Technology Section

Pak. j. sci. ind. res., vol. 34, no. 6, June 1991

IN HOUSE SOLID PHASE T-3 RADIOIMMUNOASSAY

KHAN M. SAJID AND MEHFOOZ AKHTAR
Atomic Energy Medical Centre, Multan, Pakistan

(Received August 30, 1989; revised December 1, 1990)

In order to replace the use of expensive RIA kits/bulk reagents with local technology, work with production of T3-antibodies in rabbits and measurement of antibody titres was started. The avidity of antibody with maximum titre (1:7900, determined from scatchard plot was suitably high i.e., 0.7×10^{10} lit./ mole. Coupling of this antibody to activated cellulose was done to prepare solid phase binding agent. A solid phase radioimmunoassay was then developed. Analysis of bound counts versus standard concentrations showed that the data fits well in logit-log coordinate system ($p>0.5$), and sample results correlate linearly with those of commercial RIA kits and imported bulk reagents (NETRIA, U.K.). Observed sensitivity of assay (0.2 nmol/1) compares well with commercial assays. Initially obtained high imprecision at low doses was improved with experience replacement of tracer. Between batch analysis of quality control results shows that in house assay is as good in quality as the commercial ones.

Key words: Solid phase, Radioimmunoassay, In house.

Introduction

Radioimmunoassay technique introduced by Berson and Yalow [1] has grown to a method of reliability and choice in various fields of biological sciences. It has undergone various modifications, improvements and separation methods have evolved from simple precipitation to modern solid phase techniques.

The practice of radioimmunoassay in Pakistan is limited mostly to the use of commercial kits and reagents supplied in bulk. Piyasena [2] reports that RIA kits are almost 10 times more expensive than in house assays. Sometimes serious problems arise due to irregular supply. It is, therefore, desirable to produce reagents locally to introduce in house radio immunoassay techniques.

A.E.M.C., Multan has started a programme to produce RIA reagents locally to achieve self sufficiency in this field. We have tried to set up a solid phase RIA technique by coupling locally produced T3-antibody to a solid phase matrix (activated cellulose) to use as binding agent.

Materials and Methods

In early 1988, T3-antibodies were raised in four rabbits using T3-HSA as immunogen. The titres were tested at regular interval with isotopic technique. Significant amounts of antibodies were detected in immunized animals. The optimum dilution determined from dilution curve of antiserum of rabbit, which showed maximum immune response was around 1:7900. A simple RIA method using this antiserum was developed to construct the scatchard plot [3]. The avidity of this in house antibody calculated from scatchard plot was 0.7×10^{10} lit./mole, suitable for assays quantities in the range of nmol/1[4].

Activation of cellulose was performed following procedure described by R.S. Chapman *et al.* [5]. The activated imidazolyl carbamate cellulose was filtered, washed with acetone, dried in air and finally stored at -20° until required. Coupling of Ig G to the activated cellulose [5] was as follows: To 0.2 ml of rabbit antiserum diluted to 1 ml with buffer (Barbital pH: 8.0, 0.05 M), 200 mg of activated cellulose was added and mixed. The contents were rotated end over end for 18 hrs at ambient temperature. The washing procedure to remove uncoupled and adsorbed protein was similar to that of Wide [6]. The extent of coupling of IgG was tested by checking binding of radioactive T3 to the solid phase after each washing. The protein solution removed during washing cycles was retreated with activated cellulose 2-3 times for more coupling of IgG to cellulose. Solid phase antibody was then diluted to a concentration of 5 mg solid phase (with coupled antibody) per ml buffer and stored at 4° until use.

Tracer and standards used in setting up assay were those supplied by INMOL, Lahore.

