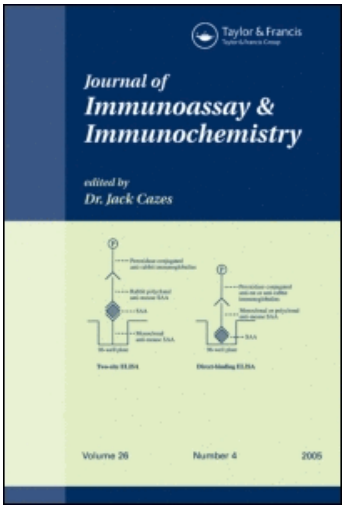

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A Rapid and Sensitive Chemiluminescent Immunoassay of Total Thyroxin with DMAE · NHS-Labeled

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Abstract: A novel chemiluminescence immunoassay (CLIA) of total thyroxin (TT4) with the acridinium ester of DMAE · NHS [2′,6′-dimethyl-4′-(n-succinimidyloxycarbonyl) phenyl-10-methyl-acridinium-9-carboxylate methosulfate] labeled has been developed. In our method, microwells were coated with anti-T4 monoclonal antibody (McAb) and DMAE · NHS synthesized by our laboratory was conjugated with streptavidin (SA). T4-BSA was conjugated with biotin-N-hydroxysuccinimide (B · NHS), T4-BSA- B · NHS and the T4 in the standard or sample competitively react with anti-T4 McAb. Streptavidin-biotin separation and enhanced techniques were applied in this assay. The presented approach shows many excellent characteristics, particularly, rapid assay process and high detection sensitivity.

Keywords: Chemiluminescence immunoassay; DMAE · NHS; Streptvaidin-biotin; Total thyroxin

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INTRODUCTION

Thyroxin is an iodine-containing hormone which is produced and secreted by the thyroid gland. Acting as a catalyst of oxidation reactions in the body, it has an important action on the regulation of metabolism. Measurement of total thyroxine provides a very important value in the diagnosis of thyroid diseases and in the evaluation of therapeutic effects. Many methods of immunoassay for total thyroxine have been developed and commercialized, including radioimmunoassay (RIA),[1] enzyme immunoassay (EIA),[2–4] fluorescence immunoassay (FIA),[5] time-resolved fluorescence immunoassay (TRFIA),[6–8] and chemiluminescent immunoassay (CLIA).[9–14]

Usually, a CLIA system includes acridinium ester, luminal, alkaline phosphatase, oxalate, and electrogenerated chemiluminescence. Comparatively, the acridinium ester system has advantages of no need for a catalyst, low natural background, high signal-to-noise ratio, facility of label, high stability of conjugation compound, and high detection sensitivity. Here we describe a novel chemiluminescent immunoassay of total thyroxin with a acridinium ester, DMAE · NHS, as label. Streptvaidin–biotin separation and enhanced techniques were applied in this assay. This is the first report of a chemiluminescent immunoassay of total thyroxin using a DMAE · NHS label combined with a streptavidin–biotin system. Because of the high luminescent signal of DMAE · NHS, the tetrameric binding feature of streptavidin, and the high affinity between streptavidin and biotin, the detection sensitivity was increased and assay time was shortened. Moreover, due to biotinylating the hapten-protein conjugate rather than biotinylating a detecting second antibody in our present approach, the assay process was simplified and facilitated which led to the assay time becoming much shorter. To our knowledge, there is no procedure reported for assaying total serum thyroxine with such a low detection limit and such a short assay time. The developed approach provides a potential and general method for chemiluminescence immunoassay of various analytes.

EXPERIMENTAL

Instrumentation

The chemiluminescence was measured with a Wallac Victor 1420 Multilabel Counter (Wallac, Finland). The UV-Visible spectrophotometer was from Varian, USA. The 1290-003 Delfia PlateShaker was from Wallac, Finland. The Shimadzu LC-6A high pressure liquid

chromatograph was from Hitachi, Japan. The 96-well plate was from NuNc, Danmark. The 1296-026 DELFIA plate shaker was from Pekin Elmer-Wallac.

Reagents and Chemicals

T4 was purchased from Sigma. T4 stands were prepared by diluting concentrated L-T4 stock solution to the desired concentration in T4-free human serum and calibrated with a commercially available RIA kit (Department of Isotope, China Institute of Atomic Energy). Anti-T4 monoclonal antibody was obtained from Medix Biochemica Co., Finland. T4-BSA conjugate was provided by our department as described in previous work.[8] 8-Anilinonaphthalene-1-sulfonic acid ammonium salt (ANS) was from Fluka. Sodium salicylate, streptavidin (SA), biotin-N-hydroxysuccinimide (B · NHS) were all from Sigma. Bovine serum albumin (BSA), normal mice serum (NMS), Tween-20, and Triton X-100 were from Sina America Biotechnology Co., China. Dimethyl formamide (DMF) was from Acros. NaN_3 was from Merck, Germany. Sephadex G-50 was from Pharmacia, Sweden. Human serum samples with T4 values measured by Ciba Corning Chemiluminescence immunoassay were kindly provided by 301 Hospital, Beijing, China. DMAE · NHS was synthesized in our laboratory.

