Radioimmunoassay for Serum Thyroxine

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SAKURADA, T., YAMAGUCHI, T., YOSHIDA, K., YAMAMOTO, M., SAITO, S. and YOSHINAGA, K. *Radioimmunoassay for Serum Thyroxine*. Tohoku J. exp. Med., 1976, 120 (2), 125-133 — A new procedure for radioimmunoassay (RIA) was applied to determine the serum concentration of thyroxine (T_4). Mixtures of T_4 -methylester hydrochloride-bovine serum albumin complex and complete Freund's adjuvant were injected to rabbits to get them immunized. Specific anti- T_4 rabbit serum, high in titer, could be obtained 18 months after the start of immunization. RIA for T_4 extracted by ethanol from serum was performed by an application of dextran-coated charcoal to separate bound and free ^{125}I - T_4 . On the other hand, RIA for T_4 performed directly in the serum containing 100 mg/100 ml of 8-anilino-1-naphthalene-sulfonic acid (ANS) gave a similar standard curve for T_4 obtained by the ethanol-extraction method. Serum T_4 values obtained by the methanol-extraction method were 9.7 ± 3.3 μ g/100 ml for normal subjects, 17.8 ± 2.9 μ g/100 ml for patients with hyperthyroidism and 0.6 ± 0.3 μ g/100 ml for those with hypothyroidism. The corresponding figures obtained by RIA in the serum containing ANS were 11.1 ± 2.1 , 19.1 ± 3.3 and 0.7 ± 0.7 μ g/100 ml, respectively. The figures positively correlated respectively to the serum T_4 values obtained by the competitive protein-binding analysis method (Tetrasrob-125) — radioimmunoassay; thyroxine; T_4 methylester hydrochloride-bovine serum albumin complex

Competitive protein-binding analysis (CPBA) (Murphy and Pattee 1964) is a method now widely used for the determination of serum thyroxine (T_4). Recently, preparation of specific anti- T_4 antibody has been successfully isolated by several authors, making it available in radioimmunoassay (RIA) for serum T_4 (Chopra et al. 1971). This report is presented to introduce our new procedure for preparing highly specific anti- T_4 antibody. The preparation has proved to be useful for the clinical use in RIA for serum T_4 .

MATERIALS AND METHODS

Preparation of antigen

Preparation of T_4 -methylester hydrochloride. T_4 -methylester hydrochloride (T_4 -MEH) was prepared by the method of Ashley and Harington (1928). 500 mg of T_4 , purified by the method of Williams et al. (1969), in 20 ml of methanol were dried by distillation with dry hydrogen chloride for 3 hr. The products were further concentrated and dried by distillation under a reduced pressure at 37°C. The resulting crystalline precipitate was washed with ethanol, ether and then dried.

Received for publication, January 22, 1976.

Preparation of T₄-MEH-bovine serum albumin complex. T₄-MEH-bovine serum albumin (BSA) complex was prepared by the method of Oliver et al. (1968). 30 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were added to 25 mg of distilled water (pH 5.5) containing 50 mg of BSA. To this, a solution of 20 mg of T₄-MEH dissolved in 2.0 ml of dimethylformamide was given in drops, keeping a pH level at 5.5. Further adding 10 mg of EDC 10 min later, the reaction mixture was stirred for 24 hr at 5°C in the dark. Finally, it was dialyzed for 48 hr against redistilled water at 5°C, and then centrifuged at 3,000 rpm at 5°C. T₄-MEH-BSA complex in the supernatant was lyophilized.

Immunological procedure

3.2 mg of T₄-MEH-BSA complex, dissolved in 1.2 ml of sterile physiological saline solution, were mixed with equivalent volume of complete Freund's adjuvant (Difco). 0.6 ml of the mixture was injected into toe-pads of rabbits. After that, 1.0 mg of T₄-MEH-BSA complex in complete Freund's adjuvant as injected intramuscularly to the back of rabbits once a month for 18 months. One week after each injection, blood was sampled from the rabbit's aural vein.

Preparation of T₄-free serum

T₄-free serum was prepared by the method of Chopra (1972) as follows: Normal human serum was incubated for 24 hr at room temperature with Amberlite IRA-100 (1.75 g/ml, Mallinckrodt). Then barbitol buffer, equivalent volume to that of serum, was mixed with slurry. And supernatant was collected after standing to obtain the T₄-free serum. With ¹²⁵I-T₄ added to the serum, the rate of T₄ elimination was examined. The result showed that the resin evidently carried off as much as 95% of the radioactivity in the original serum.

