CLINICAL EVALUATION OF A NEW INDIRECT INDICATOR OF SERUM-FREE THYROXINE CONCENTRATION

Cynthia M. Abreau, Fereidoun Azizi, Apostolos G. Vagenakis, Sidney H. Ingbar, and Lewis E. Braverman

St. Elizabeth's Hospital, Tufts Medical School, Thorndike Memorial Laboratory, Harvard Medical Service, Boston City Hospital, and Harvard Medical School, Boston, Massachusetts

A rapid, simple test for indirectly estimating serum-free T_4 concentration is described which accurately assesses thyroid function irrespective of the status of the serum T_4 binding proteins. This test is a modification of the Tetralute method for determining serum T_4 concentration. Values obtained by the present method correlate well with those obtained by another standard indirect indicator of free T_4 concentration, the thyroxine-resin T_3 index.

It is generally agreed that the concentration of free or unbound thyroxine (T_4) in serum is the single most useful clinical test in assessing thyroid function since it is the free hormone which is available to the peripheral tissues for hormonal action. Thyroxine is almost entirely bound to plasma proteins, primarily the T_4 binding globulin (TBG) (*1*). Thus the measurement of total T_4 is influenced by both the quantity of hormone secreted by the thyroid and the concentration or binding affinity of the serum T_4 binding proteins. TBG is altered in many clinical states such as an increase secondary to excess estrogens as in pregnancy and in women receiving oral contraceptives and a decrease secondary to androgenic-anabolic steroids (*1*). Thus, total T_4 concentration may be increased or decreased secondary to alterations in TBG, which may lead to a false diagnosis of hyper- or hypothyroidism. However, increases or decreases in serum TBG result in reciprocal changes in the percentage of free T_4 in serum. Many techniques for directly measuring the percentage of serum-free T_4 have been described but require prolonged dialysis of ^{131}I-labeled T_4 enriched serum (*2–4*). The concentration of free T_4 is then calculated as the product of the percentage free T_4 and total T_4 concentration. An indirect measure of the percent free T_4, the resin T_3 uptake test (*5*), obviates the necessity for measuring this parameter by direct dialysis techniques. The thyroxine-resin T_3 index (T_4-RT_3 index), the product of the resin T_3 uptake and total serum T_4 concentration, is an indirect estimate of serum-free T_4 concentration and correlates well with the direct measurement of serum-free T_4 concentration (*6*). The T_4-RT_3 index also requires two separate tests which is time consuming and incorporates the errors which may occur in carrying out each test.

In an attempt to devise a simple, one test procedure for indirectly measuring serum-free T_4 concentration, a modification of the Tetralute isotopic displacement technique* for measuring serum T_4 was devised using small Sephadex columns (*7*). This test was compared with the T_4-RT_3 index in euthyroid, hyperthyroid, and hypothyroid patients as well as in patients with alterations in serum TBG. Excellent agreement between the two tests was found and the modified Tetralute test (free T_4 equivalent) accurately predicted thyroid status.

MATERIALS AND METHODS

Basic principles. All reactions are carried out in small plastic columns containing Sephadex G-25 equilibrated with 0.1 *N* NaOH providing a pH greater than 11. A standard quantity of serum (0.3 ml) is mixed with a small volume of an alkaline solution of ^{125}I-labeled T_4 above the surface of the column, and this mixture is allowed to enter the column where the high pH results in a rupture of the T_4-serum protein bond, freeing the T_4 for binding to the Sephadex. The column is then washed with barbital buffer, pH 8.6, 0.075 *M*, which elutes the serum proteins and any free ^{125}I-iodide off the column, and lowers the pH toward 8.6. The radioactivity of the column is then measured in a well scintillation counter.

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For reprints contact: Lewis E. Braverman, St. Elizabeth's Hospital, 736 Cambridge St., Boston, Mass. 02135.

* Ames Co., Div. Miles Laboratory, Inc., Elkhart, Ind.

A standard solution of human α-globulin dissolved in barbital buffer, pH 8.6 and 0.02 ml of the test serum, are then added to the column, where at pH 8.6, binding of T_4 to the proteins is favored over that to Sephadex. The column is washed again with barbital buffer, eluting the α-globulin mixture with its bound T_4. The column is again counted to determine the proportion of the original counts retained. By using a portion of the patient's serum to help elute ^{125}I-T_4 from the Sephadex column, the serum T_4 binding capacity also becomes a factor in the percent retention of ^{125}I-T_4 on the column. The test conditions are set in such a way that the contribution a normal serum makes to the total binding capacity of the eluting mixture is approximately one-third. This condition is justified from data showing that the same percent retention can be achieved if 100% increase in binding protein is offset by a 200% increase in serum T_4 originally applied to the column. Since most euthyroid pregnancy sera have both T_4 and TBG increased by approximately 100%, the increased binding capacity of the eluting mixture contributed by the 0.02 ml of pregnant serum is offset by the increase in T_4 concentration of the pregnant serum originally applied to the column.

