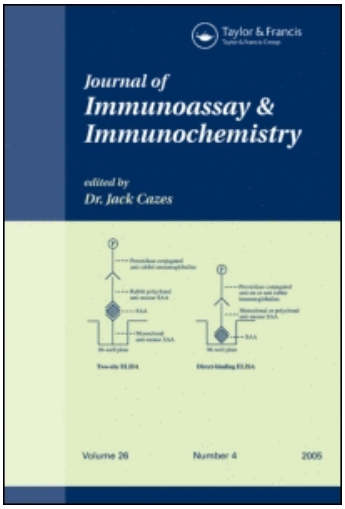

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Development of Coated Tubes RIA for Serum T_3 (Tri-Iodothyronine) for Production Scale

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Abstract: A coating procedure that could provide immobilization of antibodies, with increased binding capacity, that is cost effective, simple, robust, and appropriate for production scale application, is described. This coating approach of T_3 antibodies to the polystyrene tubes has been systematically investigated to determine its utility for the development of coated tube Radioimmunoassay (RIA) for T_3 in human serum. Further, the results obtained by the developed coating procedure are found to be comparable with those obtained by the "gold standard," the liquid phase RIA for T_3. The coating procedure is completed in three major steps, each step involving an overnight incubation. The normal rabbit γ-globulins are physically adsorbed onto the polystyrene tubes and incubated. After washing, a second antibody (goat anti-rabbit antiserum) is added and incubated. To this antigen specific antibody is added (T_3 antibody produced in rabbit) and further incubated. Finally, the non-specific sites on the tubes are saturated by the blocking solution. The concentration of normal rabbit globulin, titers of second antibody and T_3 antibody, and time required for coating are optimized to arrive at a suitable coating protocol. The coated tubes were evaluated for precision, reproducibility, and stability. Various parameters such as

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total reaction volume, incubation time and temperature, total number and volume of washings, concentration of 8-anilino-1-naphthalene sulfonic acid (ANS), and quantity of tracer per tube are optimized to arrive at a suitable standard curve. The optimized assay is validated for the quality control parameters such as intra- and inter-assay variations, recovery, and parallelism. The developed coated tubes assay had an assay range of 0.3–4.8 ng/mL with a sensitivity of 0.3 ng/mL at 90% B/B_0. Batch to batch variation in coating was $<10\%$. The coated tubes were stable up to 1 year, which is adequate for production scale.

Keywords: Coated tube RIA, T_3 Solid phase, Antibody immobilization, Production scale

INTRODUCTION

Modern immunoassay originates from the historic work of Berson and Yalow,[1] who utilized the specificity and sensitivity of the antigen–antibody reaction to quantify biomolecules, on the basis of their antigenicity. Their contribution, known as radioimmunoassay (RIA), was based on competitive inhibition. Although the popularity of RIA's grew through 1960s and 1970s, it was nevertheless troubled by the technical difficulty in separating the bound complex from the free reactant. Hence, the observation by Catt and Tregear[2] that proteins spontaneously become adsorbed to the plastic surfaces allowing the protein coated plastic tube to act as a reaction vessel, provided a convenient means of separating bound reactants from the free reactant. This technique replaced the other separation techniques due to significant advantages over them.[3] Currently, the research in the field of solid phase immobilization of antigen or antibodies is focused, worldwide, on the development of new materials and methodologies that will allow the distinct and spatially addressed coupling of biomolecules onto the solid surface. Conformational changes or orientation problems with the ligand bound onto the surface could be avoided by the use of spacers, which have initially reacted on the surface of the tube. Towards this, several technological aspects and the steps carried out in this direction are quite promising.[4–6]

