Simplification of Radioimmunoassay for Triiodothyronine: Evaluation of a New Commercial Kit

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A new triiodothyronine (T3) radioimmunoassay kit offers simplicity of method, short incubation time, specificity, and reproducibility. 125I-labeled T3 and unknown serum (or standard) are added to alkaline buffered (pH 12.4) Sephadex G-25 columns; the T3 is freed of proteins with an alkaline buffer wash, antiserum is added, and the columns are incubated at room temperature for 2 h. "Free" hormone (i.e., unbound to antiserum) remains on the column after a second wash. The radioactivity on the column is counted and related to the total activity added. The T3 standards produced a straight line on a log-log plot; reactivity of analytical-grade thyroxine was 0.6% on weight basis; and the analytical recovery of T3 added to a T3-free serum was complete. Euthyroid, hyperthyroid, and hypothyroid ranges were 0.74–2.64, 2.91–7.52, and 0.121 μg/liter, respectively. These values correlated well with both the clinical status of patients and with values obtained by an established radioimmunoassay method. The procedure is simply done; results may be obtained in less than 4 h.

Triiodothyronine (T3) in serum has been measured by several modifications of a basic radioimmunoassay (RIA) (1–4). The results have been invaluable in diagnosing thyroid dysfunction and in clarifying physiological processes (5).

A commercially prepared kit that reliably and specifically detects T3 would be welcomed by smaller laboratories that offer services to many clinicians, but do not receive the volume of requests necessary to justify preparation of the several components of the assay. In addition to accuracy, the kit must provide an uncomplicated technique and economy. We report an evaluation of a new commercial kit for radioimmunoassay of serum T3.

Materials and Methods

Kit and Procedure

Material supplied in the kit ("Seralute Total T3 RIA"; Ames Company, Elkhart, Ind. 46514) include Sephadex G-25 columns with alkaline buffer, antiserum to T3 (rabbit, lyophilized), T3 standard (lyophilized, in a matrix of bovine serum albumin), T3 buffer (anhydrous Na2HPO4, with added ethylenediamine tetraacetate), and 125I-labeled T3. The kits were stored at room temperature (23 °C) before reconstitution of the standards and antiserum, after which these reagents were frozen between uses.

Duplicate columns were used throughout this series and the mean value is reported. In the assay, 0.2 ml of serum and radioactive T3 are mixed on the column; at pH 12.4, the T3 in the serum is released from the proteins and the latter drained off the column with the highly alkaline buffer, leaving both labeled and unlabeled T3 bound to the Sephadex. Four milliliters of the T3 buffer, pH 7.4, is added and the column allowed to drain, then 0.5 ml of antiserum is passed through the column.

During a 2-h incubation at room temperature, the columns were counted in an automated gamma counter for 1 min (~40 000 counts) and equilibrium was established between labeled/unlabeled T3 and antibody/Sephadex. After incubation, the columns were washed a second time with the buffer to remove the antibody T3 complex, counted again, and these values related to respective total activity. The results, expressed as percent "free" hormone (unbound to antibody) or the reciprocal of antibody-bound hormone, are directly proportional to the unknown T3 concentration.

The directions given in the package insert recommend a three-point standard curve, prepared by pipetting different quantities of a standard solution into the columns and plotting the results. Since more points are desirable, the procedure was modified to include more than three points in the standard curve (Figure 1). For the necessary volume, standards from four kits were obtained and diluted with distilled water to give nine 0.2-ml aliquots with concentrations of 0.50, 0.75, 1.00, 1.50, 2.50, 3.00, 4.50, 6.00, and 10.00 μg/liter. Buffer (0.2 ml) added to columns instead of T3 standard provided a "zero" or trace concentration, and 125I-T3 added to columns, but without antiserum, provided the blank values. According to the manufacturer the antibody dilution was 15 000-fold, but the specific activity of the labeled T3 is uncertain.

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**Fig. 1. Standard curve, plotted as suggested by the manufacturer**

Note the nonlinearity above 3.00 μg/liter.
Clinical Material

Sera of 15 euthyroid volunteers and 19 hyperthyroid, 25 hypothyroid, and 41 euthyroid patients as well as seven subjects who were pregnant or on oral contraceptives were assayed. Diagnoses were established by clinical evaluation and other laboratory tests including serum thyroxine by competitive binding, T3 resin uptake, 24-h radioiodine uptake, and thyrotropin estimations. Assessments of the patients' courses during subsequent weeks confirmed the diagnoses.

Aliquots of sera from 82 of the subjects were sent to Bio-Science Laboratories, 7600 Tyrone Ave., Van Nuys, Calif. 91405, for measurement of T3 by the radioimmunoassay described by Pileggi et al. (6), with use of 8-anilino-1-naphthalene sulfonic acid to inhibit serum protein binding and of dextran-coated charcoal to bind the free T3. We did not specifically assess the performance of the kit with samples with low protein concentrations; if used with such samples, kit performance should be verified.

