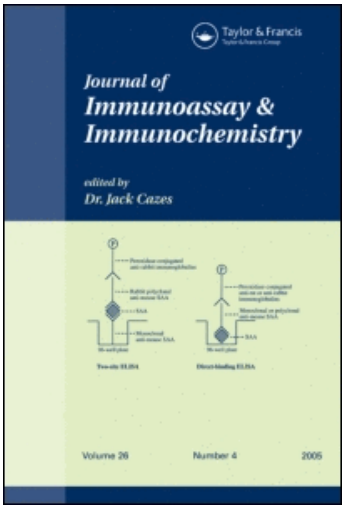

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Yok K. Tan[a]; Vipin Bhayana[a]; Anastasia Papanastasiou-diamandi[a]; Mohammad J. Khosravi[a]
[a] CyberFluor Inc., Ontario, Canada

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TRIIODOTHYRONINE UPTAKE MEASUREMENT IN SERUM BY TIME-RESOLVED FLUORESCENCE IMMUNOFLUOROMETRY

Yok K. Tan, Vipin Bhayana, Anastasia Papanastasiou-Diamandi, and Mohammad J. Khosravi

CyberFluor Inc., 179 John Street, Toronto, Ontario, Canada M5T 1X4

ABSTRACT

A solid phase competition-type fluoroimmunoassay for triiodothyronine (T3) uptake in serum is described. In the assay, exogenous free T3 binds to the unoccupied binding sites on serum thyroxine binding proteins while the remaining unbound T3 competes with immobilized T3 for binding to a soluble biotinylated anti-T3 monoclonal antibody. The bound biotinylated antibody is quantitated by the addition of streptavidin labeled with the europium chelator 4,7-bis(chlorosulfophenyl\`-1,10 phenanthroline-2,9-dicarboxylic acid (BCPDA) in the presence of excess europium. The fluorescence signal of the final complex, which is directly proportional to the number of unoccupied binding sites on thyroxine binding proteins, is then measured on the dried solid-phase with a pulsed-laser time-resolved fluorometer. The assay requires a 10 µl serum sample and a total incubation time of 90 minutes. The coefficients of variation for within-run and between-run assays ranged from 2.0 to 5.7%. Results obtained by the present method compared well with those determined by two commercial radioimmunoassays ($r > 0.9$). (KEY WORDS: time-resolved fluorescence immunoassay; fluorometer; T3 uptake; thyroid tests; monoclonal antibody; europium chelate)

INTRODUCTION

Thyroxine binding globulin (TBG) is primarily responsible for the storage and transportation of thyroid hormones in serum (1). Because of the relatively high

binding capacity and affinity of TBG for thyroxine (T4) and triiodothyronine (T3), the total levels of these hormones are influenced by changes in serum concentration of TBG which occurs in response to a host of physiological and pharmacological stimuli (2-6). To correct for alterations in TBG levels, T4 values have been normalized by calculating the Free Thyroxine Index (FTI) which relies on simultaneous measurement of total T4 and an estimate of T4 binding capacity of serum proteins. The latter has been traditionally performed by a variation of the T3 uptake test originally introduced by Hamolsky et al. (7). A number of investigators have recently questioned the value of the T3 uptake test for its being an indirect measure of TBG and for its reported inability to correct the serum T4 for the effects of high TBG concentrations (8-11). However, despite these controversies, the FTI is still one of the most widely used methods of thyroid assessment and it has been shown to correlate well with both the clinical status of the patients and the free T4 concentration (12-17).

Methods for the measurement of T3 uptake have involved exposure of the test serum to a fixed amount of radiolabeled T3 and to a variety of solid-phase materials including ion exchange resins, coated charcoal, silicate, macroaggregated albumin and solid-phase anti-T3 antibodies (18-22). Newer methodologies have incorporated enzymes, chemiluminescent, or fluorescent probes as labels in a variety of homogeneous and heterogeneous assay designs (23-26).

The fluorescent europium complexes have a number of advantages as immunological tracers compared with isotopic and other nonisotopic labels (27-30). Because the fluorescence emitted from these complexes is long lived, the tracer can be selectively detected by a gated fluorometer after the background fluorescence is allowed to resolve. The synthesis of 4,7-bis(chlorosulfophenyl)-1,10 phenanthroline-2,9-dicarboxylic acid (BCPDA), a new europium chelator, has been described recently (31). BCPDA exhibits a number of attractive properties which

have been successfully explored in developing a streptavidin-based universal detection reagent for time-resolved fluorometry. Complemented with biotinylated antibodies, the detection reagent has found wide application in time-resolved immunofluorometry of a variety of analytes (30,32-35).

