Development of Chemiluminescence Immunoassay
For the Measurement of Serum Thyroxine (T$_4$)

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Summary

We describe a simple, solid-phase chemiluminescence immunoassay for the measurement of serum T$_4$. An immunoglobulin G fraction of antibody to thyroxine was passively absorbed onto the walls of polystyrene tubes. The labeled antigen was thyroxine-aminobutylethylisoluminol. After the binding reaction (37°C for 1 hour), the solution is removed by aspiration and the antibody-bound fration was washed once with buffer. Sodium hydroxide (5mol/1,200μl) was added and the mixture incubated for 30 minutes at 60°C. Luminescence was initiated by oxidation of the label with microperoxidase-hydrogen peroxide and the signal of light emission was integrated for 10 sec. The light yield was inversely proportional to the concentration of T$_4$ in the standard or sample. An evaluation of the method gave the following values: sensitivity of calibration curve 7.5 $\pm$ 2.8 nmol/l (mean $\pm$ SD). The intra-assay precision (CV%) was 8.9, 7.3 and 5.4. The inter-assay precision (CV%) was 10.2, 8.1 and 7.1. When serum samples were assayed for T$_4$, the results obtained by solid-phase CIA and the conventional RIA agreed well (n = 35, r = 0.954).

Introduction

The measurement of total thyroxine (T$_4$) in serum has been widespread use in endoclinological research in order to assess thyroid status and monitor the treatment of function hypo- and hyper-thyrodism, since thyroid functions are related with proper growth development and sexual maturation (Kendalll Taylor, 1978 ; Van Lente & Galen, 1980).

Radioimmunoassay (RIA) has been applied to the determination of total T$_4$ in serum (Hollander et al., 1974). However, the increasing usefulness and availbility of RIA have raised several serious problems which include: i) the radioactive half-life and radiolysis of the labelled reagent, iii) health hazards associated with the use and disposal of radioactive compounds and the solvents necessary for liquid scientillation counting, iii) the high cost and maintenance of equipment, and iv) the dependence upon laboratory facilities and expertise (Collins et al., 1983; Schall and Tenoso, 1981). Consequently, in the past several years, nonisotopic immunoassays have been investigated extensively in order to overcome some of the drawbacks encountered in RIA. For the measurement of serum T$_4$, several new noninsotopic immunoassay procedures as practical alternatives to RIA have been developed (Kaplan and Chen, 1985), which have included the use of enzymatic (Ullman et al., 1975; Polmp et al., 1979; Izqierda et al., 1982) or fluorescent markers (Smith, 1977). kers (Smith, 1977).

In 1979, Schroeder et al. reported a chemiluminescence immunoassay (CIA) for serum T$_4$ using a thyroxine-isoluminol conjugate as the labelled antigen, which produced a light emission by oxidation reaction. This method was not convenient, however, for routine purposes because it employed a very complex separation system, although there was a good correlation of values with a conventional RIA (r=0.98, n=28). More recently, methods have been developed which involve the use of a solid-phase separation system(Barnard et al., 1981; Kohen et al., 1981; Eshhar et al., 1981; Kim et al., 1982).The specific IgG is adsorbed onto the surface of

polystyrene assay tubes and after the binding reaction, the incubation mixture is aspirated. Subsequently, the tubes are rinsed with buffer to remove potentially interfering substances with the concomitant reduction in back-ground chemiluminescence and free fraction. These methods have shown to be comparable in specificity, sensitivity and precision to established conventional RIA.

In this report a description is given of a heterogeneous CIA method for the measurement of T_4 in serum using antibody passively adsorbed to the walls of polystyrene tubes. The method is assessed for sensitivity, accuracy and precision. In addition, a comparison is made between values of serum T_4 as determined by CIA and an equivalent RIA.

Materials and Methods

Reagents

Bovine serum albumin (BSA Fraction V), microperoxidase (MP-11), Sepharose-4B-protein A and 8-Anilino 1-naphthalene sulphonic acid (ANSA) were purchased from Sigma London Chemical Co. Ltd., Poole, Dorset, UK. Aminobutylethylisoluminol (ABEI) used for the conjugation to T_4 was a product of LKB, Wallac Oy, Turku, Filand.