Passive adsorption of proteins on polystyrene results in the loss or alteration of antigenic epitopes and, hence, loss of binding activity of the biomolecules. In case of covalent coupling, loss of activity of the biomolecule arises during the process of coupling that may be near the reactive site of the molecule. Immunochemical immobilization, wherein anti-species antibodies (second antibody) are used for coating the reagent of interest on the solid surface, is found to be a better alternative, but this demands affinity-purified reagents, which is a disadvantage at the production level. Apart from this, a large consumption of the antibodies is required that makes the procedure costly.[7–9] Following a coating procedure that could provide immobilization

of antibodies with increased binding activity and easy adaptation to automation, which could be appropriate for production scale, we used a reported procedure[9] after suitable modification. The ability of this new coating approach was investigated with triiodothyronine (T_3) polyclonal antibody as the model. The challenge that an analytical biochemist needs to take is to develop solid phase radioimmunoassay for quantitation of analytes in biological media, which is equal to or superior to conventional liquid phase RIA, especially for very small molecules (haptens) like T_3. T_3, in spite of its presence in extremely small quantities in the human body, plays a very important role in maintaining normal metabolism; T_3 levels can be vital in the diagnosis of pathological disorders.[10,11] Systematic studies for the immobilization of the binding reagent to the polystyrene surface and development of an assay system which is simple, rugged, and requires no special equipment, such as shaker or water baths, is necessary for the development of an assay system for large scale production. Here, we report our studies on coating of T_3 antibody, evaluation of the coated tubes, assay development, and validation of the developed assay for the quantitation of total T_3 in human serum.

EXPERIMENTAL

Reagents

Normal rabbit γ-globulin was purchased from Span Diagnostics, India. Second antibody (goat anti-rabbit antiserum) was raised at Tanuvas, Madras Veterinary College, India. T_3 antibody with affinity constant as 4×10^{12} L/M was raised and characterized in-house. ^{125}I-T_3 radiotracer was prepared in-house by iodinating T_2 (Diiodotyrosine) by the conventional chloramine T method. The tracer had a specific activity of 100 MBq/microgram and a radioactive concentration of 50 MBq/L.

Polystyrene tubes were commercially procured. Tris salt, sodium carbonate, sodium bicarbonate, bovine serum albumin, and thiomersal were all of AR grade.

Coating Procedure

Various parameters, such as concentration of normal rabbit γ-globulin, titers of second antibody and T_3 antibody, blocking buffer, and washing steps were optimized to arrive at a suitable coating procedure. The first step was the physical adsorption of normal rabbit γ-globulin (0.5 mL of 0–20 mg/L) in 0.05 M bicarbonate buffer, pH 9.0 to the polystyrene tubes. The tubes were incubated overnight at room temperature, decanted, and washed twice with 1 mL of wash buffer (0.01 M Tris–HCl, pH 8.6). Then, second antibody (goat anti-rabbit antiserum) (0.5 mL of 1 : 100 to 1 : 1200) was added and

incubated overnight at room temperature. To the tubes containing second antibody solution, T_3 antiserum was added (0.1 mL of 1 : 200 to 1 : 2000), so that this is immunoadsorbed on the second antibody, which, in turn, is immunoadsorbed onto the rabbit γ-globulins and further incubated overnight at room temperature. After the incubation of second as well as primary antibody, the tubes were decanted, washed, and then saturated with blocking solution (1 mL of 0.1 M $NaHCO_3$ containing 10 g/L BSA and 0.5 g/L sodium azide) and incubated for an hour at room temperature. The tubes were then washed with wash buffer and used for optimizing the assay system.

Coated Tube Evaluation

The percentage of radiotracer bound to antibody-coated tubes was estimated, in replicate, to determine the intra-assay variation and the reproducibility in various batches of coating. The coated tubes were stored in ziplock plastic bags with desiccants at 4°C. The stability of these tubes was studied at regular intervals of time.

Assay Optimization

Various parameters, such as incubation time (1, 2, 24 hr) and temperature (room temperature and 37°C), total reaction volume (250–550 μL), quantity of radiotracer per tube (35,000–75,000 cpm), ANS concentration (0–1000 μg per tube) in the radiotracer, volume of T_3 standard or sample, and number and volume of washes were studied in detail to arrive at the optimized assay protocol.