The results obtained in each test serum are compared to that obtained in a reference solution assigned a value of 1. The reference solution is prepared by adding 0.1 *N* NaOH to a vial of lyophilized standard serum so that the alkaline solution contains 2.0 μg T_4I/100 ml. Since the solution pH is above 11, the serum reference solution has essentially no T_4 binding. The reference solution is run concurrently with the test serum. Since the reference solution has no binding capacity, one-third of the total binding capacity of the eluting mixture is lost. However, to offset this effect, only 6 ng T_4I in 0.3 ml is added to the column, rather than an average of 18 ng per column in normal serum (6.0 μg T_4I/100 ml).

Test procedure. Tetralute test kits were used throughout the procedure. The following modifications of the standard Tetralute T_4 procedure were carried out. Add 8.0 ml barbital buffer to a vial of lyophilized human α-globulin eluting reagent. The reference solution is prepared by combining the 0.1 *N* NaOH solution above four Sephadex columns and adding 2.5 ml of this solution to a vial of lyophilized standard human serum resulting in a final concentration of 20 ng T_4I/ml. All tests are carried out at room temperature.

1. Discard the top cap of the column and discard the liquid.
2. Add seven drops of ^{125}I-T_4 solution onto the column.
3. Pipette 0.3 ml serum or reference solution onto the column and swirl gently.
4. Remove and save the bottom cap of the column and place the column over a waste receptacle. Wait until no more liquid drains from the bottom of the column.
5. Add 4 ml of barbital buffer and again allow the column to drain.
6. After the column has drained, blot the tip on a paper towel, replace the bottom cap, and count the column in a well counter.
7. Pipette 0.5 ml of eluting reagent (human α-globulin) and 0.02 ml serum or reference solution onto the column and gently swirl.
8. Remove the bottom cap and allow to drain completely.
9. Add 4 ml buffer and allow to drain. Blot the tip with a paper towel, replace the bottom cap, and count the column in a well counter.
10. Calculate percent retention of test serum and reference solution as follows:

$$\% \text{ retention} = \frac{\text{final counts on the column} \times 100}{\text{initial counts on the column}}.$$

11. Free thyroxine

$$\text{equivalent (FTE)} = \frac{\% \text{ retention test serum}}{\% \text{ retention reference solution}}.$$

Thyroxine-resin T_3 index. T_4-RT_3 index was calculated as the product of the T_4I concentration (Tetralute method) and the resin T_3 uptake (Trilute method)*.

Clinical evaluation. Sera were obtained from 62 euthyroid healthy or hospitalized subjects, 20 hyper- and 21 hypothyroid subjects, 10 pregnant women and 5 patients with hereditary absent TBG. Samples of serum were analyzed in duplicate on the same day for FTE, Tetralute T_4I and Trilute resin T_3 uptake. The diagnosis of hyperthyroidism or hypothyroidism was confirmed clinically in all subjects and by ^{131}I uptake in most. The T_4 binding capacity of TBG was measured by reverse-flow paper electrophoresis in glycine acetate buffer, pH 8.6 (*8*).

RESULTS

Serum T_4I concentration (Fig. 1). The normal range for serum T_4I concentration in this laboratory is 2.6–7.6 μg/100 ml. Serum T_4I in the present euthyroid patients were within this range, averaging

* Ames Co., Div. Miles Laboratory Inc., Elkhart, Ind.

$T_4 - RT_3$ index = T_4I concentration $\times \frac{\text{resin } T_3 \text{ uptake}}{\text{mean resin } T_3 \text{ uptake in euthyroid subjects}}$.

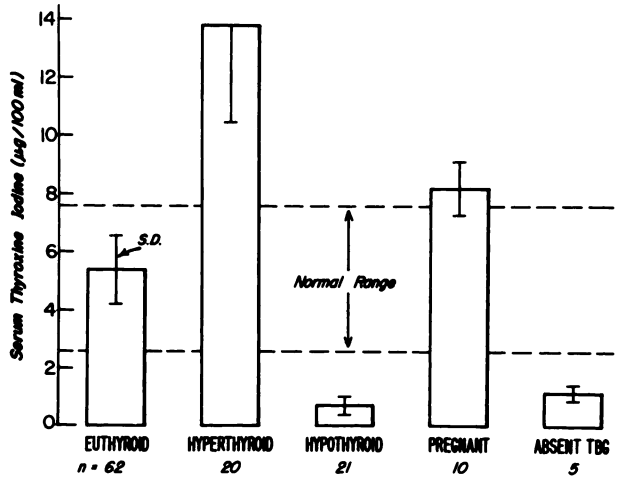

FIG. 1. Serum T_4I concentration (mean ± s.d.) in various clinical categories. n = number of subjects in each group.

