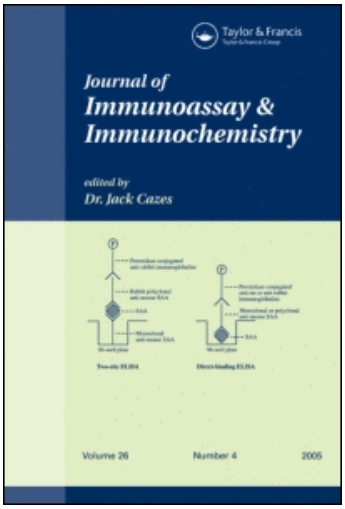

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IMPROVED ANTIBODY COATING PROTOCOL USING A SECOND ANTIBODY ANTISERUM. APPLICATION TO TOTAL THYROXIN IMMUNOASSAY

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ABSTRACT

A complete antibody coating protocol for the preparation of dry antibody coated tubes is presented. This protocol is based on a recently described antibody immobilization principle. We modify this immobilization principle in order to improve and simplify the coating procedure. In addition, we propose a drying procedure that provides long-term storage stability of the antibody coated tubes. According to the modified protocol, polystyrene plastic tubes are first coated with rabbit γ-globulins. The tubes are incubated with a sheep anti-rabbit

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IgG antiserum dilution. After incubation, antigen-specific antibody antiserum raised in rabbits is added directly into the tubes containing the sheep anti-rabbit IgG antiserum solution (difference from the original protocol). Finally, the tubes are washed, blocked, and dried following the drying procedure developed.

The suitability of the modified protocol for the development of immunoassays requiring high loading of antibody was exemplified through the development of a RIA for total thyroxin. The estimated assay characteristics (detection limit 4 μg/L, dynamic range up to 210 μg/L, within-run CV 2.7–5.7%, between-run CV 5.1–7.3%, recovery 84.4–112%, cross-reactivity for T_3 1.9%) were comparable with those provided by commercially available RIA kits for the determination of thyroxin.

INTRODUCTION

The early work of Catt and Tregear,(1) concerning the immobilization of antigen-specific antibodies onto plastic supports has found numerous applications in the development of solid-phase immunoassays.(2,3) Immobilized antibodies, however, exhibit significant loss of their binding activity.(4–6) To avoid inactivation of antigen-specific antibodies, alternative immobilization protocols, employing an immobilized second antibody, have been proposed in the literature.(5,7–10) The second antibody coating approach provides immobilized antigen-specific antibody of high binding activity with minimal reagent consumption.(7–10) However, in several applications, especially those requiring high loading of antigen-specific antibody onto the solid, an affinity purified second antibody must be used for coating.

We have developed a methodology for immobilization of antigen-specific antibodies onto plastic supports using γ-globulins from a non-immunized animal and a second antibody antiserum dilution.(11) The protocol provides immobilized antibodies with binding capacity equal to, or higher than, that obtained using affinity purified second antibody directly adsorbed onto the solid. In addition, it requires a considerably lower amount of the second antibody.

In this work, we present a modification of this antibody immobilization principle which simplifies the coating procedure and improves the quality of the antibody coated tubes. In addition, we propose a drying procedure which is appropriate for the preparation of coated tubes with

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prolonged storage stability. According to the modified protocol, polystyrene tubes are first coated with rabbit γ-globulins. Then, following decanting and washing of the tubes, they are incubated with a sheep anti-rabbit IgG antiserum dilution. After that, and without decanting and washing of the tubes, an appropriate volume of an antigen-specific antiserum, raised in rabbits, is added to the tubes, followed by further incubation. Finally, the tubes are washed, blocked, and dried. The potential application of tubes coated according to the modified protocol in immunoassays for the determination of high sample concentration analytes is demonstrated through the development of an RIA for total thyroxin in human serum samples.

