Evaluation of T4 radioimmunoassay as a screening test for thyroid function

Comparison with effective thyroxine ratio

Manjula S. Kumar, Ph.D.
Department of Immunopathology

Ali M. Safa, M.D.
Department of Endocrinology

Sharad D. Deodhar, M.D., Ph.D.
Department of Immunopathology

O. Peter Schumacher, M.D., Ph.D.
Department of Endocrinology

Various tests have been developed for thyroid function based on the measurement of total serum thyroxine (T4) level, either by direct or indirect techniques. The direct assays include competitive protein binding assay and radioimmunoassay. Radioimmunoassay of T4 provides distinct advantages over competitive protein binding assays by eliminating extraction procedures and using a smaller amount of serum. Of the indirect assays, the most commonly used screening test for thyroid function is the commercially available in vitro blood test, effective thyroxine ratio (ETR).

The present studies describe the development and clinical evaluation of a double antibody radioimmunoassay for serum thyroxine and its comparison with ETR in different thyroid disorders.

Materials and methods

Production of antiserum. Antiserum was produced in rabbits by immunizing with T4 conjugated to bovine serum albumin (BSA) using the method for triiodothyronine (T3) antibodies described by Gharib et al.[1] Four rabbits were immunized weekly with 1 mg antigen; detectable antibodies were produced in 4 weeks and ade-

quate titers in 12 weeks. Antiserum from rabbit 1 (titer 1:5000) was used for these studies.

Preparation of T4 free serum. T4 free serum was prepared by removing exogenous T4 with charcoal adsorption. Normal human serum (100 ml), to which 10 g charcoal (Norit A) was added, was stirred overnight (24 hours) at 4C. Then the charcoal was removed by centrifugation: it was centrifuged three times at 13,000 rpm for 1 hour each. The process removed over 99% of added T4 ^{125}I without making significant change in total protein concentration.

Eight-anilino naphthelene sulfonic acid (Na salt) (ANS) was obtained from K&K Laboratories, Irvine, California. One hundred fifty milligrams ANS was dissolved in 100 ml barbital buffer (0.075 M, pH 8.6). L-thyroxine free acid form, used in antibody production, and L-thyroxine sodium salt, used for standards, were purchased from Sigma Chemical Co., St. Louis, Missouri.

L-thyroxine labelled with ^{125}I (T4 ^{125}I), specific activity 100 μg/mg was obtained from Industrial Nuclear, St. Louis, Missouri. T4 ^{125}I was diluted in barbital buffer (0.075 M, pH 8.6) to give approximately 10,000 cpm/0.4 ml. To 80 ml of this solution, 10 ml ANS solution (15 mg) was added and mixed.

Radioimmunoassay. The assay was performed in duplicates in 12 × 75 mm disposable plastic tubes. The tubes were marked for nonspecific binding (NSB), zero standard, standards and unknowns. To each tube was added 450 μl T4 ^{125}I, ANS solution; 10 μl T4 free serum together with standards (50 μl of various dilutions of T4, 5 ng/ml to 50 ng/ml) were added to standards, and 10 μl patient serum was added to unknowns. Then, 100 μl antiserum dilution (approximately 40% maximum binding) was added to all the tubes except for NSB tubes. The tubes were gently mixed and incubated 1^1/$_2$ hours at room temperature (approximately 25C). The separation of the bound from the free T4 ^{125}I was obtained by the addition of 100 μl of previously titered goat anti-rabbit gamma globulin (GAR). The tubes were mixed again and incubated 18 to 24 hours at 4C, and then centrifuged at 4000 rpm for 20 minutes. The supernate was aspirated and the precipitate (bound) was counted in a well-type automatic gamma counter.

Effective thyroxine ratio. The reagents for ETR were purchased from Mallinckrodt Diagnostics, St. Louis, Missouri, and the test was performed according to the manufacturer's instructions. The normal range was 0.86 to 1.13 (mean 0.98 ± .065).

Serum thyrotrophin (TSH) and T3 were measured by double antibody radioimmunoassay. Methods previously described were used with slight modification.[1, 2] The normal range for TSH was less than 2 to 6 μU/ml (mean 2.8 ± 1.0) and for T3 was 95 to 250 ng/dl (mean 173 ± 39).