The assay was performed as follows: 50 ul of standard or unknown sera (in duplicate), 200 ul of 1125-T3 solution [15000 cpm; 8-anilino-1-naphthalene sulphonic acid (ANSA): 2mg/ml] and 50 ul of solid phase antibody (500 ug) and 300 ul assay buffer were incubated over a period of 18 hrs. The contents were then centrifuged at a speed of 3000 rpm for 20 mins. followed by decantation of the supernatent. Bound fraction was counted for 90 secs. and plotted against standard concentrations to calculate the results. RIA data processing programs supplied by I.A.E.A. for IBM compatible Personal Computer were used for data reduction. These programs prepared by R.D. Piyasena and *et al.* [2] follow the data processing philosophy and principles set out by Robert A. Dudley [8]. The mathematical and statistical manipulation in Dudleys approach are based on procedures described by D. Rodbard [9]. For comparison 30 unknown samples were analyzed by Amerlex-M T3-RIA (magnetic separation), bulk

reagent T3-RIA (double antibody; NETRIA), in house T3-RIA (coated charcoal separation) and in house T3-RIA (solid phase) alongwith quality control pools. Later, reproducibility data of 10 assay batches of each type (except charcoal based assay) was collected for between batch analysis. In late solid phase assays the old tracer (specific activity = 1000- 1200 uCi/ug) was replaced with high specific activity tracer (1800-3400 uCi/ug).

Results and Discussion

Normal standard curves relating B/T% to standard T3 concentrations for the four assay systems described above are shown in Fig. 1. The sensitivities of these assays were comparable although Amerlex-M RIA seemed to be the most sensitive one. The standard curve parameters, a,b,c,d, from the 4-parameter logistic log equation [8] as generated by data processing programs referred to above, together with the results from variance ratio test, and the results from quality control pool measurements are listed in Table 1. In case of Amerlex-M RIA, the deviation of standard dose points from the fitted line was high. This was reflected in high variance ratio value i.e., 20.05 (and hence low p). It suggests that the 4-parameter logistic model (in logit-log coordinate system) was inconsistent with the data. This effect was also seen in bulk reagent-RIA. The deviation of standard dose data in case of in house assays (coated charcoal and solid phase) was within acceptable limits implying that the data fitted well into the mathematical model (p>0.5) of curve. The results of QC pools indicate that solid phase RIA values correlate better with Amerlex-M values than those from the bulk reagent assay. Coated charcoal assay values were lowest in high concentration pool. Figure 2 shows correlation of solid phase RIA values with commercial RIA's. The imprecision profiles of the four assays are shown in Fig. 3. From these results the Amerlex assay, which uses magnetic separation appeared the most precise. The next was bulk reagent assay (double antibody). Coated charcoal assay had comparable precision in low concentration specimens but it was highly imprecise in high concentration samples. Solid phase RIA was slightly less precise in low dose region but highly precise at higher concentrations. Subsequent experience proved that a better overall precision was obtainable by the in house solid phase assay as evidenced from the cummulative precision profiles as shown in Fig. 4. The between batch quality control data of the assays is shown in Table 2.

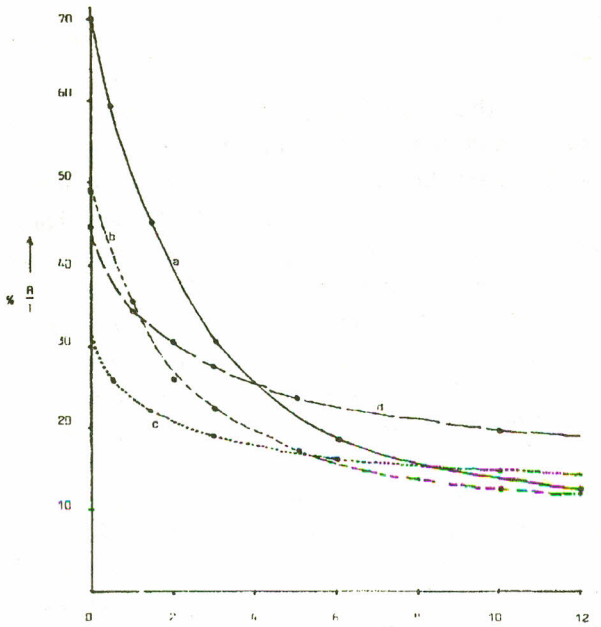

Fig 1. Standard curves obtained in four assays of different set up: (a) Amerlex-M T3 RIA: * Sensitivity = 0.08 n mol/l (b) Bulk reagent T3 RIA: Sensitivity - 0.25 n mol/l (c) Coated charcoal T3 RIA: Sensitivity = 0.2 n mol/l (d) Solid phase T3-RIA: Sensitivity = 0.2 nmol/l
*Concentration which is two standard deviations above the zero standard.