Assay Validation

The assay was validated by inter- and intra-assay variation, recovery tests, parallelism tests, and the precision profile. Human serum samples ($n = 45$) were analyzed by the optimized coated tube assay and compared with data obtained from a magnetic particles assay developed at BRIT, as well as the liquid phase assay.

RESULTS AND DISCUSSION

Antibody Coating Procedure

The normal rabbit γ-globulin concentration for coating was found to be optimal at 2 mg/L (Figure 1) (1 μg/0.5 mL per tube). The second antibody

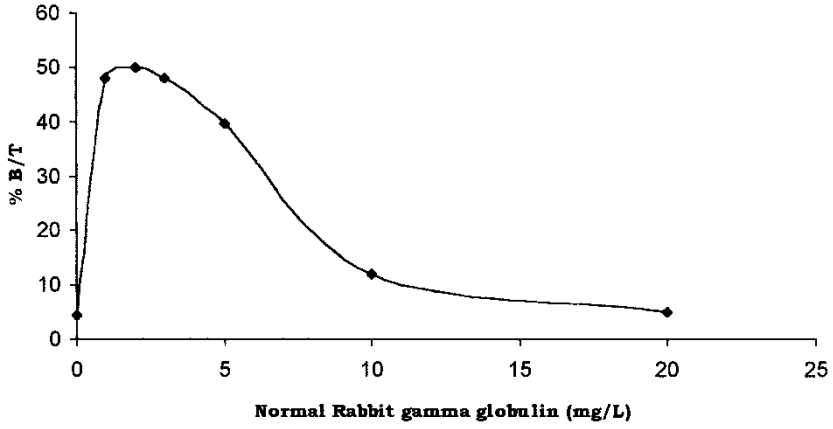

Figure 1. Effect of amount of gamma-globulin.

and T_3 antibody titers were selected as 1 : 800 (0.5 mL) and 1 : 1000 (0.1 mL), giving a final titre of 1 : 5000 for the primary antibody (Table 1). The low concentration of the three reagents shows the superiority of the developed coating approach[9] over all other types of coating procedures, such as physical adsorption and covalent coupling, hence rendering it cost effective and suitable for production purposes. An additional advantage of this coating procedure is the successful replacement of the affinity-purified antibodies by the neat serum. Two washings of 1 mL of wash buffer (0.01 M Tris–HCl, pH 8.3) each were found to be adequate for the intermediate washings for achieving good precision between the coated tubes. It was observed that blocking with 0.1 M $NaHCO_3$, containing 10 g/L BSA and 0.5 g/L sodium azide, gave a non-specific binding of $<1\%$. When the rabbit γ-globulin and

Table 1. Titres of second antibody and T_3 antibody

Second antibody titer:	1 : 100	1 : 200	1 : 400	1 : 800	1 : 1000	1 : 1200
T_3 antibody titer			$\%B/T^a$			
1 : 200	65	64	59	51	40	27
1 : 400	62	61	57	48	36	23
1 : 800	65	59	54	47	33	19
1 : 1,000	58	57	53	46	31	19
1 : 1,600	58	57	51	42	31	20
1 : 2,000	51	57	51	43	30	20

[a]% Bound counts/total counts

the T_3 antibody were allowed to incubate for 6 hr, a binding of 34% was observed when compared with 45% obtained at 24 hr incubation. Since the steps involved are extremely simple and easily amenable to automation, each of the incubations was standardized as 24 hr.

Coated Tube Evaluation

Tubes prepared and dried according to the optimized coating protocol showed an intra-assay variation (%CV of counts) of $<5\%$. Reproducibility between different batches of coated tubes ($n = 8$) showed the imprecision of percentage tracer bound at zero standard dose (%CV) to be $<7\%$. The coated tubes (both in terms of binding capacity and assay characteristics) were found to be stable for >2 years (Table 2).

