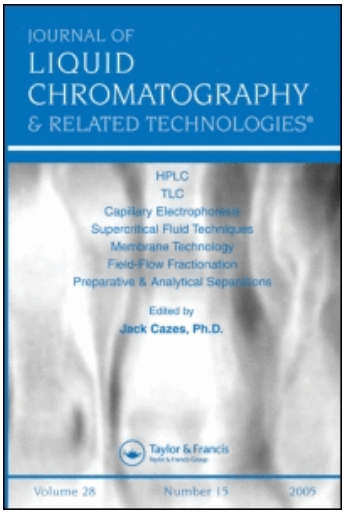

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DEVELOPMENT AND VALIDATION OF A TWO-STEP FREE THYROXINE RADIOIMMUNOASSAY BASED ON ANTIBODY COATED TUBES

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□ *We describe a two-step radioimmunoassay procedure (RIA) for the measurement of free thyroxine (fT_4) in human serum. A commercial antibody with the affinity of 5.5×10^9 L/M was used for this study. The anti-T_4 antibody was immobilized on the inner walls of the polystyrene tubes by passive adsorption through normal rabbit IgG and anti-rabbit IgG as immunobridges. The method developed uses very small amounts of antibody with minimum dilution of the sample. The assay covers the range of 0 to 87.5 pmol/L with sensitivity of 0.9 pmol/L. The intra-assay precisions were 6.3% and 7% at 6.7 and 20.7 pmol/L, respectively, for 15 replicates. The inter-assay precision at 6.4 and 24.4 pmol/L were 13% and 11.6%, respectively. The normal range established by analyzing 54 healthy volunteers was 15.3–24.7 pmol/L and that of 69 ambulatory subjects was 14.8–23.3 pmol/L. Progressive dilution of euthyroid and hypothyroid samples up to 50-fold yielded virtually constant values, whereas a registered decrease in free T_4 was observed with hyperthyroid samples. Free T_4 estimated for 75 samples, including samples from 16 pregnant female subjects, correlated well with the values obtained by a reputed commercial kit with the linear correlation coefficient of 0.84.*

Keywords ambulatory subjects, free T_4, immuno-bridges, RIA

INTRODUCTION

Sensitive measurement of thyrotropin (TSH) and estimation of free thyroxine concentration have become the first line tests for the evaluation of thyroid function. Measurement of TSH with adequate functional sensitivity clearly differentiates both overt and subclinical hypothyroidism from normal thyroid function. However, in clinical situations characterized by deranged TSH regulation, the abnormal relationship of serum TSH and

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free thyroid hormones exists, and the measurement of free thyroid hormones are essential for determining thyroid hormone status. When TSH measurements appear discordant with clinical thyroid evaluations, free T_4 measurements are helpful in identifying inaccurate TSH measurements, as well as deranged TSH regulation.[1] Among the several methods available, column chromatography-RIA, back titration method, and one-step analog assays, the two-step back titration methods are suitable for routine large scale laboratory analysis and are also unaffected by the interfering substances present in the sample. The accuracy of free hormone measurements yielded by two-step assays is dependent on a) choice of good antibody possessing suitable thermodynamic characteristics; b) intelligent assay design; and c) care in the routine performance of the assay.[2] The performance of two-step assays has been primarily constructed from reports concerning the Clinical Assays, Gamma Coat two-step method.[3] The majority of the reports show close agreement with equilibrium dialysis-RIA in all clinical states. Hence, we have used Clinical Assays kit for evaluating our developed procedure. Additionally, we have followed the guidelines recommended by the American Thyroid Association (ATA) while validating the assay system.[4]

MATERIALS AND METHODS

Reagents used in the Assay System

Assay buffer used is 0.14 M Tris-HCl buffer containing 0.9% NaCl, pH 7.4, and the wash buffer used is 0.035 M Tris-HCl buffer with 0.05% tween-20.

Antibody coated tubes were prepared by immobilizing anti-thyroxine antibody raised in rabbit through anti-rabbit IgG and normal rabbit IgG by the method described by Panagiota S. Petrou et al.,[5] with some modification. Star bottom polystyrene tubes procured from Greiner Bio One, Germany were incubated with 0.5 mL of 1 mg/L normal rabbit gamma-globulin from SIGMA Biologicals in 0.05 M sodium bicarbonate buffer, pH 9.2 for 22 hr at room temperature. On the second day, tubes were washed twice with 3 mL 0.01 M Tris-HCl buffer, pH 8.25 (wash buffer) and incubated for 22 hr at room temperature with 250 µL of 1:300 fold dilution of anti-rabbit IgG procured from Genei, Bangalore, India, in 0.15 M Tris-HCl buffer pH 8.25 containing 0.1% BSA and 0.02% thiomersol (coupling buffer). On the third day, after one wash with the wash buffer tubes were incubated with 1:1000 dilution of anti-thyroxine antibody in coupling buffer for 22 hr at room temperature. On the fourth day, the tubes were washed twice with a 3 mL wash buffer and saturated with 750 µL of 0.2 M glycine for 1 hr; tubes were washed once, drip-dried, packed

in self sealing packets, and stored at 4°C. The tubes thus prepared were evaluated for immunoreactivity, precision, extraction efficiency with normal sera, and also for stability.