EXPERIMENTAL

Chemicals, Reagents, and Instrumentation

All reagents were of analytical grade. Carrier-free $Na^{125}I$ (specific radioactivity 17 KCi/g, radiochemical purity 99.9%, iodate $< 2\%$), obtained from Nordion Europe S.A. (Belgium), was used for radiolabeling. L-Thyroxin (T_4) free acid, 3,3′,5-triiodo-L-thyronine sodium salt (T_3), Thimerosal, rabbit γ-globulins (Cohn Fraction I, II), and bovine serum albumin (BSA, fraction V, RIA grade, A-7888) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Coomasie brilliant blue G-250 was purchased from Eastman Kodak Co. (Rochester, NY, USA). Chloramine-T, mercaptoethanol, 8-anilinonaphthalene-1-sulfonic acid (ammonium salt), sodium azide, sodium salicylate, maleic acid, and all other reagents were purchased from Merck (Darmstadt, Germany). The T_4-free human serum used for the preparation of T_4 standard solutions was a product of Scantibodies Laboratory Inc. (San Diego, CA, USA). Rabbit anti-T_4 antiserum was purchased from OEM Concepts Inc. (Toms River, NJ, USA). Sheep anti-rabbit γ-globulins antiserum (second antibody antiserum) was a product of the RIA Lab., I/R-RP, N.C.S.R. "Demokritos" (Athens, Greece). Polystyrene RIA tubes (75×12 mm) were from VIVE Co. (Athens, Greece). Radioactivity measurements were performed using a 12-well γ-counter (NE-1612, Nuclear Enterprises Ltd, UK), facilitated with appropriate software for interpretation of RIA results.

T_4 Standard Solutions and Control Sera

Thyroxin standard solutions (0, 27, 57, 110, 210 μg/L) were prepared in T_4-free human serum by addition of known amounts of T_4. The

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concentration of each standard solution was verified using a commercially available RIA kit (T4 Amerlex-M, Johnson & Johnson Clinical Diagnostics Ltd, Amersam, UK). The T_4 standard solutions were stable for at least 2 months at 4°C or for 1 year at −20°C.

Three control sera were prepared by pooling human sera with low, medium, and high concentrations of T_4.

Preparation of ^{125}I-T_4

Radiolabeled T_4 was prepared by radioiodination of T_3 using a modification of the method of Greenwood et al.(12). Briefly, 20 μL of a 0.74 mM T_3 solution in ethanol were mixed with 20 μL of a 0.69 mM KI solution in 0.25 M phosphate buffer, pH 7.4 (radioiodination buffer) and 50 μL of the same buffer. Then, 10 μL of a $Na^{125}I$ solution (250 Ci/L) in radioiodination buffer and 10 μL of a 3.54 mM chloramine-T solution in the same buffer were added in the tube and the mixture was incubated for 60 s, under vortexing. The reaction was terminated by adding 10 μL of a 0.14% (v/v) mercaptoethanol solution in radioiodination buffer. The radiolabeling yield (higher than 90%) was determined by paper electrophoresis. The ^{125}I-T_4 was purified on a PD 10 column (Pharmacia LKB Biotechnology, Sweden). The radioiodination mixture (0.12 mL) was loaded onto the column which had been equilibrated with 0.05 M phosphate buffer, pH 7.4, and eluted firstly with 20 mL of the same buffer. Under these conditions, the non-reacted radioiodine was eluted, whereas the ^{125}I-T_4 was retained on the column. The retained radioiodinated hormone was then eluted using the same buffer, containing 1 g/L BSA and 0.5 g/L NaN_3. Fractions having high radioactivity and presenting high binding with the anti-T4 antibody were pooled and further diluted in 0.1 M Tris-maleate buffer, pH 8.25, containing 0.15 g/L 8-anilino-naphthalene-1-sulfonic acid (ammonium salt), 0.10 g/L sodium salicylate, and 1.0 g/L of NaN_3, to a storage activity of 0.07 mCi/L (^{125}I-T_4 radiotracer solution). The specific radioactivity of the ^{125}I-T_4, determined by the method of Chiang(13), was 230 Ci/g.

Antibody Immobilization Protocols

Proposed Protocol

Two milliliters of a 1 mg/L rabbit γ-globulins solution in 0.05 M carbonate buffer, pH 9.2 (coating buffer), were added to each tube and the tubes were incubated for 22 h at room temperature (RT). The tubes were

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washed twice with 3 mL of 0.01 M Tris-HCl buffer, pH 8.25, (washing solution). After that, 2 mL of a 1/5,000 second antibody antiserum dilution in 0.15 M Tris-HCl buffer, pH 8.25, containing 1 g/L BSA and 0.2 g/L Thimerosal (coupling buffer) were added per tube, and the tubes were incubated for 22 h at RT. Then, 100 µL of a 1/1,430 dilution of anti-T_4 antiserum (1/30,000 final dilution in the tube) in coupling buffer, containing 50 mg/L Coomasie Brilliant Blue G-250, were added per tube. The tubes were incubated for 22 h at RT. The liquid was decanted and the tubes were washed as previously. Then, 3 mL of a 0.1 M $NaHCO_3$ solution containing 10 g/L BSA and 0.5 g/L sodium azide (blocking solution) were added per tube, and the tubes were incubated for 18 h at RT. The liquid was decanted and the blocked tubes were dried overnight at 37°C. Dry tubes were kept in plastic bags, containing dessiccant, at 4°C until use. No washing of the tubes was performed prior to the assay.

