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RADIOIMMUNOASSAY FOR TRIIODOTHYRONINE

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Abstract

A sensitive and precise radioimmunoassay for measurement of triiodothyronine (T_3) in unextracted human serum has been developed by using highly specific antiserum for T_3 prepared by immunization of rabbits with T_3-human serum albumin conjugate. 8-anilino-1-naphthalene sulfonic acid has been employed in order to inhibit binding of T_3 to thyroxine binding globulin. T_3-free serum as diluent for standard line has been prepared by incubating pooled human serum with charcoal.

Different dose response lines were obtained by utilizing different preparations of charcoal treated human sera in standard portion of the assay. Two different normal ranges of serum T_3 levels of 116.7 ± 17.9 (SD) and 133.6 ± 16.6 (SD) ng/100 ml were obtained by two different charcoal treated human sera, utilizing the same samples from 50 normal subjects. Thus it is necessary to determine the normal ranges of the assay for each charcoal treated human serum.

Serum T_3 levels in 100 normal subjects ranged from 72 ng/100 ml to 158 ng/100 ml with a mean value of 111.0 ± 19.1 ng/100 ml. In 31 hyperthyroid patients serum T_3 ranged from 230 ng/100 ml to more than 800 ng/100 ml. In 15 hypothyroid patients serum T_3 ranged between 12 ng/100 ml and 63 ng/100 ml. Euthyroid subjects accompanying with hypoproteinemia showed low values.

INTRODUCTION

Various methods for the measurement of serum Triiodothyronine (T_3) have been reported, however, all of these methods were complicated and serum levels of T_3 were overestimated because of in vitro deiodination of thyroxine (T_4) to T_3[1,2]. Radioimmunoassay of T_3 was first reported in 1970 by Brown et al.[3] and thereafter several methods have been

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described by many investigators. Precise determination of T_3 in unextracted serum have become possible by adding various compounds such as thyroxine[4], tetrachlorthyronine[5,6], sodium salicylate[7,8], diphenylhydantoin [9,10], dinitrophenol[10] and 8-anilino-1-naphthalene sulfonic acid (ANS)[11] to the assay system in order to inhibit binding of T_3 to thyroxine binding globulin (TBG) or by thermal inactivation of TBG[12]. Although all of these radioimmunoassay methods of T_3 were based on the same principle, reported normal ranges of serum T_3 levels differed each other considerably.

We also have produced highly specific antiserum against T_3 by immunization of rabbits with T_3-human serum albumin conjugate and developed the sensitive and precise radioimmunoassay method for direct measurement of serum T_3. This report describes the several experiments designed to test the validity of the assay method.

MATERIALS AND METHODS

L-triiodothyronine (free acid), L-thyroxine (free acid), tetraiodothyroacetic acid, triiodothyroacetic acid, triiodothyropropionic acid and 3,5-diiodothyropropionic acid were obtained from Sigma Chemical Co. (St. Louis, U.S.A.). 3,5-diiodothyronine, 3,5-diiodotyrosine and 3-monoiodotyrosine were gifts of Teikoku Zoki Pharm. Co. (Kawasaki, Japan). Human serum albumin was purchased from Midori Juji Co. (Tokyo, Japan). Radioiodinated T_3 (^{125}I-T_3) with high specific activity was obtained from Dainabot Radioisotope Lab., LTD. (Tokyo, Japan).

T_3-human serum albumin conjugate were prepared according to Gharib et al.[13,14]. Rabbits were immunized with one mg of the above conjugate and equal volume of Freund's complete adjuvant every four weeks. Antiserum to T_3 was obtained from 10 days after the last immunization.

T_3-free serum was prepared by methods of Larsen[7] and Mitsuma et al.[11] with minor modifications. Twenty grams of charcoal (Norit A, Sigma Chemical Co.) were added per 100 ml of pooled human serum and incubated for 24 hours at 4°C. Then the mixture was centrifuged three times at 15,000 g and supernatant was collected. Supernatant was divided into small vials and kept frozen until use.

The assay procedure was showed in Table 1. Each tube contained 1.0 ml of borate buffer (0.1 M, pH 8.6) containing 0.1 per cent BSA and 0.03 per cent ANS (Eastman Kodak Co., New York, U.S.A.). For standard portion of the assay 0.9 ml of the above buffer and 0.1 ml of standard solution diluted with the above buffer were employed. Other dilutions

TABLE 1.