^{125}I labeled thyroxine (tracer) of high specific activity was prepared by iodinating triiodothyronine by chloramines-T oxidation method and purified over a sephadex-G25 gel-column. The specific activity of the tracer was determined to be around 66.6 MBq/μg by the self-displacement method.

Standards ranging from 0–87.5 pmol/L were prepared in normal human serum purchased from a local blood bank after rendering it T_4 free by charcoal stripping. FT_4 standards were prepared by spiking the free serum with a known T_4 concentration. The respective free T_4 concentrations were estimated using Clinical Assays two-step FT_4 kit. The concentrations of the standards were calculated as the mean value obtained from five different assays with the CV less than 5%. The values thus obtained were also checked by the TT_4/FT_4 correlation plot suggested by Ekins.[6]

Immunoassay kits used: Specimens collected were analyzed for T_3 and T_4 using in-house kits. TSH was estimated by Immunotech, (France) TSH IRMA kit and FT_4 was estimated using Clinical Assays two-step RIA kit.

Specimen collection: Normal serum samples were collected from 53 apparently healthy adults excluding individuals taking oral contraceptives or under hormone replacement therapy. Reference intervals for the healthy adults were TSH 0.17^{-1} 4 μIU/mL; TT_4 55^{-1} 135 ng/mL; and TT_3 0.7^{-1} 2.1 ng/mL. There were 9 hypothyroid samples, 16 pregnant female subjects, 69 ambulatory subjects, and 3 hyperthyroid samples included in this study.

Optimized Assay Protocol

Step I: 50 μL of standards and sample were incubated with 200 μL of 0.14 M tris-saline buffer, pH 7.4 in anti-T_4 antibody coated tubes for 30 mins at 37°C. The tubes were washed twice with 1 mL wash buffer and allowed to stand for 3–5 mins in the inverted position after the first wash in order to remove traces of serum proteins to obtain maximum binding with the tracer in the second incubation step.

Step II: Second incubation was carried out with 300 μL of ^{125}I labeled thyroxine at 4°C for 1 hr. It was observed that incubation at 4°C ensured the binding of tracer to unbound sites on the solid phase antibody, thus improving the sensitivity of the assay at higher FT_4 concentrations. 1 mL wash buffer was added, mixed, decanted, and activity measured in a gamma counter.

Critical Parameters Studied in Optimization of the Assay

Antibody avidity was determined by the Scatchard plot method.[7]

Extraction efficiency of antibody coated tubes: The extraction of T_4 from binding proteins by the antibody was studied using pre-equilibrated normal sera containing 1.67 KBq per 50 µL of ^{125}I labeled thyroxine. 50 µL of this sera spiked with tracer was added to the tubes and the procedure mentioned in step I was followed. Percentage activity associated with the solid support with respect to total counts was expressed as percentage extraction efficiency.

Imprecision of the antibody coated tubes were determined by randomly selecting 5% of the tubes from each rack and incubating with ^{125}I labeled thyroxine for 1 hr at 4°C as shown in the optimized assay protocol Step II. The %CV of the bound activity was calculated.

Steps taken to avoid the interference of binding proteins with tracer during the second incubation: Variations in the volume of wash buffer and the numbers of washes were carried out to ensure complete removal of serum after the first incubation.

Analytical performance characteristics: Sensitivity, reproducibility, and accuracy were studied using a zero calibrator and in-house quality control pools. Interference due to cross-reactants such as T_2, T_3, and phenytoin was studied. Drift was also assessed by analyzing a sample at regular intervals in an assay volume of 75 samples.

Effect of varying sample volume: The first incubation of samples with the antibody coated tubes was performed with varying volumes of the sample, ranging from 10 µL to 100 µL.

Effect of sample dilution on FT_4 measurement: Sera from hypo-, normal, and hyperthyroid patients were diluted serially from 2 to 20 fold with tris-saline buffer, pH 7.4. Diluted samples were assayed along with the undiluted serum samples for FT_4 concentration by the standardized assay protocol.

