A SIMPLE AND RAPID RADIOIMMUNOASSAY OF TRIIODOTHYRONINE IN UNEXTRACTED SERUM

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A simple, accurate, and rapid procedure for radioimmunoassay (RIA) of triiodothyronine (T_3) is described. Serum is denatured by sodium trichloroacetate and the T_3 released is allowed to react with ^{125}I-T_3-labeled T_3 antiserum. The displaced ^{125}I-T_3 is rapidly taken up by an anionic-resin sponge, and this uptake is linearly related to serum T_3 concentration. The entire assay procedure was completed in the same tube in about 1 hr. The T_3 antibody was specific, and T_4-to-T_3 conversion in the assay did not occur. Recovery of exogenously added T_3 was virtually complete. The mean T_3 concentration in 92 euthyroid subjects (142 ng/100 ml) is comparable to the values described in a number of radioimmunoassays for T_3.

The usefulness of triiodothyronine (T_3) along with thyroxine (T_4) measurement for proper clinical assessment of thyroid function and for other physiologic and metabolic thyroid studies has underscored the significance of serum triiodothyronine determination in man (*1*). Determination of T_3 by radioimmunoassay (T_3-RIA) is the only practical way, as the alternative procedures (gas chromatography, saturation analysis after separating T_3 and T_4 by chromatography, etc.) are too involved for routine use and might even be misleading. Hence, new T_3-RIA procedures have been continually appearing (*2–15*). Many of these T_3-RIAs, however, entail time-consuming and cumbersome details; in addition, to eliminate interference from thyroxine-binding globulin (TBG), blocking agents must be employed and such an approach may not be altogether satisfactory (*12,16*).

We recently described a simple and rapid procedure for T_4-RIA that did not employ TBG-blocking agents; instead, sodium trichloroacetate was used to denature serum and extract T_4, and anionic-resin sponges permitted rapid separation of bound and unbound hormone (*17*). Substituting T_3 antiserum for T_4 antiserum in essentially the same assay system affords an equally simple T_3-RIA that can be completed in the same tube in about 1 hr.

MATERIALS AND METHODS

High-specific-activity ^{125}I-T_3 (590 μCi/μg) was obtained from Abbott Laboratories (North Chicago, Ill.). No significant decomposition was observed within 2 weeks (by chromatography). The procedure used to conjugate T_3 with bovine serum albumin is similar to that described in detail by Oliver et al (*18*): 25 mg of T_3 conjugate (0.5 ml), emulsified in an equal volume of Freund's adjuvant, is injected intramuscularly in rabbits weekly for 3 weeks, and T_3 antisera are harvested 6 weeks after primary immunization. Antiserum was premixed with ^{125}I-T_3 (5 μg/100 ml), and after overnight equilibration the mixture was diluted 2000-fold (final dilution 3000) as determined from preliminary trials. Labeled antiserum (i.e., premixed with tracer amounts of ^{125}I-T_3) proved stable for 2 weeks (period tested), as shown by the superimposability of standard curves. Thyronine-free plasma was prepared as described previously (*17*). For preparing T_3 standards, T_3 free acid (Sigma Chemical Co., St. Louis, Mo.) was dried overnight at 40°C, and appropriate amounts in 0.25% albumin solution were then added to thyronine-free plasma to provide final T_3 concentrations of 25, 50, 100, 200, 400, 600, and 800 ng/100 ml. Then TCA–NaOH (sodium trichloroacetate diluted with enough excess NaOH to reach pH 12) was prepared by adding three parts of 0.5 *N* NaOH to one part 20% trichloroacetic acid.

Received Aug. 7, 1975; revision accepted Nov. 4, 1975.

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T_3-RIA. The principle of the technique consists in denaturing serum T_3 binding sites by TCA–NaOH; T_3 liberated from the native protein-binding sites then reacts with ^{125}I-T_3-labeled antibody and displaces ^{125}I-T_3, which is taken up by the resin sponge. This displacement (sponge ^{125}I-T_3 uptake) is linearly related to T_3 present in serum or standards.

