Radioimmunoassay of Triiodothyronine

TOSHIRO SAKURADA, SHINTARO SAITO, TORU YAMAGUCHI, MAKIKO YAMAMOTO, REIKO DEMURA, HIROSHI DEMURA, SOITSU FUKUCHI, KATSUMI YOSHIDA and TATSUO TORIKAI

Department of Internal Medicine, Tohoku University School of Medicine, Sendai


Recent remarkable progress in radioimmunoassay made it possible to prepare the antisera against low-molecular-weight non-protein materials by conjugation of them to proteins or peptide carriers (Oliver et al. 1968; Abraham 1969; Mayes et al. 1970). Specific antibodies against triiodothyronine (T₃) have been reported in some papers (Brown et al. 1970; Gharib et al. 1970; Ekins et al. 1970; Chopra et al. 1971; Mitsuma et al. 1971; Lieblich and Utiger 1972; Larsen 1972).

In the present study, highly specific antiserum against T₃ has been prepared in rabbits with T₃-methyl ester hydrochloride (T₃-MEH) conjugated to bovine serum albumin (BSA), and a clinically useful method has been investigated for the radioimmunoassay of T₃.

MATERIALS AND METHODS

1) Preparation of T₃-MEH

T₃-MEH was prepared by a modified method of Ashley and Harington (1928). Three hundreds mg of l-T₃ (Sigma Co.) purified by the method of Williams et al. (1969) were covered with 20 ml of methanol and a dry distillation was carried out for 3 hrs being saturated with a vigorous stream of dry hydrogen chloride. The solution was then concentrated by distillation under a reduced pressure. The crystalline precipitate was filtered off, washed with ethanol and ether, and dried. The reaction products were estimated by the infrared spectrophotometry by KBr method.

2) Preparation of antigens

T₃-MEH-poly-1-lysine complex was prepared by the method of Brown et al. (1970). T₃-MEH-BSA complex was prepared using a slightly modified method of Oliver et al. (1968)

Received for publication, November 13, 1972.

329
as follows: 50 mg of BSA were dissolved in 25 ml of water at pH 5.5. After the solution was filtered, 30 mg of EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride] and 20 mg of T3 MEH dissolved in 2 ml of dimethylformamide were added dropwise while stirring at pH 5.5. Ten min later, 10 mg of EDC were added. The solution was stirred at 5°C in the dark for 24 hours. The reaction mixture was dialysed for two days against redistilled water. The suspension was centrifuged and the soluble protein was lyophilized. Ten mg of T3-MEH-BSA complex were dissolved in 5 ml of 0.1 M NaOH and estimated by the ultraviolet spectrophotometry.

3) Immunological procedure

T3 MEH-poly-l-lysine complex (750 μg) or T3 MEH-BSA complex (3.2 mg) was dissolved in 0.8 ml of sterile physiological saline solution. They were mixed with aliquot volume of complete Freund's adjuvant and the mixtures were injected into toe-pads of rabbits (0.4 ml/toe-pad). Rabbits were boosted with 750 μg of T3 MEH-poly-l-lysine complex or 1 mg of T3 MEH-BSA complex in complete Freund's adjuvant once a month for one year. Ten days after the last injection blood was withdrawn from the treated rabbits.

4) Extraction of T3 from serum

After adding 2.0 ml of methanol to 0.1 ml of serum in a small test tube, it was shaken by volume mixer for 5 sec, then left standstill for 15 min at room temperature. After further shaking for 5 sec, the tube was centrifuged at 3,000 r.p.m. at 5°C for 15 min. Supernatant was dried with a light stream of nitrogen. To dissolve the residue at the bottom of the test tube, 1 ml of barbital buffer (0.05 M, pH 8.6) containing 0.5% BSA was added.

5) Assay procedure

a) T3 extracted by ethanol from 0.1 ml of serum was dissolved in 1.0 ml of barbital diluent.

b) 0.01 ml of dinitrophenol (DNP, dissolved in water-ethanol (1:1, pH 8.0) in 1.2 × 10⁻³ M in serum) or diphenylhydantoin (DPH, dissolved in the water (pH 12) containing 40% propylene glycol and 10% ethanol in 5.8 × 10⁻³ M in serum) was added to 0.1 ml of crude serum diluted with 0.89 ml of barbital diluent.