Original Protocol

Anti-T_4 antibody coated tubes were prepared following a previously described procedure,(11) appropriately modified to meet the requirements of the T_4 assay. In brief, the steps followed up to the incubation of the tubes with the second antibody solution were same as those described in the proposed protocol. After incubation for 22 h at RT with the second antibody solution, the liquid was decanted and the tubes were washed with 2×3 mL of washing solution. Then, 2 mL of a 1/30,000 dilution of anti-T_4 antiserum in coupling buffer were added per tube and the tubes were incubated for 22 h at RT. After washing and blocking of the tubes, as in the proposed protocol, the tubes were washed twice prior to use.

T_4-Assay Procedure

Twenty microliters of T_4 standard solutions (0–210 µg/L), or serum sample, and 2 mL of ^{125}I-T_4 radiotracer solution were added to tubes coated with anti-T_4 antibodies and mixed by vortexing. The tubes were incubated for 2 h at RT. The liquid phase was then decanted and the tubes were left inverted for a few minutes. The radioactivity bound onto the solid was measured in the γ-counter. The binding capacity of the solid-phase antibody was calculated as the fraction of radiotracer bound in presence of T_4-free serum (Bo). For the construction of the calibration curve, the (Bs/Bo) values were plotted against the T_4 concentration of the standard solutions, on a semi-logarithimic scale, and the data were subjected to linear regression fit

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by the least-squares method (Bs represents the radioactivity bound onto the solid in the presence of standard solutions containing known amounts of unlabeled T_4).

Comparison with Other T_4-Assays

Two commercially available solid-phase RIA kits for the determination of total T_4, namely the T4 Amerlex-M RIA (Johnson & Johnson Clinical Diagnostics Ltd, Amersam, UK), and the T4-SOLID-RIA (N.C.S.R. ''Demokritos'', Athens, Greece) kit were used for the comparison studies. The T_4 Amerlex-M RIA kit utilizes a sheep anti-T_4 antibody bound to magnetizable polymer particles, whereas the T4-SOLID RIA kit is based on rabbit anti-T_4 antibody adsorbed onto polystyrene tubes.

RESULTS

Optimization of the Proposed Coating Protocol

Conditions such as the duration and the temperature of the various incubation steps, as well as the pH and the ionic strength of the buffers used, were optimized for each of the coating steps. The selection of the optimum conditions for each of the steps was based on the binding capacity values of the immobilized anti-T_4 antibody and the analytical characteristics of the assay. Considering the first step of the immobilization procedure, specifically, adsorption of the rabbit γ-globulins onto the inner surface of the polystyrene tubes, the pHs of the coating solutions examined ranged between 7.0 and 9.5. Sodium phosphate, Tris-HCl, and sodium carbonate buffer solutions were used. The ionic strength of the buffers used ranged between 0.01 and 0.15 mol/L. Maximum immunoreactivity values were obtained using a 0.05 mol/L carbonate buffer, pH 9.2. Optimum duration and temperature conditions for this coating step were established by immobilizing rabbit γ-globulins from 3 to 72 h at 4, 22, and 37°C. Maximum immunoreactivity values were reached after 10 and 16 h at 37 and 22°C, respectively, and remained unchanged for up to 72 hours of incubation. On the other hand, at least 48 h of incubation were required to obtain maximum immunoreactivity values when the incubation was carried out at 4°C. Thus, 20–24 hours of incubation at RT (22°C) were adopted in the final coating protocol in order to facilitate the handling of the tubes.

