Radioimmunoassay

Improved Method for the Measurement of Serum Thyroxine

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Methodology for the measurement of thyroxine in unextracted human serum is described. Antibodies were prepared by immunization of rabbits with thyroxine-human serum albumin (T_4-HSA) conjugates. Bound and free labeled hormones are separated using the double antibody technique. The validity of the assay has been demonstrated by the excellent recovery of exogenous T_4 added to various serum samples and by production of a curve obtained by various dilutions of a hyperthyroid serum which is parallel to the standard curve. Radioimmunoassay T_4 values ranged from 4.5 to 12.0 μg/100 ml in 172 euthyroid patients. In all clinical states, serum T_4 values obtained by radioimmunoassay afforded excellent agreement (correlation coefficient, 0.95) with those obtained by competitive protein binding assay. Advantages of the method over commercially available radioimmunoassay kits or the competitive protein binding assay are outlined.

The value of serum thyroxine determinations as a screening test for thyroid function has been well documented (*1, 2*). The technique of radioimmunoassay (RIA) as introduced by Yalow and Berson over 14 years ago has found wide application in the measurement of hormones involved in thyroid function (*3-6*). Radioimmunoassay of thyroxine has the advantages over the competitive protein-binding assay, as described by Murphy, in that it does not require extraction of serum samples and only a small volume of serum (20 μl) is required for the test (*7*). Although there are many commercial kits available to determine serum thyroxine concentration, they have limited range of measurement and are very expensive when large numbers of tests are required. Price per tube of commercial kits varies from $0.70 to $1.50, regardless of volume of patient sera tested.

This report describes a procedure for the radioimmunoassay of thyroxine which has greater sensitivity than the competitive protein-binding assay and is a rapid, specific, and thoroughly validated method for measurement of thyroxine in unextracted serum.

Materials and Methods

Materials. Thyroxine antiserum was obtained from rabbits immunized with T_4 conjugated to human serum albumin (HSA) exactly as described in a previous paper for a T_3 assay (*8*). The antiserum used in this study was obtained two months after the rabbit had received four 1-mg injections of T_4-HSA conjugate spread over a seven-week period. Iodine-125-thyroxine with a specific activity of 50–75 μCi/μg was obtained from Abbott Laboratories, North Chicago, IL; 8-anilino-1-naphthalene sulfonic acid (ANS), a fluorescent dye with the general formula $C_6H_5NHC_{10}H_6SO_3H$, was obtained from Eastman Kodak Co., Rochester, NY; reagent grade Na-L-T_4 was obtained from Sigma Chemical Co., St. Louis, MO; and goat antirabbit serum (GARS) was supplied by the Clinical Aerospace Research Laboratory, USAF.

Detection and specificity of T_4 antibody. Antibody to T_4 was detected by reaction of 250 pg ^{125}I-T_4 with varying quantities of T_4 antibody for 1½ hr at 37° C. Separation of antibody-bound and free ^{125}I-T_4 was accomplished by addition of GARS. The antiserum concentration which bound 50% of the tracer was selected for use in the T_4 RIA. Specificity of T_4 antiserum was primarily assessed by studying the displacement of ^{125}I-T_4 from the T_4 antibody by various doses of the most potential cross-reacting substance, T_3.

Determination of quantity of ANS required to prevent thyroxine-binding globulin (TBG) interference. Various concentrations of ANS were incubated with ^{125}I-T_4, T_4 antiserum, and serum samples from eu-, hyper-, hypothyroid, and pregnant patients. The object of using ANS in the assay of T_4 is twofold: (A) to displace T_4 bound to TBG in the patients' serum so that the T_4 is free to react with the T_4 antibody, and (B) to prevent binding of ^{125}I-T_4 to TBG so that the ^{125}I-T_4 is available for binding to the T_4 antibody.

Patient dilution curve. A hyperthyroid serum was assayed at various increasing quantities from 0.005 to 0.025 ml to check for parallelism with the standard curve.