In this report we describe the first time-resolved immunofluorometric assay for thyroid hormone binding capacity in serum. In the assay a known amount of exogenous T3 added to serum sample binds to the available binding sites on thyroid hormone binding proteins. The remaining unbound T3 competes with T3 immobilized on white microtiter wells for binding to a soluble biotinylated monoclonal anti-T3 antibody. After washing, the biotinylated antibody bound to the solid-phase T3 is quantitated by a bridge reaction with the streptavidin-based detection reagent in the presence of excess europium. The fluorescence of the final complex on the dried solid-phase is then measured using an automated time-resolved fluorometer.

MATERIALS AND METHODS

Chemicals and Reagents

Streptavidin, bovine serum albumin, bovine globulin, and bovine thyroglobulin were from Sigma Chemical Co., St. Louis, MO 63178. Sulfosuccinimidyl 6-(biotinamido) hexanoate (NHS-LC-Biotin) was from Pierce Chemical Co., Rockford, IL 61105. Europium (III) chloride hexahydrate was from Aldrich Chemical Co., Milwaukee, WI 53233. White opaque microtitration strips (12-well) are products of Dynatech Laboratories Inc., Alexandria,VA 22314. The europium chelator 4,7-bis(chlorosulfophenyl)-1,10 phenanthroline-2,9-dicarboxylic acid (BCPDA) was synthesized as described previously (31).

The assay buffer was a 0.05 mol/l Tris solution of pH 7.8 containing 9 g NaCl, 1 g BSA and 0.5 g sodium azide per liter. The streptavidin-europium buffer was a 0.05 mol/l Tris solution of pH 7.2 containing 9 g NaCl, 40 g BSA and 0.5 g sodium azide per liter. The wash solution was a 9 g/l NaCl solution containing 0.5 ml of polyoxyethylenesorbitan monolaurate (Tween 20) per liter. Stock free T3 (10 mg/l) was made in NaOH, 100 mmol/L.

Calibrators

For calculating unknowns, three levels of human serum-based calibrators corresponding to low, normal and high T3 uptake values were used. Calibration curves were constructed by plotting established % T3 uptake values versus fluorescence units. These calibrators were products of Scantibodies Laboratories, Santee, CA 92071.

Monoclonal Antibody and Biotinylation Procedure

A monoclonal antibody to T3 was purchased from O.E.M. Concepts Inc., Toms River, NJ 08755. The antibody was stored at 4 °C in 0.1 mol/l phosphate buffer containing 0.05% sodium azide until use.

The biotinylation procedure has been described previously (32). After dialysis against a 0.1 mol/l bicarbonate solution containing 0.5 g sodium azide per liter, the biotinylated antibody was tested at various dilutions to determine the optimal concentration for the assay. The stock antibody is stable for at least 12 months when stored at 4 °C.

Preparation of the Tracer (Streptavidin-Thyroglobulin-BCPDA-Eu^{3+})

The preparation of BCPDA-labeled streptavidin-thyroglobulin protein conjugate is described in detail elsewhere (34). The working solution of the

BCPDA-labeled streptavidin-thyroglobulin conjugate contained 0.3 mg/l streptavidin and 40 μmol/l $EuCl_3$.

Coating Microtiter Wells with Globulin-T3 Conjugate

The T3-globulin conjugate, synthesized according to the procedure of Alexander and Jennings (36), was immobilized by adsorption onto the wells of microtiter strips as described in (35). 100 μl per well of the T3-protein conjugate in the coating buffer at a protein concentration of approximately 5 μg/ml was used for coating.

Specimens and Comparative Methods

Clinical serum samples stored at 4 °C for no longer than 5 days were obtained from The Toronto Western Hospital, Toronto, Canada. Lyphocheck immunoassay control sera (human) levels I, II, and III were from Bio-Rad, Clinical Division, Richmond, CA 94801. Ligand control sera (human) levels A, B, and C were obtained from Ciba Corning Diagnostic Corp., Irvine, CA 92714.

Correlation studies were performed using two commercially available kits., the Coat-A-Count® [^{125}I] T3 uptake (Diagnostic Products Corp., Los Angeles, CA 90045) and the GammaCoat™ [^{125}I] T3 uptake kit (Dade, Cambridge, MA 02139). These kits employ anti-T3 antibody coated tubes as the secondary binder. Radioactivity counting was performed with the LKB 1272 Clinigamma counter (Wallac Oy, Turku, Finland). TBG contents were measured by time-resolved immunofluorometry (37) using the FIAgen™ TBG kit (CyberFluor Inc., Toronto, Canada, M5T 1X4).