Sources of sera. Serum specimens were obtained from 50 euthyroid, healthy subjects, 19 men and 31 women, aged 18 to 55 years. Specimens were also obtained from 10 euthyroid women taking oral contraceptives and from 24 pregnant women (third trimester).

A total of 69 serum specimens were obtained from hypothyroid patients, 52 women and 17 men, aged 13 to 74 years. Forty-six patients were clinically hypothyroid, whereas 23 had a clinical diagnosis of probable hypothyroidism which was later confirmed by elevated TSH levels. Etio-

logically, the patients were classified in four categories: idiopathic hypothyroidism, Hashimoto's thyroiditis, post ^{131}I therapy, hypothyroidism, and post-thyroidectomy hypothyroidism.

Serum specimens were also obtained from 56 hyperthyroid patients, 38 women and 18 men, aged 11 to 72 years. Fifty of these patients had a diagnosis of Graves' disease with hyperthyroidism, two had hyperthyroidism associated with Hashimoto's thyroiditis, two had adenomatous goiter, and two patients were diagnosed as T3 thyrotoxicosis with elevated T3 levels but normal T4 and ETR tests. Patients who were partially treated or taking estrogen or oral contraceptives were not included.

Results

Specificity. The antiserum was tested for the cross reaction with L-triiodothyronine (L-T3), diiodotyrosine (3'5' T2) and monoiodotyrosine (3 T1). With L-T3 the cross reaction was 4.8%; with 3'5' T2 and 3 T1 no significant cross reaction was noted (*Fig. 1*).

Effect of ANS on the antigen antibody reaction. In the presence of T4 free serum (20 μl) the antibody binding dropped from 50% to 6%. With the addition of ANS the binding was restored, provided the ANS concentration was between 15 μg to 75 μg. Addition of greater amounts of ANS also had the effect of inhibiting the binding (*Fig. 2*).

Effect of T4 free serum on the antigen antibody reaction. When increasing amounts of T4 free serum were added to the tubes containing antiserum and labelled T4, a gradual inhibition of labelled T4 bound to antibody was observed so that binding gradually decreased from 52% to 6%. The addition of 75 μg ANS resulted in restoring binding to 46% (*Fig. 3*).

Two standard curves were run simultaneously with and without 20 μl T4 free serum. The addition of T4 free serum shifted the curve slightly toward the lower side. Because of this difference in the curve, the addition of T4 free serum equivalent to unknown sample was considered a desirable step in the assay.

Reproducibility. The interassay variation was checked for low, medium, and high level T4 samples.

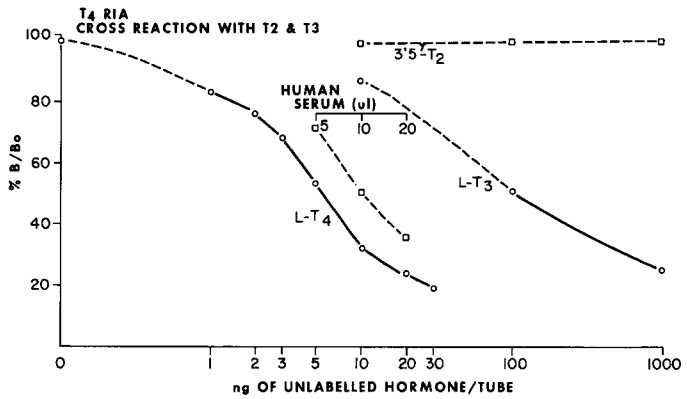

Fig. 1. Inhibition of labelled T4 bound to T4 antiserum by addition of increasing amounts of unlabelled T4, and also cross reaction of T4 antiserum with L-T3 and T2. Serum of hyperthyroid patients run in three dilutions.