To each assay system in a) and b), 0.1 ml of ¹²⁵I-T3 (less than 25 pg as T3, specific activity 70-450 μCi/μg, Abbott Co. or Mallinckrodt Co.) in barbital diluent and 0.1 ml of antiserum diluted with barbital diluent (1:1,000-50,000) were added. This gave total volume of 1.2 ml in each assay system. The mixtures were incubated at 5°C for 18 hrs. Then 0.2 ml of dextran-coated charcoal (charcoal 5.0 g and dextran 0.5 g in 400 ml of water) was added to them. One hr after light shaking at room temperature for 5 min, the mixtures were centrifuged at 3,000 r.p.m. for 15 min at 5°C, then radioactivities of the supernatant and the precipitate were counted in the well-typed scintillation counter (Aloka, Japan). Percentages of bound ¹²⁵I-T3 were calculated.

RESULTS

Spectrophotometry of the T3-MEH and the T3-MEH-BSA complex.

Absorption of carbonyl ester observed at 1,735 cm⁻¹ in the infrared spectrophotometry made it clear that T3-MEH was produced by acid-methanol method (Ashley and Harington 1928) (Fig. 1).

As shown in Fig. 2, when the reaction products obtained by EDC method (Oliver et al. 1968) were estimated by the ultraviolet spectrophotometry, the absorption maxima of T3-MEH-BSA complex were observed at 290 and 320 nm,
Radioimmunoassay of Triiodothyronine

Fig. 1. Infrared spectrum of T3-methyl ester hydrochloride.

Fig. 2. Ultraviolet spectra of T3-MEH-BSA complex and BSA.

1. T3-methylester hydrochloride (T3-MEH)
2. T3-MEH against BSA blank
3. T3-MEH-BSA complex
4. BSA
5. T4-MEH
BSA alone at 290 nm, T₃-MEH alone at 320 nm and T₃-MEH-BSA complex against BSA blank at 320 nm. Absorption maximum of thyroxine methyl-ester hydrochloride prepared with the same method as T₃-MEH was observed at 324 nm. These results showed that T₃-MEH-BSA complex was prepared.

Titer of antiserum (Fig. 3)

When T₃-MEH-poly-l-lysine complex was employed as an antigen to produce antibody in rabbits, the titer of antiserum obtained 6 months later was very low. Serial dilutions of anti-T₃ serum, which was obtained from rabbit being immunized with T₃-MEH-BSA complex, showed progressively decreased binding of ¹²⁵I-T₃. Neither ¹²⁵I-T₄ and anti-T₃ serum nor ¹²⁵I-T₂ and normal rabbit serum showed significant binding (Fig. 3).

Fig. 3. T₃-antibody titration curve.

- - - - - 25 pg ¹²⁵I-T₃+anti-T₃ serum
- - - - 25 pg ¹²⁵I-T₄+anti-T₃ serum
- - - - - 25 pg ¹²⁵I-T₃+normal rabbit serum
TABLE 1. Cross reaction between anti-T₃ serum and T₃ analogue

<table>
<thead>
<tr>
<th>T₃ analogue</th>
<th>Relative activity</th>
<th>T₃ analogue</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>l-T₃</td>
<td>1.00</td>
<td>Tetrac.</td>
<td>0.0013</td>
</tr>
<tr>
<td>l-T₄</td>
<td>0.0021</td>
<td>T₄ form.</td>
<td>0.0014</td>
</tr>
<tr>
<td>T₃ form</td>
<td>0.066</td>
<td>l-MIT</td>
<td>0.000670</td>
</tr>
<tr>
<td>T₃ prop.</td>
<td>0.081</td>
<td>l-DIT</td>
<td>0.000059</td>
</tr>
<tr>
<td>Triac.</td>
<td>0.061</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Analogue studies (Table 1)

Relative activities of T₃ analogues were assessed by determining the amount of analogue required to cause a 50% decrease in the binding of ¹²⁵I-T₃ and comparing to the amount of l-T₃ required to produce the same decrease in binding of ¹²⁵I-T₃. As shown in Table 1, relative activities of triiodothyroformic acid, triiodothyropropionic acid and triiodothyroacetic acid were considerably remarkable. But the relative activity of l-T₄ was only 0.0021, and those of l-MIT and l-DIT were negligible.

Standard dose response curve of T₃ obtained by DNP- or DPH-addition method (Figs. 4 and 5)

When l-T₃ dissolved in ethanol-2N ammonia water (9:1) was assayed in the present assay system, a good standard curve was obtained as shown in Fig. 4. But by the addition of serum of cretinism in which no T₃ was supposed to be

Fig. 4. Effect of serum on the T₃ standard curve.
1. Serum of cretinism
2. Cohn fraction IV-9
3. Serum of TBG deficient patient
4. T₃ standard curve
Fig. 5. Effect of serum, DNP and DPH on the T₃ standard curve.
1. T₃ standard curve
2. 1+DNP
3. 1+DPH Serum of a patient with cretinism
4. 1+DPH
5. 1+DNP Serum of a patient with cretinism
6. 1+Serum of a patient with cretinism

present, the dose response curve tended to be low and flat.