Immunoassay Procedure

Before commencing the assay, dilute the stock T3 and the biotinylated anti-T3 antibody solutions in the assay buffer to produce a final concentration of 8 ng/ml and 500 ng/ml respectively. Pipet 10 µl of samples in duplicate and 100 µl of the assay buffer containing diluted T3 and the antibody into the T3 conjugate coated wells. After the wells have been shaken for 1h at room temperature, remove the reaction mixture and wash the wells 4 times with the wash solution. Add 100 µl per well of the diluted tracer solution and incubate the wells for 30 min at room temperature. Wash the wells as above and dry for 10 minutes using cool air. The fluorescence of the final complex on the dried solid phase (bovine globulin-T3-antibody-biotin-streptavidin-thyroglobulin-BCPDA-Eu^{3+}) is then measured on the CyberFluor™ 615 Immunoanalyzer (CyberFluor Inc., Toronto, Canada, M5T 1X4).

RESULTS

Assay Optimization

Effects of sample size, antibody and free T3 concentrations. We examined wells coated with T3 conjugate (500 ng/well) for binding to increasing concentrations of the biotinylated antibody in the presence of a varied amount of exogenous T3 and a constant volume of sample (10 to 50 µl). We observed increased binding of the antibody to the solid-phase when the relative amount of the exogenous T3 was decreased. Conversely, decreased binding occurred with increasing concentrations of the exogenous T3. As expected, the assay response was also related to the volume of sample used. A proportional increase in antibody binding was observed when the sample volume was increased. Finally, using a sample size of 10 µl, a

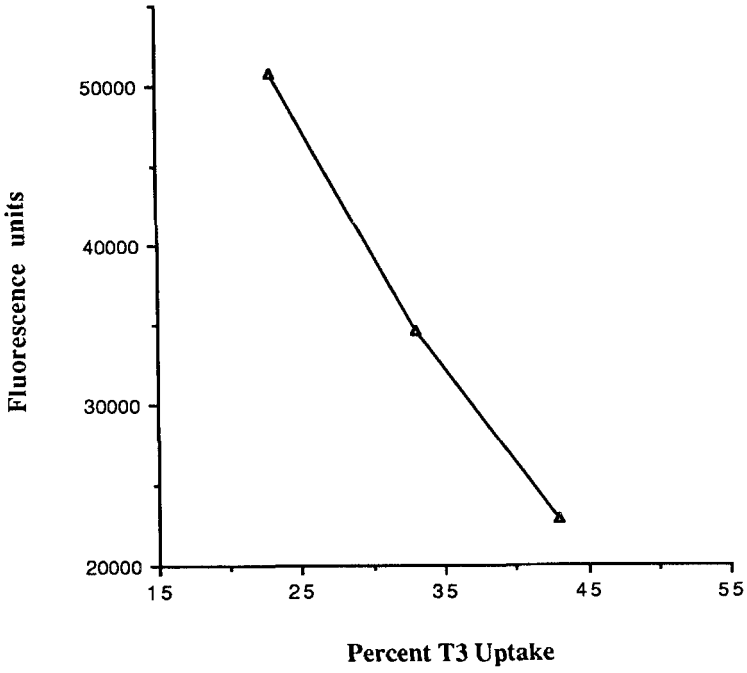

FIGURE 1. Typical calibration curve of the proposed T3 uptake assay. Each point represents the mean fluorescence of duplicates.

concentration ratio of 1 (exogenous T3) to 62 (biotinylated antibody) was found to be the most favorable combination in terms of linearity of the assay response and sensitivity of the standard curve in distinguishing between normal, hypothyroid and hyperthyroid samples. A typical standard curve of the assay is shown in Figure 1.

Effect of incubation time. We also investigated the effect of incubation time on assay performance. Using a 30 min second incubation step, there was a progressive increase in fluorescence signal when the antibody binding step was increased from 30 to 120 min. However, as Table 1 indicates, the T3 uptake values obtained for

TABLE 1

Effects of Antibody Incubation Time on the T3 Uptake of Sera from Hypothyroid, Euthyroid and Hyperthyroid Individuals

	Percent Uptake (% T3U)			
Sample	30 min	60 min	90 min	120 min
1	23.0	23.9	22.6	25.2
2	30.8	30.4	33.2	35.0
3	32.7	30.5	34.2	32.5
4	37.7	35.9	36.4	38.1
5	45.0	42.3	42.9	41.4
6	47.0	49.8	50.0	47.5

normal, hypothyroid and hyperthyroid serum samples remained relatively constant. We selected a 60 min duration for the first step because the fluorescence signal generated was relatively high and the rate of its increase was somewhat slower with further incubation. The time course of the second incubation step was also examined. Binding of the labeled streptavidin increased continuously with incubation time up to 30 min. As the rate of increase was much slower with further incubation, the 30 min period was selected for the tracer addition step (data not shown).