Similarly, the pH, the temperature, and the duration of immunoadsorption of the second antibody onto the immobilized rabbit γ-globulins

(second step) were optimized. Amongst the different pHs (7.2 to 8.5) and ionic strengths (0.01 to 0.25 mol/L) tested, the higher binding capacity and the best coating reproducibility was obtained using a 0.15 mol/L Tris-HCl buffer, pH 8.25 ± 0.05. The immunoadsorption proceeded more rapidly at RT than at 4 or 37°C and maximum binding capacity values were reached after 18 hours of incubation. Thus, 20–24 hours of incubation at RT was finally adopted for this step.

In the third step, the immunoadsorption buffer was same as in the previous step, since only a small volume of the anti-T_4 antibody solution was added into the tubes containing the second antibody solution (100 μL to 2 mL). Maximum binding capacity values were achieved by incubation for 18 h at RT.

The concentration of rabbit γ-globulins required for the first coating step, in order to obtain maximum binding capacity values, was estimated. It was found that maximum binding capacity values were obtained using a rabbit γ-globulins solution of 1 mg/L for all the second antibody antiserum dilutions tested. Thus, this concentration of rabbit γ-globulins was adopted in the proposed coating protocol.

In order to select anti-T_4 and second antibody antiserum dilutions appropriate for our application, a range of anti-T_4 antiserum dilutions was tested in combination with several second antibody antiserum dilutions. Our target was to determine an anti-T_4 antiserum dilution that could provide adequate binding capacity value for a wide range of second antibody antiserum dilutions. As is shown in Figure 1, for each anti-T_4 antiserum dilution there was a different range of second antibody antiserum dilutions which provided maximum binding capacity values. This range of second antibody antiserum dilutions drifted to higher dilutions as higher anti-T_4 antiserum dilutions were used. In addition, the range of second antibody antiserum dilutions for which maximum binding capacity values were obtained was becoming constantly narrower as higher anti-T_4 antiserum dilutions were used. Nevertheless, for final anti-T_4 antiserum dilutions in the tube, higher than 1/50,000 the binding capacity values obtained were relatively low, even when optimum second antibody antiserum dilutions were employed. Adequate binding capacity values for our application can be obtained using an anti-T_4 antiserum dilution equal to, or lower than, 1/40,000. For our application, we selected an anti-T_4 antiserum dilution of 1/30,000 because it provided adequate binding capacity values for a wider range of second antibody antiserum dilutions (1/2,000–1/10,000) than the 1/40,000 dilution. Concerning the second antibody, in our final protocol, we selected a 1/5,000 dilution, which provided maximum binding capacity values with the selected anti-T_4 antiserum dilution. Coomasie brilliant blue G-250 was added to the anti-T_4 antibody solution in order to facilitate the

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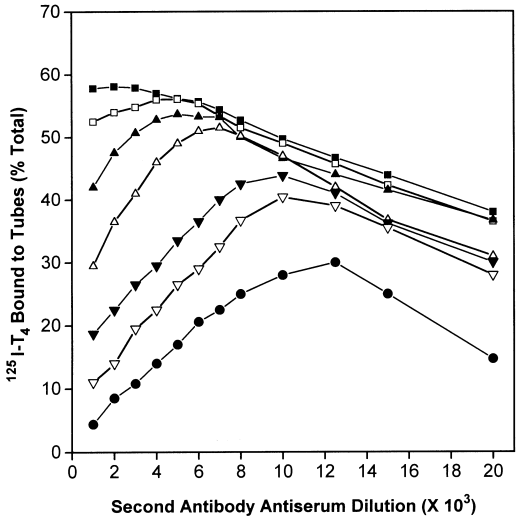

Figure 1. Effect of the second antibody antiserum dilution on the binding capacity of the immobilized anti-T_4 antibody. The dilutions of the anti-T_4 antiserum used were: 1/10,000 (■); 1/20,000 (□); 1/30,000 (▲); 1/40,000 (△); 1/50,000 (▼); 1/60,000 (▽); and 1/120,000 (●). The concentration of rabbit γ-globulins used for the initial coating of the tubes was 1 mg/L.

handling of the tubes. We found that the addition of this dye at the concentration described under Experimental did not affect the binding capacity of the antibody coated tubes.

The selected combination of anti-T_4 and second antibody antiserum dilutions resulted in T_4 RIA with appropriate analytical characteristics. In addition, the between-batch coating reproducibility obtained following these conditions was excellent, since 10 batches of anti-T_4 antibody coated tubes (500 tubes per batch) prepared over a period of 1 year had a mean binding capacity value $\pm$ 1SD of $58.8 \pm 3.4\%$.