Procedure for Immunoassay of T_3 in Human Serum

Sample	0.1 ml	Standard Solution*	0.1 ml
Borate Buffer (0.1 M, pH 8.6 0.1 % BSA, 0.03 % ANS)	1.0 ml	Borate Buffer	0.9 ml
		Charcoal Treated Human Serum	0.1 ml

↓

^{125}I-T_3 Solution**	0.1 ml
Anti-T_3 Serum (1:2,200)**	0.1 ml

↓ Incubation for 20 hr at 4°C

0.2 % Dextran Coated 2 % Charcoal ***	0.2 ml

↓ Incubation for 20 min at 4°C

↓ Centrifuge for 15 min at 3,000 rpm

↓ Aspirate Supernatant

↓ Count Precipitate

* Diluted with borate buffer (0.1 M, pH 8.6) containing 0.1 % BSA and 0.03 % ANS.
** Diluted with borate buffer (0.1 M, pH 8.6) containing 0.1 % BSA.
*** Suspended with borate buffer (0.1 M, pH 8.6) containing 0.1 % BSA.

were made with 0.1 per cent BSA in 0.1 M borate buffer. A 2 per cent of charcoal (Norit A) suspension with 0.2 per cent dextran (T-70, Pharmacia Fine Chemical Co., Uppsala, Sweden) was prepared in 0.1 M borate buffer (pH 8.6) at room temperature. After incubation for 20 hours at 4°C, 0.2 ml of the charcoal suspension was added to each incubation tube under constant stirring at room temperature and stood 20 minutes at 4°C. Following centrifugation for 15 minutes at 4°C the supernatant was aspirated and precipitate was counted in an automatic gamma counter (Model AL-201, Dainabot Radioisotope Lab., LTD., Tokyo, Japan).

L-thyroxine was purified by gel filtration on the column (Sepadex LH-20, 1.1×60 cm) according to Williams et al.[15].

Serum T_3 concentration were measured in 100 normal subjects, untreated 31 hyperthyroid and 15 hypothyroid patients and euthyroid subjects accompanying with hypoproteinemia caused by various non-

endocrine diseases. Normal subjects had no abnormalities in routine laboratory examinations. Hyperthyroid and hypothyroid patients had typical clinical manifestations and their diangoses were confirmed by other thyroid function tests. Elevated serum TSH levels were noted in all of hypothyroid subjects by the radioimmunoassay of TSH. Details of the radioimmunoassay of HTSH were previously described elsewhere[16].

RESULTS

A typical dose response line showed straight line from 37.5 to 800 pg of T_3 on a semilogarithmic scale (Figure 1).

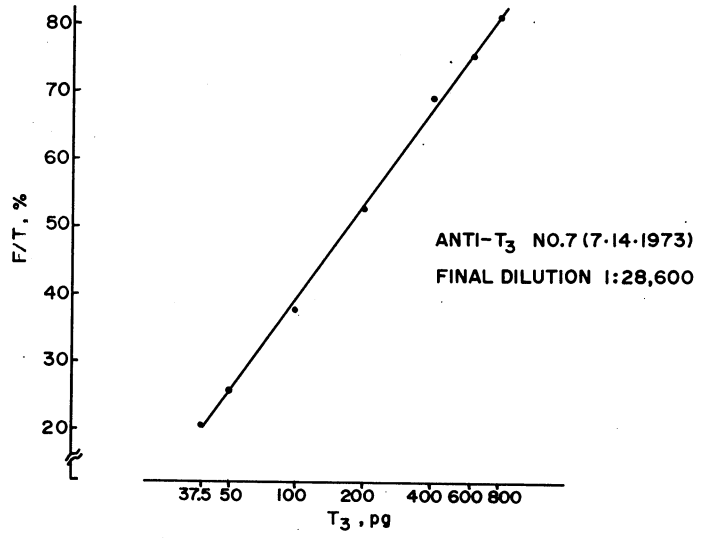

Fig. 1. A typical dose response line. A straight dose response line was obtained from 37.5 pg to 800 pg of T_3 on a semilogarithmic scale.

