A solid phase radioimmunoassay for free triiodothyronine in serum:assay development and validation

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Abstract A solid phase radioimmunoassay for free triiodothyronine in serum was developed based on double-antibody coated tubes. The method was turned out to be reliable with good reproducibility, higher sensitivity and easy performance. The measurable range of FT_3 in serum was 1.2 to 38 pmol/L. The mean coefficients of variation within and between assays were 1.79%~3.18% and 4.72%~9.31%, respectively. The FT_3 concentrations in euthyroid serum as determined by this method were 2.8 to 7.8 pmol/L. The FT_3 values determined by this new method correlated well with those measured by a commercial radioimmunoassay (r=0.853).
Keywords Free triiodothyronine (FT_3), Solid-phase, Radioimmunoassay, T_3 analog, Double-antibody

1 Introduction

The non-protein bound proportion of the total triiodothyronine is assumed to be responsible for the physiological action of thyroid hormones, and measurements of this free triiodothyronine (FT_3) plasma concentrations are widely used to evaluate the thyroid status.[1] Previous methods used for FT_3 detection, including equilibrium dialysis[2], ultrafiltration[3] and chromatography[4] are technically demanding and time consuming, and consequently unsuitable for routine clinical use. Many new practical methods, based on various analytical approaches, such as two-step back-titration[5], labeled analog[6] and labeled antibody[7], have been reported. Among them, the direct analog base method has gained relatively wide acceptance. In 1991, we cooperated with Nanyang Medical Radioimmunoassay Center and developed an one-step direct method for FT_3[8], based on the labeled analog of triiodothyronine[9], and have made it commercially available. However, the separation of antibody bound fraction of FT_3 from unbound in that assay turned out to be a tedious procedure, especially for mass clinical samples, and difficult to automatize the operation.

Recently we developed a simple and convenient solid-phase radioimmunoassay for FT_3. In this assay a double-antibody was employed to coat tubes as the solid-phase for separating the bound from the unbound. Thus, centrifugation can be avoided. Aspirating liquid from tubes can complete separation simply. The serum concentration of FT_3 in sample can then be measured through its competition with the labeled analog for the limited number of antibody binding sites on the solid surface of tubes. ^{125}I counts bound to the solid phase are inversely proportional to sample or standard FT_3 concentration.

2 Materials and methods

All assay were performed in 12 mm× 60 mm polystyrene tubes with asteroid bottom. Counting was done with a SN 682B gamma counter.

Antiserum the first antibody was raised in rabbit with T_3 conjugated with Bovine serum albumin (BSA) as the antigen. The detail about antiserum, FT_3 standards and control sera were described before[8]. The second antibody was obtained from immunizing sheep with rabbit IgG and purified chromatographically for coating tubes. ^{125}I labeled T_3 analog was prepared in our laboratory as described by Tong Guozhong, *et al.*[9]. Washing buffer was 0.01mol/L phosphate buffer at pH7.4. Blocking solution was 1% BSA in washing buffer. Stable solution composed of 2.5% sucrose in washing buffer.

Antibody coated tubes 0.6 mL of the second antibody in the concentration of 7.3 mg/L

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and with purity above 90% was added to each polystyrene tube and incubated one hour at room temperature. After incubation and careful removal of the coating solution by suction, washing buffer was added. After aspirating the washing buffer, 0.6 ml anti-T_3 serum in the dilation of 1:160000 was added and incubated overnight. After washing, the coated tubes were blocked with blocking solution for three hours and followed by an overnight incubation with the stable solution at room temperature. Afterwards, the solution was decanted and the tubes were dried in vacuum at room temperature. In the end, these double antibody coated tubes were sealed and stored under moisture free condition at 4°C.

2.1 Assay procedure

The assay procedure is summarized in Table 1.

Table 1 Assay procedure-summary

Tube kinds	NSB* Second-Ab coated	Standards Double-Ab coated	Samples Double-Ab coated
Standards	100	100	
Samples/controls			100
^{125}I analog	400	400	400
Incubation for 3 hours at 37°C water-bath, Decanting the solution before Counting with a γ counter for at least 1 min per tube.			

Notes: For total counting tube, it can be any normal tubes. *Nonspecific binding (NSB) was determined in tubes coated with all components but the anti-serum to T_3 as in the coating protocol described above.

2.2 Effect of albumin

The bound of labeled T_3 analog to albumin was studied by measurement of albumin-added serum at various albumin concentrations. The albumin-added serum was prepared by adding 200 mg of human serum albumin (HSA) to 1 mL of a pooled specimen of serum from euthyroid subjects at first and then diluting the albumin-added serum with various amount of non-added pooled serum. Interference effects were quantified from the decreased percentage of radioactivity bound to antibody at various albumin concentrations.

