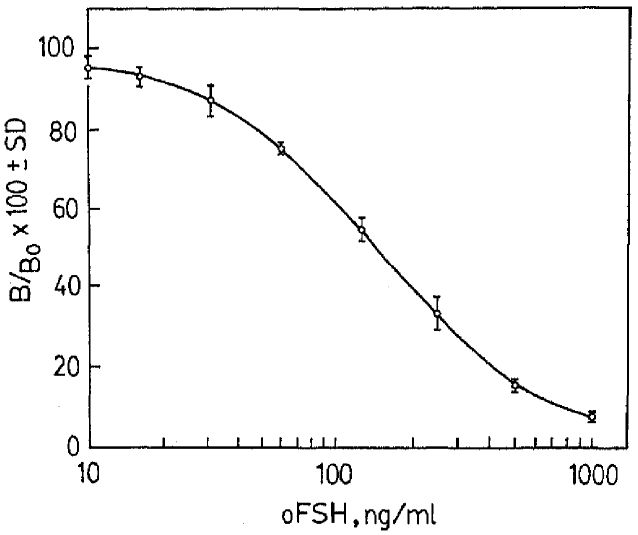

Figure 5. Displacement profile in oFSH RIA's using a/s (for oFSH) immobilised on plastic tube through -IAIA bridge. The -IAIA bridges were coated with 1/100,000 a/s (0·6 ml). A total of 5 assays were done over a period of 2 weeks using plastic tubes coated with the a/s. The bars show the SD of specific binding at each concentration of the hormone.

assays. They are superimposable indicating good reproducibility of the assay. Figure 6 presents displacement analysis in hLH assay system using -IA coated a/s. Inset in the figure shows the B/Bo *vs* concentration plot of 4 such assays (curve a). These results clearly indicate that the solid phase assay is quite consistent and reproducible and is independent of the nature of the bridge (-IA or -IAIA) used for the adsorption of the a/s. Displacement of ^{125}I-hLH by a human serum is also shown in the inset (curve b), which is parallel to normal displacement.

Tables 4 and 5 show the effect of normal serum (human, sheep and rabbit) and recovery of the exogeneously added hCG from serum samples. The recovery is quantitative with negligible interference from serum. Figure 7 (curves a, b and c) shows the displacement profile with immobilised a/s (on plastic through immunochemical bridge) in the absence of serum (curve a), and presence of 100 µl of human serum (curves b and c). Inset in the figure shows the plot of B/Bo *vs* hormone concentration for ally the 3 cases in the figure. Absolute overlapping clearly