Crossreactivities with various L-triiodothyronine and L-thyroxine analogues were shown in Table 2. Significant crossreaction with commercial T_4 was noted, but this crossreaction with T_4 became negligible after purification of T_4 by gel filtration (LH-20). Triiodothyroacetic acid and triiodothyropropinic acid showed considerable crossreactions. No crossreactions were observed with monoiodotyrosine and diiodotyrosine upto the concentration of 50 μg/100 ml.

Effects of ANS on euthyroid and pregnant sera were shown in Figure 2. Maximum effects of ANS were observed at the concentration of more than 200 μg/0.1 ml of serum to inhibit binding of T_3 to TBG under the

TABLE 2.
Crossreactivity with Thyroid Hormone Derivatives

Compounds	Crossreactivity, %
L-Thyroxine (Sigma)	0.32
L-Thyroxine (purified)*	0.012
Tetraiodothyroacetic acid	1.38
Triiodothyroacetic acid	50.4
Triiodothyropropionic acid	32.0
3,5-Diiodothyropropionic acid	0.25
3,5-Diiodothyronine	1.48
3,5-Diiodotyrosine	0
3-Monoiodotyrosine	0

* Purified by gel filtration on the column (Sephadex LH-20).

described conditions. Constant amount of labeled hormone and diluted antiserum was incubated for 20 hours at 4°C without TBG or serum sample against increasing amount of ANS. Free and antibody bound labeled hormone was separated by charcoal method (charcoal 4 mg). Effects of ANS on the binding of the hormone to antibody was estimated. An increased charcoal adsorbed radioactivity was noted at larger quantities of ANS (more than 400 μg/tube). Therefore 300 μg/tube of ANS was employed in the following examinations.

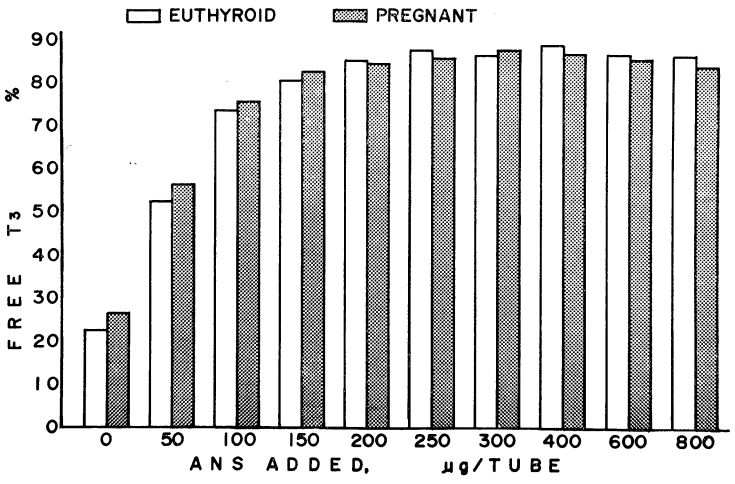

Fig. 2. The effects of ANS on binding of ^{125}I-T_3 to TBG. A maximum inhibiting effects of ANS were noted at the concentration of 200 μg per 0.1 ml of euthyroid or pregnant serum.

The reproducibility of the assay was satisfactory (Table 3). Average coefficient of variances of within assay and between assays were 3.5 and 7.4 per cent respectively. These coefficient of variances were appeared to be increased slightly at lower and higher T_3 concentrations.

TABLE 3.
Precision and Reproducibility of the T_3 Immunoassay

Sample	T_3		CV
	ng/100 ml		%
Within assay (N=6)			
A	61		2.0
B	122		3.1
C	418		5.5
		average	3.5
Between assays (3 different occasions)			
D	33		7.1
E	66		10.1
F~J (5 samples)	98~122		5.1
K	573		7.3
		average	6.3

The mean recovery was 104 per cent in quadriplicate when 100 pg of T_3 was added to a serum (144 ng/100 ml) and 99 per cent when 247 pg was added to the same sample.

Several dilutions of hyperthyroid serum were made with charcoal treated human sera and a linear line which was superimposed on a dose response line on a semilogarithmic scale was obtained (Figure 3).