Systems based on differential migration are expensive and less practicable, whereas those based on adsorption and fractional precipitation are associated with significant misclassification errors [10,11] which results in inefficiency and lack of ruggedness. Double antibody methods may be

TABLE 1. CURVE PARAMETERS AND QUALITY CONTROL POOL RESULTS OBTAINED IN FOUR ASSAYS BASED ON DIFFERENT SEPARATION SYSTEMS

	Curve parameters						Quality control pool results					
	A	B	C	D	VR*	P	A	%CV	B	%CV	C	%CV
Amersham-m T3 RIA (Magnetic separation)	69.78	1.14	2.21	3.4	20.05	0.001	0.46	3.1	1.96	2.5	3.11	0.91
Bulk reagent t3 ria (Double antibody method)	48.26	0.82	2.57	0.06	6.60	0.05	0.3	8.7	1.40	10.6	2.7	7.33
Modified	70.06	0.7	0.88	3.50	0.89	NS	0.6	3.51	1.49	8.04	2.6	6.9
Coated charcoal T3-RIA (In house)	30.0	1.34	2.47	11.07	1.38	NS	0.79	1.1	1.66	5.7	1.92	3.9
Solid-phase T3-RIA (In house)	41.3	0.80	4.64	2.17	1.08	NS	0.75	3.4	1.85	6.3	2.74	4.0

*Variance ratio.

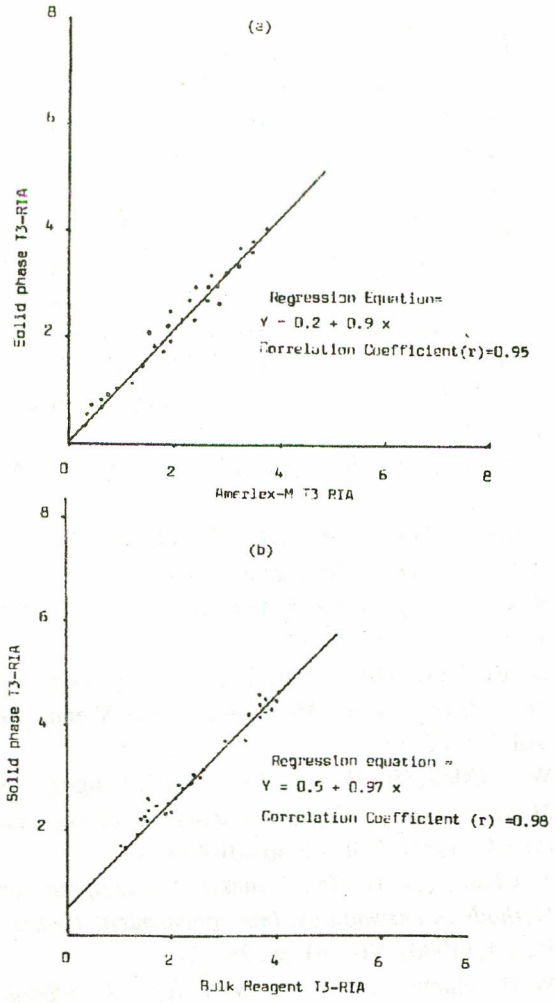

Fig 2. Correlation of solid phase RIA values with values obtained in commercial assays (a) solid phase assay value vs Amerlex-M values (b) solid phase assay values vs bulk reagent assay.

sensitive to interference by complement [12] although these methods are efficient, simple and applicable. The extensive use of solid phase methods in advanced immunoassys [13] is due to high efficiency, low misclassification errors and simple protocols. A loss of antibody avidity (and hence assay sensitivity) due to coupling of antibody to solid phase is compensated by improved precision resulting from high efficiency of separation. A choice of solid phase material with suitable physical characteristics can avoid the need of mixing during incubation [5].