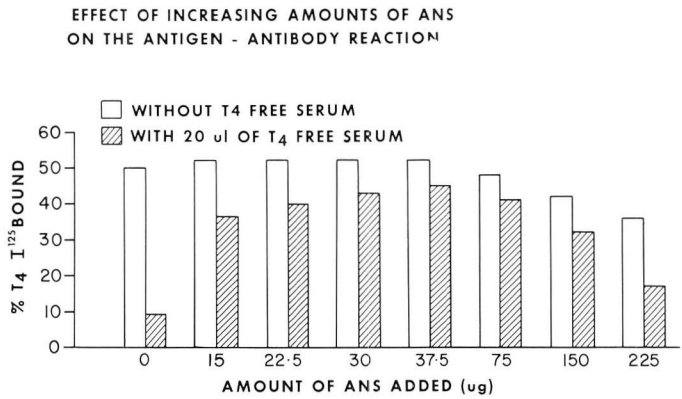

Fig. 2. Effect of increasing amounts of ANS on the antigen-antibody binding in the presence and absence of 20 μl of T4 free serum.

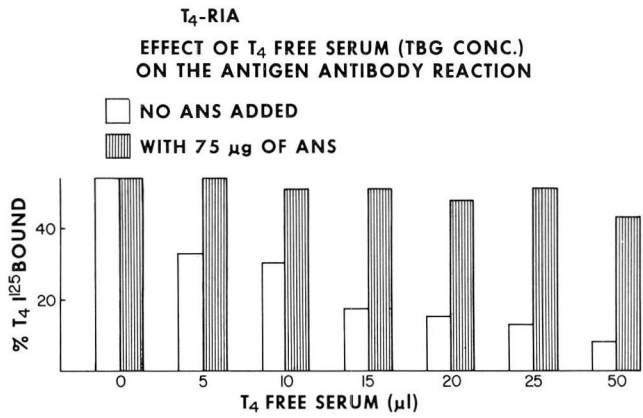

Fig. 3. Effect of increasing amounts of T4 free serum (TBG concentration) on the antigen-antibody reaction in the presence and absence of 75 μg of ANS.

Three control sera were repeated in 10 different assays. The mean values for these sera were 0.63 ± 0.1 (SD) μg/dl; 10.3 ± 1.0 μg/dl; and 21.9 ± 1.3 μg/dl with the coefficient of variation 15.8%; 9.7% and 5.9%, respectively.

Recovery of added T4. The accuracy of the assay was tested by adding 10 and 50 ng of T4 to 1 ml T4 free serum. The mean recovery was found to be 94 ± 11% (n = 10) and 99.5 ± 9.5% (n = 10), respectively.

Clinical evaluations

Normal controls. In 65 euthyroid subjects the mean T4 level was 6.4 ± 1.2 μg/dl with a range of 4 to 9 μg/dl. In 10 euthyroid women taking oral contraceptives, the mean T4 level was 7.9 ± 1.5 μg/dl with a range of 5.2 to 11 μg/dl, and in 24 euthyroid pregnant women (third trimester) the average T4 level was 9.4 ± 1.5 μg/dl with a range of 6 to 12 μg/dl (*Table*).

Hypothyroid patients. In 69 primary hypothyroid patients the mean

Table. Serum T4 concentrations in various clinical studies

Thyroid status	No. of patients			Serum T4	
	Male	Female	Total	M ± SD μg/dl	Range μg/dl
Euthyroid	24	41	65	6.4 ± 1.2	4.0–9.0
Euthyroid (oral contraceptives)		10	10	7.9 ± 1.5	5.5–9.5
Euthyroid (3rd trimester of pregnancy)		24	24	9.4 ± 1.5	6.0–12.0
Hypothyroid, primary	17	52	69	1.7 ± 1.3	0.2–5.0
Hyperthyroid	18	38	56	14.4 ± 4.6	8.5–30.0

M = mean; SD = standard deviation.

T4 level was 1.7 ± 1.3 (less than 0.2 to 5 μg/dl). The TSH level was elevated in all these patients with the mean level of 75.6 ± 50 μU/ml with a range of 20 to 240 μU/ml. Sixty-four (93%) of these patients had subnormal T4 levels and only 5 (7%) had T4 levels in low normal range (4 to 5 μg/dl). A good inverse correlation was observed between TSH and T4 (r = 0.71).

Hyperthyroid patients. In 56 clinically hyperthyroid patients the mean T4 level was 14.4 ± 4.6 with a range of 8.5 to 30 μg/dl. Fifty-three (95%) of these patients had elevated T4 levels, only 3 (5%) had levels in the high normal range (8.5 to 9 μg/dl).