When 0.1 ml of the serum of a thyroxine-binding globulin (TBG) deficient patient (T₄-binding capacity was null) was added to the present assay system, a curve of percentage of bound ¹²⁵I-T₃ which was similar to the standard curve of T₃ was obtained. When 0.1 ml of solution of Cohn fraction IV-9 (dissolved in 20 mg/100 ml in barbital diluent), known to be rich in TBG (Tata 1961), was added to the present assay system, the value of percentage of bound ¹²⁵I-T₃ tended to become low.

When the dose of 1.2×10⁻³ M of DNP (Wolff et al. 1961) or 5.8×10⁻³ M of DPH (Oppenheimer et al. 1961) which is known to be a binding-inhibitor of T₄ to thyroxine-binding protein (TBP) was mixed with the serum of cretinism, a considerably steep curve was obtained in percentages of bound ¹²⁵I-T₃ (Fig. 5).

When 1.2×10⁻³ M of DNP was added to 0.1 ml of serum of hyperthyroid patient to investigate the change of elutable fraction of T₃ (Sakurada et al. 1969) (per cent of free T₃) by micro-column of Sephadex G-25, the value increased from 6.4×10⁻¹ to 85.4×10⁻¹ per cent.
Radioimmunoassay of Triiodothyronine

Fig. 6. Effects of increasing concentration of unlabeled T₃ and of serial dilutions (×1, ×2, ×4, ×8 and ×16) of a serum of hyperthyroid patient (serum T₃ 1,600 ng/100 ml) on binding of labeled T₃ to antiserum (final dilution of antiserum 1:600,000, methanol-extraction method).

Standard dose response curve of T₃ obtained by the methanol-extraction method (Fig. 6).

The extraction rate of T₃ from serum by methanol was 85±2%.

Fig. 6 showed the standard dose response curve of T₃ which was added to the serum of cretinism and then extracted by the methanol-extraction method.

The dilution curve of serum T₃ of hyperthyroid patient was parallel to the standard curve (×1; 1600, ×2; 750, ×4; 420, ×8; 210 and ×16; 110 ng/100 ml).

In vitro addition of T₄ in the dose of 4,000 to 20,000 ng/100 ml to the normal serum (serum T₄ 10.4 μg/100 ml) had no influence on estimated T₃ concentration (Table 2).

<table>
<thead>
<tr>
<th>Added T₄ (ng/100 ml)</th>
<th>Estimated T₃ (ng/100 ml)</th>
<th>Added T₄ (ng/100 ml)</th>
<th>Estimated T₃ (ng/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>129</td>
<td>12,000</td>
<td>126</td>
</tr>
<tr>
<td>4,000</td>
<td>128</td>
<td>18,000</td>
<td>129</td>
</tr>
<tr>
<td>8,000</td>
<td>132</td>
<td>20,000</td>
<td>124</td>
</tr>
</tbody>
</table>
Fig. 7. Correlation between serum T₃ values obtained by methanol-extraction method and those by DPH-addition method.

Correlation coefficient between serum T₃ values obtained by the methanol extraction method and those obtained by the DPH addition method was 0.97 (Fig. 7).

Correlation between serum T₃ values obtained by the methanol extraction method and those obtained by the DPH addition method (Fig. 7).

Mean serum T₃ values in the methanol extraction method and those in PDH...
Radioimmunoassay of Triiodothyronine

Fig. 9. Effect of replacement therapy on the serum T₃, BMR and plasma TSH in a case of hypothyroidism.

Fig. 10. Effect of replacement therapy on the serum T₃, BMR and plasma TSH in a case of hypothyroidism.

addition method in euthyroid, hyperthyroid and hypothyroid patients were 111±19 and 126±48, 508±220 and 440±180, and 46±15 and 41±24 (SD) ng/100 ml, respectively.

Recovery rates obtained by the addition of 100 ng/100 ml of l-T₃ to the serum containing 126 ng/100 ml of T₃ were 98.5±6.4 (SD) in the methanol-extraction method and 96.7±7.1% in the DPH-addition method.

The change of serum T₃ in a hyperthyroid patient during the treatment with 1-methyl-mercaptoimidazole (Mercazol) (Fig. 8)

In the daily administration of 30 mg of Mercazol to a hyperthyroid patient, whose serum T₄ value was 24.6 μg/100 ml and serum T₃ value was 260 ng/100 ml, the levels of serum T₄ and T₃ became 16.0 μg/100 ml and 200 ng/100 ml respectively one week later. The levels of serum T₄ and T₃ became normal accompanying the normalization of BMR one month later.