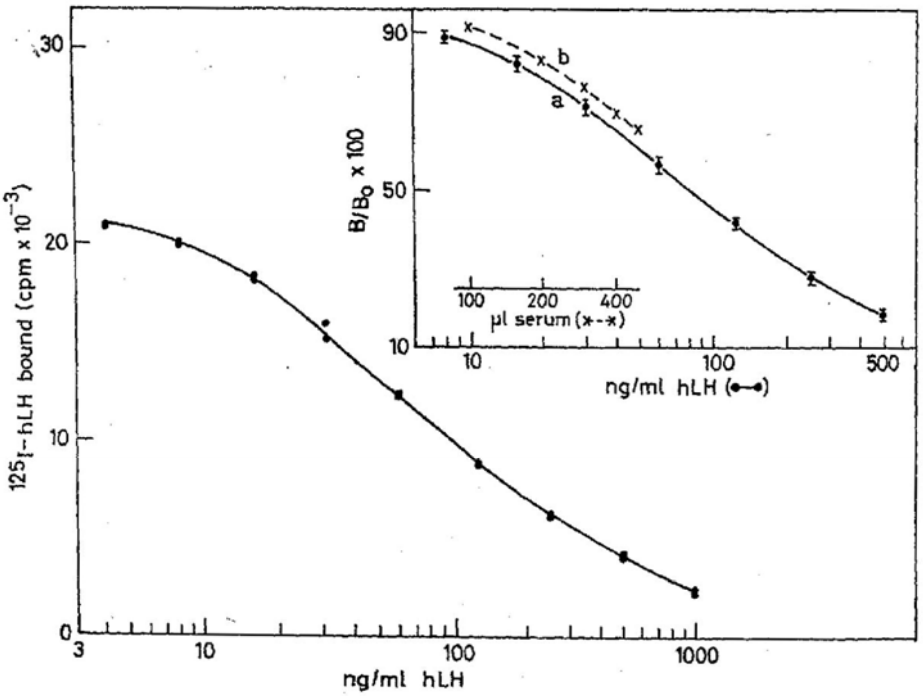

Figure 6. Representative displacement profile in solid phase RIA for hLH using immobilised a/s on -IA bridge· Inset shows the B/Bo *vs* concentration plot for 4 such assays (curve a). Displacement of ^{125}I-hLH in one of the assay by different volumes of a human serum sample obtained from WHO for a qc program (curve b). Vertical bars represents the ± SD at each concentration of the hormone used for displacement.

Table 4. Recovery of hormone (hCG) by RIA in the presence of normal sheep and rabbit serum (type of bridge used is -IA).

Serum added	hCG added exogeneously (ng)	Specific binding (%)	ng hormone recovered	Recovery (%)
Sheep (100 µl)	0	100*	—	—
	8	70·2	9·0	112
	31	44·7	34·0	109
	125	22·1	125·0	100
Rabbit (100 µl)	0	100*	—	—
	8	76·0	7·5	94
	31	50·2	27·0	87
	125	21·5	130·0	104

*Specific binding in the presence of sheep and rabbit serum with respect to the buffer, sp. binding was 94 and 97% respectively.

demonstrates that the binding characteristics of the ^{125}I-hCG to the adsorbed a/s is same in the absence and presence of serum samples. Table 6 shows the recovery of the exogeneously added hLH to human serum samples described under figure 7. Recovery is quantitative from serum samples carrying basal hLH also. Curve d in figure 7 and curve b in figure 6 show the displacement pattern by varying volumes of human serum samples and is parallel to normal displacement profile.

Table 5. Recovery of hormone (hCG) from human serum samples*.

No. of samples	Exogeneously added hCG (ng)	Recovery ng ± SD	Recovery (%)
6	200	210 ± 38	105
10	100	110 ± 20	105
2	50	60	120

*Random serum samples from different individuals. Specific binding of ^{125}I-hCG in the presence of these human serum samples was found to be 99·5 ± 5% (100 μl serum, n=18) compared to the binding in the buffer (type of the bridge -IAIA).