Establishment of normal range: FT_4 values defining the central 95% reference range was obtained from 54 normal healthy volunteers' samples comprised of 24 female and 30 male volunteers. All subjects were considered euthyroid by the biochemical thyroid function tests, T_3, T_4, and TSH. 69 ambulatory subjects were also included in this study.

Comparison with the commercial kit: The FT_4 values obtained with the developed system was compared with Clinical Assays Kit values. 76 serum samples including 53 normal volunteers' samples, 2 hypo samples, 1 hyper sample, and 20 pregnant females' samples were included in this study.

RESULTS

The antibody with the average affinity of 5.5×10^9 L/M was used in the study. The antiserum had two different population of antibodies with the affinities estimated to be 1.4×10^{10} L/M and 1.1×10^9 L/M by Scatchard plot method.

The mean T_4 extraction efficiency calculated for 19 batches were 0.65%, with the standard deviation of 0.08%. The imprecision in tracer binding to antibody coated tubes was expressed as percentage CV obtained with bound counts, which was found to be 2.9 with the standard deviation of 0.8 for 19 batches. The antibody coated tubes were stable for more than a year at 2–8°C with respect to immuno-reactivity and precision.

Failure to drain the tubes completely leads to very less binding as shown in the Table 1. Increasing the volume of wash buffer improved the binding only marginally. But, washing twice improved the binding considerably, yet complete utilization of the tracer has not occurred. After the first incubation of the antibody with the sample, the tubes were washed with 1 mL wash buffer and the tubes were left in the inverted position for 3–5 min. before the second wash. This ensured the complete utilization of tracer avoiding the interference due to the carrier proteins.

Analytical Performance of the Assay

Minimum detection limit: The average minimum detection limit of the assay obtained from 5 different assays was found to be 0.9 pmol/L with the standard deviation of 0.24 pmol/L.

Assay precision: The intra assay precision for 15 replicates of two serum pools were estimated to be 6.3% at 6.7 pmol/L and 7% at 20.7 pmol/L. The Inter assay precision was estimated from 15 different runs as 13% at 6.4 pmol/L and 11.6% at 24.4 pmol/L.

TABLE 1 Effect of Washing After Step I

Volume and Number of Washings	Tracer Added Immediately After Washings	With 3–5 min. in the Inverted Position After One Wash Before Tracer Addition	With 3–5 min. in the Inverted Position Before Second Wash
1 mL	13.6%	25.0%	–
1 mL + 1 mL	40.9%	–	49.0%
2 mL	20.7%	31.6%	–
2 mL + 2 mL	42.7%	–	48.0%

Maximum binding obtained without serum was 48%.

Assessment using external quality control serum: The inter assay precision estimated from 15 different runs using Bio-Rad controls at three levels were 11.8%, 8.75%, and 11.6% at 5.15, 24.2, and 61.85 pmol/L, respectively. The values mentioned by Clinical Assays (Two-step) kit in the External Quality control assessment chart for the same Bio-Rad controls at three levels were 5.7 ± 1.5, 23.5 ± 6.2 and 65.6 ± 23.5 pmol/L, respectively. These values are closer to the values obtained using the developed procedure as shown in Table 2.

Analytical recovery: Analytical recoveries at four different FT_4 concentrations were estimated to be 102% at 6.4 pmol/L, 94% at 15.2 pmol/L, 97% at 21.9 pmol/L, and 97% at 33.5 pmol/L.

Study on drift: In an assay with the sample volume of 75, a sample with the mean FT_4 concentration of 8.1 pmol/L was analyzed seven times after every 10 samples with mean SD of 0.58 and CV of 7%, indicating the absence of drift in the estimation.

Figure 1 shows %B/Bo at standard concentrations 3.2, 6.4, 12.7, 21.9, 46.3, and 87.5 pmol/L for 27 assay runs with one standard deviation. The %CV estimated for 27 different runs on different dates with different batches of reagents particularly tracer and antibody coated tubes and were estimated to be 3.9, 5.6, 6.7, 6.9, 7.5, and 7.8% for the concentrations mentioned above, respectively.

Clinical Validity Parameters

One of the important parameters for a valid free hormone measurement of minimum dilution of the test sample during the reaction is ensured. During the reaction, 50 µL of the sample gets diluted to 250 µL, i.e., the test sample gets diluted 5 fold in the reaction mixture.

Effect of sample dilution on FT_4 measurement: Figure 2 shows how FT_4 estimations in various patient's sera changes with progressive dilution. Most sera except thyrotoxic patient sera were robust to dilutions up to 50 fold, i.e., 10 fold additional dilution over normal assay conditions giving virtually constant values. Hypothyroid sera even after a dilution of 100 fold yielded an FT_4 estimate of almost 91.7% of the original value; whereas, the thyrotoxic sample showed continuous decline in FT_4 values over dilution.