Assay Optimization

The optimized standard curve had an assay range of 0.3–4.8 ng/mL with a 2 hr incubation at room temperature. The optimum assay protocol is given in Table 3. At the end of incubation, the tubes were washed twice with 1 mL of wash buffer. The high counts per tube prevented any imprecision that may arise due to coating or at the user's end in the range that differentiates the normal from hypothyroid or hyperthyroid patient samples. Thus, in the crucial range of T_3 i.e., 0.7–2.4 ng/mL, a difference of $\sim$10,000 cpm is observed in the bound counts. The optimum amount of ANS required was 100 μg/0.2 mL tracer (Figure 2). Thus, the optimized assay for the estimation of T_3 in human serum sample is a simple, two-step assay with a short

Table 2. Stability of coated tubes

T_3 standard concentration (ng/mL)	Duration ($\%B/T^a$)				
	0th day	2 months	6 months	12 months	2 year
0	37	43	43	41	49
0.3	32	37	38	37	44
0.6	30	33	34	35	40
1.2	25	27	28	28	33
2.4	20	23	24	20	25
4.8	13	14	15	13	17

[a] T_3 tracer used for studying the stability was from different batches.

Table 3. Optimized assay protocol

T_3 antibody coated tubes + T_3 standard/sample (50 μL) + $^{125}I - T_3$ (200 μL)
Incubate the tubes for 2 hr at room temperature
Decant and wash the tubes twice with 1.0 mL wash buffer. Blot for 5 min and count them in NaI(Tl) scintillation counter

incubation of only 2 hr, which is very user friendly. A typical coated tube T_3 standard curve is shown (Figure 3).

Assay Validation

The developed coated tube based RIA was validated for various parameters. The intra-assay coefficient of variation ($n = 10$) was 5–14% and the inter-assay coefficient of variation ($n = 25$) was 8–15%. The range and sensitivity of this assay were established from its precision profile[12] (Figure 4). The range of the assay was found to be 0.3–4.8 ng/mL. Over this range of T_3 concentration, the error in the measured dose remained <10%. Recovery of known standard added along with the samples was found to be between 85 and 116% (Table 4). In order to check samples and the T_3 standard similarity, a high dose T_3 sample was appropriately diluted as 1 : 2, 1 : 4, 1 : 8, respectively, with the T_3-free serum and assayed along with the sample. The values obtained on dilution correlated well with the dilution factor, indicating

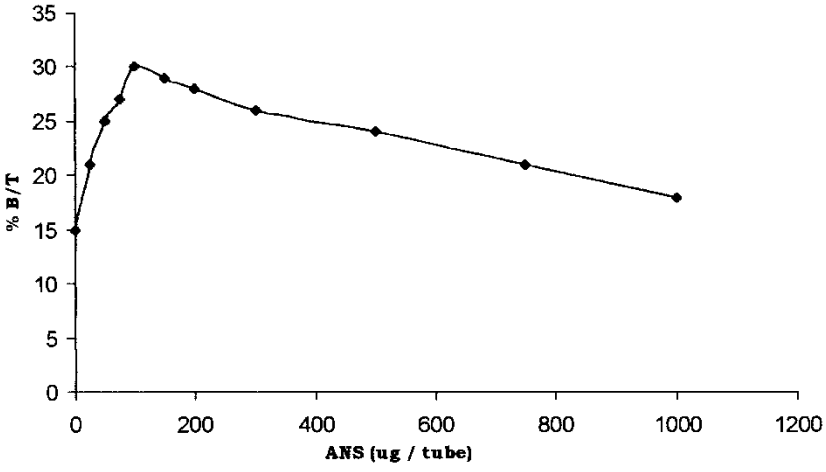

Figure 2. Effect of ANS concentration in tracer.

