Simultaneous Measurement of Triiodothyronine and Thyroxine in Unextracted Serum Samples Using a Double-Tracer Radioimmunoassay Method

Mary L. Brown, Therian Hannah,* David T. Kopp, Gladys Stephens, and Robert F. Carretta

Wilford Hall USAF Medical Center, Lackland Air Force Base, Texas

A method is described for the simultaneous measurement of triiodothyronine (T_3) and thyroxine (T_4) in 0.04 ml of unextracted serum. Antibodies were prepared by immunization of rabbits with T_3 and T_4 conjugated with human serum albumin. Bound and free labeled hormones were separated by the double-antibody technique, and 8-anilino-1-naphthalene sulfonic acid was used to inhibit binding of the two hormones to thyroxine-binding globulin. The validity of the assay using ^{125}I-T_3 and ^{131}I-T_4 is shown by the excellent recovery of T_3 and T_4 added to serum and by the finding that curves obtained by assaying various dilutions of a hyperthyroid serum run parallel to the standard curves. In all clinical states, serum T_3 and T_4 values obtained using the double-tracer radioimmunoassay method were in excellent agreement with those obtained by single-tracer RIA techniques. The combined T_3–T_4 method appears to be accurate, sensitive, and specific, thus making the assay highly desirable as a technical time-saver.

J Nucl Med 18: 300–304, 1977

Major improvements in the measurement of the thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) have been accomplished recently through radioimmunoassay techniques. Since knowing both T_3 and T_4 plasma concentrations is useful in the diagnosis of thyroid disease, it would be convenient to have both laboratory values available at the same time. Since both T_3 and T_4 labeled with either ^{125}I or ^{131}I are commercially available, the simultaneous measurement of these hormones is entirely feasible. This paper describes such a method.

MATERIALS AND METHODS

Antibodies to T_3 and T_4 were prepared by injecting into rabbits T_3 and T_4 that had previously been conjugated to human serum albumin by a carbodiimide reaction (*1,2*). The cross-reactivity of T_4 to the T_3 antiserum was less than 0.1%, and that of T_3 to the T_4 antiserum was approximately 2.0%. The second or precipitating antibody was prepared by injecting rabbit gamma-globulins into goats. The ^{125}I-T_3 was obtained with a specific activity of 350–544 mCi/mg,* and the ^{131}I-T_4 with 20–50 mCi/mg.† Human serum free from T_3 and T_4 was prepared as described in a previous paper (*3*).

Quantity of ANS‡ required to prevent TBG interference. Various concentrations of 8-anilino-1-naphthalene sulfonic acid (ANS) were incubated with ^{125}I-T_3, ^{131}I-T_4, T_3 and T_4 antisera, and serum samples from euthyroid, hyperthyroid, hypothyroid, and pregnant patients.

T_3–T_4 double-tracer radioimmunoassay procedure.

1. Reagents were added to disposable 10 × 75-mm polystyrene tubes as follows:
   a. 0.6 ml of PBS buffer, pH 7.4, containing 0.25% HSA and 0.05 *M* EDTA.

Received Feb. 6, 1976; revision accepted Sept. 29, 1976.

For reprints contact: Major Mary Lou Brown, Nuclear Medicine Service, Wilford Hall USAF Medical Center, Lackland AFB, TX 78236.

* Present address: Nuclear Medicine Service, Veterans Administration Hospital, Durham, NC.

b. 0.04 ml of 4.5% HSA or T_3–T_4-free human serum, to control and standard curve tubes, respectively.
c. Ten-point standard curves (each point in triplicate) were constructed for T_4 concentrations ranging from 0.1 to 10 ng per tube and T_3 concentrations from 10 to 500 pg per tube.
d. 0.04 ml of the unknown serum (in duplicate).
e. 0.1 ml of the tracer solution containing 25 pg of ^{125}I-T_3, 400–900 pg of ^{131}I-T_4, and 400 μg of ANS in PBS–HSA–EDTA buffer.
f. 0.1 ml of antibody solution containing 1:2,500 dilution of T_3 antiserum, 1:150 dilution of T_4 antiserum, and 2% normal rabbit serum in PBS–HSA–EDTA buffer.

2. The tubes were vortexed and incubated at 37°C for 1.5 hr.

3. The assay tubes were removed from the water bath and incubated at 4°C for 30 min.

4. Precipitation of the antibody-bound ^{125}I-T_3 and ^{131}I-T_4 was accomplished by adding 0.1 ml of a previously titered (1:10) goat antirabbit serum to all tubes. The tubes were vortexed again and incubated at 4°C for 18–24 hr.