Procedure. (A) First, 50 μl of serum or T_3 standards are pipetted into polypropylene tubes (15 × 86 mm). (B) Then 0.2 ml of TCA–NaOH solution is added to each tube, followed by shaking for 2 min. (C) Next, 0.5 ml of ^{125}I-T_3-labeled T_3 antiserum is added, followed by a further 2-min mixing in the automatic shaker, and the hapten–antibody mixture is allowed to equilibrate at 37°C for 30 min (final reaction mixture, pH 7.2). After equilibration, the tubes are transferred to an ice bath (0–1°C).* (D) Ten minutes after equilibration in ice bath, anionic-resin sponges (Abbott Laboratories) are introduced into each tube and squeezed with a plastic plunger (Abbott Laboratories). The radioactivity in a few tubes (usually six) is counted to represent initial activity. (E) Exactly at 30 min, sponge incubation is terminated by aspirating excess fluid, using a hollow plastic aspirator (Abbott) connected to a vacuum source through flexible tubing. The sponges are then washed three times by filling the tube with distilled water, squeezing the sponge, and aspirating the fluid each time. The washing procedure removes only bound ^{125}I-T_3, and unbound ^{125}I-T_3 abstracted into the resin sponge by ion exchange is not affected. The radioactivity in the sponge is subsequently determined (final activity). The sponge uptake of radioactivity (final activity/initial activity) is linearly related to triiodothyronine present in standards or test serum up to a concentration of 400 ng/100 ml or more, depending on assay conditions and dilution of antisera.

The T_3 extraction recovery studies were based on the rationale that, once the T_3–protein bond is effectively destroyed by TCA–NaOH, unbound T_3 would be readily abstracted by the resin sponge. Serum was labeled with ^{125}I-T_3 and the experimental procedure was repeated except for the addition of antibody. In control experiments, labeled test serum was replaced by ^{125}I-T_3-labeled gamma-globulin or labeled buffer. Then 50 μl of ^{125}I-T_3-labeled serum, 50 μl of ^{125}I-T_3-labeled gamma-globulin, or 50 μl of ^{125}I-T_3-labeled buffer was pipetted into tubes followed by the addition of 0.2 ml of TCA–NaOH. After mixing for 2 min, 0.5 ml of phosphate buffer was added and mixed, and ^{125}I-T_3 uptake was determined as described.

TABLE 1. EFFECT OF VARYING PERIODS OF EQUILIBRATION OF LABELED T_3 ANTISERUM WITH T_3, IN THE REACTION MIXTURE AT 37°C, ON SPONGE ^{125}I-T_3 UPTAKE (%) IN T_3 STANDARDS*

T_3 standards (ng/100 ml)	Duration of Ag–Ab equilibration (min)			
	15	30	60	120
0	24.7	23.6	24.2	24.8
25	25.5	24.5	25.8	25.9
50	26.6	25.6	27.1	26.6
100	29.8	27.9	27.9	28.4
200	34.3	33.1	33.7	34.5
400	43.5	44.5	44.2	45.6
600	50.6	52.1	51.1	51.1
800	54.2	56.8	56.4	57.6
Response range between 0–800-ng standards (units)	29.5	33.2	32.2	32.8

* All values shown are means of triplicates.

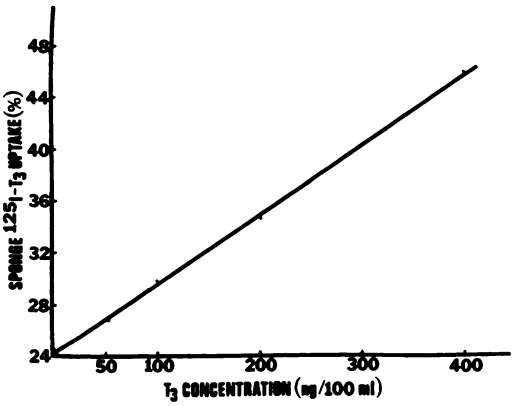

FIG. 1. Representative T_3 dose–response curve.

RESULTS

When sponge ^{125}I-T_3 uptake was plotted against T_3 standards, a linear dose–response curve was obtained (Fig. 1). The possible effects of various factors on standard curve were investigated as follows.

Equilibration (at 37°C) of ^{125}I-T_3-labeled antiserum with T_3 in the reaction mixture. Results are given in Table 1. From the similarity of ^{125}I-T_3 uptake values corresponding to T_3 standards (0–600 ng/100 ml), at 15 and 120 min, it is apparent that a 15-min equilibration period with the antibody alone is sufficient. Similar results were noted with human sera, which also indicates that equilibration with antibody up to 2 hr at 37°C apparently does not result in T_3 formation from T_4 present in sera. The

* Ice-bath incubation is not critical. Incubation at ambient temperature may also be carried out, provided the standards are run along with sera.

zero value (no T_3) at 15 min was the same as that noted at 120 min after equilibration, indicating that even prolonged equilibration at 37°C does not compromise the integrity of labeled T_3 antiserum, as shown by the absence of spontaneous release of ^{125}I-T_3 from labeled antiserum.