Extraction of T₄ from serum

2.0 ml of ethanol were added to 0.1 ml of test serum or T₄-free serum mixed with standard T₄. After shaking for 10 sec in a volume mixer, they were left at room temperature for 15 min, then again shaken for 10 sec, and finally centrifuged at 3,000 rpm at 5°C. The supernatant was dried under nitrogen gas at 37°C. To the dried residue, 0.8 ml of barbitol buffer (0.05 M, pH 8.6) containing 0.5% BSA was added.

Indirect RIA of T₄

T₄, extracted from 0.1 ml of serum and then dissolved in 0.8 ml of barbitol buffer (0.05 M, pH 8.6) containing 0.5% BSA, was mixed with 0.1 ml of anti-T₄ rabbit serum (1:500) and 0.1 ml of ¹²⁵I-T₄ (50 pg/0.1 ml). Each mixture was incubated at 5°C for 18 hr and then given 0.2 ml of dextran-coated charcoal; 5.0 g of charcoal (Shirasagi, Takeda) and 0.5 g of dextran (molecular weight 70,000, Pharmacia) suspended in 200 ml of water. The aqueous suspension was shaken for 5 min, then left for 1 hr at room temperature, and finally centrifuged at 3,000 rpm at 5°C for 15 min.

Radioactivities in the supernatant (bound ¹²⁵I-T₄, *B*) and in the precipitate (free ¹²⁵I-T₄, *F*) were counted with a well-typed scintillation counter (Aloka, Japan). Per cent *B* (%*B*) and *F* (%*F*) were calculated applying the following equations: %*B* = $\frac{B}{B+F} \times 100$, and %*F* = $\frac{F}{B+F} \times 100$.

Thin-layer chromatography (Sakurada et al. 1968) was performed to examine the purity of ¹²⁵I-T₄ used (specific radioactivity 230 Ci/g, Mallinckrodt). With a trivial share of less than 1% identified each for triiodothyronine (T₃) and inorganic iodine, the purity of the ¹²⁵I-T₄ was confirmed to be satisfactory.

Direct RIA of T₄

RIA for T₄ was carried out on normal serum, without the extraction of T₄ from serum,

on one hand, and with the addition of 8-anilino-1-naphthalene-sulfonic acid (ANS) to the serum at the dose of 10, 50 and 100 mg per 100 ml of serum, on the other.

RESULTS

Effect of dilution of dextran-coated charcoal on %B

Estimations were made to identify an optimum concentration of dextran-coated charcoal for absorption of free $^{125}\text{I-T}_4$ in the incubation system. Two-fold or more dilution of dextran-coated charcoal evidently caused the decrease in the absorption of free $^{125}\text{I-T}_4$, showing an increase in %B (Fig. 1). Therefore, it was decided in the present RIA to use dextran-coated charcoal without dilution.

Working time of dextran-coated charcoal related to %F

After the mixture of antigen and antibody was incubated for 18 hr at 5°C , free and bound $^{125}\text{I-T}_4$ were separated from each other with the addition of dextran-coated charcoal. The leaving of the incubation mixture with dextran-coated charcoal at room temperature disclosed a %F plateau reached in 50 min, while at 5°C , %F remained low until after 60 min when its plateau was finally attained (Fig. 2). Thus, centrifugal separation of free and bound $^{125}\text{I-T}_4$ was decided to be performed 60 min after the addition of dextran-coated charcoal at room temperature.

Fig. 1. Effect of dilution of dextran-coated charcoal on %B. A 400-fold diluted anti- T_4 serum was employed.

Fig. 2. Effect of working time of dextran-coated charcoal on %F.

Titer of anti- T_4 rabbit serum

Immunization of rabbits with T_4 -MEH-BSA complex indicated %B as 13% in the serum diluted 100-fold three months after the start of immunization (Fig. 3). The titer curves for anti- T_4 rabbit serum were observed rising with months.

Fig. 3. T_4 -antibody titration curves obtained 3, 4 and 5 months after the initial immunization. $\bullet$ --- $\bullet$, anti- T_4 serum + ^{125}I - T_4 (25 pg); $\triangle$ — $\triangle$, normal rabbit serum + ^{125}I - T_4 (25 pg); $\circ$ — $\circ$, anti- T_4 serum + ^{125}I - T_3 (25 pg).

TABLE 1. Cross reaction between anti- T_4 serum and T_4 analogues

T_4 analogue	Relative activity	T_4 analogue	Relative activity
L- T_4	1.0	T_3 -prop	0.000011
L- T_3	0.0029	Triac	0.000029
T_4 -form	0.00050	L-MIT	0.0000006
Tetrac	0.00060	L-DIT	0.0000007
T_3 -form	0.000021		

Cross reactions between T_4 analogues and T_4

In order to identify the cross reactions between T_4 analogues and T_4 , the amount of L- T_4 causing the ^{125}I - T_4 binding rate, %B, of 50% was compared with that of each T_4 analogue showing the same effect (relative activity).