5. The tubes were centrifuged at 5,000 rpm (5,700 g) for 20–30 min in a refrigerated centrifuge; the supernatant was then aspirated and discarded. The precipitate (antibody-bound T_3 and T_4) was counted for 1–3 min in a dual-channel gamma counter with teletype.|| Punched tape from the teletype was entered into a calculator system containing a four-parameter logistic programmed cassette¶ whereby data from both channels are automatically processed and recorded.

Validation of the T_3–T_4 double-tracer assay.

Recovery experiments. Unlabeled T_3 and T_4 representing various typical thyroid states were added to sera in concentrations ranging over 100–375 ng/100 ml and 2.5–18.8 μg/100 ml, respectively.

Patient dilution curve. Hyperthyroid serum was assayed at five dilutions ranging from 10–50 μl. The amount of serum was kept constant using T_3–T_4-free serum.

Serum specimens. Sera were obtained from euthyroid healthy volunteers, normal pregnant women, and hyper- and hypothyroid patients. The blood was allowed to clot; the serum was then separated by centrifuging and was frozen at —20°C unless it was to be assayed within 3 days. The T_3 and T_4 serum concentrations were measured using both single-tracer and double-tracer radioimmunoassay procedures.

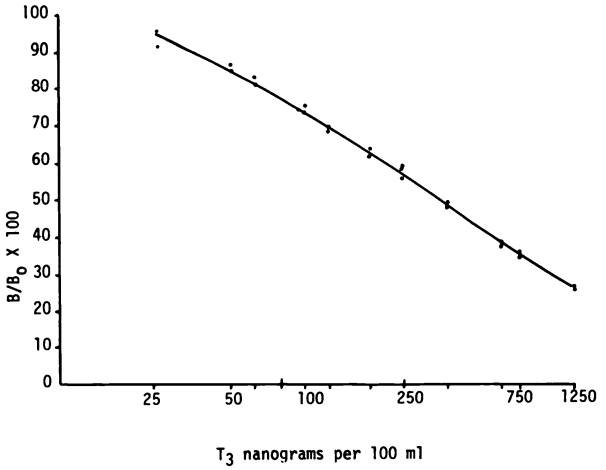

FIG. 1. Dose–response curve for T_3, prepared in T_3–T_4-free serum and ANS, showing inhibition of ^{125}I-T_3 binding by unlabeled T_3.

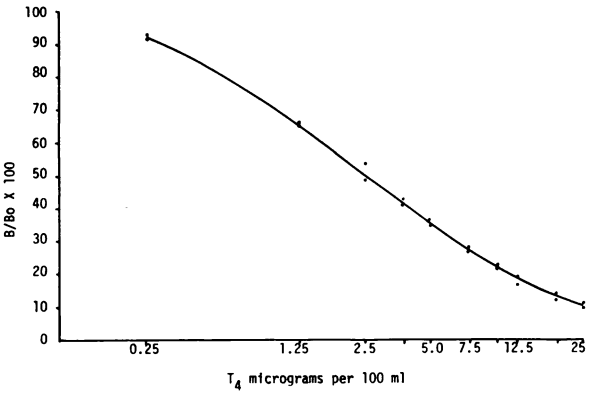

FIG. 2. Dose–response curve for T_4, prepared in T_3–T_4-free serum and ANS, showing inhibition of ^{131}I-T_4 binding by unlabeled T_4.

RESULTS

ANS concentration. Concentrations ranging over 70–1,400 μg of ANS were added to sera containing different quantities of TBG: 400 μg of ANS was found to be sufficient to block the binding of ^{125}I-T_3 and ^{131}I-T_4 to TBG and to displace endogenous T_3 and T_4 bound to TBG. A detailed discussion of this procedure and the use of ANS in the assay of the thyroid hormones may be found in an earlier paper (*3*).

Standard curves. Figure 1 illustrates a typical T_3 dose–response curve covering a range of 25–1,250 ng/100 ml. Sensitivity of the T_3 curve over 12 assays was generally 20 pg, which corresponds to a T_3 concentration of 50 ng/100 ml.

The T_4 dose–response curve is shown in Fig. 2. Measurable T_4 concentrations ranged over 0.25–25 μg/100 ml. Sensitivity of the T_4 curve over 12 assays was generally 0.2 ng, which corresponds to a T_4 concentration of 0.5 μg/100 ml.