Two buffers were used: i) 0.07M barbitone buffer for coating of antibody, prepared by dissolving 14.4g sodium barbitone in 1 liter doubly distilled water containing 0.1% sodium azide, pH 9.5, ii) 0.05M barbitone assay buffer used for the dilution of conjugate and standard, prepared by disssolving 10.31g sodium barbitone in 1 liter doubly distilled water containing 0.1% BSA and 0.1% sodium azide, pH 8.6.

Microperoxidase was dissolved in doubly distilled water and the stock solution (1mg/ml) was stored at 4°C until required. The working solution was 20μl/ml (50 fold dilution). The oxidant solution was prepared by adding 100μl of hydrogen peroxide (30%, Sankoku Chemical Co., Japan) to 10ml doubly distilled water.

Antibody-Coated Tubes

Rabbit anti-T_4 sera were kindly donated by Dr. G.

Barnard from King's College Hospital Medical School, London Univ. UK and Dr. M. Pazzagli from Endocrinology Unit, Florence University Italy. These sera were purified by Sepharose-4B-protein A affainity column (Kim et al., 1982). An IgG fraction of the antisera were suitably diluted in 0.07M barbitone buffer. Aliquots (0.2ml) of this solution were added to polystyrene tubes (LP3; Luckham Ltd., Burgess Hill, Sussex, UK). After an overnight incubation at 4°C, the buffer was aspirated to waste and saline containing 0.3% BSA was added to each tube. After an incubation of 30 minutes or 1 hour at room temperature the solution was aspirated to waste and the tubes were stored at 4°C until required.

Sample Collection

Serum samples were provided by Prof. K.J. Ryu from Medical School of Yon-Sei University. Blood samples were obtained by venepuncture from patients attending an outpatient clinic, transferred into collection tubes and allowed to clot. After centrifugation the serum was removed and stored at -20°C. Repeated freezing and thawing was avoided during assay periods.

Preparation of the Labelled Antigen

The labelled antigen-thyroxine-aminobutylethylisoluminol (T_4-ABEI) was synthesized essentially according to the method of Schroeder et al. (1979).

Chemiluminescence Reaction and Measurement

We add 0.2ml of 5 mol/l sodium hydroxide to each tue and the contents were incubated at 60°C for 30 minutes and another 10 minutes at room temperature. A quantity of 0.1ml microperoxidase solution (20μg/ml) were added to the assay tube, which was then placed in the luminometer in front of the photomultiplier. The chemiluminescence reaction was initiated by the rapid injection of 0.1ml of the diluted hydrogen peroxide into the assay tube using automatic microdispenser.

The light emitted was measured with an LKB Lumi-

nometer Model 1250 connected with the automatic dispenser (Hook and Tucker Instruments Ltd., Crydon, Surrey, UK) to inject the solution of hydrogen peroxide. The signal of the light emission was integrated over 10 seconds and expressed by the unit of mV.

Immunoassay Procedures

Solid-phase CIA: $25\mu l$ of serum of standard were added in duplicate to the assay tubes coated by T_4 antibody. Subsequently, 0.15ml of T_4-ABEI (400pg/0.15ml in 0.5M barbitone buffer containing 1mg/ml of AASA) were added, and the mixture was incubated at 37°C for 1 hour. The reaction mixture was removed by aspiration with vaccum pump and the tubes were washed again with 1ml of 0.9% saline solution. The standard used for CIA was the same as that for RIA (range 12.9-30.9nmol/1).

Solid-phase RIA: For RIA, we used Coat-A-Count total T_4 assay kit (Diagnostic Products Corporation). The assay was performed according to the procedure described in the given manual.

Calculation of Results

The unknown values were derived from the calibration curves (% B/Bo against concentration of T_4, nmol/1).