2.3 Other RIA kit

The immunotech (France) FT_3 RIA (labeled antibody) kit was applied according to the producer's statement.

3 Result

3.1 Coating equilibrium status

A:The kinetics of the coating of the second antibody was studied by assaying zero standard and samples that reacted in tubes which had been incubated with the second antibody for various time intervals, and comparing their percentages of the tracer bound to the solid phase. The maximum binding was reached in less than 10 minutes.

B: In order to clarify the nature of the first antibody binding to the second antibody, 160000 times diluted anti-T_3 rabbit serum were incubated for different time intervals with the second antibody coated tubes prepared by the standard protocol, and then the bound percentages of tracer in both zero and maximum standards were measured. The results shown in Fig.1 indicated that an incubation period of 24 h was needed for the maximum binding. Under such condition the bound percentage of the tracer reached its maximum for both standards. It also implied that the formation of the conjugate from these two antibodies reached its maximum.

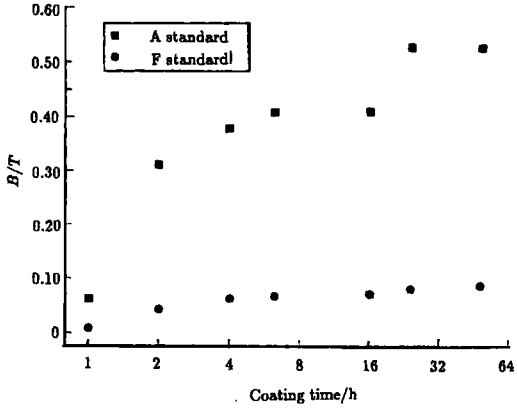

Fig.1 Kinetics of anti-T_3 serum bound to second antibody

A standard is aero standard, F standard is standard with FT_3 concentration at 38 pmol/L

3.2 Assay characters

3.2.1 Assay equilibrium As shown in Fig.2, maximum bound of tracer was achieved for zero standard and the standard containing 38 pmol/L FT_3 after 3h of incubation.

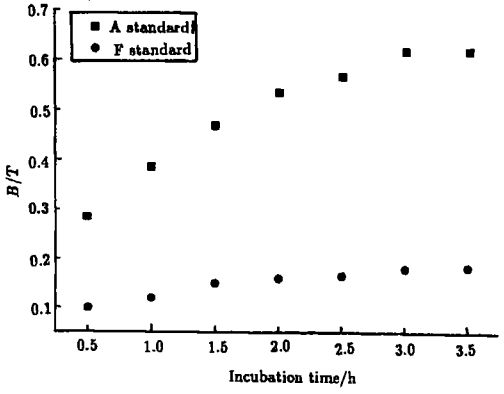

Fig.2 kinetic status of the assay

3.2.2 Standard curve The B/B_0 ratio was plotted vs. FT_3 concentration of the standards to construct the standard curve in Fig.3. B and B_0 are radioactivities of standards or samples and the zero standard, respectively. The fitting parameters were B_0=56.8, A=1.5162, B=-0.7644 r=0.999 ED_{75}=11.727, ED_{50}=7.268, ED_{25}=30.592. The non-specific binding was 0.05% to 0.8%, lower than that of routine methods. The range covered by FT_3 assay standards is 0 to 38 pmol/L. The minimum detectable dose, calculated as two standard deviations of 10 duplicate determinations of the zero standard counts, was 0.18 pmol/L.

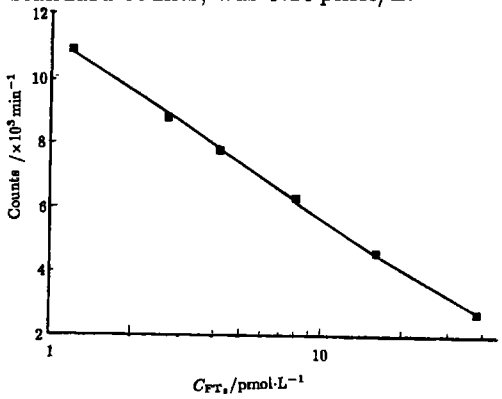

Fig.3 The standard curve

Precision as shown in Table 2, the within assay coefficient of variation was less than 5%. The between assay coefficient of variation was within 10%.

Table 2 The reproducibility for FT_3

	$X \pm SD$/pmol·L^{-1}	CV/%
	3.704±0.27	2.8
Intra-assay (n=10)	5.374±0.41	1.79
	18.698±0.45	3.18
	3.293±0.36	7.9
Inter-assay (n=10)	7.526±0.37	4.72
	14.77±0.51	9.31

3.2.3 Reference interval The FT_3 concentration in serum from 200 healthy samples was 4.5±1.7 pmol/L and the normal range was calculated as 2.8 to 7.8 pmol/L.