Variation in interval of resin sponge incubation. When ^{125}I-T_3 uptake corresponding to various T_3 standards was compared at 15, 30, 45, and 60 min after sponge incubation, the 30-min incubation provided the best linear response up to 400 ng.

Effect on T_3-RIA of elapsed period after TCA–NaOH addition. Replicate T_3 standards were extracted with TCA–NaOH for 2 min and left at room temperature for 0, 30, 60, and 120 min, after which labeled T_3 antiserum was added and the assay completed. The values for zero and T_3 standards at all these intervals were the same, showing that in human serum TCA–NaOH releases T_3 from T_3-binding proteins instantaneously.

In another experiment, TCA–NaOH was added to two T_4 standards (15 and 20 μg/100 ml) and two hyperthyroid sera, and the samples were left in a 37°C water bath 1–4 hr, followed by addition of ^{125}I-T_3-labeled antiserum and completion of the assay. The ^{125}I-T_3-uptake values noted in T_4 standards and in serum samples at 1 hr were similar to those noted at 4 hr, again showing that even with prolonged TCA–NaOH extraction at 37°C under highly alkaline conditions, T_4 present in sera is not converted to T_3, since any new T_3 formed would be expected to react with ^{125}I-T_3-labeled immune serum and thus increase the sponge ^{125}I-T_3 uptake.

Effect on T_3-RIA of moderate excess of TCA–NaOH. A twofold excess of TCA–NaOH extractant had no deleterious effect on the assay. Use of 0.1–0.3 ml extractant in the assay resulted in standard curves that could be superimposed.

Serum T_3 extraction efficiency of TCA–NaOH. The ^{125}I-T_3 uptakes, 30 min after sponge incubation in the extraction medium containing ^{125}I-T_3-labeled sera and in labeled controls, are shown in Table 2. The ^{125}I-T_3 uptake in buffer was 92%, and the failure to obtain 100% within this interval (i.e., 30 min) can be attributed partly to the low temperature (0–1°C) used for sponge incubation. The control with gamma-globulin (which does not actively bind T_3) showed 90% uptake. Since protein concentrations in sera and gamma-globulin were approximately equal, T_3 extraction recovery values were compared with the latter (Table 2). Since there is 96% (mean) abstraction of radioactivity by a secondary binding site such as the resin (Table 2), in the presence of specific and high-affinity T_3-binding protein, such as the antibody, virtually all unbound T_3 in the system, after treatment of serum by TCA–NaOH, should react with antibody almost instantaneously. Therefore, after TCA–NaOH extraction in the assay, T_3 antibody clearly becomes the only binding agent, and serum T_3-binding proteins no longer compete or bind T_3. Additional corroborative investigations are noted below.

Comparison of TBG inactivation by thermal denaturation and TCA–NaOH on T_3-RIA. The direct evidence for lack of TBG interference in T_3-RIA was obtained in experiments where TBG was inactivated by thermal denaturation (*12*). Instead of

TABLE 2. SERUM T_3 EXTRACTION EFFICIENCY OF TCA–NaOH*

^{125}I-T_3-labeled sera and controls	Sponge ^{125}I-T_3 uptake at 30 min† (%)	Extraction recovery‡ (%)
Controls		
Buffer	92.3 ± 1.01	
Gamma-globulin	90.4 ± 1.12	
Sera		
Hypothyroid	88.3 ± 0.74	97.7
Normal	87.6 ± 1.27	96.9
Pregnancy	85.5 ± 1.43	94.6
Hyperthyroid	86.2 ± 1.24	95.3

* Based on recovery of ^{125}I-T_3 from ^{125}I-T_3-labeled serum, or labeled controls, as determined by sponge ^{125}I-T_3 uptake. Data based on ten different samples in each category.
† Mean ± 1 standard deviation.
‡ Expressed in reference to gamma-globulin.