The coating buffer was 0.05 mol/L sodium carbonate buffer (pH 9.5). The blocking buffer was 0.05 mol/L phosphate buffer (pH 7.4), containing 0.9% NaCl, 1% BSA, 0.04% NaN_3. The assay buffer was 0.1 mol/L Tris-HCl (pH 8.6), containing 0.06% ANS, 0.2% sodium salicylate, 0.9% NaCl, 0.1% BSA, 0.05% NaN_3, 0.03% Tween-20, 0.05% NMS. The washing buffer was 0.01 mol/L phosphate buffer (pH 8.0), containing 0.9% NaCl, 0.05% Tween-20. The labeling buffer was 0.1 mol/L $NaHCO_3$ (pH 9.0). The purifying buffer was 0.1 mol/L phosphate buffer (pH 7.0), containing 0.9% NaCl.

Preparation of DMAE · NHS-SA

6.5 mmol/L DMAE · NHS solution was prepared by dissolving a definite amount of DMAE · NHS in DMF. 90 μg SA, 200 μL labeling buffer, and 10.5 μL 6.5 mmol/L DMAE · NHS were mixed well in a 2 mL brown glass bottle. After 2 hours incubation at room temperature, the reaction was terminated by adding 100 μL 10 g/L lysine and allowing it to stand for 15 minutes at room temperature. The unreacted DMAE · NHS was separated from the conjugate of DMAE · NHS-SA by size-exclusion chromatography on a 1×25-cm column of Sephadex G-50, eluting

with purifying buffer, and monitoring the protein peak at 280 nm with Shimadzu LC-6A high pressure liquid chromatograph. Chemiluminescence intensity and protein concentration were determined on a Multilabel Counter and UV-Visible Spectrophotometer, respectively. Fractions having high chemiluminescence intensity and protein concentration were pooled and stored at -20°C after adding 1% BSA. The molar ratio of DMAE · NHS to SA was determined to be 4.2, obtained as the amount of DMAE · NHS conjugated to SA divided by the amount of SA.

Preparation of B · NHS-T4-BSA

167 μL 6mg/mL T4-BSA was first dialyzed against labeling buffer overnight. The T4-BSA was then transferred to a clean brown glass bottle. Then, 70 μL 2.84 mg/mL B · NHS in DMF solution was added into the bottle and the mixture was incubated for 2.5 h at room temperature, under vortexing. Then, saturated ammonium sulfate solution was added to the bottle to precipitate the B · NHS-T4-BSA conjugate. After centrifuging and decanting, purifying buffer was added to dissolve the precipitate. Then, the solution was dialyzed against purifying buffer overnight and stored at -20°C after adding 1% BSA.

Preparation of Surface Antibody

To each microwell, 150 μL coating buffer containing 1.2 μg Anti-T4 monoclonal antibody was added and incubated for 24 h at 4°C. After twice washing with washing buffer, the microwells were blocked by incubation with 200 μL of blocking buffer for 2 h at room temperature. After decanting the solution, the microwells were allowed to thoroughly dry, placed in plastic bags, vacuumed, sealed tightly, and stored at 4°C.

Serum TT4 CLIA

After careful optimization, the serum TT4 CLIA based on acridinium-ester-labeled and streptvaidin-biotin-separated-enhanced was performed as follows: B · NHS-T4-BSA and DMAE · NHS-SA were first diluted with assay buffer to the ratio of 1:350 and 1:100, respectively. Duplicate 50 μL of T4 standards or serum samples and 100 μL B · NHS-T4-BSA diluted solution were pipetted into the microwells coated with anti-T4 monoclonal antibody. The mixture was incubated for 20 minutes at room temperature with slow shaking. The microwells were washed 4 times with

washing buffer. Then, 150 μL DMAE · NHS-SA diluted solution was added to each well and incubated 10 minutes at room temperature with slow shaking. After washing 6 times, the chemiluminescence was measured for 1 s on Wallac Victor 1420 Multilabel Counter. TT4 concentrations of serum were calculated from the standard curve.

RESULTS AND DISCUSSION

Standard Curve and Detection Limit

A typical standard curve is shown in Fig. 1. The B/B_0 value decreases with increasing T4 concentration, showing a typical characteristic of competitive-restrained, while the T4 standard concentration was in the range of 15–240 ng/mL. The detection limit of the assay was 0.56 ng/mL, defined as the T4 concentration corresponding to the mean chemiluminescence reading of zero standard ($n = 24$) minus two times the standard deviation.

Usually, non-competitive immunoassays were used to detect most large antigens because of their high sensitivity and specificity, simple assay optimization, and wide dynamic range. However, competitive immunoassays are widely used to measure a variety of analytes, especially the haptens with a small molecular size, and the large antigens when there is limited supply of antibodies. Three typical configurations of solid-phase competitive immunoassays have been extensively employed: (1) immobilized primary antibody, (2) immobilized surface antigen, and (3) immobilized antibody of a second anti-species.

