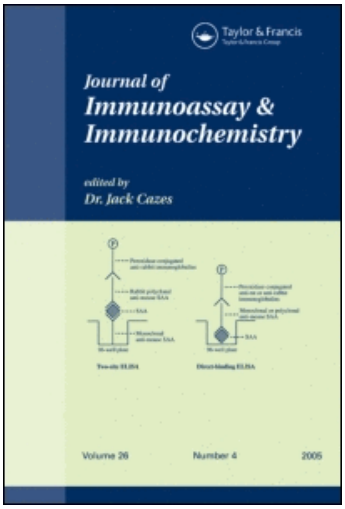

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Yok K. Tan[a]; Mohammad J. Khosravi[a]; Eleftherios P. Diamandis[ab]
[a] CyberFluor Inc., Ontario, Canada [b] Department of Clinical Biochemistry, University of Toronto, Ontario, Canada

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TIME-RESOLVED IMMUNOFLUOROMETRIC ASSAY FOR THYROXINE-BINDING GLOBULIN IN SERUM

Yok K. Tan[1], Mohammad J. Khosravi[1], and Eleftherios. P. Diamandis[1,2]
[1]CyberFluor Inc., 179 John Street, Toronto, Ontario, Canada M5T 1X4
[2]University of Toronto, Department of Clinical Biochemistry
100 College Street, Toronto, Ontario, Canada M5G 1L5

ABSTRACT

We describe a new "sandwich"-type immunofluorometric assay for thyroxine-binding globulin (TBG) in serum. The assay involves a solid-phase monoclonal antibody immobilised in white microtiter wells, and a soluble biotinylated monoclonal antibody that reacts with the captured TBG molecules. Addition of streptavidin labeled with the europium chelator, BCPDA (4,7-bis(chlorosulfophenyl)-1,10 phenanthroline-2,9-dicarboxylic acid), and excess europium results in the formation of a highly fluorescent product. The fluorescence signal of the final complex is quantitated on the dried solid-phase with a pulsed-laser time-resolved fluorometer. The assay requires a 151-fold sample pre-dilution and a total incubation time of 90 minutes. It has a broad dynamic range of 0 - 100 mg/L and a minimum detection limit of 0.4 mg/L. The coefficients of variation for within-run and between-run assays averaged 4.5% and 5.4%, respectively. The mean analytical recovery of TBG added to serum was 103%. Results obtained by this method correlated well with those determined by a commercial radioimmunoassay ($r = 0.96$, $n = 112$) and by an immunoradiometric procedure ($r = 0.95$, $n = 131$). (KEY WORDS: time-resolved fluorescence immunoassay; thyroxine binding globulin; thyroid tests; monoclonal antibodies; europium chelate)

INTRODUCTION

Thyroxine-binding globulin (TBG), an acidic glycoprotein with a molecular weight of about 60,000 daltons (1), is the principal carrier protein for thyroxine (T_4) and triiodothyronine (T_3) in serum. Together with thyroxine-binding

prealbumin (transthyretin) and albumin, they maintain a small unbound fraction of circulating T_3 and T_4 at a constant level. As TBG serum concentration may be influenced by factors such as genetic predisposition (2-6), pregnancy (3-7), severe illness (5,6,8), fasting (9,10), drugs and hormones (7,11), correct interpretation of values for serum T_4 requires simultaneous measurement of serum TBG concentrations. For this reason, the commonly used T_3 uptake tests based on the estimation of TBG binding capacity, rather than its concentration, have been traditionally conducted together with the measurements of serum total T_4 to yield the "Free Thyroxine Index" (FTI). The FTI correlates well with both the clinical status of patients and the free T_4 concentration (12). However, a better and more reliable method of thyroid assessment may be derived from the concentration ratio of total T_4 and TBG as measured directly (13-17). Unlike the FTI the T_4 : TBG ratio is able to correct the serum T_4 for extreme concentrations of TBG. In addition to aiding thyroid function assessment, measurement of serum TBG is currently of interest as a biochemical index to follow the clinical course of patients with serious nonthyroidal illnesses (18,19). A low and progressively decreasing serum TBG may predict a poor prognosis in these patients.