Results

Light Yield of T_4-ABEI Conjugate

In order to see the properties of synthesized T_4-ABEI conjugate (see the structure in Fig. 1) as a chemiluminescent tracer, the detection limit of the conjugate was determined as shown in Fig. 2. It was calculated that the detection limit, defined as twice mean the background chemiluminescent signal, was 1.2pg.
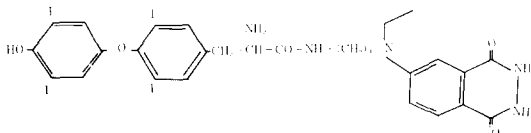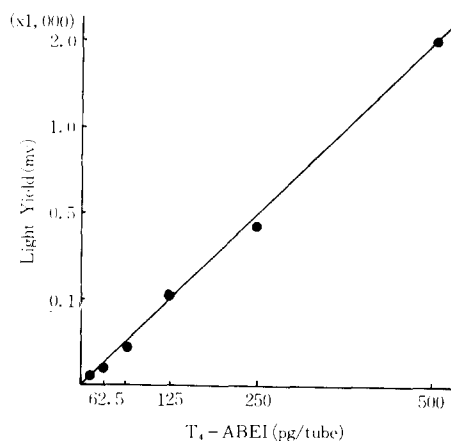

Fig. 1. The proposed structure of T_4–ABEI

Fig. 2. Determination of light yield of T_4-ABEI conjugate

Antibody Dilution Curve

The optimum dilution of anti-T_4 IgG was determined by coating the polystyrene tubes with serial dilutions of the 0.07M babitone buffer (range 1:200, v/v to 1:12,800, v/v). Alquots of $150\mu l$ T_4-ABEI (400pg) were incubated in the presence and absence of $25\mu l$ of authentic T_4 (309 nmol/1). The results are shown in Fig. 3 and it was concluded that the optimal dilution with sufficient binding and displacement was 1:1,000 (v/v).
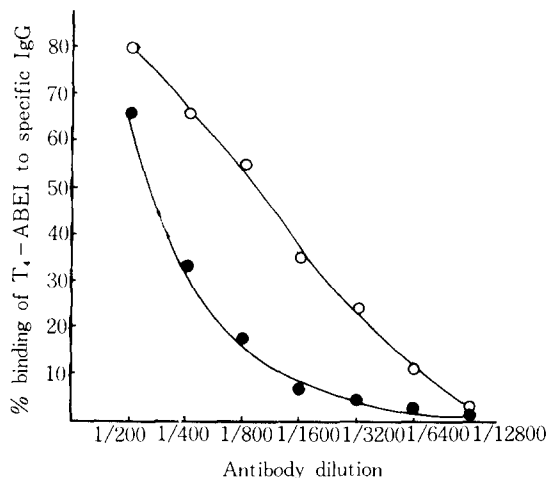

Fig. 3. Antibody dilution curve of T_4 in the presence (o–o) and absence (o–o) of authentic T_4
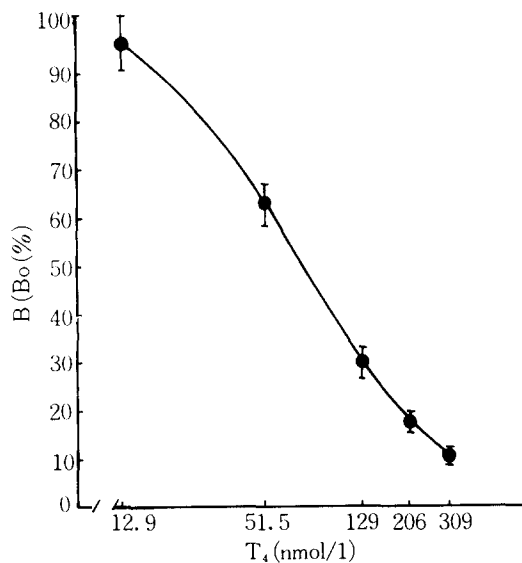
Fig 4. Typical calibration curve of T₄

Fig. 5. Correlation between RIA and CIA

Sensitivity

Typical calibration curves at the optimal concentration of antibody (1:1,000, v/v) is shown in Fig. 4. The minimum concentration of T_4 that could be significantly distinguished from zero (mean minus 2S.D.) was calculated from three calibration curves. The values (mean ± S.D) obtained was 7.5 ± 2.8 nmol/l.