Thyroxine RIA procedure. Reagents were added to disposable 10 × 75-mm polystyrene tubes as follows:

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1. 800 μl of phosphate buffered saline, pH 7.4, containing 0.5% HSA and 0.05 *M* EDTA was employed.
2. 20 μl of 4.5% HSA was added to the 0% control tubes, the 100% control tubes, and the tubes containing the standard curve.
3. Delivery of the T_4 standard solution was such that the concentration ranged from 0.1 to 7.5 ng per tube (corresponding to a range of 0.5–37.5 μg/100 ml) for a nine-point standard curve. In the case of the unknowns, 20 μl of serum was used. Each point on the standard curve was assayed in triplicate and the unknown in duplicate.
4. 0.1 ml of the PBS buffer which contained 0.25 ng ^{125}I-T_4 and 200 μg ANS was added to all tubes.
5. 0.1 ml of a 1:300 initial dilution of T_4 antiserum containing 2.5% normal rabbit serum in the PBS buffer was added to all tubes except the 0% control tubes. The tubes were vortexed and incubated in a 37° C water bath for 1½ hr and then at 4° C for 30 min.
6. Precipitation of ^{125}I-T_4 bound to antibody was accomplished by adding 0.1 ml of a previously titered GARS to all tubes, vortexing, and incubating at 4° C for 20–24 hr.
7. The tubes were centrifuged at 3000 rpm for 30 min, with the supernate aspirated and discarded. The precipitate (antibody-bound T_4) was counted for 1–2 min in a Searle Radiographics automatic gamma counter.
8. A standard curve was plotted on semilog paper. The percent of ^{125}I-T_4 precipitated in the patient serum samples was compared to that precipitated by known amounts of unlabeled T_4. Each assay contained a complete standard curve and two sets of control tubes. The first control contained labeled T_4, but no antibody (0% controls). The radioactivity contained in the precipitates of the 0% control tubes was averaged and subtracted from the counts in the precipitates of all other tubes in the assay. These counts were taken to be nonspecifically bound or trapped in the final precipitate. The second set of control tubes contained antibody to T_4 and labeled T_4, but no unlabeled T_4 (100% controls). The precipitates from the 100% control tubes contained from 50 to 60% of the total ^{125}I-T_4 when the final dilution of antibody was 1:3000. The ^{125}I-T_4 contained in the precipitates of all tubes containing standards or patient sera was expressed as a percent of the activity in the 100% control tubes.

Clinical material. Sera were obtained from euthyroid healthy volunteers, normal pregnant women, and hyper- and hypothyroid patients being treated or having follow-up studies at the Wilford Hall USAF Medical Center. The blood was allowed to clot, and the serum was separated by centrifugation and frozen at −20° C, unless assayed within two days.

Competitive protein-binding assay (CPBA). Thyroxine serum concentrations were determined in a group of patients using RIA and also the Murphy procedure to determine correlation between the two methods (*7*).

Results

Antibody specificity. A dose response or standard curve was constructed using unlabeled T_3 to test its ability to inhibit the binding of ^{125}I-T_4 to T_4 antibody. There was no inhibition of ^{125}I-T_4 binding to the T_4 antibody with T_3 concentrations as great as 1.0 ng per tube. Since 20 μl of serum is used in the assay, 1.0 ng of T_3 would be equivalent to a T_3 concentration of 5000 ng/100 ml. Therefore, the T_4 serum concentration would not be affected by any T_3 serum concentration likely to be seen in clinical practice.

Concentration of ANS. Concentrations ranging from 45 to 700 μg were added to sera containing

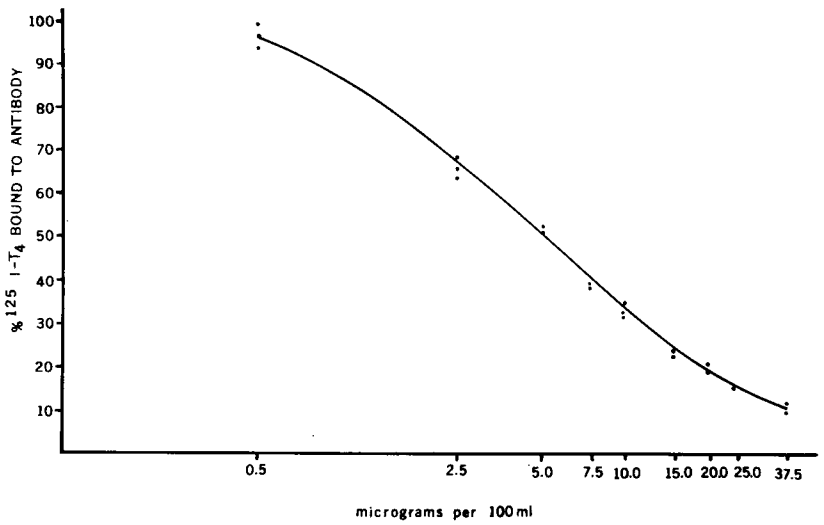

FIG. 1. Dose-response curve; inhibition of binding of ^{125}I-T_4 by unlabeled T_4.