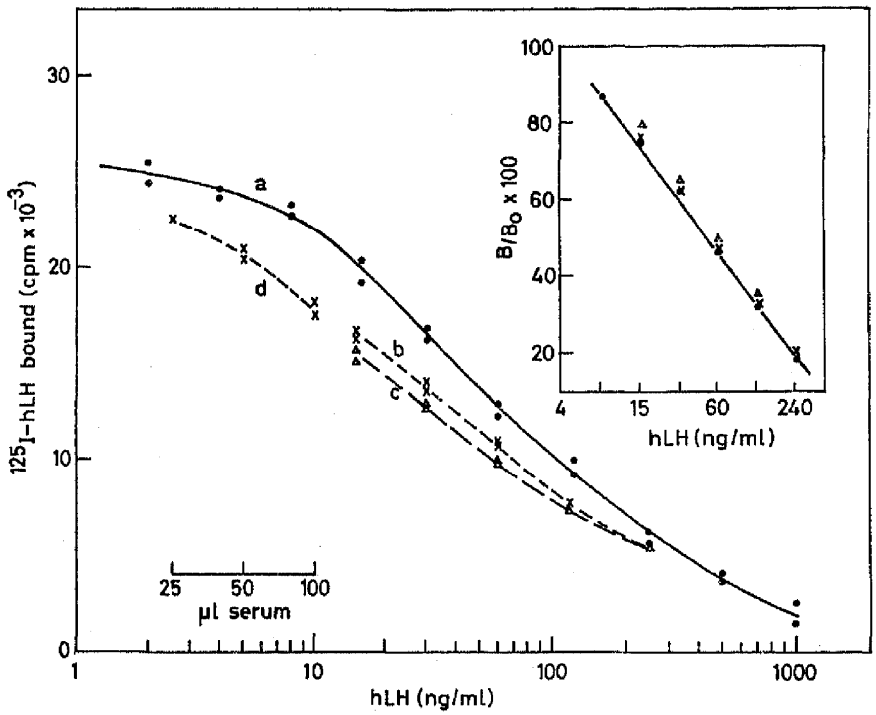

Figure 7. Displacement profile and recovery of hLH in solid phase RIA. The type of the bridge used was -IA. Curve (a), Normal displacement profiles; curves (b) and (c), displacement profiles in hLH RIA in the presence of 100 μl of two serum samples 1 and 2 with basal hLH levels of 11 and 19 ng/ml respectively; curve (d), displacement of ^{125}I-hLH by a human serum sample. Inset represents B/Bo*100 *vs* hLH concentration for curves (a), (b) and (c) respectively. The nature of the samples used is explained under table 6.

Table 7 shows a comparison of quantitative aspects of a/s adsorbed on the plastic tube either by direct coating or through -IA bridge. The results clearly demonstrate that the adsorption of a/s in both the direct method and through the bridge per tube corresponds to about 150–200 μl (1/100,000 dilution) and this gives about 20–25% greater binding than 100 μl of a/s (1/100,000 dilution). It is to be noted that

Table 6. Recovery of hormone (hLH) from human serum samples containing basal hLH*.

Serum number	Exogeneously added hLH (ng)	Serum hLH (blank)	Total hLH measured (ng)	Recovery (%)
1	0	11	11	—
	15	11	30	113
	30	11	46	110
	60	11	70	97
	120	11	130	99
	250	11	250	96
2	0	19	19	—
	15	19	36	105
	30	19	55	112
	60	19	85	107
	120	19	150	108
	250	19	280	104

*Both the serum samples used were from two female volunteers during 10–14 days of the cycle. hCG a/s was used for the measurement of hLH using ^{125}I-hLH as tracer and hLH for displacement (type of the bridge -IA).

Table 7. Efficiency of a/s adsorption and comparison of ^{125}I-hCG binding between adsorbed and normal a/s.

Type of bridge	Vol. of a/s used for adsorption (μl-dilution)	a/s adsorbed[a]# (%)	Vol. of a/s adsorbed[b] (μl/dilution)	I-hCG bound cpm
Direct	600 – 1/20,000	5	150 – 1/100,000	28,000
-IA[c]	600 – 1/200,000	60	180 – 1/100,000	30,000
normal[d]	—	—	100 – 1/100,000	22,000

[a]This was calculated by the binding of ^{125}I-hCG to the a/s left after adsorption.
[b]This also represents the volume of the a/s that is used for binding the ^{125}I-hCG.
[c]Data for -IA and normal binding are taken from figure 3.
[d]This represents the binding that is obtained in the conventional binding done under same conditions.

the adsorption of a/s by immunochemical bridge is near quantitative (60%) compared to the direct method which is less than 5%.

Discussion

The data presented clearly demonstrates that the adsorption of antibody through immunochemical bridge is satisfactory. It is seen from table 1 that consistency of binding to immunochemically adsorbed a/s is comparable to the binding observed in the liquid phase (conventional method). The consistency is far superior to that observed with the tubes coated with a/s directly. This observation is true for several hormones as shown in the table (hCG, oFSH and hLH).

Immunochemically coated tubes are stable at 4°C for atleast 3 months, and probably longer. This observation makes it viable to have a large number of tubes

coated and stored at 4°C and use it as required. Making -IA or -IAIA bridge is a batch operation and as many as 1000–2000 tubes can be coated within 4 h with minimal fatigue. In the whole operation the only place where care needs to be exercised is in the first step (addition of normal rabbit serum). Subsequent steps are much like in enzyme-linked immunosorbent assay operations.

Competitive binding analysis of direct a/s, and immunochemically adsorbed a/s on plastic tubes (table 2, figures 1 and 3) clearly show that duplicates in the conventional method and immobilised a/s method are comparable. The sensitivity of the assay using immunochemically coated tubes is of the same order as that of the conventional method.