As shown in Table 1, the relative activity for L- T_3 was only 0.29% against 100% for T_4 . Further lower values were obtained for tetraiodothyroformic acid (T_4 -form), tetraiodothyroacetic acid (Tetrac), triiodothyroformic acid (T_3 -form), triiodothyropropionic acid (T_3 -prop) and triiodothyroacetic acid (Triac). Extremely lower values were identified for monoiodo-L-tyrosine (L-MIT) and diiodo-L-tyrosine (L-DIT).

Standard curves for serum T_4 obtained by the extraction and direct methods

Fig. 4 shows a T_4 standard curve obtained by the ethanol-extraction method in the anti- T_4 serum immunized for 18 months and diluted 500-fold. The values of %B estimated from the curve were 8.0% for T_4 at 50 $\mu\text{g}/100$ ml and 17.5% for T_4 at 1.0 $\mu\text{g}/100$ ml.

Standard curves of serum T_4 obtained by the direct method in T_4 -free serum are shown in Fig. 5. RIA performed for T_4 in the T_4 -free serum added with standard

Fig. 4. Effect of increasing concentrations of unlabeled T_4 and serial dilutions ($\times 1$, $\times 2$, $\times 4$ and $\times 8$) of a serum of hyperthyroid patient (serum T_4 $32 \mu\text{g}/100 \text{ ml}$) on binding of labeled T_4 to antiserum 18 months after the initial immunization. (final dilution of antiserum 1:5,000, methanol-extraction method)

Fig. 5. T_4 standard curves obtained by the direct ANS-addition method and ethanol-extraction method.

Direct method: 1, T_4 -free serum + T_4 ; 2, T_4 -free serum + T_4 + ANS (10 mg/100 ml); 3, T_4 -free serum + T_4 + ANS (50 mg/100 ml); 4, T_4 -free serum + T_4 + ANS (100 mg/100 ml).
Extraction method: 5, T_4 -free serum + T_4 .

T_4 showed a high level of %B, with the curves appearing flat. With the addition of ANS at increasing doses of 10, 50 and 100 mg/100 ml, the standard curves became closer to that for serum T_4 obtained with the ethanol-extraction method;

the difference was no longer significant in the curve with 100 mg/100 ml of ANS.

Effect of serial dilutions of serum

Serum containing 32.0 $\mu\text{g}/100\text{ ml}$ of T_4 was diluted by barbital buffer to see the effect of dilution of serum on RIA of T_4 in the methanol-extraction method. As shown in Fig. 4, the dilution curve of serum T_4 was paralleled to the standard curve ($\times 1$, 32.0; $\times 2$, 16.1; $\times 4$, 7.8; and $\times 8$, 4.3 $\mu\text{g}/100\text{ ml}$).

Recovery rate of T_4

To the normal serum with a T_4 level of 6.2 $\mu\text{g}/100\text{ ml}$ identified in the direct method, T_4 was added progressively and RIA was performed for the responding serum levels of T_4 at increasing doses of 5, 10 and 20 $\mu\text{g}/100\text{ ml}$ linked to determined T_4 levels of 12.0, 16.0 and 26.0 $\mu\text{g}/100\text{ ml}$, respectively, showing recovery rates of 113, 98 and 94%, with a mean of 102%.

Fig. 6. Correlation of T_4 values obtained by radioimmunoassay (extraction-method) and CPBA assay.

Fig. 7. Correlation of T_4 values obtained by radioimmunoassay (direct method) and CPBA assay.

Correlation between serum T_4 values obtained by RIA and those by CPBA

Figs. 6 and 7 show correlation between serum T_4 values obtained by RIA and those by CPBA (Tetrasorb-125, Dainabott). In normal subjects, the mean values of serum T_4 obtained by RIA with the extraction and direct methods were 9.7 ± 3.3 and 11.1 ± 2.1 $\mu\text{g}/100$ ml, respectively, while a value of 11.1 ± 2.1 $\mu\text{g}/100$ ml was obtained by CPBA. In cases of hyperthyroidism, the corresponding figures were 17.8 ± 2.9 and 19.1 ± 3.3 $\mu\text{g}/100$ ml, respectively, while the value obtained by CPBA was 17.9 ± 2.4 $\mu\text{g}/100$ ml. In cases of hypothyroidism, the figures were 0.6 ± 0.2 and 0.7 ± 0.7 $\mu\text{g}/100$ ml, while 1.9 ± 0.7 $\mu\text{g}/100$ ml was obtained by CPBA.

DISCUSSION

Thyroid hormones were usually identified as protein-bound iodine (PBI) (Barker et al. 1951) or in specific cases as butanol extractable iodine (Benotti and Benotti 1963). Assay for PBI, however, inevitably involves both T_4 and T_3 , and moreover, would be affected considerably by MIT, DIT and inorganic iodine in the blood. These disadvantages together with troublesome measuring procedure have made the assay for PBI inconvenient in a routine test for blood thyroid hormones.