Analytical techniques that have been developed for measuring TBG include radioimmunoassay (20,21), immunoradiometric assay (22), rocket immunoelectrophoresis (23), nephelometic assay (24), enzyme immunoassay (25-27), immunoturbidimetric assay (28,29), and the single radial diffusion method (30). Some of these assays are commercially available. Here we describe the first time-resolved immunofluorometric assay for the determination of TBG in serum. In the assay, TBG is captured with a monoclonal anti-TBG antibody which is non-covalently immobilised in the wells of microtiter strips. A second biotin-labeled monoclonal antibody is employed to detect captured TBG molecules. The degree of binding of the biotinylated antibody, which is proportional to the TBG

concentration in the serum sample, is quantitated by a bridge reaction with streptavidin labeled with the europium chelator, 4,7-bis(chlorosulfophenyl)-1,10 phenanthroline-2,9-dicarboxylic acid (BCPDA), in the presence of excess Eu^{3+}. The resulting fluorescent complex (antibody-TBG-antibody-biotin-streptavidin-BCPDA-Eu^{3+}) is then measured on the dry solid phase by time-resolved fluorescence spectroscopy. The assay and its performance characteristics are discussed below.

MATERIALS AND METHODS

Chemicals and Reagents

Bovine serum albumin (BSA, RIA grade), bovine γ-globulin, streptavidin, and polyoxyethylenesorbitan monolaurate (Tween 20®) surfactant were all products of Sigma Chemical Co., St. Louis, MO 63178. Europium (III) chloride hexahydrate and sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC-Biotin) were purchased from Aldrich Chemical Co., Milwaukee, WI 53233, and Pierce Chemical Co., Rockford, IL 61105, respectively. White opaque 12-well microtiter strips, Microfluor™, were from Dynatech Laboratories Inc., Alexandria, VA 22314. All other chemicals were of highest quality obtainable.

The coating buffer is a 0.01 mol/L Tris buffer, pH 8.2, containing 0.1 mol/L NaCl. The blocking buffer is 0.1 mol/L sodium bicarbonate, pH 8.3, containing 10 g of BSA and 0.5 g of sodium azide per liter. The assay buffer is a 50 mmol/L Tris solution of pH 7.8 containing, per liter, 9 g of NaCl, 0.5 g of sodium azide, 5 g of BSA, and 0.5 g of bovine globulin. The streptavidin-europium buffer is a 50 mmol/L Tris solution of pH 7.2, containing 4×10^{-5} mol/L $EuCl_3$, 9 g NaCl, 40 g BSA, and 0.5 g sodium azide, per liter. For wash solution we used a 0.05% (v/v) Tween 20® in 9 g/L NaCl solution.

TBG Standards

Highly purified human TBG was obtained from Scripps Laboratories, San Diego, CA 92131. Calibration standards with concentration up to 100 μg/mL were prepared in a 50 mmol/L Tris buffer, pH 7.5, containing 0.15 mol/L NaCl, 0.05% sodium azide, 7% BSA and 1% γ-globulin. They were stored at 4 °C until use.

Anti-TBG Antibodies

Two mouse anti-TBG monoclonal antibodies were used. The antibodies were purified from ascites fluid using the Protein-A-agarose method (Affi-Gel® Protein A MAPS® II kit; Bio-Rad Chemical Division, Richmond, CA 94804) and stored at 4 °C. These monoclonal antibodies are available from CyberFluor Inc.

Specimens and Controls

Human serum samples were obtained from Mount Sinai Hospital, Toronto, Ontario, and stored at -20 °C until analysis. For controls, we used the human serum-based Lyphochek® Immunoassay Control levels I, II, and III obtained from Bio-Rad Laboratories, Anaheim, CA 92806. They were aliquoted and stored frozen at -20 °C

Biotinylation of Antibody

One of the affinity-purified anti-TBG monoclonal antibodies was biotinylated according to the procedure previously described (31). After dialysis, to remove the unconjugated biotin, the biotinylated antibody was tested at several dilutions to determine its optimal assay concentration. The stock biotinylated antibody preparation is routinely diluted 200-fold with the asssay buffer to give a working concentration of approximately 5 μg/mL.

Preparation of BCPDA-Labeled Streptavidin

Streptavidin was labeled with the Eu^{3+} chelator, BCPDA, according to the procedure described by Chan et al. (32). The synthesis of BCPDA has been described previously (33). Before use, the BCPDA-streptavidin conjugate was diluted with the streptavidin-europium buffer to give a working solution containing 3 μg/mL streptavidin.

Preparation of Antibody-Coated Microtiter Wells

Coating of the microtiter wells with the anti-TBG capture monoclonal antibody was carried out according to the procedure described previously (31,32). The wells were coated by adding 100 μL per well, of a 10 μg/mL antibody in the coating buffer.