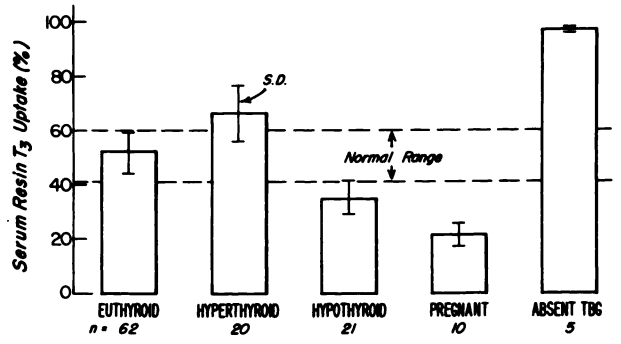

FIG. 2. Serum resin T_3 uptake values (mean ± s.d.) in various clinical categories. n = number of subjects in each group. Normal range as reported by manufacturer.

5.4 ± 1.2 μg/100 ml (mean ± s.d.). The serum T_4I concentration in 20 hyperthyroid patients was elevated, averaging 13.8 ± 3.4 μg/100 ml, while T_4I was decreased in 21 patients with hypothyroidism, averaging 0.5 ± 0.5 μg/100 ml. As expected, the serum T_4I concentration was elevated in eight of ten pregnant subjects (8.2 ± 0.9 μg/100 ml) due to the increase in the T_4 binding capacity of TBG. In five male subjects with absent serum TBG, the serum T_4I concentration was decreased (1.2 ± 0.2 μg/100 ml).

Serum resin T_3 uptake (Fig. 2). Normal values for resin T_3 uptake as reported by the manufacturer are 43–60% for males and 41–55% for females. Values in 15 euthyroid males averaged 56.6 ± 9.6% and in 47 euthyroid females, 50.7 ± 6.3%. Some of these euthyroid subjects were sick with various illnesses. Thus, it was not surprising that the resin T_3 uptake was abnormal in 13 (8 increased and 5 decreased) since T_4 binding may be abnormal in various acute and chronic illnesses (*1*). The resin T_3 uptake was increased in 18 of 20 hyperthyroid patients, averaging 66.1 ± 10.5%. The two patients with normal resin T_3 uptakes were receiving oral contraceptive pills, and the T_4 binding capacity of TBG was elevated in both (32.2 and 32.3 μg T_4/100 ml, normal value 21.3 ± 3.6). The resin T_3 uptake was decreased in 20 of 21 patients with hypothyroidism (34.9 ± 5.3%), was low in all 10 pregnant subjects (21.9 ± 4.1%), and was strikingly increased in the 5 patients with absent TBG (96.4 ± 0.6%).

Thyroxine-resin T_3 index (Fig. 3). The T_4-RT_3 index in the 62 euthyroid subjects ranged from 3.3 to 9.4, averaging 5.7 ± 1.3. The index was elevated in all hyperthyroid patients (20.6 ± 10.1) and decreased in all hypothyroid patients (0.4 ± 0.3). Eight of the ten pregnant subjects had a normal T_4-RT_3 index, averaging 3.8 ± 0.8. The abnormal values in two pregnant subjects were only slightly low (2.8 and 2.7) and far above the hypothyroid range. Values in the patients with absent TBG were somewhat low, averaging 2.2 ± 0.4, but all were above the hypothyroid range.

Free thyroxine equivalent (Fig. 4). Values for the euthyroid subjects ranged from 0.74 to 1.30 with

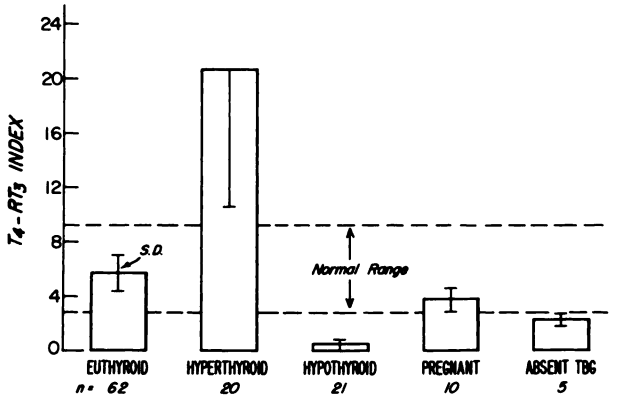

FIG. 3. T_4-RT_3 index (mean ± s.d.) in various clinical categories. n = number of subjects in each group.