Comparison of T4 and ETR test. Besides T4, ETR was also measured in the same 69 hypothyroid and 56 hyperthyroid patients. In the hypothyroid group the mean ETR was 0.81 ± 0.052, and in the hyperthyroid group the mean was 1.26 ± 0.12. Fourteen patients (20%) from the hypothyroid group and nine patients (16%) from the hyperthyroid group had ETR levels in the normal range. Of the 14 hypothyroid patients who had normal ETR (0.86 to 0.96), only five had normal T4 levels (4 to 5 μg/dl). TSH levels were elevated in all these patients (20 to 190 μU/ml). Eight of these patients had a diagnosis of idiopathic hypothyroidism, three had Hashimoto's thyroiditis, and two had hypothyroidism due to thyroidectomy and post ^{131}I treatment, respectively.

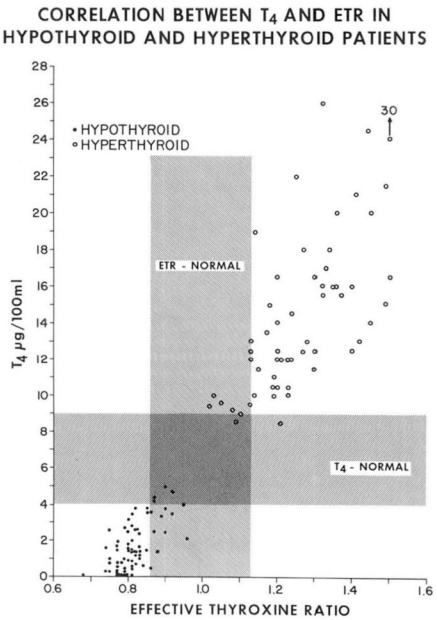

Fig. 4. Correlation between serum T4 and ETR in 125 patients with hypothyroidism and hyperthyroidism.

Of the nine clinically hyperthyroid patients who had normal ETR levels, seven had a diagnosis of Graves' disease and elevated T4 levels. Two had normal T4 but significantly elevated T3 (440 and 380 ng/dl), thus leading to a diagnosis of T3 thyrotoxicosis.

The correlation between ETR and TSH in the hypothyroid patients was poor with the correlation coefficient (r) of 0.43. A positive correlation was observed when T4 and ETR levels were compared in hypothyroid and hyperthyroid patients separately, and the correlation coefficient in the hypothyroid group was 0.61 and in the hyperthyroid group was 0.63.

Discussion

In previous studies antibodies to T4 had been produced in rabbits by immunizing with human thyroglobulin[3] or beef thyroglobulin[4] which showed 10% and 13% cross reaction with T3, respectively. In our experience, coupling T4 with bovine serum albumin using the method of Gharib et al[1] described for T3 gave an antiserum with greater specificity showing 4.8% cross reaction with T3.

Different TBG blocking reagents have been used for T4 RIA.[4, 5] In our experience ANS is effective, but the amount of ANS must be carefully titered according to the amount of serum sample used. Higher concentration of ANS can inhibit antibody binding with labelled antigen itself. Addition of T4 free serum equivalent to the sample size, to the standards was found to shift the standard curve slightly; therefore, it was considered a desirable step in the assay.

The euthyroid range (4 to 9 μg/dl) we obtained appeared to be slightly lower when compared with others. Chopra[3] and Larsen et al[4] observed a higher euthyroid range; the levels reported by Mitsuma et al[5] appeared to be quite comparable with our results.

Serum TSH levels have been found to be most diagnostic for primary hypothyroidism.[3, 6] From the 69 primary hypothyroid patients who had elevated TSH levels, 93% were found to have subnormal T4 levels. A good inverse correlation was observed between TSH and T4 in these patients. This indicates the diagnostic significance of T4 assay in these patients.

T3 thyrotoxicosis has been reported by a number of investigators.[7–9] In our study of 56 clinically hyperthyroid patients, two patients who had normal T4 and ETR levels were found to have significantly elevated T3 levels which led to the diagnosis of T3 thyrotoxicosis. This indicates the need of T3 determinations in hyperthyroid patients with normal T4 levels.