Assay Procedure

The assay was performed by adding 50 μL of 151-fold diluted (in distilled water) standards, controls, or patient samples into the antibody-coated wells in duplicate. This was followed by the addition of 100 μL per well of the biotinylated antibody working solution. The wells were then shaken continuously for 1 hour at room temperature on a mechanical shaker (Dynatech Laboratories). The reaction mixture was aspirated and the wells washed three times with the wash solution using a 12-well aspirating-washing device. 100 μL of the BCPDA-labeled streptavidin working solution was then added to each well. After a 30-minute incubation at 37 °C, the wells were washed three times and dried for 10 minutes on a cool-air drying device (CyberFluor Inc.). The fluorescence signal of the complex on the dried solid-phase was then measured on the CyberFluor 615™ Immunoanalyser which has automated data reduction capability.

Comparison Methods

Two commercially available kits were used as comparison methods. The first was a radioimmunoassay (Nichols Institute Diagnostics, San Juan Capistrano, CA 92675) with ^{125}I-human TBG as the radioactive tracer. The other was a "ligand partitioning" sandwich assay (Immo Phase®; Corning Medical, Medfield, MA 02052) using ^{125}I-T4 as the tracer. Radioactivity counting and data reduction were performed with the LKB-Wallac 1272 Clinigamma counter (Wallac Oy, Turku, Finland).

RESULTS

Dynamic Range and Detection Limit

Figure 1 depicts a typical concentration-response curve and precision profile of the assay. The calibration curve is almost linear for TBG concentrations up to 100 mg/L. Beyond this point a plateau in fluorescence intensity is attained. There is no evidence of the high-dose "hook" effect even at concentrations as high as 220 mg/L. As the precision (within-run) profile shows, the working range of the assay is between 2 and 100 mg/L, with a coefficient of variation (CV) of < 6% for 12 replicate determinations at each standard point. The detection limit of the assay, as calculated from the mean fluorescence plus three standard deviations of the zero standard for 14 replicate analyses, was found to be 0.4 mg/L.

Precision

The precision of the assay was evaluated by repeat analyses of a tri-level commercial serum control. As summarised in Table 1, within-run CVs for mean TBG levels of 36.4, 28.0, and 23.1 mg/L were 4.0, 4.2, and 5.4% respectively. At the same levels of TBG, between-run CVs determined in five successive runs were

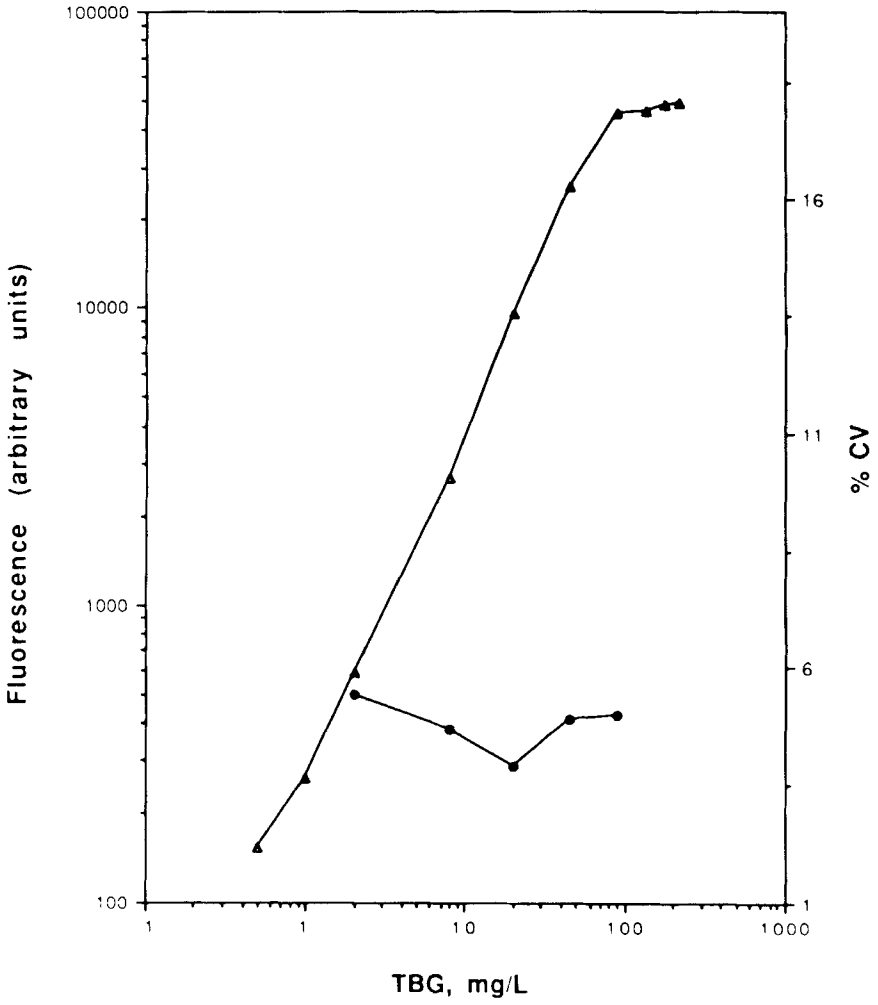

FIGURE 1. Concentration-response curve (Δ) and precision profile (•) of the TBG assay