Comparison with the Original Protocol

We compared the modified coating protocol with the original one in terms of the binding capacity values and the intra-batch coating reproducibility they provided. In both protocols, the anti-T_4 antiserum dilution used for the preparation of the tubes was 1/30,000.

As is shown in Figure 2, similar maximum plateau binding capacity values were reached by both protocols as the second antibody antiserum

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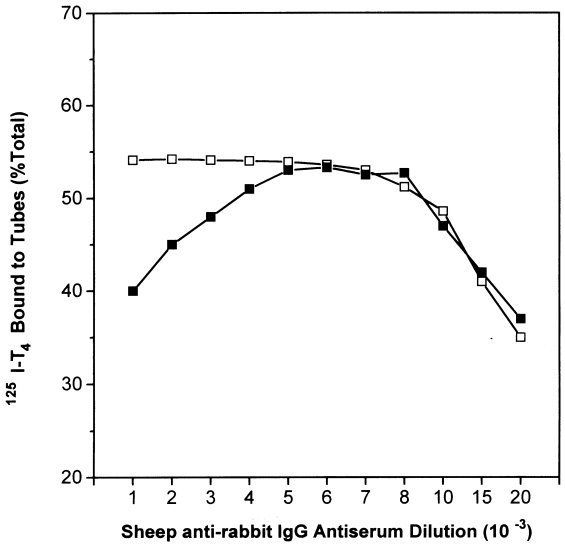

Figure 2. Comparison of the binding capacity values of the anti-T_4 antibody immobilized onto plastic tubes at a 1/30,000 antiserum dilution following: (a) the proposed coating protocol (■); and (b) the original protocol (□), using second antibody antiserum dilutions ranging from 1/1,000 to 1/20,000.

dilution was decreased from 1/20,000 to 1/7,000. Following the proposed coating protocol, the plateau binding capacity values were reserved for second antibody antiserum dilutions down to 1/4,000, whereas, using more concentrated second antibody antiserum dilutions (1/4,000 to 1/1,000), the binding capacity values were gradually decreased. On the other hand, following the original protocol, plateau binding capacity values were retained for second antibody antiserum down to 1/1,000.

In order to evaluate the intra-batch coating reproducibility of the two coating protocols, 10 batches of anti-T_4 antibody coated tubes (at least 500 tubes per batch) were prepared following either of the coating protocols within a period of one year. The coating reproducibility was determined by assaying, in a single run, at least 100 tubes, randomly selected from each batch, in presence of T_4 zero standard. We found that the intra-batch reproducibility obtained using both protocols was $\leq 5\%$. However, the mean intra-batch CV $\pm$ 1SD provided by the modified protocol for the 10 batches of tubes was $2.8\% \pm 0.7\%$, and the corresponding value obtained by the original protocol was $3.9\% \pm 0.8\%$. T-test analysis of the results showed that, at the 0.01 level, these values were significantly different ($t = 3.347$; $n_1 = 10$, $n_2 = 10$, $p = 0.01$).

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Blocking and Drying of the Anti-T_4 Antibody Coated Tubes

The effect of blocking of the tubes on their binding capacity values was investigated. Several blocking agents, such as bovine serum albumin (BSA), gelatin, and casein were tested. It was found that blocking with any of these agents caused a marginal increase in the binding capacity values of the tubes. Tubes blocked with BSA, however, had significantly lower CV values (less than 5%) compared with tubes blocked with gelatin or casein (CVs 4–12%). Thus, BSA was selected as the blocking agent in the final protocol.

The conditions for the preparation of dry antibody coated tubes were evaluated with respect to the binding capacity they provided after drying, and to their stability during long-term storage. The tubes were dried either at RT or at 37°C. The duration of the drying procedure was varied from 3 to 18 h. Dried tubes were kept at 4°C in presence of desiccant. It was found that the binding capacity values of the tubes, after drying, were decreased by approximately 5% compared with the binding capacity values of the wet tubes, no matter what was the duration of the drying. Therefore, the drying procedure was finally judged on the basis of the storage stability of the dried tubes. For this purpose, the binding capacity of tubes dried either at RT or at 37°C for 18 h was determined in 15-day intervals, over a period of 1 year. It was found that tubes dried at 37°C preserve more than 85% of their binding capacity value after 1 year of storage at 4°C. By contrast, tubes dried at RT preserved only 70% of their initial binding capacity value over the same period of time. Thus, in the final protocol, we adopted drying of the tubes at 37°C for 18 h.