The diagnostic value of ETR in thyroid disease seems to be equal to that of free thyroxine index, since it is not influenced by changes in thyroxine binding proteins in serum. Comparison of ETR with free thyroxine index and with absolute free thyroxine in serum have been reported,[10, 11] but a comparison with total T4 in a large number of patients has not been reported before. We found a positive correlation between these two tests when hypothyroid and hyperthyroid patients were compared separately. As far as the diagnostic value of these tests is concerned, ETR test gave less satisfactory results than T4. With ETR, 20% of hypothyroid patients and 16% of hyperthyroid patients had values in the normal range. Similar results with this test have been reported.[11] In contrast, total T4 in these patients was found to be more diagnostic. In addition to this, a better inverse correlation was found between serum T4 and TSH than ETR and TSH in hypothyroid patients.

Thus, in our experience, T4 ra-

dioimmunoassay appeared to be a reproducible and reliable screening test for hypothyroidism and hyperthyroidism, especially when used in appreciation of alterations in thyroid binding proteins.

Summary

A radioimmunoassay for T4 has been evaluated as a screening test for hypothyroidism and hyperthyroidism by comparing T4 levels with the ETR test.

In 69 patients with primary hypothyroidism the mean T4 level was 1.7 ± 1.3 μg/dl, and the mean ETR was 0.81 ± .05. Ninety-three percent of these patients had clearly subnormal T4 levels, whereas 7% were in the low normal range; 20% had normal ETR. In 56 clinically hyperthyroid patients the mean T4 level was 14.3 ± 4.6 μg/dl and the mean ETR was 1.2 ± 0.12. Only 5% of these patients had high normal T4 levels; 16% had normal ETR. The correlation coefficient between T4 and ETR in the hypothyroid group was 0.61 and in the hyperthyroid group was 0.67.

References

1. Gharib H, Ryan RJ, Mayberry WE: Radioimmunoassay for triiodothyronine (T3): I. Affinity and specificity of the antibody for T3. J Clin Endocrinol Metab **33:** 509–516, 1971.
2. Mayberry WE, Gharib H, Bilstad JM: Radioimmunoassay for human thyrotrophin; clinical value in patients with normal and abnormal thyroid function. Ann Intern Med **74:** 471–480, 1971.
3. Chopra IJ: A radioimmunoassay for measurement of thyroxine in unextracted serum. J Clin Endocrinol Metab **34:** 938–947, 1972.
4. Larsen PR, Dockalova J, Sipula D, et al: Immunoassay of thyroxine in unextracted human serum. J Clin Endocrinol Metab **37:** 177–182, 1973.
5. Mitsuma T, Colucci J, Shenkman L, et al: Rapid simultaneous radioimmunoassay for triiodothyronine and thyroxine in unextracted serum. Biochem Biophys Res Commun **46:** 2107–2113, 1972.
6. Taunton OD, McDaniel HG, Pittman JA Jr: Standardization of TSH testing. J Clin Endocrinol Metab **25:** 266–277, 1965.
7. Sterling S, Refetoff S, Selenkow HA: T3 thyrotoxicosis; thyrotoxicosis due to elevated serum triiodothyronine levels. JAMA **213:** 571–575, 1970.
8. Mitsuma T, Nihei N, Gershengorn MC, et al: Serum triiodothyronine; measurements in human serum by radioimmunoassay with corroboration by gas-liquid chromatography. J Clin Invest **50:** 2679–2688, 1971.
9. Rappaport B, Ingbar SH: Production of triiodothyronine in normal human subjects and in patients with hyperthyroidism; contribution of intrathyroid iodine analysis. Am J Med **56:** 586–591, 1974.
10. Mincey EK, Thorson SC, Brown JL: A new parameter of thyroid function—the effective thyroxine ratio. J Nucl Med **13:** 165–168, 1972.
11. Kølendorf K, Siersbaek K, Nielsen JM, et al: Evaluation of thyroid status with the "effective thyroxine ratio" (E.T.R.). Acta Endocrinol **77:** 250–256, 1974.