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A simple time-resolved fluoroimmunoassay of total thyroxine in serum

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SUMMARY. We describe a non-isotopic heterogeneous competitive immunoassay of total thyroxine in serum. Thyroxine, released from its binding proteins by merthiolate (thimerosal), competes with immobilised thyroxine (thyroxine-bovine globulin conjugate) for binding to a monoclonal biotinylated antibody. The amount of biotinylated antibody bound, which is inversely related to the amount of thyroxine in the sample, is then quantified by adding streptavidin labelled with the europium chelator 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) in the presence of excess Eu^{3+}. The complex formed (bovine globulin-thyroxine-antibody-biotin-streptavidin-BCPDA-Eu^{3+}) is measured on the solid-phase by time-resolved fluorescence. The assay is simple to perform and its characteristics are similar to those of other currently used immunoassay techniques.

One of the most frequently requested tests in the immunoassay laboratory is total thyroxine (T_4). The original methods pioneered by Ekins,[1] which employed thyroxine-binding globulin (TBG) as the binder, were replaced by techniques which used polyclonal antibodies. More recently, monoclonal antibodies for T_4 with sufficient affinity and specificity became available and they are increasingly used.[2]

Radioimmunoassays for T_4 are well established.[3,4] However, the recent trend is to replace radioactive nuclides with alternative labels to avoid the well-known disadvantages of radioactivity.[5] Numerous methods for total T_4 have been published in which fluorescent, luminescent or other labels have been employed. Homogeneous immunoassays based on fluorescence polarisation, enzymes or other methodologies are now widely used.[6–10]

In this paper we describe a time-resolved fluoroimmunoassay of total T_4 in serum using the newly synthesised europium chelator 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA)[11] as label. In the assay, serum total T_4 released from the binding proteins by the addition of merthiolate (thimerosal) competes with immobilised T_4 (T_4-bovine globulin conjugate adsorbed on white microtitration wells) for binding to a soluble biotinylated monoclonal anti-T_4 antibody. After washing, the degree of binding of the biotinylated antibody to the solid-phase, which is inversely related to the T_4 concentration in the sample, is quantified by a bridge reaction with BCPDA-labelled streptavidin in the presence of excess Eu^{3+}. The fluorescent complex (bovine globulin-T_4 antibody-biotin-streptavidin-BCPDA-Eu^{3+}) is measured on the dry solid-phase by pulsed excitation with a nitrogen laser (337·1 nm), monitoring the specific Eu^{3+} fluorescence at about 615 nm during a preselected time window (time-resolved measurement). The analytical characteristics of the new assay are similar to those of other immunological T_4 assays that are currently commercially available.

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Materials and methods

INSTRUMENTATION

For solid-phase time-resolved fluorometric measurements we used the CyberFluor 615™ Immunoanalyzer. Radioactivity counting was performed with the LKB Wallac 1275 Minigamma counter.

CHEMICALS AND SOLUTIONS

The europium chelator BCPDA was synthesized as described elsewhere.[11] Streptavidin and bovine

serum albumin (BSA) were purchased from Sigma Chemical Co, St Louis, MO 63178, USA. $EuCl_3$ hexahydrate was from Aldrich Chemical Co, Milwaukee, WI 53201, USA. Sulfosuccinimidyl-6-(biotinamido)hexanoate (NHS-LC-biotin) was from Pierce Chemical Co, Rockford, IL 61105, USA. All other chemicals were from Sigma. White opaque 12-well microtitre strips were products of Dynatech Labs, Alexandria, VA 22314, USA.