Consistency of the tube assay is definitely acceptable (figures 5 and 6) where consolidated displacement analysis of 5 assays are presented. The mean values obtained within an assay and between assays is within limits as in the conventional method (222+10 ng/ml, and 217+15 ng/ml), Scatchard plot of displacement analysis by homogeneous (conventional) and plastic tube methods show that the plots are linear in both cases. However K_a in the plastic tube method appears to be little on the lower side. Apparent capacity for the plastic tube is about 4 ng/tube. Considering that the reaction between the hormone and antibody in the plastic tube assay is nonhomogeneous, neither the capacity nor the affinity can be taken seriously.

Interference from serum on specific binding to the coated a/s is insignificant. Recovery of the hormones added exogeneously to serum sample (human, sheep and rabbit, 100 μl) are quantitative clearly demonstrating that this method has a potential application in measuring hormones in serum samples. The fact that parallel displacement is seen for human serum sample in hLH measurement justifies the above conclusion (figure 7). In addition the binding characteristic of the ^{125}I-hLH to a/s coated tube is not affected by the presence of serum is clear from the inset in figure 3. The above conclusion is also drawn from the data shown in table 4 for sheep and rabbit serum. Thus serum interference in the assay is ruled out.

The above assay system is economical in terms of double antibody as well as primary a/s. The quantity of the double antibody required in this system per tube is 100-fold less than the requirement in the liquid phase assay. It is also very economical in terms of the a/s required. For example in hCG a/s the requirement in the liquid phase would be 100 μl of 1/100,000 dilution. The volume of a/s adsorbed immunochemically on the plastic tube is hardly equivalent to 150–200 μl (1/100,000; dilution-60% of 600 μl of 1/200,000 a/s is adsorbed), this giving about 20% more binding than in the liquid phase. In the direct method of coating 600 μl of 1/20,000 dilution of a/s is required which is about 30 times more, only about 5% or less being adsorbed, the remaining 95% becoming nonfunctional because of inactivation of a/s at such high dilutions in the absence of proteins. Such inactivation of a/s for both hCG as well as oFSH has been observed in our laboratory. Thus immunochemical method of adsorption of the a/s is extremely economical in terms of primary a/s in comparison to any other method of immobilisation known so far.

The type of immunochemical bridge does not appear to have any preference in RIA. The recovery of the hormone in -IA, AIA and -IAIA bridges are all excellent, stability and consistency comparable and interference from serum sample insignificant. Hence technically there is little to choose among the 3. -IA bridge would require least manipulations and hence probably the most useful practically

-IA bridge to rabbit and monkey systems have worked well (for hCG and oFSH respectively) and there is no reason to believe that other systems may present problems. Manipulation wise this method is also very simple. Once the coated tubes are available the operations are minimal involving addition of bluffer, standard or sample and ^{125}I-hormone. There is no requirement of the centrifugation nor the manipulations for the separation of the bound hormone from the free hormone.

Thus a/s coated plastic tubes through immunochemical bridge has all the technical advantages of double antibody method. In addition it has the following other advantages: (i) requires 100-fold less of double antibody, (ii) does not require centrifugation, (iii) has much less experimental manipulation and (iv) extremely economical on the a/s, almost nearly the same as in the conventional liquid phase assay. Added to these practical advantages are that the a/s coated tubes are stable for atleast 90 days. Hence a mass scale preparation of a/s coated tubes can be undertaken.

On the basis of these data we believe that this method of RIA is an alternative to expensive double antibody method. The method retains all the technical excellence of the conventional method and possess other practical advantages of economy and ease of operation.

References

Chakrabarti, R., Murthy, G. S. and Jagannadha Rao, A. (1985) *J. Biosci.,* **7,** 145·
Leif Wide (1981) *Methods Enzymol.,* **73,** 203.
Murthy, G. S., and Moudgal, N. R. (1986) in *Proceedings of the Endocrine Society of India* (in press).
Murthy, G. S. and Moudgal, N. R. (1987) *J. Biosci.,* **12,** 23.
Murthy, G. S., and Friesen, H. G. (1985) *J. Biosci.,* **7,** 134.
Parsons, G. H. Jr. (1981) *Methods Enzymol.,* **73,** 224.
Scatchard, G. (1949) *Ann N. Y. Acad. Sci.,* **51,** 660.