Results

The standard curve, plotted as suggested by the Ames Co. (Figure 1) for percent retention, was essentially linear from 0–3.00 µg/liter, but nonlinear for greater concentrations. Figures 2 and 3 show additional plots of standard responses (% T3 bound vs. dose on a logarithmic scale and % T3 bound on a logit scale vs. dose on logarithmic scale, respectively).

The responses obtained from serially diluted samples of serum from a hyperthyroid patient parallel the standard curve (Figure 3); the reactivity of analytical-grade thyroxine (Sigma Chemical Co., St. Louis, Mo. 63178) was about 0.6% that of triiodothyronine by weight. Analytical recovery (mean of duplicates) of AR grade T3 added to T3-free serum was 97% at 0.50 µg/liter, 101% at 1.50 µg/liter, and 103% at 5.00 µg/liter.

Tryptiodothyronine binding by antibody was plotted vs. duration of incubation (Figure 4); equilibrium was approached in 2 h. The coefficient of variation for 10 aliquots of a pooled serum was 8% at a mean concentration of 1.88 µg/liter. The inter-assay CV of a sample tested on eight different occasions was 13.6% at a mean concentration of 1.27 µg/liter.

Figure 5 summarizes the results of the clinical study. 95% confidence limits for the euthyroid range were found to be 0.84–2.64 µg/liter, while hyperthyroid and hypothyroid individuals had T3 ranges of 2.91–7.52 µg/liter and 0–1.21 µg/liter, respectively. As expected, abnormally high serum T3 concentrations (2.24 ± 0.53 µg/liter) were found in sera of women who were pregnant or were taking oral contraceptives.

Results of assays for T3 in patients' sera at Bio-Science Laboratories (6) (normal range, 0.90–1.90 µg/liter) correlated well with those obtained by the kit method (r = 0.945), but the values by the latter procedure were about 1.3-fold higher.
Discussion

Use of the three point (0.75, 3.00, and 6.00 μg/liter) standard response curve advocated by the manufacturer would have limited precision; furthermore, for values exceeding 3.00 μg/liter, extrapolation of a straight line would give erroneous results (Figure 1). When plotted on a semilog graph, it is apparent that values of less than 0.50 μg/liter and exceeding 8.00 μg/liter (Figure 2) became imprecise.

Log-logit transformation of data (Figure 3) permits ease in using the standard curve to determine values for serum samples and to compare the displacement of T3 with that recorded for potential interfering substances, such as thyroxine. It would also permit computer analysis (7). The parallelism with the standard curve for serially diluted hyperthyroid serum indicates the specificity of the assay. The reactivity of thyroxine, which paralleled that of T3, may be due to its presence as a contaminant in the analytical-grade thyroxine and is not too different from that reported by others (3, 8).

The inter-assay CV of 13.6% compares well with that obtained by Patel et al. (8) and Pileggi et al. (6), but greater than that reported by Sekadde et al. (9). However, according to Challand et al., an inter-assay CV of less than 6% is rarely achieved for immunoassay procedures (10).

Equilibrium is approached in 2 h and no significant change occurs between 2 and 3 h of incubation; however, the time to attain equilibrium for sera containing increased concentrations of thyroid-binding globulin was not determined. With this possible exception, time of incubation does not appear to be critical after 2 h.

The overlap was between values obtained for sera from euthyroid and hypothyroid patients (Figure 5), which may reflect physiological compensatory changes in the latter subjects. Abnormally high T3 concentrations would be expected in sera that harbor increased thyroid-binding proteins, an expectation verified in the results for women who were pregnant or were receiving estrogen-containing drugs.

T3 values by the present method correlate well with those obtained by an established radioimmunoassay procedure (6) and with the clinical status of the patients. The consistently higher concentrations obtained by use of the kit may result from better separation of T3 from the binding proteins by the alkaline pH than by 8-anilino-1-naphthalene sulfonic acid (Bio-Science Laboratories method), a supposition implied by the kit's normal range, which approaches that observed after thermal inactivation of thyroxine-binding globulin, a technique thought to be most effective in separating T3 from binding proteins (11).

The procedure is quite simple and can be completed in less than 4 h. Euthyroid and hyperthyroid subjects are reasonably accurately distinguished. Precision is lost in the hyperthyroid range, but individuals with subnormal thyroid function may have, in any case, somewhat variable concentrations of serum T3. It would be advisable to use a standard curve based on at least six concentrations of T3, with the highest value exceeding 7.00 μg/liter (12)—indeed, use of eight points is advocated for optimum precision (13).

References