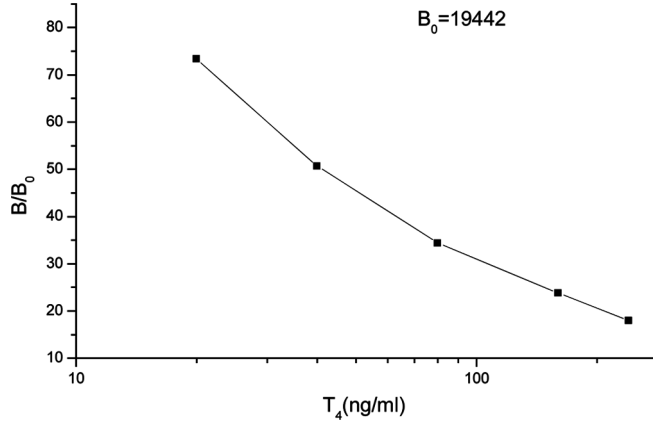

Figure 1. Typical standard curve of the present T4 assay.

Approach (3) involves more assay steps and requires the preparation and use of a highly active surface second anti-species. Approach (2) suffers from the inability to measure high analyte concentration. Comparatively, approach (1) overcomes the shortcomings from approaches (2) and (3), and has been successfully employed in our present method.

Precision

The within-run precision of the assay was determined by assaying three control sera, corresponding to different levels of T4 (mean T4 concentrations of 15, 59.3, and 122 ng/mL), in 12 replicates in a single assay. For the determination of the between-run precision, duplicate measurements of these control sera were performed in 12 different runs. The within-run CVs were 4.5, 5.6, and 4.0% (mean), and the between-run CVs were 5.4, 10.3, and 5.9%, respectively.

Recovery

The recovery was assessed by analyzing human serum samples before and after the addition of known concentrations of exogenous T4 (20, 80, and 240 ng/mL). The measured increase in the T4 concentration of the sample, expressed as percentage of the expected increase, was determined as the recovery of the assay. The recovery of added exogenous T4 was found to be 104.7, 97.6, and 112.2%, respectively.

Dilution Test

The dilution linearity of the assay was determined by assaying serum samples serially diluted with T4-free human serum. There was a good

Table 1. Dilution of the samples with T4-Free human serum

	Dilution factor					
Sample	Original	2	4	8	16	32
Expected	61	30.50	15.25	7.63	3.81	1.91
Observed	61	31.70	17.40	9.51	4.43	2.11
Expected	335	167.50	83.75	41.88	20.94	10.47
Observed	335	172.60	86.90	41.30	24.30	13.40

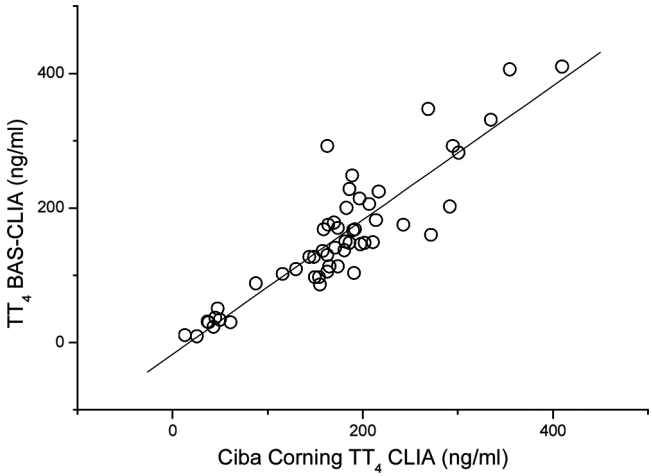

Figure 2. Correlation between the present method and Ciba Corning CLIA.17 samples from healthy people, 30 samples from hyperthyroid patients, and 9 samples from hypothyroid patients.

agreement between the expected and the measured values, as is shown in Table 1.

Specificity

The specificity of the assay was evaluated by determining the cross-reaction values for a number of chemically related compounds or metabolites of T4. The cross-reactivity for each of these compounds was expressed as the percent ratio of the amount of T4 which corresponded to 50% of zero standard binding divided by the amount of the compound that corresponded to the same level of binding. 3,5-Diiodo-L-thyronine, 5,5-diphenylhydantoin, and triiodothyronine (T3) were tested; they exhibited cross-reactivity values less than 0.02%, except for T3, which was 1.5%.

Correlation

For comparison, 56 clinical samples were assayed by the present method and by the commercially available Ciba Corning CLIA T4 kit. The values obtained by the present method were in good agreement with those obtained by the commercial Ciba Corning CLIA, as shown in Fig. 2. The linear regression equation is as follows: $y = 0.998x - 3.02$, $r = 0.962$.

CONCLUSIONS

In summary, we have introduced a novel chemiluminescence immunoassay of total thyroxin. Comparatively, the presented method shows excellent characteristics, such as rapid assay process with two incubations totalling 30 minutes, and 0.56 ng/mL of low detection limit. All the quality criteria required for clinical measurement were fulfilled. This approach, as a potential general method, is believed to be applicable for determination of other analytes besides total thyroxin.

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