In addition, it was found that the T_4 RIA calibration curves obtained over a period of 1 year, using a single batch of tubes dried according to the proposed protocol, were almost identical (Figure 3).

Analytical Characteristics of the T_4 Assay Developed

An RIA for the determination of total T_4 in human serum samples was developed using dry anti-T_4 antibody coated tubes prepared according to the proposed coating approach.

The detection limit of the RIA developed, determined as the analyte concentration corresponding to the mean radioactivity value of 20 replicates of zero standard -2SDs (95% confidence), was 4.0 μg/L, and the useful analytical range was 6–210 μg/L.

The within-run precision of the assay was determined by assaying three control sera, corresponding to different levels of T_4 (mean T_4 concentrations of 51.6, 96.0, and 134 μg/L), in 12 replicates in a single assay.

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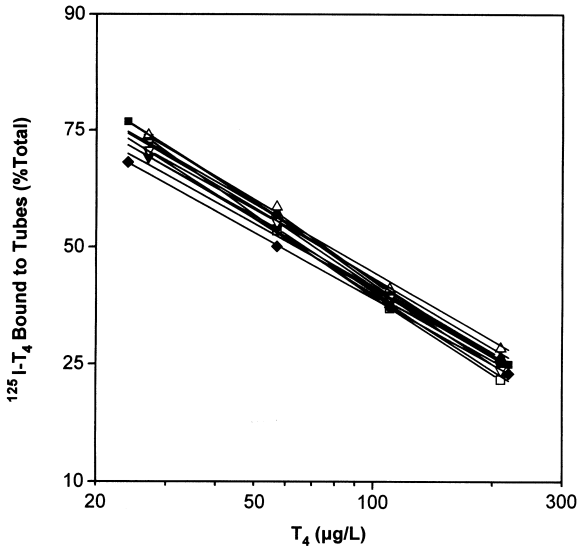

Figure 3. T_4 RIA calibration curves obtained within a 1-year period using tubes prepared by the proposed protocol. Tubes were run 1 (■), 15 (○), 45 (△), 90 (▽), 120 (◆), 270 (□), and 365 days (▲) after their preparation.

The within-run CV of the assay was found to range between 2.7% and 5.7%. For the determination of the between-run precision, duplicate determinations of the same control samples were performed in 15 different runs. The between-run CV ranged between 5.1% and 7.3%.

The recovery was assessed by analyzing human serum samples before and after the addition of known concentrations of exogenous T_4. The measured increase in the T_4 concentration of the sample, expressed as percentage of the expected increase, was determined as the recovery of the assay. The recovery of added exogenous T_4 was found to be between 84.4 and 112% (Table 1).

The dilution linearity of the assay was determined by assaying serum samples serially diluted with T_4-zero calibrator. As is shown in Figure 4, there was a good agreement between the measured and the expected values which were derived from the concentrations of T_4 in the undiluted samples.

The specificity of the assay was evaluated by determining the cross-reaction values for a number of chemically related compounds and metabolites of T_4. The cross-reactivity for each of these compounds was expressed as the per cent ratio of the amount of T_4 which corresponded to 50% of zero standard binding divided by the amount of the compound that corresponded to the same level of binding. Amongst the substances tested,

Table 1. Recovery of Exogenous T_4 Added to Human Serum Samples

Sample	Initial Value (μg/L)	Added Value (μg/L)	Expected Value (μg/L)	Measured Value (μg/L)	Recovery %
1	43.6	27.0	70.6	73.7	111
		55.8	99.4	93.6	89.6
		74.3	117.9	123.3	107
2	68.4	27.0	95.4	94.5	96.7
		55.8	124.2	116.8	86.7
		74.3	142.7	149.5	109
3	98.2	27.0	125.2	121.0	84.4
		55.8	154.0	149.6	92.1
		74.3	172.5	181.6	112