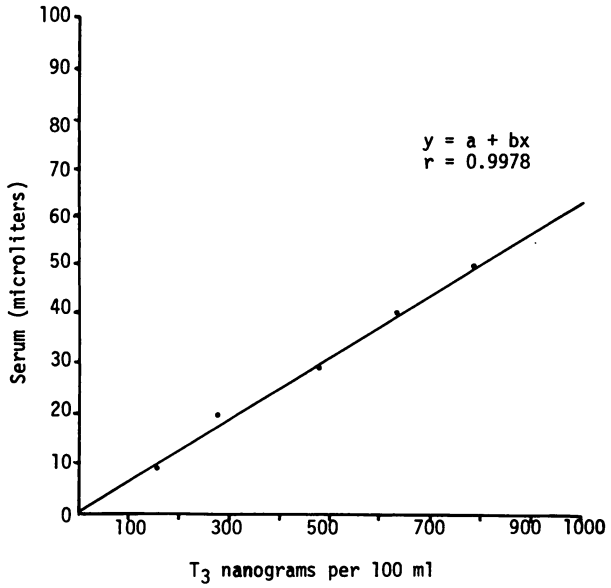

FIG. 3. Hyperthyroid serum assayed at various dilutions versus T_4 concentration. Coefficient of correlation was 0.9978.

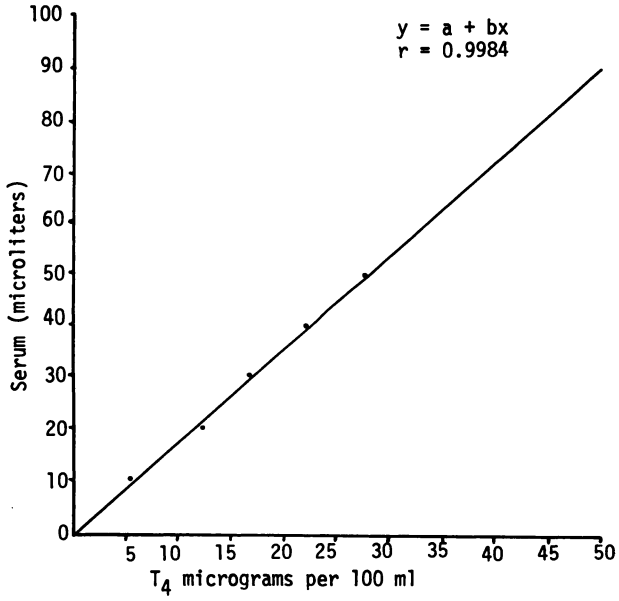

FIG. 4. Hyperthyroid serum assayed at various dilutions versus T_4 concentration. Coefficient of correlation was 0.9984.

Nonspecific binding of the labeled hormones was 2–3%.

Recovery of T_3 and T_4 added to serum. Unlabeled T_3 was added to euthyroid, hypothyroid, and normal pregnant serum in concentrations of 100–375 ng/100 ml, and the recovery was 99.9, 97.5, and 102.6%, respectively.

Unlabeled T_4 was added to euthyroid, hypothyroid, and euthyroid pregnant sera in concentrations of 2.5–18.8 μg/100 ml; the recovery was 100.3, 96.4 and 100.5%, respectively.

Patient dilution curve. Figures 3 and 4 depict the correlation between the amount of serum assayed and the T_3 and T_4 concentrations measured. Correlation for the T_3 measurement was 0.9978, and for the T_4 measurement 0.9984.

Single-tracer RIAs compared with double-tracer T_3–T_4 procedure. Sera from 74 patients representing the various thyroid states were assayed in both single- and double-tracer T_3 and T_4 RIA systems, and the correlation between the two systems was 0.97 for T_3 and 0.99 for T_4 (Figs. 5 and 6). Single T_3 and T_4 radioimmunoassay determinations were performed as described in previous papers (*2,3*).

Reproducibility of the T_3–T_4 RIA. Interassay variability was evaluated by duplicate measurements of Lederle RIA Control Serum I and II, lot numbers 392-111 and 392-112, respectively. The T_3 concentration of Lederle Control Serum I was 129 ± 10 ng/100 ml; the coefficient of variation (CV) over 12 assays was 7.9%. Control Serum II assayed at 255 ± 21 ng/100 ml (CV = 8.3%).

The T_4 concentration of Lederle Control I was 7.8 ± 0.45 μg/100 ml; coefficient of variation over 12 assays was 5.8%. Control Serum II assayed at 14.2 ± 1.2 μg/100 ml (CV = 8.4%).