3.2.4 Comparison with other RIA method FT_3 values detected with this new solid phase assay and immunotech FT_3 RIA kit were compared in Fig.4. The FT_3 concentrations in serum determined by the present method (y) are linearly correlated with those determined by Immunotech RIA kit (x): Y=3.747+1.35X. With r=0.853 for 67 samples.

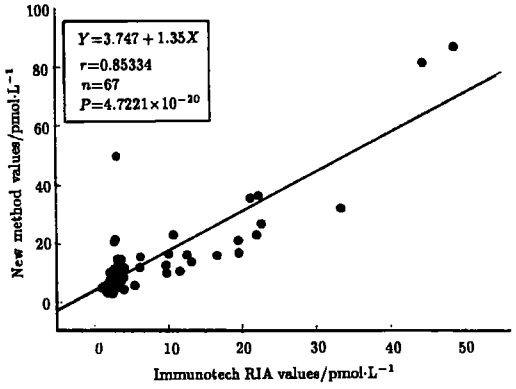

Fig.4 Correlation between FT_3 values in serum determined by the present method and immunotech RIA kit

3.2.5 Influence of HSA The influence of HAS concentration on the FT_3 value determined by the present procedure is shown in Fig.5. The percentage of radioactivity bound to antibody in the presence of HSA is shown on the left ordinate and FT_3 value determined by this new method is on the right ordinate. Under 90 g/L HSA in serum, the B/B_0 ratio of the counts was scarcely affected by the progressive adding of albumin and the bias estimated for this new assay is approximately 0.045 pmol/L of FT_3 per gram of albumin per liter under 90 g/L HSA.

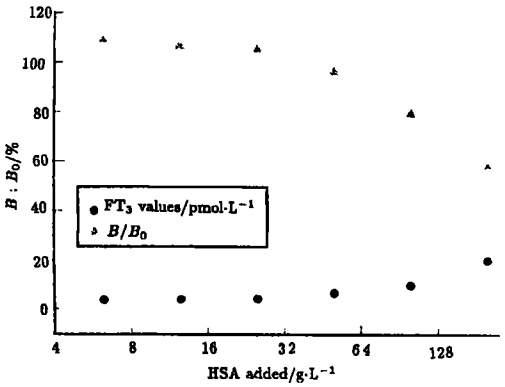

Fig.5 Effect of various concentrations of albumin

4 Discussion

The basis of this new method is based on the competition between radiolabeled T_3 analog and unlabeled FT_3 in samples or standards for the specific antibody binding sites. The method for the separation of the bound from the unbound plays an important role in determining the reproducibility, speed and ease of performance of the assay. This new assay has shown a very low non-specific binding (<1%), a higher sensitivity than the preceding method and good reproducibility. Furthermore, the use of solid-phase tubes for separation has obviated the tedious procedures of precipitation and centrifugation. The separation can be performed simply by decanting, thus it has greatly simplified the analyzing procedure, minimized the variations brought by different performer, and sped up the routine clinical analysis.

Another advantage of this new assay is the utilization of its double-antibody coated tubes. In those assays with single antibody coated tubes, when antiserum covalently linked to solid carriers, the activity of the antiserum is suspected to be lost to some extent. Besides, the amount of the anti-serum added to each assay tube must be kept constant in binding ability and suitable for the specific radioactivity of the tracer each time to ensure the precision, which proved to be a crucial but tedious work for a kit. In our double- antibody coated tubes, the antibody covalently linked to the solid carrier is the easily obtainable second antibody. The precious first anti-serum can be preserved and a minor variation of the amount of second antibody will not significantly affect the precision or it can be corrected easily by adjustment of the concentration of the first antibody subsequently added. Besides, double-antibody coated tubes can be used versatilely in other assays, if the first anti-serum to T_3 is displaced by other antiserums that from the same animal species as T_3-BSA.

As the normal HAS concentration in serum is 30~50 g/L, doubling the serum albumin concentration in euthyroid patients, FT_3 values in this new assay are not sensitive to the added albumin for up to 90 g/L. This indicates that the pre-existing equilibrium in the serum between the thyroid hormones and its binding proteins is not significantly disturbed, and for most clinical and laboratory samples FT_3 values determined with present assay will not be interfered significantly by the variation in serum albumin. This agrees with the results reported by Wilkins *et al.*[10], that the interference of albumin with RIA for FT_3 was not important for most laboratory samples, because the binding of ^{125}I-labeled T_3 analog to serum albumin was insignificant.

In summary, we have developed a new solid phase RIA system, with double-antibody coated tubes. This method as applied to FT_3 analysis was found to be reliable, and to be able to give a lower non-specific binding, better reproducibility and higher sensitivity as well. Its ease to perform is more convenient for the mass routine clinical diagnosis.

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