Precision

The precision of the assay is shown in Table 2. Within-run precision was determined by assaying 24 replicates of clinical samples and control sera in a single assay. Between-run precision was assessed by performing repeat analysis of the

TABLE 2

Within- and Between-run Precision

		% Uptake		
Sample	n	Mean	SD	CV (%)
Within-run				
1	24	23.4	0.89	3.8
2	24	32.5	1.28	3.9
3	24	36.3	1.28	3.5
4	24	45.7	0.93	2.0
Between-run				
1	20	17.3	0.96	5.6
2	20	21.3	1.22	5.7
3	20	30.0	1.13	3.8
4	20	36.0	1.27	3.5
5	20	40.8	1.54	3.8
6	20	50.3	2.18	4.3

samples in duplicate on 20 different occasions during a two week period. The overall coefficients of variation ranged from 2.0 to 5.7%.

Interference Studies

Serum samples supplemented with hemoglobin, lipids and bilirubin were tested for possible effects on assay performance. We found no significant interference at concentrations up to 10 g/l (hemoglobin), 20 g/l (lipids), and 200 mg/l (unconjugated bilirubin).

Relation between TBG Concentration and Percent T3 Uptake

To assess the rate of change in T3 uptake at various TBG concentrations, we assayed samples from 126 euthyroid patients with TBG concentrations ranging

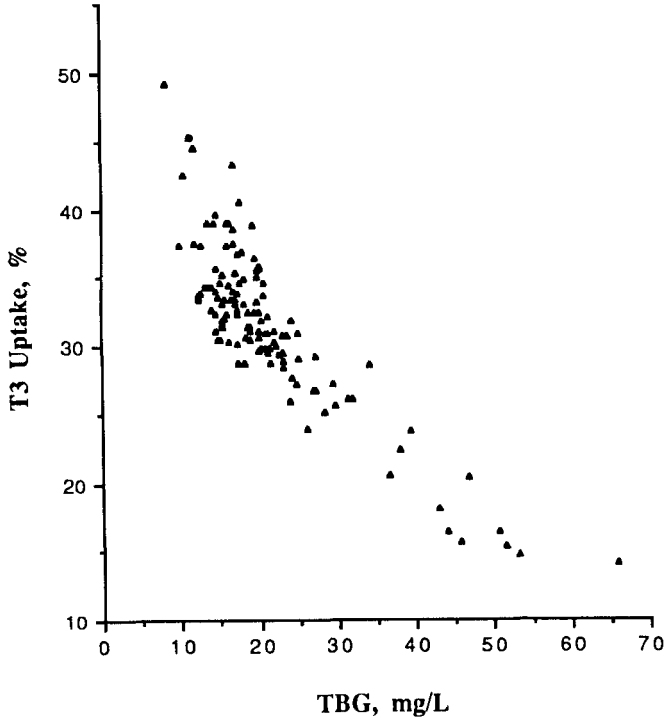

FIGURE 2. Relationship between percent T3 uptake and TBG concentration in sera from euthyroid patients.

from approximately 8 to 66 mg/l. As shown in Fiqure 2, there is a typical hyperbolic relationship between the two measured parameters, with the assay demonstrating a sufficient change in response to a wide range of TBG concentrations.

Correlation Studies

To assess accuracy, we analyzed 95 clinical samples by the present method (y) and by two commercially available radioimmunoassay procedures (x). As