Following the proposal of saturation analysis for measurement of serum T_4 by Ekins (1960) and the introduction of CPBA for determining serum T_4 by Murphy and Pattee (1964), various ways of measuring serum T_4 have been developed in stead of PBI assay.

As for the RIA for serum T_4 , there have been some reports: Chopra et al. (1971) using human thyroglobulin, Beckers et al. (1972) with bovine thyroglobulin, Mitsuma et al. (1972) combining T_4 with albumin, and Larson et al. (1973) with beef thyroglobulin and producing specific antibodies against T_4 . It is indisputable that the use of T_4 as an antigen is theoretically preferable in making the T_4 -antibody, because there are other iodinated amino acids in addition to T_4 in the thyroglobulin. Therefore, in the present study, following our previous result with RIA for serum T_3 (Sakurada et al. 1972), T_4 -MEH was prepared and its complex with BSA was used for immunization of rabbits.

In the lastly obtained antiserum, the titer of T_4 -antibody was high with 1:5,000 in final dilution, comparing with 1:300 reported by Chopra (1972), 1:500 by Beckers et al. (1972) and 1:1,000 by Mitsuma et al. (1972).

Cross reaction of T_4 analogues with T_4 , represented as relative activity, showed almost negligible values, ranging from the highest 0.29% for L- T_3 down to 0.00006% for L-MIT. These results indicate that T_4 analogues have little influence on RIA for serum T_4 .

As shown in Fig. 4, standard curves of T_4 obtained by the methanol-extraction method was distinctively favorable. The extraction rate of T_4 from serum, estimated by the addition of ^{125}I - T_4 , was 85 ± 4 (S.D.)%. The procedure of methanol-extraction and of drying-up of the extract can be done easily. But if the extraction procedure could be spared, RIA of T_4 would become more convenient.

In RIA for serum T_4 without a extraction, dinitrophenol (Sakurada et al. 1972), diphenylhydantoin (Sakurada et al. 1972), tetrachlorothyronine (Mitsuma et al. 1971), ANS (Chopra 1972) and salicylate (Larson 1971) are used as the binding inhibitor against T_4 with T_4 -binding globulin (TBG). A direct RIA for serum T_4 gave a high and flat curve for %B, as evident in Fig. 5, showing that RIA for serum T_4 without extraction procedure is inadequate. As reported by Chopra (1962) and Mitsuma et al. (1972), RIA for T_4 in T_4 -free serum containing ANS demonstrated that standard curves for T_4 were quite flat with 10 mg/100 ml of ANS, yet insufficient even with 50 mg/100 ml, but satisfactory with 100 mg/100 ml, fairly agreeing with the curve for T_4 obtained by the ethanol-extraction. This results indicate that an addition of 100 mg/100 ml of ANS to serum can fairly spare the process of extraction for RIA for serum T_4 .

When standard T_4 at the concentrations of, 5, 10 and 20 μ g/100 ml were added to the serum in which T_4 was 6.2 μ g/100 ml and then T_4 in the serum was estimated by RIA with the direct method, the recovery rate of T_4 from the serum was satisfactory. Furthermore dilution of serum had no influence on the serum T_4 value obtained by RIA with the methanol-extraction method.

With regard to the relationship between T_4 values obtained by RIA and those by CPBA, Larson et al. (1973) and Mitsuma et al. (1972) noted a favorable correlation between the two. Chopra (1972) observed a good correlation between the two when serum T_4 values were obtained by extraction, but noticed the inconsistency when the values were estimated by RIA directly in the serum containing ANS; the T_4 values were fairly correlated to PBI values, but tended to be higher than T_4 values obtained by CPBA. For such differences, Chopra suspected the possibility that " T_4 -like" substance as well as T_4 was determined by RIA.

In the present study, the comparison of T_4 values between those obtained by RIA with the direct method and those by CPBA showed no distinctive difference for normal cases, but indicated the values obtained by RIA to be slightly higher in the case of hyperthyroidism and lower in the case of hypothyroidism than those obtained by CPBA. The differences in these results might be attributed to the specificity of antibody, to the difference of measuring methods and to the so-called " T_4 -like" substance, though any definite explanation has not yet been given.

The serum T_4 values obtained by the ethanol-extraction method somehow lower than those by the direct method. Further experiments on this problem will be required, but there might remain the possibility that ANS could not inhibit completely the binding of T_4 with TBG.

The present method of RIA for T_4 using anti- T_4 antibody obtained by T_4 -MEH-BSA complex is clinically favorable with its simple procedure available for fast indicating accurate serum T_4 values in as small an amount of sample serum as 0.1 ml.

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