The coating buffer was a 50 mmol/L Tris solution of pH 7·80. The blocking buffer was a 50 mmol/L sodium phosphate solution of pH 7·4 containing 9 g NaCl, 1 g gelatin and 1 mL polyoxyethylenesorbitan monolaurate (Tween 20) per litre. The T_4 assay buffer was a 50 mmol/L Tris-HCl solution of pH 7·8 containing 20 g merthiolate, 10 g BSA, 9 g NaCl and 0·5 g sodium azide per litre. The streptavidin-europium dilution buffer was a 50 mmol/L Tris-HCl solution of pH 7·8 containing 9 g NaCl, 40 g BSA, 0·5 g sodium azide and 4×10^{-5} moles of Eu^{3+} per litre. The wash solution was a 9 g/L NaCl solution containing 0·5 mL Tween 20 and 0·5 g sodium azide per litre.

Thyroxine standards were in T_4-free serum prepared as described by Chard.[12] The T_4 monoclonal antibody was purchased as a 1 mg/mL solution in 0·1 mol/L phosphate buffer of pH 7·40 from Chemicon International, El Segundo, CA 90245, USA. A 1×10^{-3} mol/L $EuCl_3$ stock solution was prepared in 0·01 mol/L HCl.

LABELLING OF STREPTAVIDIN WITH BCPDA

The preparation of highly labelled streptavidin after its conjugation to BCPDA derivatised thyroglobulin is described in detail elsewhere.[13] Briefly, we synthesised a streptavidin-thyroglobulin-BCPDA complex (SA-TG-BCPDA) as follows. We derivatised streptavidin with the heterobifunctional reagent sulpho-succinimidyl-4- (N-maleimidomethyl) -cyc- lohexane-1-carboxylate (sulpho-SMCC). We incorporated protected thiol groups on the bovine thyroglobulin molecule using the reagent N-succinimidyl-s-acetylthioacetate (SATA). We then incorporated approximately 160 BCPDA molecules on the derivatised thyroglobulin molecule. We reacted the activated streptavidin with the activated labelled thyroglobulin (after deprotection of thiol groups with hydroxylamine) to obtain the conjugate SA-TG-BCPDA. This conjugate was isolated from unreacted strepavidin with gel filtration chromatography and then diluted in streptavidin-europium dilution buffer to give a concentration of 0·30 mg/L in terms of streptavidin. This tracer solution also contained 4×10^{-5} M Eu^{3+}.

BIOTINYLATION OF THYROXINE MONOCLONAL ANTIBODY

The antibody solution (1 mL, containing 1 mg antibody) was dialysed twice in 5 L of a 9 g/L NaCl solution at 4 °C and then diluted 1 : 1 with a 0·5 mol/L carbonate buffer of pH 9·1. The solution was then concentrated to 0·5 mL by centrifugation in a Centricon™ 30 Microconcentrator (Amicon Canada, Oakville, Ontario L6H 2B9, Canada). To this solution was added 2 mg of NHS-LC-biotin dissolved in 50 μL dimethyl sulphoxide and the mixture incubated for 2 h at room temperature. The solution was then dialysed twice in 5 L of a 9 g/L NaCl solution at 4 °C and its volume adjusted to 1·0 mL with the same solution (stock). For the assay, the stock antibody solution was diluted 800-fold in the assay buffer (working antibody solution). The stock antibody solution was stable for at least 6 months and the working antibody solution for at least 2 weeks at 4 C.

PREPARATION OF BOVINE GLOBULIN-THYROXINE CONJUGATE

The procedure is similar to the one used by Alexander and Jennings[14] for the preparation of T_3-bovine serum albumin conjugates and it is based on the water-soluble carbodiimide method. We used 20 mg of T_4 instead of 40 mg of T_3. After conjugation, the product was dialysed extensively in a 9 g/L NaCl solution to remove unreacted T_4. Finally, the product was assayed for total protein with the BioRad protein assay and stored at 4 °C as a 1–3 mg/mL solution.

COATING OF MICROTITRE STRIPS

The strips were coated overnight at room temperature with 100 μL of a solution of T_4-bovine globulin conjugate in coating buffer. After coating, the plates were rinsed once with wash solution, blocked for 1 h at room temperature with 200 μL of blocking buffer, washed twice and air dried overnight at room temperature. When stored in sealed plastic bags at 4 °C with dessicant, in the dark, they were stable for at least 6 months.