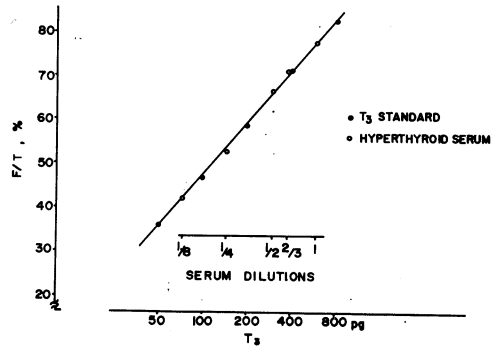

Fig. 3. Dilution test of a hyperthyroid serum. Several dilutions of hyperthyroid serum were made with charcoal treated human serum and assayed for T_3. The estimated T_3 concentration in this hyperthyroid serum was 588 ng/100 ml. ○: represent mean of duplicate determinations of dilution test.

Five different dose response lines were obtained by utilizing five different charcoal treated human sera as diluent for standard portion of the assay (Figure 4). Each charcoal treated human serum was prepared in a similar manner described above and less than 1 per cent of tracer ^{125}I-T_3 added to pooled human serum before incubating with charcoal remained in each charcoal treated human serum. Two normal T_3 ranges of 116.7±17.9 (SD) and 133.6±16.6 (SD) ng/100 ml were obtained by using two different charcoal treated human sera as diluent for standard portion when the same serum samples from 50 normal subjects were assayed.

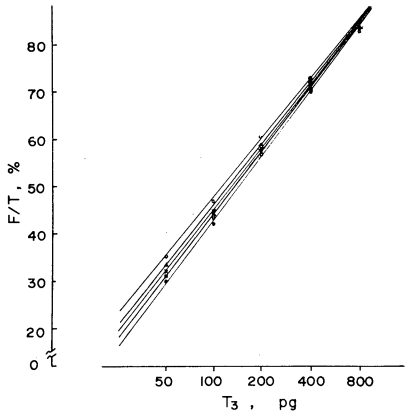

Fig. 4. The effects of charcoal treated human serum on dose response lines. Although each charcoal treated human serum was prepared in a similar manner, five different dose response lines were obtained. Protein concentrations and electrolyte contents of these charcoal treated human sera were similar.

Serum T_3 levels of 100 euthyroid, 31 hyperthyroid and 15 hypothyroid subjects assayed with the same charcoal treated human serum were illustrated in Fig. 5. In 100 euthyroid subjects T_3 values ranged from 72 to 158 ng/100 ml, with a mean value of 111.0±19.1 ng/100 ml. In 31 hyperthyroid patients the estimated T_3 levels ranged from 230 ng/100 ml to more than 800 ng/100 ml. In 15 hypothyroid patients serum T_3 values ranged between 12 and 63 ng/100 ml. There was no overlap among these three groups. Generally, T_3 values obtained by this methed correlated with other thyroid function tests. But in euthyroid subjects accompanying with hypoproteinemia the assayed T_3 values were low whatever condition caused hypoproteinemia, and the T_3 values were considerably proportional to the degree of hypoproteinemia (Figure 6).

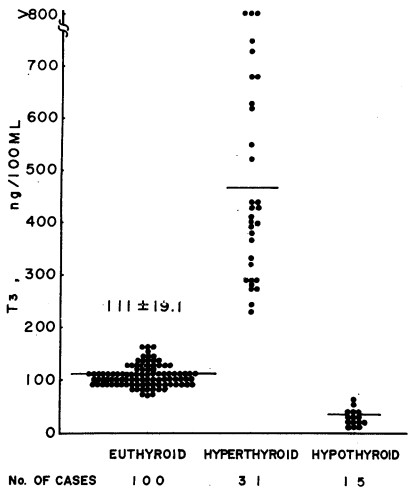

Fig. 5. Serum T_3 concentrations in euthyroid, hyperthyroid and hypothyroid subjects. Mean values are indicated by horizontal solid lines.