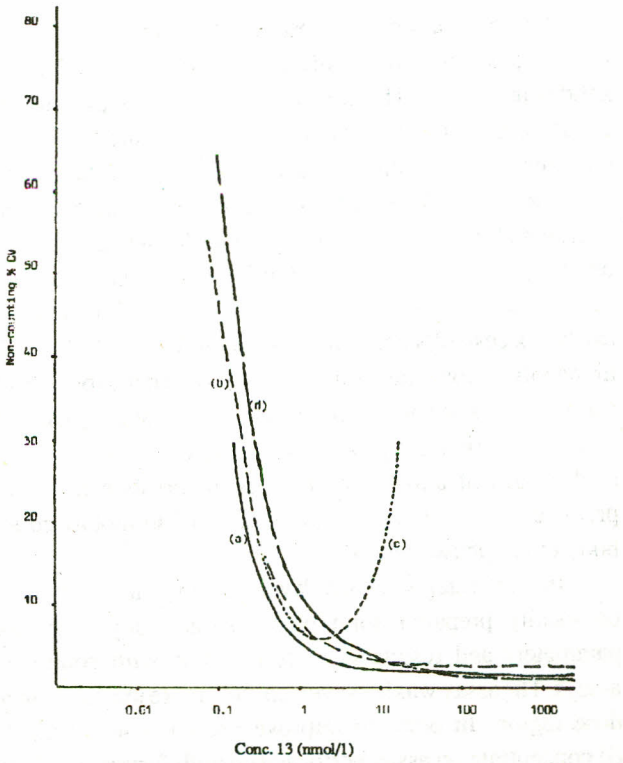

Fig. 3. Imprecision profiles of the assays: (a) Amerlex - M RIA ; (b) Bulk reagent RIA ; (c) coated Charcoal RIA ; (d) Solid phase RIA

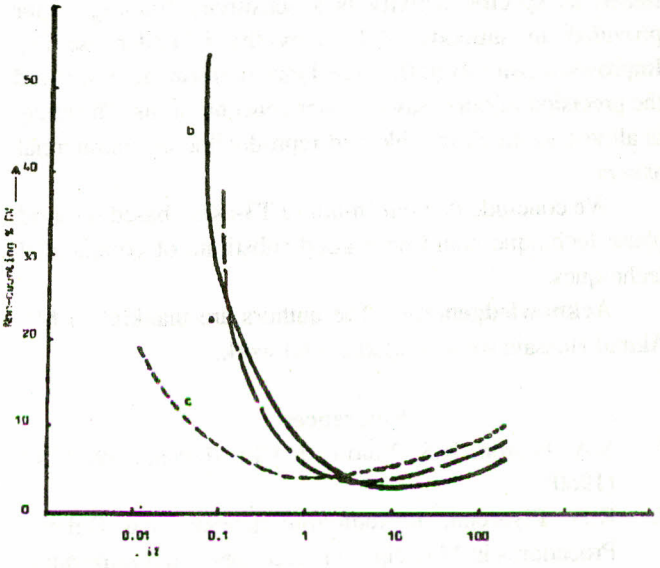

Fig. 4. Commulative imprecision profiles (10 Assays) (a) Amerlex M RIA ; (b) Bulk Reagent RIA ; (c) Solid phase RIA

TABLE 2. REPRODUCIBILITY DATA OF ASSAYS

	Average			Average			Average		
	Control -1	Drift (%)	BBCV (%)	Control -2	Drift (%)	BBCV (%)	Control -3	Drift (%)	BBCV (%)
Bulk reagents RIA	0.58	3	20.2	1.58	0	7.3	4.14	-1	12.0
Solid phase RIA	0.46	12	22.4	1.47	1	12.2	4.64	-1	10.2
Amerlex-M RIA	0.77	16	16.8	1.98	5	9.5	4.50	-3	8.6

Note: All controls assayed in duplicate in beginning, middle and end of each 100 test tube assay. Concentration expressed in n. mol/l. Control values are mean of 10 assay means.

* between Batch coefficient of variation.