ASSAY PROCEDURE

Before the assay, the strips were washed twice with the wash solution. Ten microlitres of standard or serum samples (in duplicate) were pipetted into each well and 100 μL of the working biotinylated antibody solution added. The strips were then shaken in an automatic shaking device

at room temperature for 60 min. The strips were then washed three times with wash solution and 100 μL of working tracer reagent (streptavidin-BCPDA-Eu^{3+}) was added. After incubation for 30 min at 37°C, the strips were washed three times with wash solution and dried with a stream of air. Surface fluorescence was measured on a Cyber-Fluor 615 Immunoanalyzer. The instrument has an automatic data reduction capability and results along with the calibration curve were printed automatically as soon as the readings were complete (~5 min for a 96-well plate).

COMPARATIVE METHOD

For comparison studies we used the Coat-a-Count Total T_4 RIA kit (Diagnostic Products Corporation, Los Angeles, CA 90045, USA).

Results

ASSAY OPTIMISATION

We constructed calibration curves with varying amounts of thyroxine-globulin conjugate added to the wells during coating. We observed (working with a constant antibody dilution, see below) that by increasing the amount of coating, the fluorescence reading of the zero standard (Bo) increased. However, at higher but still subsaturating amounts of coating, the sensitivity of the calibration curve decreased and the non-specific background fluorescence (due to adsorption of labelled streptavidin) increased. As a compromise, we chose to coat the microtitration wells with 500 ng/well of the T_4-globulin conjugate.

The effect of antibody dilution on the shape of the calibration curve was also studied. As expected, the sensitivity of the calibration curve improved at higher antibody dilutions. The 800-fold dilution was chosen as optimum and the resulting calibration curve is shown in Fig. 1. At even higher dilutions (data not shown) the sensitivity improved further but the curve started to become relatively flat at T_4 concentrations of 155–260 nmol/L (12–20 μg/dL), narrowing the working dynamic range.

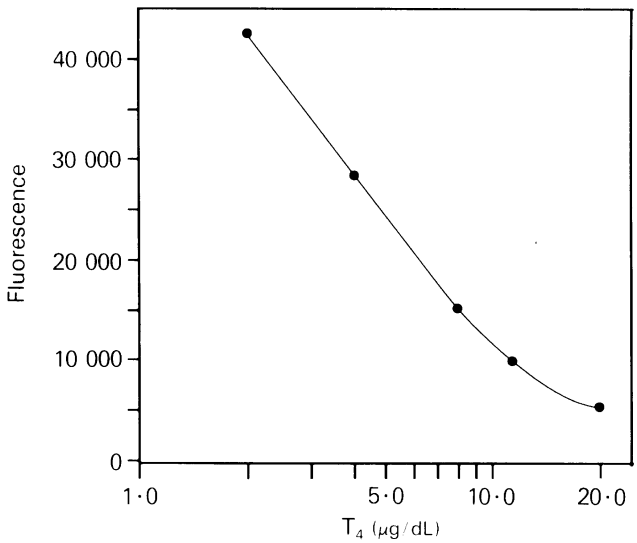

FIG. 1. Calibration curve of the proposed T_4 assay. (Conversion factor: nmol/L = μg/dL × 12·87)

TABLE 1. Precision of the thyroxine assay

	Mean (nmol/L)	SD (nmol/L)	CV (%)
Within-run (n = 15)			
A	42·0	2·32	5·5
B	100·6	2·19	2·2
C	211·1	5·41	2·6
Day-to-day (n = 18)			
D	40·4	4·63	11·5
E	98·5	3·73	3·8
F	215·2	11·84	5·5

We optimised the incubation times of the assay so as to obtain accurate and reproducible results. The optimum combination was found to be 60 min at room temperature with shaking for the antibody-binding step and 30 min at 37°C (air-oven) for the streptavidin-binding step. The assay was not sensitive to accurate timing since binding did not increase significantly after the specified incubation times.