Intra-assay variability was evaluated by duplicate determinations of a euthyroid serum assayed as four pairs, and the CVs were found to be 1.9% and 1.6% for T_3 and T_4, respectively.

Clinical results. The T_3 and T_4 concentrations in patients representing various typical thyroid states are shown in Tables 1 and 2, respectively. The mean

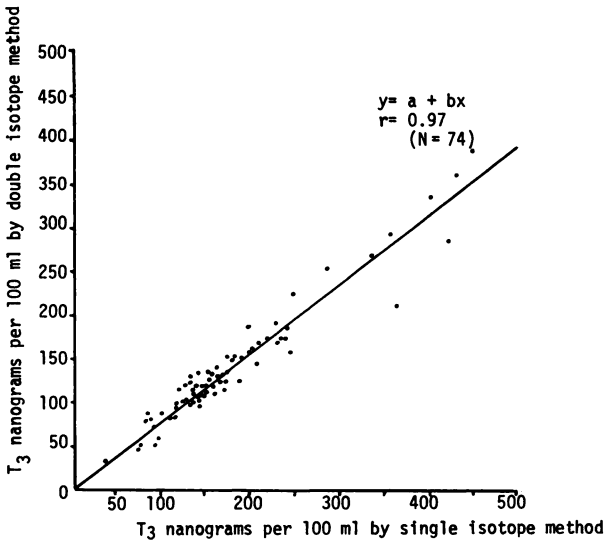

FIG. 5. Correlation between results obtained for T_3 using single-tracer and double-tracer RIA systems.

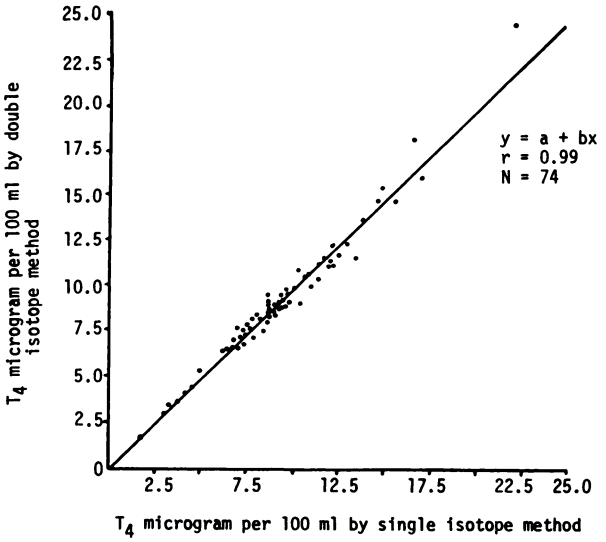

FIG. 6. Correlation between results obtained for T_4 using single-tracer and double-tracer RIA systems.

T_3 concentration in 84 euthyroid subjects was 130 ± 29 ng/100 ml (mean ± s.d.). The T_4 concentration was 8.7 ± 1.4 μg/100 ml.

Shortened T_3–T_4 double-tracer assay. For those laboratories that want to obtain T_3–T_4 results in a single work day, it is possible to do so simply by increasing the amount of carrier normal rabbit serum and goat antirabbit serum used in the assay. For example, in our laboratory we increased our carrier NRS to 2.5% and reduced the incubation periods as follows:

1. Primary incubation period was reduced to 1 hr at 37°C.
2. One-tenth milliliter of a 1:6 dilution of goat antirabbit serum was added; the tubes were vortexed and incubated at 4°C for 1.5–2 hr.

Regardless of which incubation period was used, standard curves were superimposable, and there was no change in values of pooled sera representing hypo-, hyper-, and euthyroid states. Table 3 shows results obtained on these pooled sera over 12 different assays using long and short incubation times.

TABLE 1. SERUM T_3 CONCENTRATIONS IN VARIOUS GROUPS OF PATIENTS

Clinical status	No.	T_3* (ng/100 ml)	Range (ng/100 ml)
Euthyroid	84	130 ± 29	80– 213
Hypothyroid	21	45 ± 23	12– 115
Hyperthyroid	17	397 ± 213	251–1178
Euthyroid pregnant	54	222 ± 42	114– 294

* Mean ± s.d.

TABLE 2. SERUM T_4 CONCENTRATIONS IN VARIOUS GROUPS OF PATIENTS

Clinical status	No.	T_4* (μg/100 ml)	Range (μg/100 ml)
Euthyroid	84	8.7 ± 1.4	6.5 –12.0
Hypothyroid	21	2.6 ± 1.4	0.39– 4.8
Hyperthyroid	17	18.2 ± 6.2	12.2 –39.3
Euthyroid pregnant	66	12.8 ± 2.1	7.5 –16.4

* Mean ± s.d.