The changes of serum T₃ and plasma TSH value in hypothyroid patients treated with thyroid hormones (Figs. 9 and 10)

One week after the administration of 25 μg of l-T₃ to a hypothyroid patient, whose values of serum T₄ and T₃ were 3.3 μg/100 ml and 49 ng/100 ml, respectively
and plasma TSH (Demura et al. 1969) was 51 μU/ml, serum T₃ increased to 282 ng/100 ml and plasma TSH was inclined to decrease. When the daily dose of l-T₃ was increased to 100 μg, plasma TSH was suppressed completely and serum T₃ value was elevated to 385 ng/dl (Fig. 9).

When 25 to 100 μg of l-T₃ were administered daily to another hypothyroid patient, serum T₃ increased gradually as plasma TSH decreased and BMR elevated (Fig. 10).

After the daily administration of 200 to 300 μg of l-T₄ to those patients, serum T₄ values were moderately higher than pretreatment values.

**DISCUSSION**

By means of the paper, column, thin-layer and gas chromatographies, several normal serum T₃ values were obtained such as 630±130 (SD) (Sakurada et al. 1969), 450±40 (Hollander 1968), 330±70 (Nauman et al. 1967) and 220±27 ng/100 ml (Sterling et al. 1969). In these methods, it is said that there remain some problems on the conversion of T₄ to T₃, the accuracy of the separation of T₄ and T₃, and the appearance of derivatives of them (Oddie et al. 1971). Dussault et al. (1971) reported that normal serum value was 98±48 ng/100 ml by the double column chromatography.

In succession to the paper of Brown et al. (1970), there have been some reports on the radioimmunoassay for T₃. Normal serum T₃ values described in these reports were 218±55 (SD) (Gharib et al. 1971), 147±27 (Lieblich and Utiger 1972), 139±23 (Mitsuma et al. 1971), 120 (Ekins et al. 1970), 110±25 (Larsen 1972) and less than 100 ng/100 ml (Chopra et al. 1971). Normal T₃ values obtained by the present study were 111±19 (SD) and 126±48 ng/100 ml.

In the present paper, T₃-MEH was combined with poly-l-lysine or BSA. In the former only a slight elevation of titer of antibody was observed, while in the latter a high titer of antibody was obtained one year after the immunization. Chopra et al. (1971) reported that thyroglobulin was the most suitable antigen for preparing the antibody against T₃.

In the radioimmunoassay for T₃, the cross reaction between anti-T₃ serum and l-T₄ should be minimum. The relative activity of l-T₄ in the present study was 0.0021. This value is comparable to previous ones; for example, they were less than 0.0002 (Mitsuma et al. 1971), 0.0036 (Gharib et al. 1971), 0.0014–0.0134 (Lieblich and Utiger 1972), 0.013 (Chopra et al. 1971) and 0.02–0.05 (Brown et al. 1970).

T₃ value of the serum, after in vitro addition of 20,000 ng/100 ml of l-T₄, was almost equal to the one of untreated serum.

As shown in Table 1, T₃ analogues had considerably high relative activity, but none of them are known to exist in the normal human serum. The relative activity of both l-MIT and l-DIT was negligibly low.

The addition of crude serum to the present assay system caused a suppressed flat dose response curve of percentage of bound ¹²⁵I-T₃ as mentioned by Mitsuma et
Radioimmunoassay of Triiodothyronine

As the results of study with the serum of TBG-deficient patient and Cohn fraction IV-9, TBG was proved to be concerned with this effect. DPH (Lieblich and Utiger 1972), tetrachlorothyronine (Mitsuma et al. 1971) and salicylate (Larsen 1972) have been employed to inhibit the binding of T3 to serum protein. In the present study, DNP as well as DPH gave good result for this purpose. It is interesting that DNP is the inhibitor of binding of T4 to TBPA (Wolff et al. 1961) to which no T3 is proved to bind (Tata et al. 1961). A remarkable increase in the elutable fraction of T3 after the addition of DNP indicated the increase in free T3 value. These results might show that TBP (Miyai et al. 1968) including TBG inhibits the immunoreaction.

Elevated plasma TSH value in patients with hypothyroidism was completely suppressed by the administration of 100 μg/day of l-T3.

The serum T3 value was elevated remarkably at an earlier stage of administration in one case, while no increase in serum T4 value was observed.

As shown in Figs. 9 and 10, moderately elevated T3 values were obtained in hypothyroid patients receiving replacement therapy with l-T4. This might be due to peripheral conversion of administered T4 to T3 as reported by Braverman et al. (1970).

References