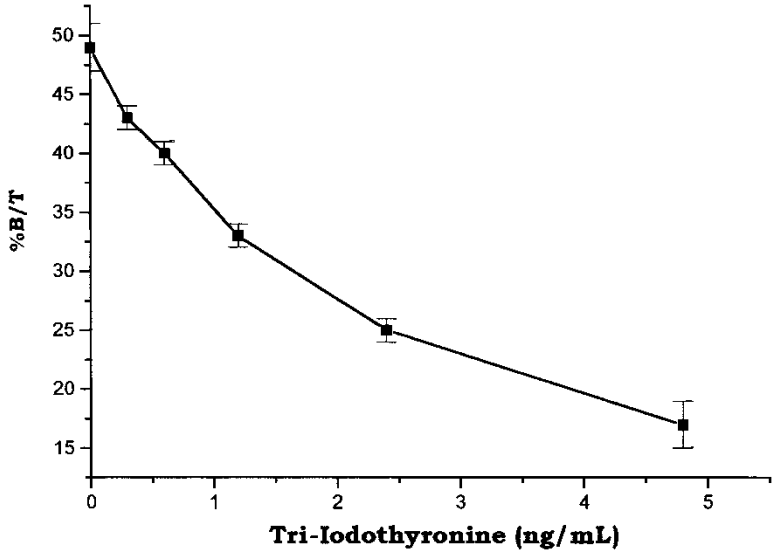

Figure 3. A typical standard curve.

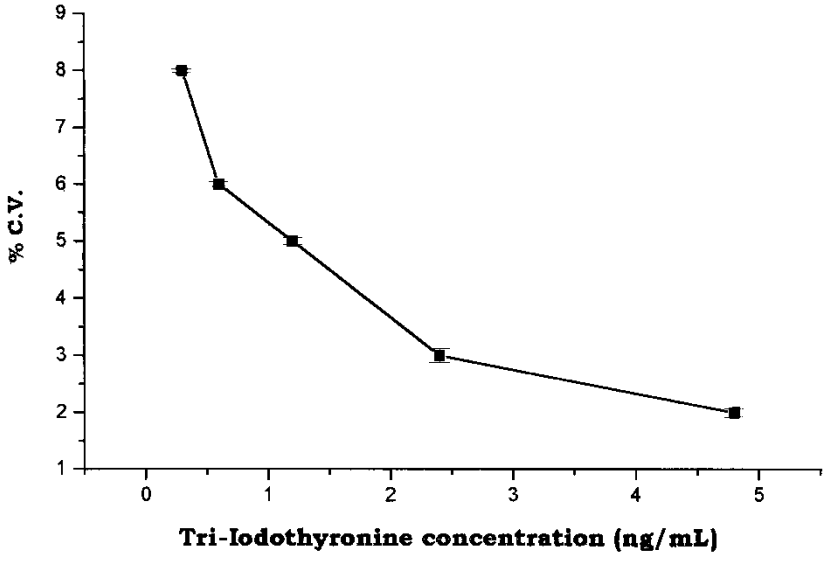

Figure 4. Precision profile.

Table 4. Recovery studies

Sample value (ng/mL)	T_3 standard added (ng/mL)	Expected value (ng/mL)	Observed value (ng/mL)	Recovery (%)
1.7	0.6	1.15	0.97	85
	1.2	1.45	1.68	116
	2.4	2.05	1.86	91
1.36	0.6	0.98	1.02	104
	1.2	1.28	1.35	105
	2.4	1.88	1.97	105
4.77	0.6	2.38	2.65	111
	1.2	2.98	3.18	106
	2.4	3.58	3.38	95

the sample and the standard similarity. Serum sample analysis ($n = 45$) was performed using the developed coated tube RIA and compared with those obtained from liquid phase RIA, as well as magnetic particles RIA for T_3. Correlation with liquid phase RIA showed $r = 0.967$ with a regression line $y = 0.8x + 0.1$ ng/mL (Figure 5) and the correlation with magnetic particles RIA showed $r = 0.957$ with a regression line $y = 0.923\text{x} - 0.066$ ng/mL (Figure 6).