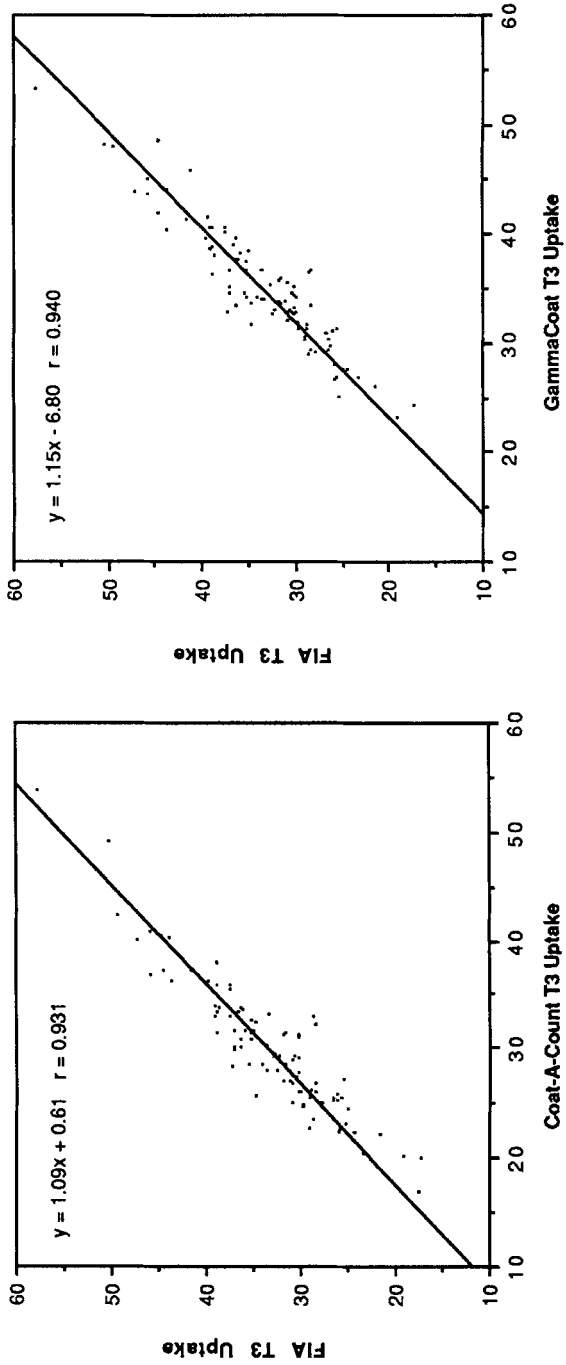

FIGURE 3. Comparison of results between the proposed method and two commercial radioimmunoassay procedures for 95 serum samples.

shown in Figure 3, there is good correlation between the results of the present method and those of the comparative kits.

Reference Range

We analyzed 114 clinically normal serum samples obtained from a mixed population of euthyroid individuals for T3 uptake values. The values ranged from 25.2 to 41.0%, with a mean and standard deviation of 32.9 and 3.23% respectively. The median was 32.6%. The proposed reference range of the present assay defined as the mean ± 2 S.D. was 26 to 39%.

DISCUSSION

Time-resolved fluoroimmunoassays are recognized as a practical alternative to the traditional methodologies involving radioactive labels in both clinical and research laboratories. A number of reports have recently reviewed the merits of this technology (27-30) and its application in the immunoassay of a diverse group of analytes has been described (29,30,32-35). The potential of the fluorescent lanthanide chelate complexes as versatile immunological and nonimmunological tracers is further demonstrated by their recent application in the development of double-label (38) and homogeneous time-resolved (39) immunoassays.

The present assay integrates a universal detection system based on BCPDA labeled streptavidin derivatives with a biotinylated monoclonal antibody in a competetive assay format for T3 uptake in serum. The procedures for multiple fluorescence labeling of streptavidin with BCPDA through a protein bulking agent (thyroglobulin) and use in time-resolved fluoroimmunoassays are detailed elsewhere (34).

The performance characteristics of the proposed assay are comparable to those reported for the most commonly used T3 uptake assays (16, 40). The assay is precise with coefficients of variation ranging from 2 to 5.7%. We did not observe any significant interference from hemoglobin, lipid or bilirubin on assay performance. Furthermore, varying the incubation time of the assay did not significantly influence assay results.

In comparison to the previously published methods (16), the present assay exhibits a marked change in response to a wide range of TBG concentrations (from approximately 8 to 66 mg/l). The rate of change in uptake values is more pronounced at lower TBG concentrations but slows down in response to elevated TBG levels (Figure 3). The linear regression analysis demonstrated a good correlation between results obtained by the present method and and those of two commercially available radioimmunoassays.

The preliminary normal range expressed as percent T3 uptake established in our laboratory for a mixed population of euthyroid males and females was found to be 26 to 39%. This is similar to the normal range reported for T3 uptake tests by other investigators (40).

In summary, we have described a new nonisotopic immunoassay for routine monitoring of T3 uptake in serum. The assay exhibits good performance characteristics and has a protocol which is easy to perform. It works with a 10 µl sample volume and requires a total incubation time of 90 min.

Requests for reprints should be sent to Dr. Mohammad J. Khosravi, CyberFluor Inc., 179 John Street, Toronto, Ontario, Canada M5T 1X4

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