DISCUSSION

Major improvements in the measurement of T_3 and T_4 have been accomplished by the addition of ANS, a fluorescent dye that blocks the interference of thyroxine-binding globulin in the radioimmunoassay system (*4,5*). This eliminates the alcohol extraction step required in the older thyroxine assays using competitive protein binding.

The double-tracer radioimmunoassay appears to offer an accurate, sensitive, and efficient method for the simultaneous determination of serum T_3 and T_4 concentrations. The excellent recovery of exogenous

TABLE 3. COMPARISON OF ASSAY RESULTS ON POOLED SERA USING LONG AND SHORT INCUBATION PERIODS

		Original incubation period			Shortened incubation period		
Assay	Pooled sera	Mean*	s.e.m.	CV†	Mean*	s.e.m.	CV†
T_3	Euthyroid	130	2.7	7.2%	132	2.5	6.6%
T_3	Hypothyroid	53	2.3	15.1%	56	1.3	8.3%
T_3	Hyperthyroid	330	4.9	5.1%	331	4.6	4.8%
T_4	Euthyroid	7.9	0.12	5.1%	8.0	0.12	5.3%
T_4	Hypothyroid	2.2	0.04	6.0%	2.2	0.02	3.6%
T_4	Hyperthyroid	16.7	0.24	4.9%	16.9	0.25	5.2%

* T_3 (ng/100 ml); T_4 (μg/100 ml).
† Interassay variability over 12 assays.

T_3 and T_4 added to various serum samples, the excellent correlation coefficient for different dilutions of hyperthyroid serum versus the amount measured, and the satisfactory inter- and intra-assay variability all serve to validate this RIA procedure. Specificity of the antibodies is such that there was no significant cross-reactivity of T_4 in the T_3 measurements, and vice versa. This was confirmed by the excellent correlation between results obtained for T_3 and T_4 using single- and double-tracer RIA systems.

The T_3 and T_4 serum concentrations in euthyroid subjects reported here are in reasonable agreement with those reported for another double-tracer procedure in which charcoal is used to separate bound and free labeled hormone (*6*). Simultaneous assay of T_3 and T_4 provides an approximately 50% reduction in man-hours over the two single-tracer methods. In spite of the 8-day half-life of ^{131}I-T_4, we have been able to use this preparation for 2 weeks without any detrimental effects on the assay.

FOOTNOTES

* Abbott Laboratories, North Chicago, Ill.

† Amersham/Searle, Arlington Heights, Ill.

‡ 8-Anilino-1-naphthalene sulfonic acid (Sigma Chemical Co., St. Louis, Mo.).

|| Packard Model 5260, Downers Grove, Ill. Experiments were conducted to determine the correction factor needed to correct the ^{125}I-T_3 counts for the ^{131}I-T_4 Compton effect. Because of the extremely high count rate of the high-specific-activity ^{125}I-T_3 compared with the relatively weak ^{131}I-T_4, we determined that Compton correction was not required. No significant difference in clinical values was observed between samples that had been corrected and those that had not.

¶ Hewlett–Packard 9830A calculator (Waltham, Mass.).

** Supplied as free acids by Sigma Chemical Co.

REFERENCES

1. BROWN ML: Radioimmunoassay and its application in the measurement of triiodothyronine. *Am J Hosp Pharm* 30: 336–339, 1973

2. BROWN ML, HANNAH T: An improved method for the measurement of serum thyroxine. *J Nucl Med Technol* 4: 34–37, 1976

3. BROWN ML, METHEANY J: Use of 8-anilino-1-naphthalenesulfonic acid in radioimmunoassay of triiodothyronine. *J Pharm Sci* 63: 1214–1217, 1974

4. CHOPRA IJ, HO RS, LAM R: An improved radioimmunoassay of triiodothyronine in serum: Its application to clinical and physiological studies. *J Lab Clin Med* 80: 729–739, 1972

5. CHOPRA IJ: A radioimmunoassay for measurement of thyroxine in unextracted serum. *J Clin Endocrinol Metab* 34: 938–947, 1972

6. MITSUMA T, COLUCCI J, SHENKMAN L, et al.: Rapid simultaneous radioimmunoassay for triiodothyronine and thyroxine in unextracted serum. *Biochem Biophys Res* 46: No. 6, 2107–2113, 1972