TABLE 2 Assessment of the System Using External Quality Control Serum

Bio Rad Control Serum	Values given by Clinical Assays Kit (pmol/L)	Values Obtained with Our System (pmol/L)
I	5.7 ± 1.5	5.15 ± 1.26
II	23.5 ± 6.2	24.2 ± 4.4
III	65.6 ± 23.5	61.85 ± 14.9

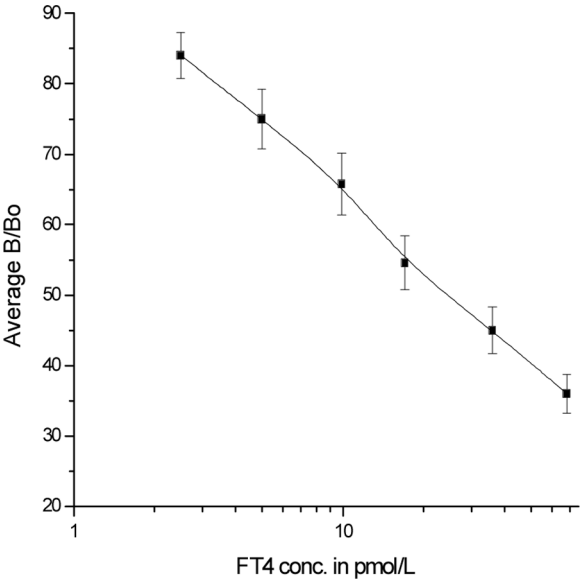

FIGURE 1 Dose response curve (n = 27).

Effect of varying sample volume: Varying sample volume such as 10, 25, 50, 75, and 100 µL yielded similar values as expected at 5.9 pmol/L and at 21 pmol/L with the intra-assay CV of 2% and 7%, respectively.

Normal range estimation: The central 95% euthyroid reference range, using 54 normal healthy volunteers, was found to be 15.3–24.7 pMol/L.

The range established for 69 ambulatory patients using our method was found to be 14.8–23.3 pMol/L and that of 12 pregnant females' samples was estimated to be 14.8–20.3 pMol/L. The distribution of FT_4 concentration in various groups of patients is shown in Figure 3.

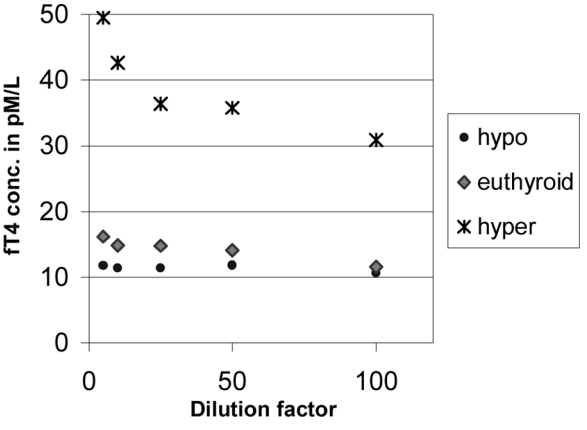

FIGURE 2 Effect of serum dilution on FT_4 measurement.

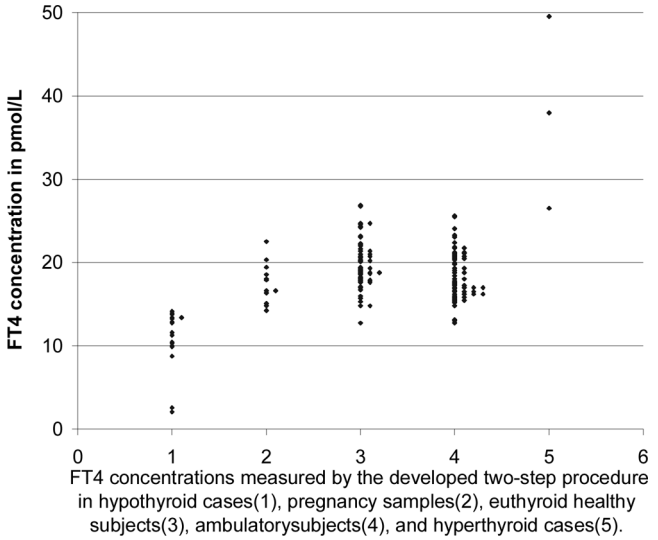

FIGURE 3 Distribution of FT_4 concentrations in various clinical states.