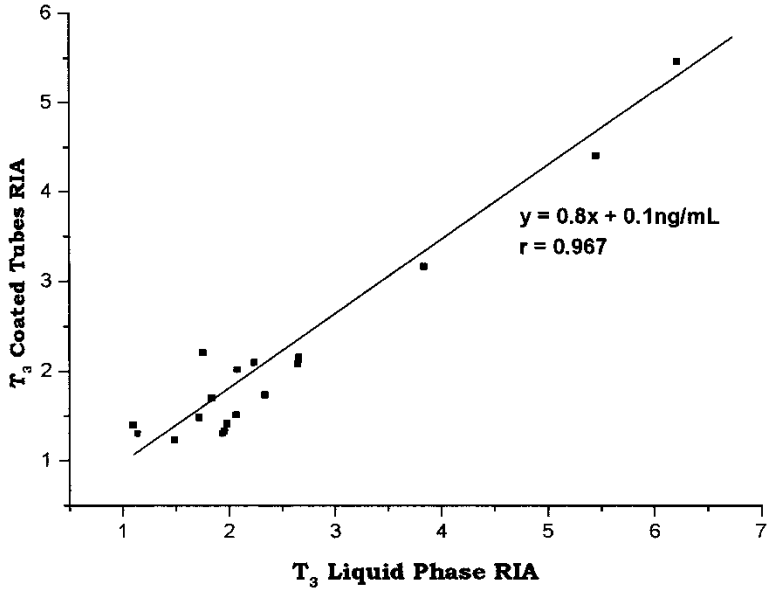

Figure 5. Correlation of T_3 coated tubes RIA with T_3 liquid phase RIA.

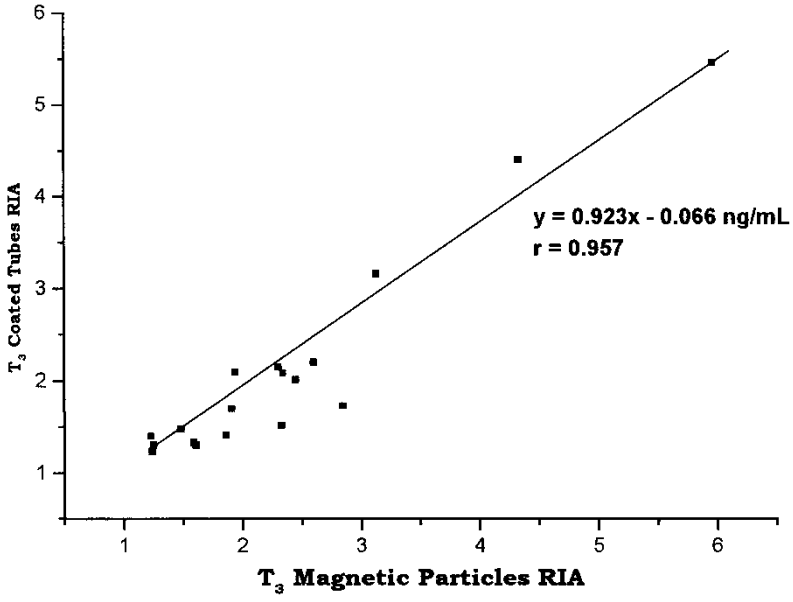

Figure 6. Correlation of T_3 coated tubes RIA with T_3 magnetic particles RIA.

CONCLUSION

The present method of coating using normal rabbit γ-globulin, second antibody, and primary antibody is found to be superior with respect to precision and stability. The use of normal rabbit γ-globulin and second antibody helps the antigen specific recognition sites to line up more easily and also increases the binding capacity, reducing the steric hindrance problem. Since this method uses a small amount of primary antibody for coating, it is highly cost effective for production. Also, the optimized coating procedure is easily amenable to automation and, hence, it is appropriate for the production of T_3 coated tube RIA kits.

The T_3 assay developed and validated using this coated tube approach is user friendly and the results obtained for the estimation of serum T_3 are highly reliable.

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