TABLE 1. Recovery of T_4 Added to Sera

Patient sera	Initial serum T_4*	T_4 added	T_4 expected/recovered†	Percent recovered
Estrogen (#2221)	13.0	2.5	15.5/15.8	101.9
		5.0	18.0/17.2	95.5
		10.0	23.0/22.3	96.9
		15.0	28.0/27.2	97.1
		20.0	33.0/32.1	97.2
		25.0	38.0/35.3	92.8
Euthyroid (#2202)	8.4	2.5	10.9/11.0	100.9
		5.0	13.4/13.7	102.2
		10.0	18.4/18.1	98.3
		15.0	23.4/22.2	94.8
		20.0	28.4/27.8	97.8
		25.0	33.4/33.1	99.1
Hyperthyroid (#2083)	14.6	2.5	17.1/16.6	97.1
		5.0	19.6/18.6	94.9
		10.0	24.6/23.7	96.3
		15.0	29.6/27.3	92.2
		20.0	34.6/34.5	99.7
		25.0	39.6/39.2	99.0
Hypothyroid (#1250)	2.1	2.5	4.6/4.4	95.7
		5.0	7.1/7.2	101.4
		10.0	12.1/11.6	95.9
		15.0	17.1/16.8	98.2
		20.0	22.1/22.7	102.7
		25.0	27.1/27.3	100.7
Average				97.8 ± 2.9

All T_4 concentrations are in micrograms per 100 ml.

†*Mean of duplicate determinations.*

TABLE 2. Thyroxine Concentrations in Dilutions of Serum From a Hyperthyroid Patient (GK-1147)

Amount of serum assayed (ml)	T_4* (μg/100 ml)
0.005	5.1
0.010	8.6
0.015	14.6
0.020	19.1
0.025	22.4

Mean of duplicate determinations.

different quantities of TBG and T_4, e.g., sera of hypothyroid, euthyroid, and pregnant patients. Maximum percent binding of ^{125}I-T_4 to T_4 antiserum occurred when the ANS concentration was approximately 200 μg per 0.020 ml of serum in all samples. Thus, 200 μg ANS is added to all tubes in the T_4 RIA.

Figure 1 shows a typical standard curve. The minimum detectable T_4 concentration for 12 assays was generally 0.9 μg/100 ml. Between-assay coefficient of variation of control serums in 12 assays was 5.8% and 8.4% at T_4 concentrations of 7.8 μg/100 ml and 14.2 μg/100 ml, respectively.

Validation of the T_4 RIA. Sera of pregnant, euthyroid, hypothyroid, and hyperthyroid patients were enriched with T_4 in concentrations ranging from 2.5 to 25.0 μg/100 ml. The recovery of added T_4, shown in Table 1, averaged 97.8 ± 2.9%. Table 2 shows the results of T_4 estimates in several dilutions of serum from a hyperthyroid patient. Estimates of the serum T_4 from various dilutions were quite comparable to those obtained by assay of full-strength serum.

Comparison of T_4 RIA and T_4 CPBA. Figure 2 shows the results of analysis of serum samples using