TABLE 1

Precision of the TBG Assay With Commercial Quality Control Sera

Sample	TBG, mg/L Mean	SD	CV, %
Within-run (n = 12)			
1	36.4	1.4	4.0
2	28.0	1.2	4.2
3	23.1	1.2	5.4
Between-run (n = 10)			
1	33.4	1.4	4.3
2	28.4	1.6	5.5
3	22.8	1.7	7.6
Between-day (n = 16)†			
1	34.8	1.4	3.9
2	30.0	1.7	5.8
3	24.8	1.6	6.5

† over a period of one month

4.3, 5.5, and 7.6% respectively. For between-day precision, the same control samples analysed during a 30-day period gave CVs ranging from 3.9 to 6.5%.

Recovery and Dilution Linearity

The analytical recovery of the method was assessed by assaying serum samples which had been spiked with three different concentrations of TBG. As shown in Table 2, the recoveries of TBG ranged from 94 to 114%, with a mean ± S.D. of 103 ± 8.2%.

To evaluate dilution linearity, four serum samples were diluted up to 1208 fold with distilled water and then assayed in triplicates. TBG values of samples at the standard 1:151 dilution were used to calculate the expected values of the other diluted samples in the same series. As shown in Table 3, the observed

TABLE 2

Analytical Recovery of TBG Added to Four Serum Samples

Sample	Added mg/L	Measured	Recovery mg/L	Recovery %
1	0	18.9	-	-
	5.5	25.1	6.2	113
	10.0	30.0	11.1	111
	17.0	35.4	16.5	97
2	0	22.8	-	-
	5.5	28.7	5.9	107
	10.0	32.4	9.6	96
	17.0	38.1	15.3	90
3	0	31.9	-	-
	5.5	38.2	6.3	114
	10.0	42.9	11.0	110
	17.0	49.0	17.1	101
4	0	35.2	-	-
	5.5	40.6	5.4	98
	10.0	45.3	10.1	101
	17.0	51.2	16.0	94
			Mean ± SD	103 ± 8.2%

concentrations of TBG decreased linearly with increasing dilution. The correlation between the expected and observed values was excellent ($r > 0.99$).

Interference Studies and TBG Saturation

We studied the effect of hemolysis, lipemia, dilantin and salicylate on the performance of the assay, by supplementing serum samples with varying

TABLE 3

Dilution Linearity of the TBG Assay†

	Dilution			
Sample	1/151	1/302	1/604	1/1208
Sample 1				
Expected	-	12.7	6.4	3.2
Measured	25.4	12.5	5.6	3.1
Sample 2				
Expected	-	13.0	6.5	3.3
Measured	26.0	14.2	6.9	3.5
Sample 3				
Expected	-	19.9	10.0	5.0
Measured	39.8	20.2	9.5	4.6
Sample 4				
Expected	-	23.3	11.7	5.8
Measured	46.6	24.8	10.9	5.4

† All TBG values in mg/L

concentrations of hemoglobin, lipids, dilantin and salicylate. We found no significant effect on TBG results from these compounds at concentrations up to 2 g/L (hemoglobin), 50 g/L (lipids), 200 mg/L (dilantin), and 2g/L (salicylate). Additionally, saturation of TBG with exogenous T_4 (up to 100 μg/dL) was found to have insignificant effect on TBG concentrations.