PERFORMANCE

The detection limit of the assay calculated from the point which is 2 standard deviations of the zero standard below the response of the zero standard was 6·4 nmol/L (0·5 μg/dL). The precision of the assay is shown in Table 1. The recovery of thyroxine added to serum samples in amounts from 31 to 162 nmol/L (2·4 to 12·6 μg/dL) in three

TABLE 2. Dilution of samples with high thyroxine concentrations (nmol/L)

Sample	Dilution			
	Nil	2-fold	4-fold	8-fold
1 Observed	290	151	79	44
Expected	—	145	72	36
2 Observed	232	118	58	35
Expected	—	116	58	29

TABLE 3. Cross-reactivity studies with the monoclonal thyroxine antibody

Compound	% Cross-reactivity*
L-Thyroxine	100
D-Thyroxine	100
3,3′,5-Triiodo-L-thyronine	2·9
3,3′,5-Triodothyroacetic acid	0·8
Phenylbutazone	<0·1
Propylthiouracil	<0·1
Diphenylhydandtoin	<0·1
3,5-Diiodo-L-thyronine	<0·0001
3,3-Diiodo-L-tyrosine	<0·0001
3-Iodo-L-tyrosine	<0·0001

* Cross-reactivity is expressed as the percent ratio of the thyroxine concentration to the cross-reacting substance concentration at 50% inhibition of maximum binding.

different sera ranged from 87% to 114% with a mean of 99·2 ± 8·6% (11 additions). The dilution linearity of two samples with high T_4 values suggests that the assay is free from any serum matrix effects (Table 2). No major cross-reacting compounds were identified from the cross-reactivity studies with the monoclonal antibody (Table 3).

The comparison of results between the proposed method and one widely used radioimmunoassay procedure is shown in Fig. 2. The regression equation was: y (present method) $= 12{\cdot}9 + (0{\cdot}994 \times \text{RIA})$ nmol/L $(1{\cdot}07 + (0{\cdot}994 \times \text{RIA})$ μg/dL), $r = 0{\cdot}97$, $n = 78$.

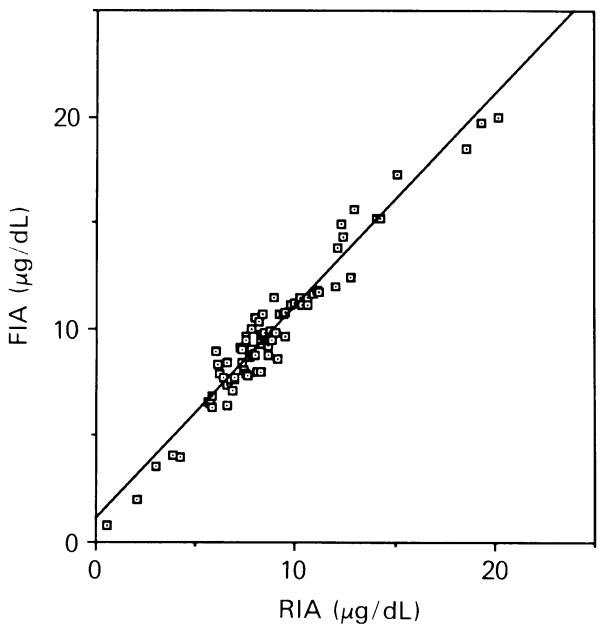

FIG. 2. Comparison of the proposed method with a widely used radioimmunoassay procedure for 78 serum samples. (Conversion factor: nmol/L = μg/dL × 12·87)