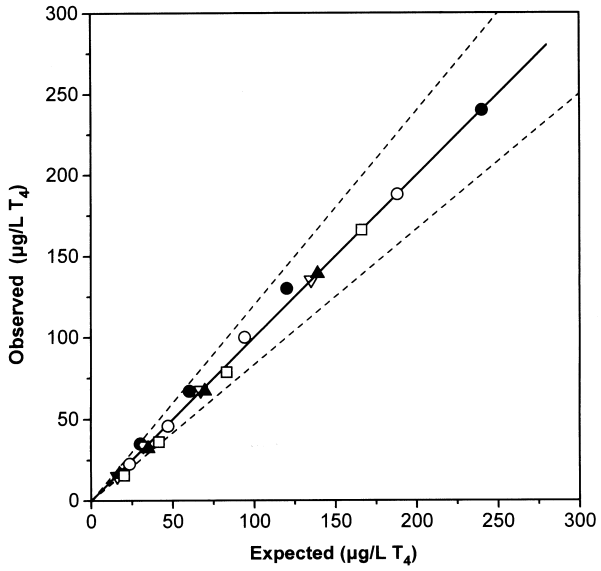

Figure 4. Serial dilution of serum specimens in zero calibrator. Serial dilutions were performed on each of five serum specimens. Average dilution recoveries across all dilutions for each specimen ranged from 86.3 to 114%, with a grand mean across all specimens and dilutions of 98.9%. (—), ±20% tolerance limits for reference.

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3,5-diiodo-L-thyronine, 3,5-diiodo-L-tyrosine, 3-iodo-L-tyrosine, 5,5-diphenylhydantoin, and phenylbutazone exhibited cross-reactivity values less than 0.03% while, for T_3, the cross-reactivity value was 1.9%.

Sera from 57 apparently euthyroid subjects were assayed in order to determine the range of the normal values. The mean value of T_4 concentrations determined was 79.2 ± 31.1 ($\pm$ 2SD) μg/L and the normal range was calculated to be 48.1–110.3 μg/L.

The results obtained by assaying serum samples with the developed assay were in good agreement with the results obtained by two commercially available RIA kits for the determination of total T_4, namely the T4 Amerlex-M RIA (Johnson & Johnson Clinical Diagnostics Ltd, Amersam, UK) and the T4-SOLID RIA (N.C.S.R. "Demokritos") kit (Figures 5A, 5B).

DISCUSSION

Recently, we presented an antibody coating method that provided high binding capacity values of immobilized antibodies using a second antibody antiserum instead of affinity purified antibody.(11). Here, we present a modification of this method which simplifies the coating procedure and improves the quality of the antibody coated tubes. In addition, we propose a procedure for the preparation of dry antibody coated tubes that is an important issue when large-scale production is considered. Thyroxin was selected as model analyte in our study, in order to investigate the potential of the modified protocol for the development of solid-phase immunoassays for analytes of high sample concentration.

According to the original protocol, γ-globulins from a non-immunized animal of the same species as that from which the antigen-specific antibody has been developed are absorbed onto the solid. After removal of excess γ-globulins, a second antibody solution is added to the tubes. Following incubation, unbound second antibody is removed and antigen-specific antibody solution is added into the tubes. The modification proposed here concerns the addition of the antigen-specific antibody to the tubes already incubated with the second antibody, without prior decanting of the second antibody solution.

Our results indicated that, using optimized combinations of anti-T_4 and second antibody antiserum dilutions, binding capacity values adequate to perform a T_4 RIA were achieved following the modified protocol (Figure 1). In fact, the maximum binding capacity values obtained were similar to those obtained following the original protocol (Figure 2). However, decreased binding capacity values, compared with those obtained by the original protocol, were observed when second antibody