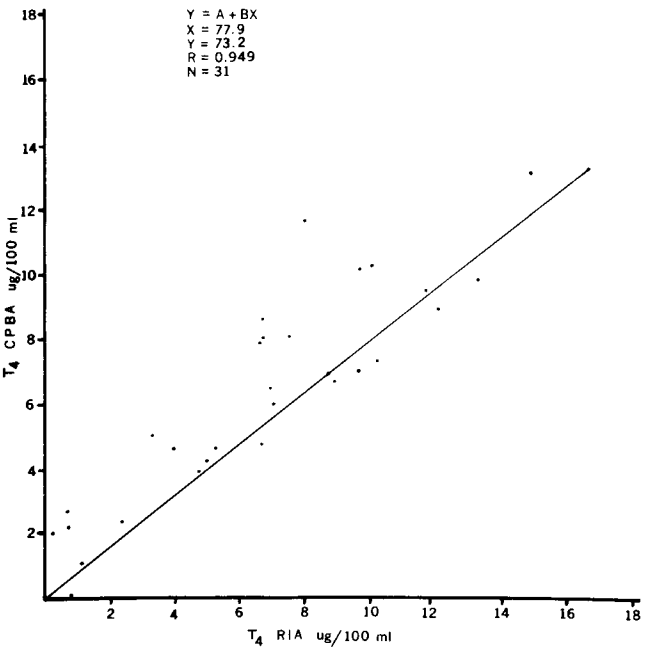

FIG. 2. Correlation between T_4 values as assayed by RIA and competitive protein-binding technique.

TABLE 3. Serum T_4 Concentrations

Clinical status	No.	Range (μg/100 ml)	Mean range (μg/100 ml)
Euthyroid	172	4.5–12.0	7.7 ± 1.5
Hyperthyroidism	17	8.0–22.0	16.3 ± 4.5
Hypothyroidism	16	0.8–5.4	2.2 ± 1.3
Normal Pregnant	66	7.5–16.4	12.8 ± 2.1

radioimmunoassay and the competitive protein-binding assay. Patients with low, normal, and high T_4 values are included in the group. The regression analysis was performed on a Wang calculator (model 720). The correlation coefficient was 0.95, showing excellent agreement between the two techniques.

Clinical results. Thyroxine levels were determined in patients proven clinically eu-, hyper-, and hypothyroid and in a group of normal pregnant patients. The results of these determinations are shown in Table 3. Only one patient in the hypothyroid group had a T_4 serum concentration that overlapped with the euthyroid group. In hyperthyroid patients, two patients had T_4 levels in the euthyroid range.

Discussion

Major improvements in the RIA of serum T_4 have been accomplished by the use of ANS. Specific antibodies to T_4 are easily produced by use of the T_4-HSA conjugate, thus making the assay inexpensive to develop and maintain. The excellent recovery of exogenous T_4 added to various serum samples and the virtually identical values of T_4 calculated from measurements of five different dilutions of the same serum all serve to validate this RIA procedure.

This T_4 RIA has four major advantages over the competitive protein-binding assay: (A) it has greater sensitivity (0.9 μg/100 ml RIA versus 2–3 μg/100 ml CPBA); (B) it offers greater accuracy (straight-line graph is obtained from 0.5–25 μg/100 ml by RIA method versus 2–12 μg/100 ml by CPBA); (C) it does not require an extraction procedure; and (D) only a small volume of serum is required (very important consideration in pediatric patients).

Our T_4 serum assay, as compared to most commercially available T_4 kits, is also very inexpensive, requiring only the purchase of ^{125}I-T_4 every six to eight weeks.

References

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RIA Questions and Answers

1. Is it really necessary to run a standard curve with each set of patient samples?

   Unless you have found a method which is unaffected by changes in time, temperature, reagent deterioration, and a host of other variables, you must run a standard curve to obtain the best possible test results.

2. Is it acceptable to extend a standard curve beyond its maximum measured concentration to read out a high answer?

   Most curves lose sensitivity at elevated concentrations even though this may not be readily apparent as a result of the plot format. An elevated answer would more accurately be reported out as "greater than 8 ng/ml," in which 8 ng/ml is the most concentrated standard point. If an exact answer is necessary, the sample should be diluted and retested.

3. How reproducible should a semi-automatic pipette be?

   For good quality work, this type of pipette should have a coefficient of variation less than 3%.

4. Where can information be obtained regarding external quality control programs for radioimmunoassay?

   Proficiency survey information is available from:

   The College of American Pathologists, 7400 Skokie Boulevard, Skokie, IL 60076

   and

   William Shaw, Immunochemistry Section, Clinical Chemistry Div., Bureau of Laboratories, Center for Disease Control, Atlanta, GA 30333.

If you have any questions regarding RIA procedures, please write to Patricia Weigand, Nuclear Medicine Service, VA Hospital, Philadelphia, PA 19104. Selected questions and answers will be published in future issues of the JNMT.