Bias

Increasing amounts of authentic T_4 were added to a serum of low T_4 concentration, which had been analysed previously by RIA, and the samples were re-analysed by CIA. The results are shown in Table 1.

Comparison of Solid-Phase Ria for Serum T_4 with Solid-Phase CIA

The concentrations of serum T_4, as determined by CIA(x) and RIA(y) in 35 samples of serum (Fig. 5), gave a linear regression equation of $y=0.98x + 1.48$ (r=0.954).

Precision

An estimate of the intra-assay variation was obtained by analysing 12 replicate from three serum pools within a single assay. The corresponding values for inter-assay variation were obtained from the measurement of T_4 in aliquots from the three serum pools used for internal

Table 1. Measurement by CIA of T₄ in *serum containing additive amounts of analyte

Amount added (nmol/1)	Expected value (nmol/1)	Observed value (nmol/1)	Recovery (%)	Bias (%)
10.0	30.0	34.0	113.3	+13.3
30.0	50.0	55.0	110.0	+10.0
80.0	100.0	93.0	93.0	− 7.0
100.0	120.0	110.0	91.6	− 8.4
200.0	220.0	215.0	97.7	− 2.3
Mean		101.1		+ 1.1

* Serum sample contained 20 nmol/1

Table 2. Intra- and Inter-assay varation in serum T_4 measurement

Sample	No. of determinations	nmol/1 (mean±SD)	Coefficient of variation(%)
(1) Intra-assay variation			
Pool 1	12	42.5±3.8	8.9
Pool 2	12	102.3±7.5	7.3
Pool 3	12	223.7±12.3	5.4
(2) Inter-assay variation			
Pool 1	10	40.8±4.2	10.2
Pool 2	10	98.5±8.0	8.1
Pool 3	10	215.9±15.3	7.1

quality control over a period of six months. The results are shown in Table 2.

Discussion

This paper describes a solid-phase immunoassay procedure for serum T_4 based on monitoring chemiluminescence. The present method requires three steps: i) passive adsorption of specific IgG onto the walls of the polystyrene tubes ii) incubation of the antibody-coated tubes with the chemiluminescent marker conjugate and with standards and samples iii) removal of the antibody-free fraction by aspiration and subsequent measurement of the light emission of the bound label with H_2O_2-microperoxidase oxidation system at pH 13. The evaluation data presented in this report indicate that the solid-phase CIA of T_4 has similar and comparable characteristics to the RIA in terms of sensitivity, accuracy and precision. This method combines the advantages of a stable, sensitive non-isotopic label and an immunoassay that is not significantly affected by background interference since potentially interfering luminescent compounds are removed by aspiration after the binding reaction.

In recent years, attempts have been made to develop non-isotopic immunoassays that are as sensitive, accurate, and precise as the conventional RIA (Schall and Tenoso, 1981). We have been successful in developing simple CIAs for steroids (Collis et al., 1983; Kim et

al., 1982), steroid glucuronides (Collins et al., 1983; Weerasekera et al.., 1981; Barnard et al., 1981) and, most recently, the potentially more sensitive immuno-chemiluminometricassay (ICMA) for peptide hormone HCG, which involves labeled antibody (Barnard et al., 1985). There is no doubt that CIA has already achieved a status comparable with that of RIA in terms of sensitivity and reliability. Indeed, grater sensitivities than those possible with conventional RIA have already been achieved with antigens labeled with isoluminol, which have a relatively low quantum efficiency. With the development of new, more efficient labels, simpler oxidation systems, and more reliable separation system (e.g., using magnetizable particles attatched to the antibodies), the possibility of improving sensitivities and precision should become a reality. In addition, the availability of extremely reliable yet relatively cheap and simple instruments will facilitate the acceptability of luminescence techniques for the researches in life science.

Although serum samples taken from humanbeing were used for the evaluation of CIA, the method presented here can be applied to the animal as well and various kinds of compounds to be assayed if their corresponding antibodies are available. CIA of the milk progresterone is under investigations for the early diagnosis of pregnancy in cow.

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