Discussion

Currently, the most promising alternative immunoassay methods use fluorescent or luminescent probes and enzymes as labels. The heterogeneous techniques have potential sensitivities equivalent to or better than radioimmunoassays (RIA) and immunoradiometric (IRMA) procedures. The same techniques can be used for small and large molecules and for analytes present in serum from micromolar to picomolar concentrations. Time-resolved fluorescence is now becoming an established analytical technique in the field of non-isotopic immunoassay. This technique is based on the measurement of delayed fluorescence emitted by the rare earth metals, and especially Eu^{3+}, when the metal is complexed with appropriate organic chelators.[15] The attractive properties of the fluorescent rare earth chelates and their suitability in immunoassay are discussed in detail elsewhere.[5,16] Wieder, Soini, Hemmila and their colleagues pioneered the use of Eu^{3+} as a potential immunological label.[15 18] However, despite the excellent sensitivity of the assay configuration in which antibodies labelled with Eu^{3+} are used, there are some disadvantages, especially the vulnerability to exogenous Eu^{3+} contamination from seed and dust, skin surfaces, etc[5,19], the need to dissociate Eu^{3+} from the immunological complex before measurement in solution, the instability of tracer reagent on reconstitution, and the fact that the final fluorescent complex is not stable for long periods of time.

Recently, we synthesised the new europium chelator BCPDA.[11] BCPDA can be introduced covalently into proteins[19] and complex Eu^{3+} to form fluorescent adducts. We designed a new chemical system suitable for time-resolved immunoassays in which BCPDA is used as the immunological label and excess of Eu^{3+} is used to form the fluorescent complex. With this configuration, we achieved freedom from exogenous Eu^{3+} contamination, fluorescence was measured directly on the solid-phase without the need for dissociation, and there was very long stability of working reagents and stability of the fluorescence on the dry solid-phase for months.

Exogenous Eu^{3+} contamination is avoided because the label (the concentration of which is proportional to the T_4 concentration in the sample) is BCPDA and not Eu^{3+}. Eu^{3+} is added in excess in the tracer reagent solution to form a 1:1 complex with BCPDA. However, if the potential sensitivities for measuring either Eu^{3+} in the presence of excess chelators or BCPDA in the presence of excess Eu^{3+} (proposed configu-

ration) are compared, the first system is advantageous. This is due to the fact that with excess Eu^{3+} only the 1:1 BCPDA:Eu^{3+} complex can be formed, and this has a lower fluorescence quantum yield than the complexes of the Eu^{3+}-$(BCPDA)_n$ type ($n=2$ or 3).[11,20] However, the sensitivity of the proposed system for measuring analytes in biological fluids can match or even exceed the sensitivity of the previous system if proper amplification is used.[13] The sensitivity of the method used is not exploited to its limits in the proposed thyroxine assay because this analyte is present at relatively high concentrations in serum.

The proposed assay configuration (chemical system) can be used routinely for the measurement of many analytes in biological fluids if it is combined with a fluorimeter/analyser suitable for time-resolved fluorescence measurements directly on the dry solid-phase (microtitre wells) and having data reduction capabilities. Such an instrument has been developed recently.[5] The complete analytical system has already been used successfully for the immunoassay of alpha-fetoprotein[21] and chorionic gonadotrophin[22] in serum.

The procedure employed for T_4 immunoassay uses the immobilised antigen approach with soluble biotinylated antibody and not the more widely used immobilised antibody approach. The first configuration has several advantages. Antigen-protein conjugates can be prepared easily with well-established procedures in the same manner as immunogens for antigens are produced. Biotinylated antibodies are also prepared easily and they are stable reagents. In addition, they are suitable as complementary reagents with a universal detection system based on a labelled streptavidin. Antigen-enzyme or antigen-fluor conjugates are not needed. The advantages of the biotin-streptavidin system as applied to the proposed assay configuration are summarised elsewhere.[5]

The proposed T_4 assay can be completed in less than 2 h, including the plate-measuring step and data reduction. The instrument is capable of reading up to 384 wells in less than 10 min, making the methodology attractive especially for laboratories with high workloads.

In conclusion, the performance characteristics of the proposed T_4 assay are similar to those of other immunoassay techniques. The assay is simple to perform, rapid and directly amenable to automation. In the proposed assay design, a europium chelator and not europium is used as label resulting in a contamination-free system. The same system can be used for the assay of other analytes in biological fluids.

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