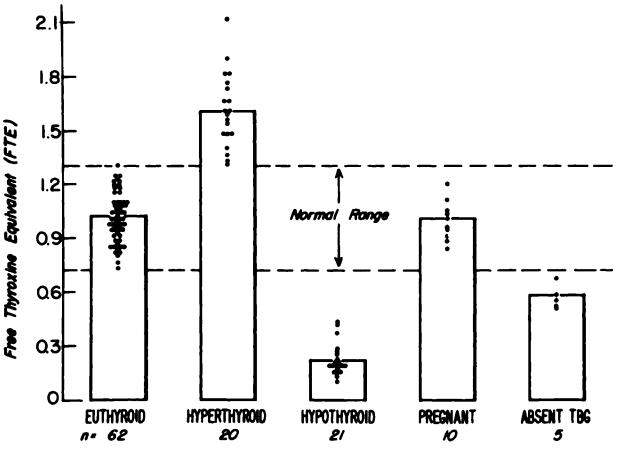

FIG. 4. Free T_4 equivalent in individual subjects in various clinical categories. n = number of subjects in each group.

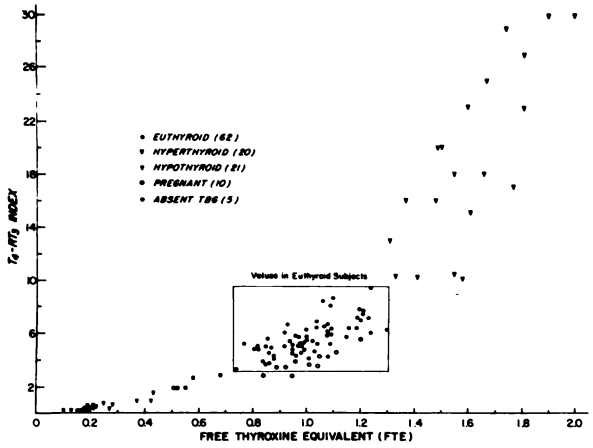

FIG. 5. Comparison of T_4-RT_3 index and free T_4 equivalent in individual subjects in various clinical categories.

a mean of 1.01 ± 0.13. All values in the hyperthyroid patients were increased (1.61 ± 0.20) and all values in the hypothyroid patients decreased (0.22 ± 0.09). The test was normal in the pregnant subjects, averaging 1.0 ± 0.1. Although values for the FTE were decreased in the five subjects with absent TBG (0.57 ± 0.07), none were in the hypothyroid range. Duplicate tests in all patients were very similar, the range of difference varying from 0 to 0.09 with a mean difference of 0.04 ± 0.02.

As can be seen in Fig. 5, the FTE and T_4-RT_3 index in individual subjects accurately differentiated patients with hyper- or hypothyroidism from euthyroid subjects, irrespective of abnormalities in serum TBG. Both values were increased in patients with hyperthyroidism and decreased in hypothyroid patients, while values in pregnant patients were normal. Values in subjects with absent TBG were somewhat low but not decreased into the hypothyroid range.

DISCUSSION

A rapid, simple, accurate method for indirectly estimating the concentration of free T_4 in small quantities of serum has been described which correlates well with the T_4-RT_3 index, obtained as the product of the serum T_4I concentration and resin T_3 uptake. Other one-test methods for indirectly estimating serum-free T_4 similar to the present test have been described which appear to be as accurate in assessing thyroid function but are more cumbersome and time consuming (*9–11*). In contrast to these methods, the present test requires only 0.32 ml of serum, no extraction of serum with ethanol, and no centrifugation or use of a rotary mixer. All steps are carried out within the small Sephadex column, and 28 tests, including the reference solution, can be completed in approximately 90 min. Since it is desirable to also measure the total serum T_4 concentration, the same test materials may be used and the standard Tetralute method carried out. (A further modification of the present method was suggested by H. J. Dworkin and others at the 19th annual Society of Nuclear Medicine meeting, July 1972 in Boston. This modification permits the determination of total T_4 by the Tetralute method, followed by the addition of a small quantity of the patient's serum and barbital buffer to the same column used for that patient's total T_4 determination, followed by a wash using barbital buffer. The column is again counted for residual radioactivity and the percent retention of ^{125}I-T_4 calculated by dividing this final count by the initial count obtained in the standard Tetralute method. The percent retention is then compared with that obtained in a concurrently run euthyroid normal serum pool. This ratio will be similar to that obtained by the present method. This new modification is now under investigation.)

Although the five patients with absent TBG in the present study had values for both the T_4-RT_3 index and FTE below the normal range, they were not in the hypothyroid range. This finding was not unexpected since we and others have reported slightly low values for serum-free T_4 concentration when measured by equilibrium dialysis (*12,13*). The present method will require further evaluation, especially in patients receiving replacement or suppressive doses of T_3, drugs such as phenylhydantoin, salicylates, and phenobarbital which inhibit T_4 binding or increase hepatic disposal of T_4 by increasing hepatic microsomal enzyme activity, and androgenic-anabolic steroids resulting in moderate decreases in the T_4 binding capacity of TBG.

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