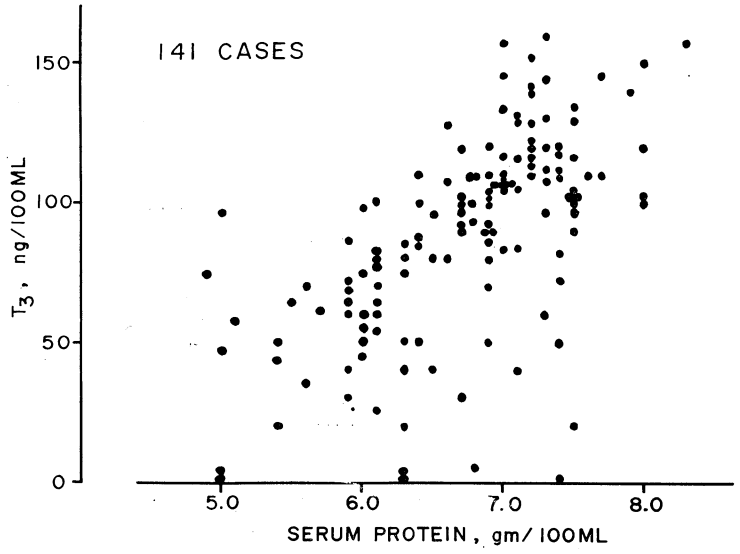

Fig. 6. Serum protein concentrations and serum T_3 levels. Low serum T_3 levels were observed in hypoproteinemic subjects.

DISCUSSION

Althouth slight crossreaction (0.012 per cent) with L-T_4 was observed in the assay system after gel filtration on the Sephadex column (LH-20),

this was negligible for clinical use as already stated by others[9,12]. The nature of this crossreaction was not verified yet, however, the presence of small amounts of T_3 converted from T_4 during the fractionation or slight intrinsic crossreactivity of T_4 might be responsible for this reaction[9]. Triiodothyroacetic acid and triiodothyropropionic acid showed considerable crossreactions in the assay sytem. But these crossreactions are not significant in clinical use, because these substances are thought to be not present in human serum.[6,10]

In the measurement of T_3 in unextracted human serum, various regents such as thyroxine[4], tetrachlorthyronine[5,6], sodium salicylate[7,8], diphenylhydantoin[9,10], dinitrophenol[10] and ANS[11] have been used in order to inhibit the binding of T_3 to TBG. ANS have been employed in the assay and our data revealed that ANS was most effective at the concentration of more than 200 μg/0.1 ml of serum to inhibit the binding of T_3 to TBG. This amount of ANS was slightly larger than that reported by others[11], who described that 150 μg of ANS/0.1 ml of serum was enough to inhibiting the binding of T_3 to TBG. Although exact mechanism of this differences was not understood, this might be due to the specificity of the assay system as stated by others[17]. On the other hand, in TBG-free system much ^{125}I-T_3 remained unbound with anti-T_3 serum when ANS was added in larger quantities. As regards this phenomenon two possibilities were considered. First, ANS crossreacted with anti-T_3 serum, second, ANS inhibited the binding of ^{125}I-T_3 to anti-T_3 serum. The former is unlikely, because the amount of the unbound ^{125}I-T_3 did not changed as the concentration of ANS was increased. Although the precise mechanism of ANS on the reaction between T_3 and anti-T_3 serum remained to be verified at the moment, the latter cause appeared to be more likely as stated by others[17]. In order to eliminate the effect described above, the amount of ANS to be added should be lower than 400 μg/0.1 ml of serum.

The T_3 values observed in normal, hyperthyroid and hypothyroid subjects are in agreement with those reported by other investigators[5,6,7,9,10,11,18], although slightly different values were obtained by utilizing different charcoal treated human sera in our T_3 assay. Values are somewhat higher than those reported by Chopra et al.[4] and lower than those described by Gharib et al.[14] and Sterling et al.[12]. As described by other investigators[6,18], there was no overlap among normal, hyperthyroid and hypothyroid subjects.

Charcoal treated human sera was used in the standard portion of the assay to obtain the same protein concentration in the assay tube. Five

different charcoal treated human sera were prepared and five different dose response lines were obtained. When the same serum samples from euthyroid subjects were run in the assay against two different charcoal treated human sera, two different normal ranges of T_3 value were obtained. As described above, normal ranges of reported T_3 radioimmunoassays differed each other considerably. Differences in T_3 free human sera may explain this phenomenon. Thus it is necessary to determine the normal ranges of the assay for each charcoal treated human serum.

Moreover, patients accompanying with hypoproteinemia showed low T_3 values, although they were in euthyroid state. It was thought that these patients had a small amounts of T_3 bound to TBG caused by TBG deficiency and so measured total T_3 levels were low in spite of normal amount of free T_3. The exact nature of this phenomenon remained to be verified.

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