Comparison Studies

TBG concentrations of clinical serum samples assayed in duplicate by the present method (y) and by two commercial kits (x) were compared. As shown in Figure 2, results obtained by the present method (FIA) correlated well with those

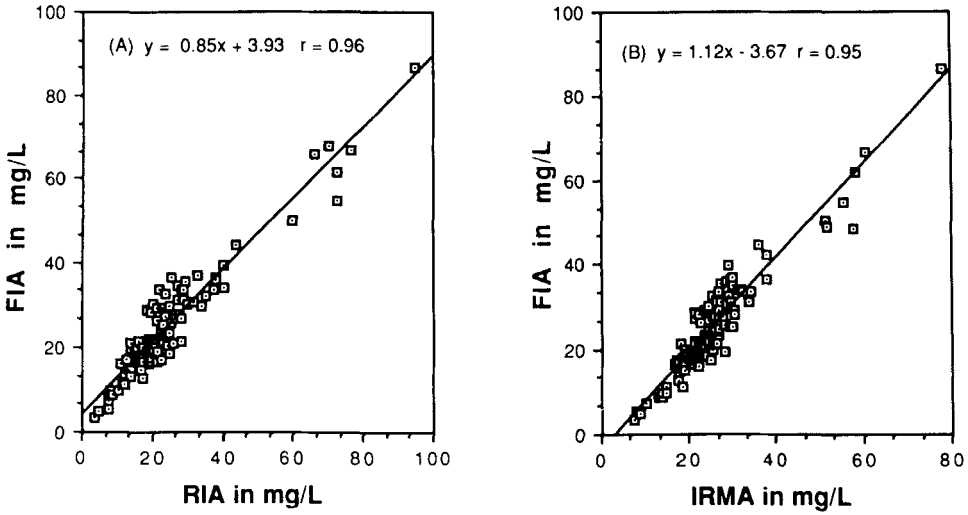

FIGURE 2. Comparison of the proposed procedure with a radioimmunoassay (A) and an immunoradiometric assay (B) for 112 and 131 serum samples, respectively.

determined by a radioimmunoassay (RIA), and by an immunoradiometric (IRMA) procedure. The regression equations were: y (FIA) = 0.85x(RIA) + 3.93, r = 0.96 (n = 112), and y (FIA)= 1.12x(IRMA) - 3.66, r = 0.95 (n = 131).

DISCUSSION

Several methods are currently available for the measurement of TBG concentration in serum (20-30). Isotopic immunoassays (20-22), although satisfactory in terms of performance, suffer from the disadvantages that are related to the use of radioactivity. Among the non-isotopic assays, the enzyme (25-27) and turbidimetric (28,29) immunoassays are compromised by the lower sensitivity and narrower dynamic range in comparison to the proposed procedure. In general, time-resolved fluorescence immunoassays with europium chelates as labels have a broad

dynamic range and sensitivities equivalent to or better than those obtained with radioactive labels (34-36). In the present assay, a europium chelator is used as label and excess europium is added to create the fluorescent complex. This assay design is different from previously reported time-resolved fluoroimmunoassay systems in which europium is used as label. In the present design, the problems of europium contamination have thus been eliminated. Another feature of the proposed assay is the use of the biotin-streptavidin system. This bridge method has a number of advantages including signal amplification and reagent stability. Furthermore, it constitutes a universal detection system and its use offers versatility and simplicity (31,32,34).

The performance characteristics of the present assay are comparable to or better than those reported previously (20-30). Unlike many other methods, it has a dynamic range of up to 100 mg/L. Thus, values for serum TBG as high as those found in pregnancy are readily measured using the proposed dilution. The well known high-dose "hook" effect commonly associated with sandwich-type assays (31) was found to be absent in this assay, even at concentration of TBG as high as 220 mg/L. Furthermore, TBG in serum of TBG-deficient subjects can also be easily quantitated due to the low detection limit of the procedure (0.4 mg/L). However, in this particular assay, detection limit is not a major consideration since the levels of TBG in serum are normally high enough to require sample pre-dilution. Thus, detection level can be manipulated by selecting the proper sample dilution.

The accuracy of the method, as assessed by recovery, dilution and comparison studies is satisfactory. The precision is similar to other well established methodologies for TBG. We believe the use of two monoclonal antibodies is preferable to the use of one antibody and labeled thyroxine in devising immunometric assays for TBG. This is because TBG concentration can be

underestimated if the serum sample contains thyroid hormone binding inhibitors (e.g., unsaturated free fatty acids, drugs and hormones). Such inhibitors are potentially present in patients with nonthyroidal illnesses (37).

In conclusion, the TBG assay reported here represents a new non-isotopic immunoassay based on time-resolved fluorescence measurements. The assay has performance characteristics similar to or better than those of the previously reported procedures, and it compares well with radioimmunological methods. These characteristics, coupled with the simplicity of the assay procedure, make the assay well suited for routine monitoring of TBG concentration in clinical laboratories to derive the T4/TBG ratio, and in population screening for the assessment of thyroid status.

Requests for reprints should be sent to Dr. E. P. Diamandis, CyberFluor Inc., 179 John Street, Toronto, Ontario, Canada M5T 1X4

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