TABLE 3. COMPARISON OF T_3-RIA VALUES IN SERA DENATURED THERMALLY (60°C) OR BY TCA–NaOH

Sera	T_3 concentration (ng/100 ml)	
	Thermally denatured	TCA–NaOH denatured
Euthyroid	178	190
	156	154
	170	162
	177	175
	240	235
	110	123
Hypothyroid	48	62
	55	50
	73	83
	40	39
Pregnancy	212	207
	172	185
	206	204
	190	195
Hyperthyroid	625	650
	262	260
	300	270
	440	455

adding 0.2 ml of TCA–NaOH as done in our procedure, 0.2 ml of phosphate buffer was added to serum samples as well as T_3 standards, followed by addition of ^{125}I-T_3-labeled antiserum. The samples were left in a 60–65°C water bath for 3 hr, after which they were transferred to a refrigerator. Following overnight cold (6°C) equilibration, the assay was completed in the usual manner. In some experiments, assays were completed immediately after thermal inactivation, i.e., without overnight cold equilibration. No differences in T_3 values were found, whether or not thermally inactivated sera were subjected to overnight cold equilibration. Accordingly most assays were completed immediately after thermal inactivation. The values noted are compared with the T_3 values obtained by our regular procedure using TCA–NaOH to denature serum proteins (Table 3). The values obtained in all sera by either procedure are virtually the same, showing that the presence of TBG in unextracted serum does not interfere with T_3-RIA, and that TCA–NaOH denaturation* of TBG is as effective as thermal denaturation.

Sensitivity, precision, accuracy, and validity of T_3-RIA. The sensitivity of the technique was at least 25 ng/100 ml (or 12.5 pg/tube), since the use of this T_3 standard consistently caused a significant displacement of label from ^{125}I-T_3-labeled antibody. Concentrations of T_3 between 0 and 25 ng/100 ml were not tested. Interassay precision of 20 determinations in normal (142 ng/100 ml), hypothyroid (60 ng/100 ml), and hyperthyroid (330 ng/100 ml) sera showed coefficients of variation of 9.5%, 13.6%, and 5.6%, respectively. In determining accuracy, T_3 concentrations added were allowed to equilibrate with sera overnight prior to the assay. Table 4 shows the T_3-RIA values found before and after exogenous T_3 addition. At all levels of added T_3 there was virtually complete recovery in all sera despite TBG variation, the range being 93–109%.

To study T_4 cross-reaction, T_4 standards (5 and 10 μg/100 ml) were used in the assay, and the equivalent T_3 values were 7.5 and 45 ng/100 ml, respectively. Thus, the cross-reactions were 0.1% and 0.4%, respectively. Essentially similar results were obtained with 15- and 20-μg T_4 standards, and the average cross-reaction was approximately 0.4%. Negligible interaction with the antibody was found with 3:5 diiodothyronine, monoiodotyrosine, and diiodotyrosine. The T_3-RIA determinations in eightfold dilution of T_3 standards, in T_3-free plasma, or in saline agreed well with the values of undiluted standards (Table 5). Similar results were noted in the serum sample subjected to fourfold dilution.

TABLE 4. T_3 RECOVERY IN T_3-RIA PROCEDURE

Sera	(A) Endogenous serum T_3 (ng/100 ml)	(B) T_3 added exogenously (ng/100 ml)	(C) Serum T_3 after exogenous T_3 addition (ng/100 ml)	Recovery (%) (C — A)/B
Normal				
No. 1	108	250	365	102
No. 2	150	250	388	95
Hypothyroid				
No. 1	50	300	353	101
No. 2	35	250	300	106
Hyperthyroid				
No. 1	300	500	768	94
No. 2	400	300	680	93
Pregnancy				
No. 1	200	800	1070	109
No. 2	150	800	1020	109

T_3-RIA in normal and abnormal thyroid states. The T_3-RIA values (mean in ng/100 ml ± 1 s.d., with the ranges) were 142 ± 29.8 (82–217) in 92 normal subjects; 220 ± 82.1 (110–348) in 16 pregnant women; 55 ± 32.1 (8–128) in 19 hypothyroid subjects; and 411 ± 188 (222–970) in 18 hyperthyroid subjects. The T_3 values in normal and hyperthyroid subjects were clearly separated, but some overlap was found between normal and hypothyroid values, as noted by several other investigators (*5,8,19*). Among other possible factors, it has been suggested that excess thyroid-stimulating hormone (TSH) secretion in early primary hypothyroidism may stimulate T_3 in preference to T_4 (*19,20*), and the evidence from recent in vitro investigations of TSH effects on T_4-to-T_3 ratios (*21*) seems compatible with this suggestion.

DISCUSSION

The results make it clear that the RIA technique described for T_3 determination is an accurate, sensitive, reliable, and valid procedure. Furthermore, it has completely avoided such cumbersome and time-consuming steps as pre-extraction, centrifugation, column preparation, transfer of assay reactants for free- and bound-hormone separation, etc. The assay can be completed in the same tube in about 1 hr. For elicit-

* The effectiveness of TCA–NaOH in denaturing TBG in serum under the assay conditions described was also checked by TBG radioimmunoassay (Nichols Institute for Endocrinology, Wilmington, Calif.). The TBG concentration in a serum pool before denaturation was 3.6 mg/100 ml, whereas TBG was not detectable after TCA–NaOH extraction.