Microcrystalline cellulose, a cheap and readily available material, is most commonly employed as general purpose solid phase matrix. The old methods used to couple antisera to cellulose gave health, cost and time problems [14-16]. The commercial preparations available in bulk and kit form are often prohibitively expensive [5]. These limitations can be overcome by using 1,1'-carbonyld iimidazole (CDI), a compound introduced by G.S. Bethal *et al.* [17] for activation of agarose beads in affinity chromato- graphy. The compound can be successfully and safely used to activate cellulose. Here imidazolyl carbamate groups are introduced into polysaccharide in an organic solvent environment [5]. Subsequently, the remaining imidazole groups are reacted with peptide amino acid groups of anti-bodies in aqueous alkaline medium to produce N-alkylcarbamate derivatives of immobilized antibody (solid phase antibody).

In our initial work we developed in-house assay based on locally prepared solid phase T3-antibody. The assay parameters and results were comparable with commercial assays. The assay was however, slightly more imprecise in low dose region. In order to improve precision we decided to (i) concentrate on assay performance with further experience (ii) replace the old tracer with relatively high specific activity tracer, as specific activity is a sensitivity limiting factor provided an antibody of high avidity is under use [3]. Improved sensitivity (0.07 nmol/1) and performance improved the precision of our assay in lower concentrations. The assay is almost as much reliable and reproducible as commercial assays.

We conclude that our in-house T3-assay based on solid phase technique could be a good substitute of commercial techniques.

Acknowledgement. The authors are thankful to Mr. Akmal Hussain for all experimental work.

References

1. S.A. Besron, R.S. Yalow, J. Clin. Invest., **39**, 1157 (1960).
2. R.D. Piyasena, In:Radioimmunoassay and Related Procedures in Medicine (Proceedings of a Symposium, IAEA Vienna, 1982). pp. 659-671.
3. J.I. Thorell, S.M. Larson, *Radioimmunoassay and Related Techniques. Methodology and Clinical Applications* (The Mosby Company, Saint Louis, 1973) pp. 15-18.
4. Ray Edwards, *Immunoassay.* An Introduction (Willium Heinemann Medical Books, London, 1985) pp. 22-23.
5. R.S. Chapman, R.M. Sutherland and J.G. Ratcliff, In: W.M. Hunter, and J.E.T. Corrie. (eds). *Immunoassay for Clinical Chemistry* (Churchill Livingstone, Edinburgh London Melburn and Newyork, 1983). 2nd ed., pp. 178-179.
6. L. Wide, J. Porath, (Acta Endocrinol Suppl; **142**, 207 (1969).
7. I.A.E.A. TECHDOC-509 PC/RIA User's Manual (I.A.E.A, July, 1988). version 1.0, p1.
8. R.A. Dudley, I.A.E.A-TECHDOC 346 (I.A.E.A, Vienna, 1985). pp. 31-65.
9. D. Rodbard, D.M. Hutt, In:*Radioimmunoassay and Related Procedures in Medicine* (I.A.E.A, Vienna, 1974). Vol. **1**, pp.165.
10. W.D. Odell, (In: H. Van Vunakis, J.J. Langone. (eds), *Methods in enzymology. Immunochemical Techniques, Part A,* (1980). Vol. 70, pp.401-426.
11. T. Chard, (In: H. Van Vunakis, J.J. Langone. (eds), *Methods in enzymology. Immunochemical Techniques Part A,* (1980). Vol. 70, pp. 280-291.
12. W.M. Hunter, In: D.M. Weir (ed), *Handbook of Experimental Immunology* (Blackwell, Oxford, 1978), Vol. **1**, 3rd ed., pp. 14.4-14.40.
13. L. Wide, J. Porath, Biochem. Biophys. Acta., **130**, 257(1966).
14. C.G. Sanderson, D.V. Wilson, Immunology, **20**, 1061 (1971).
15. A.E. Gurevich, O.B. Kuzolleva, A.E. Tumanova, Biochemistry, pp. 803 (1962).
16. R. Axen, J. Porath, S. Ernback, Nature., **214**, 1302 (1967)
17. G.S. Bethel, J.S. Ayers, W.S. Hancock, M.T. Hearn, J. Bio. Chem., **254**, 2572 (1979).