Comparison with the commercial kit: FT_4 values obtained for 76 serum samples using the developed procedure were compared with that obtained using clinical assays kit as shown in Figure 4. The following relationship was obtained for FT_4 values from developed procedure(y) regressed against

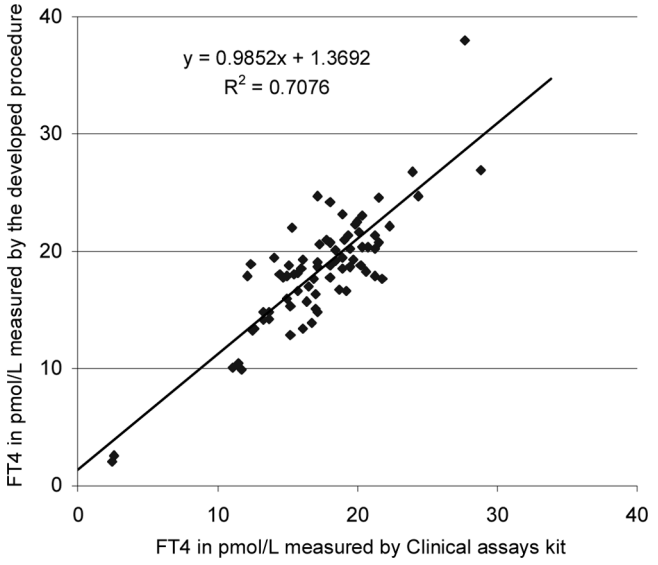

FIGURE 4 Correlation between FT_4 concentrations measured by Clinical Assays kit and the developed procedure.

Clinical Assays (two-step) values(x) for 76 samples: $y = 0.9852\ x + 1.0639$ ($r = 0.84$), SE = 2.107, slope = 0.985.

DISCUSSION

We have described, herein, the development and validation of FT_4 assay, which is well suited for the routine laboratory practices. The antibody coating approach adapted here described by P.S. Petrou et al.[5] has the advantage of using raw antiserum without the requirement of affinity purification. Use of glycine instead of bovine serum albumin for saturation of antibody coated tubes yielded similar results with respect to nonspecific binding and maximum binding, but with the added advantage of low cost and elimination of albumin from the assay system, which is a prerequisite for the validity of the free T_4 assay.

The two-step assay is, by definition, undoubtedly independent of the influence of the serum proteins and their bound T_4. One of the potential problems encountered is the need to rigorously remove all the traces of serum proteins before the second incubation with tracer. This is taken care by 1 mL washing followed by draining for 5 min and washing once again before the addition of the tracer. This way the binding of tracer with the antibody was made comparable with the percentage obtained without serum. The second problem encountered with the two-step assay is the possibility of back-displacement of T_4 bound in the first incubation by competition with labeled probe during the second incubation was tackled by the second incubation being carried out at 4°C.

The important aspects provided by ATA for optimization of FT_4 assay protocol were followed.

1. Antibody with sufficiently high affinity constant was used, yet, it was not as high as recommended by Roger Ekins.[2] There were several reports stating the use of slightly low affinity antibodies in the development of valid two-step assays without compromising assay performance.[7,8–11] The antibody used in this study has the average affinity of 5.5×10^9 L/M, whereas the antibody used by the Clinical Assays kit is still less, i.e., 2.5×10^9 L/M, as per the kit instruction manual Lot: 8/01. This is in accordance with the works reported by Christifides and Sheehan[12] that the affinity originally demanded for validity in free T_4 assays can be relaxed without compromising the assay performance.
2. Sufficiently low concentrations of antibody was used to ensure that <1% of the total hormone from the normal sample was extracted, thus avoiding significant perturbation of the equilibrium between bound and free hormone.[2]

3. Minimum dilution of the sample was ensured to avoid any perturbation in the equilibrium existing between bound and free.[2,4,9,10]
4. Gelatin was used instead of BSA in the assay system in order to avoid interference in samples from non-thyroidal illness due to the added albumin.[7] Because it contains no extra albumin which bind to T_4, assay responds to serum dilution as expected on the theoretical grounds.[9,13–16]

In practice most commercial FT_4 RIA kits are so optimized in order to meet the performance and convenience of modern clinical chemistry laboratories. According to T.A. Wilkins, the absence of FT_4 constancy with dilution is not always indicative of an invalid assay. However, the dilution test can sometimes be useful for assessing the degree of T_4 extraction by the antibody in the free T_4 assays.[17]

CONCLUSION

In conclusion, we have developed a free T_4 assay system as user-friendly as a commercial kit at the same time satisfying the validity parameters required for a valid free T_4 assay.

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