TABLE 5. EFFECT OF DILUTION ON T_3-RIA*

Dilution factor	T_3 Standard 1		T_3 Standard 2		Serum	
	in T_3-free plasma	in saline	in T_3-free plasma	in saline	in T_3-free plasma	in saline
Undiluted	3000	3000	2000	2000	500	500
1:1 (1/2)	2950	2810	1810	1900	425	460
1:3 (1/4)	2900	2960	1900	1825	410	—
1:7 (1/8)	2975	2460	1820	—	—	—

* Values (ng/100 ml) obtained with diluted sera are corrected for appropriate dilution.

ing T_3 antibodies, T_3-albumin conjugate was satisfactory, and the antibody titer is fairly comparable to that of other workers who have used similar conjugated immunogen. In addition, the negligible T_4 cross-reaction with T_3 antibody shows not only that T_3 can be measured satisfactorily, but also the absence of measurable T_4-to-T_3 conversion in the assay.

In the RIA of T_3 in unextracted serum, various solvents have been used to prevent serum-protein binding of T_3 (principally by TBG). These include ANS (*4*), tetrachlorothyronine (*3*), dilantin (*7*), sodium salicylate (*5*), and ethylmercurithiosalicylate (merthiolate, Ref. *2*). Dinitrophenol and diazepam have also been used in some studies. While successful assays can be developed by the careful use of blocking agents, some workers have only reported varying degrees of success (*9,13*), whereas others (*12,16*) have expressed strong reservations regarding the use of many of these TBG-blocking agents. Alternative techniques of TBG inactivation and T_3 extraction have also been resorted to, e.g., thermal denaturation, enzymatic cleavage, etc. Use of TCA–NaOH extractant as in the present assay offers certain attractive features: (A) the denaturation of TBG is instantaneous; (B) despite the rapidity of extraction, TCA–NaOH facilitates fairly uniform T_3 recovery from all samples; (C) as the ability of serum proteins to bind T_3 is lost, there is no active or residual binding of T_3 tracer—in contrast with TBG-blocking assays, where the potential for such binding does exist.

Serum T_3 concentration in euthyroid subjects as noted in the present study (142 ng/100 ml) is virtually the same as that noted by several groups of investigators (*3,6–9,11*) and is closely comparable to the values in the majority of T_3-RIAs. Our mean value is also in close agreement with that of Patel and Burger (*8*), who used serum extracts in their assay, and that of Surks et al (*6*), who used column chromatography to separate proteins. In addition, the mean value found by this assay is close to that observed in gas–liquid chromatography (137 ng/100 ml), as described by Mitsuma et al (*3*), who made appropriate corrections for T_4-to-T_3 conversion in their technique. In the extreme, our mean T_3 values are 35% lower than those reported by Gharib et al (*2*) and 48% higher than those reported by Alexander and Jennings (*10*). The discrepancy between results has been attributed to a variety of causes, some of which are variation in iodine concentration among subjects; the differing TBG-blocking agents used in the assay; the widely varying techniques for separating free and bound hormone; and such other experimental conditions as sensitivity, specificity of the antibody, etc. Sterling and Milch (*12*) suggested that the order of discrepancies in T_3 concentrations between workers is similar to that noted when one reads the values from standard curves prepared in buffer or T_3-free serum. In influencing T_3 values the importance of the nature of the protein solution used to prepare T_3 standards has also been stressed (*15*). Moreover, recent investigations show that age could be an important factor, since T_3 concentration has been reported to be reduced in subjects with increasing age (*19*). Finally, Savoie et al (*14*) have suggested that different T_3 antisera may varyingly cross-react with reverse T_3 in serum, which may contribute to apparent differences in mean T_3 values in normal subjects as noted in different investigations.

ACKNOWLEDGMENTS

The author is indebted to various personnel at Abbott Laboratories who extended cooperation in this study, particularly I. D. Smith and J. P. Miller for providing materials and equipment. The author is also indebted to numerous physicians and other scientific colleagues for providing serum specimens and supporting clinical diagnoses, and in particular, the assistance of W. L. Florsheim, H. W. Wahner, G. A. Hagen, V. V. Row, R. Volpe, I. B. Perlstein, and R. Burstein is gratefully acknowledged.

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