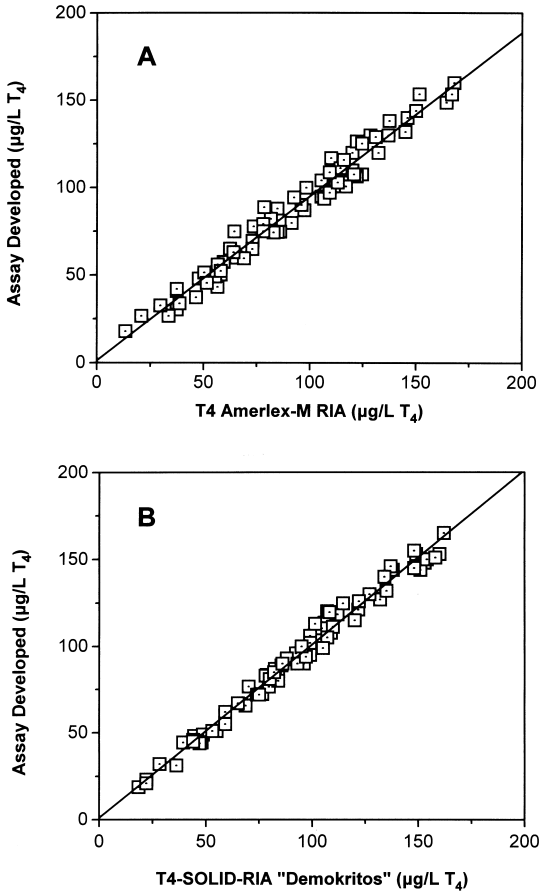

Figure 5. Correlation between the T_4-RIA developed (y) and two commercially available RIA kits (x) for the determination of total T_4: (A) For the T4 Amerlex-M RIA kit, $y = 0.94x + 1.4$, $n = 72$, $r = 0.984$; (B) For the T4-SOLID RIA kit, $y = 1.00x + 1.1$, $n = 76$, $r = 0.991$. All serum samples were run in duplicate.

dilution lower than that provided maximum binding capacity values was used. This difference between the two protocols arises from the difference in the immobilization principle. When relatively high concentrations of second antibody are used in the modified protocol, its unbound fraction consumes a significant part of the added anti-T_4 antibody. Thus, the amount of the anti-T_4 antibody that is available for coupling with the solid-phase second antibody is reduced, affecting negatively the binding capacity values.

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Following the proposed protocol, excellent reproducibility of the binding capacity values both within a single preparation, as well as between different preparations of antibody coated tubes, was achieved. The proposed coating protocol provided better within-batch reproducibility, compared with the original one. This was indicated by comparing 10 different batches of tubes prepared following each of the protocols. This finding is of great importance, especially when commercial application of antibody coated tubes is considered.

The long-term storage stability of the antibody-coated tubes was another issue of consideration in this work. We found that the best results were obtained when the tubes were blocked with BSA and subsequently dried overnight at 37°C. Tubes prepared and dried according to the proposed protocol presented great stability, both in terms of binding capacity and assay characteristics. Using these tubes, similar T_4 RIA calibration curves were obtained over a period of 1 year, although several batches of radiotracer and T_4 standard solutions were used (Figure 3). Concerning the stabilization of the antibody coated tubes prior to drying, several agents, such as sucrose, maltose, trehalose, and sorbitol, that have been proposed as protein stabilizers during drying or freeze-drying procedures, were examined.(14,15) For the stabilization experiments, after blocking, the anti-T_4 antibody coated tubes were incubated for several time periods (1–24 h) with solutions of the above-mentioned agents at concentrations up to 3% (w/v) in 0.1 mol/L sodium bicarbonate, pH 8.5. We found that, in all cases tested, the treatment of the anti-T_4 antibody coated tubes with these reagents, although improved marginally the binding capacity values, it affected negatively the reproducibility of the assay (data not shown). Thus, treatment of the tubes with such a stabilizing agent was not adopted in the final protocol.

The modified coating protocol decreases the labor required for the preparation of the tubes, compared with the original one. This is due to the elimination of the washings after immobilization of the second antibody involved in the original protocol. In addition, in order to facilitate the handling of the tubes, we added a colored substance (Coomasie Brilliant Blue G-250) to the dilution buffer of the anti-T_4 antiserum. The color facilitates the discrimination of the tubes containing the anti-T_4 antibody from those not containing it.

The total T_4 RIA, developed using tubes prepared according to the proposed protocol, had appropriate sensitivity and was precise and accurate as was indicated from the recovery (Table 1) and the linearity experiments (Figure 4). In addition, the T_4 values obtained by assaying human serum samples with the RIA developed were in good agreement with those obtained by two commercially available solid-phase RIA kits for the determination of total T_4 in human serum (Figure 5A, 5B).

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In conclusion, here we present an improved version of a previously described immobilization procedure for the preparation of antibody coated polystyrene tubes using a second antibody and antigen-specific antibody antisera. The proposed coating protocol provides dry antibody coated tubes with high binding capacity and excellent reproducibility. In addition, it is appropriate for the development of RIAs for analytes of high sample concentration. Thus, it should be helpful for the development of solid-phase immunoassays for other analytes.

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