EVALUATION OF SERUM THYROXINE

BY RADIOIMMUNOASSAY

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The results of radioimmunoassay for serum thyroxine levels have been compared with conventional saturation analysis techniques in normal euthyroid subjects, patients with thyroid dysfunction, and pregnant women. The results were equivalent except in the hyperthyroid patients in whom the mean values with radioimmunoassay were about 50% higher than with competitive protein-binding assay (CPBA). This difference may be of practical value for differentiating between hyperthyroidism and hyperestrogenism in patients with elevated serum thyroxine levels.

Progress during these last years in saturation analysis techniques (1,2) has made possible the development of several methods for the determination of serum levels of thyroid hormones, thyroxine (T₄), and triiodothyronine (T₃), and the evaluation of the properties of their specific binding proteins. The two main advantages of competitive protein-binding assay (CPBA) are: first, the concentration of the circulating thyroid hormones and of their free fraction can be directly measured and, second, problems of radiation safety are avoided because no radioisotope is administered to the patient.

For a long time, no radioimmunoassay (RIA) was developed for measuring T₄ and T₃ because these iodoamino acids have no antigenic properties. Recently, however, it has been shown that by linking T₄ or T₃ to polypeptides or albumin (3-6) or by using thyroglobulin (7-9) it is possible to obtain antibodies against T₄ or T₃. A paper by Chopra (10) appeared on the radioimmunological determination of T₄, while this work was in progress. In the present study, our own experience in the determination by radioimmunoassay of the T₄ levels present in minute amounts (25 μl) of unextracted serum is described.

MATERIALS AND METHODS

General description of the radioimmunological procedure (RIA). Two main problems have to be solved to make possible the RIA for T₄ determination. First, it is essential to displace completely all the thyroxine bound to the thyroxine-binding proteins, principally thyroxine-binding globulin (TBG), if the time-consuming step of T₄ extraction from the serum is to be avoided. Second, TBG has also to be completely blocked in order to avoid any competitive binding between TBG and the T₄ antibodies. The addition of anilino-1-naphthalene-sulfonic acid (ANS) to all standards and unknown samples solves these problems (10). The thyroxine-binding pre-albumin is blocked by using barbital buffer as in the CPBA assays (11,12).

Thyroxine-binding antiserum. Beef thyroglobulin from commercial sources (Sigma and Nutritional Biochemical Corp., U.S.) was used to produce the antibodies. The immunization procedure proposed by Chopra, et al was followed using 10 mg of thyroglobulin in Freund's adjuvant given subcutaneously (8). The T₄ antibodies were detected using the routine RIA procedure described below, the antiserum being tested at different dilutions.

Routine RIA procedure. The serum samples (25 μl) or the appropriate dilutions of reagent grade Na-I-T₄, (Mann Research Lab., U.S.) for the cali-

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ibration curve are preincubated in duplicate for 1 hr at room temperature in 375 μl of 0.075 M barbital buffer, pH 8.6, containing 2% normal rabbit serum (NRS) in the presence of an excess of ANS (50 μl of a 6-mg/ml solution), radioactive 1-thyroxine (specific activity 50–80 mCi/mg, Abbott Laboratories, U.S.), 10,000 cpm corresponding to 0.2 ng T₄, approximately. The purity of the ¹²⁵I-T₄ is always checked by appropriate paper chromatography. Then 100 μl of a 1:100 dilution of the T₄-binding rabbit antiserum in barbital buffer (final dilution: 1/500) are added. Appropriate amounts of buffer are added to have a final volume of 500 μl in each test tube, and the incubation is carried out for another 1-hr period at room temperature. This antiserum dilution gives a final 40–45% binding of the radioactive hormone after addition of the second antibody, i.e., 50 μl of goat antirabbit gamma globulin (Antibodies Inc., U.S.) added at the end of the second hour of the RIA. The amount of goat antirabbit gamma globulin is chosen which will precipitate maximally the radioactive antigen (T₄) bound to the antibody (anti-T₄). The whole mixture is then kept overnight at +4°C. At each step of the procedure the tubes are carefully mixed. Separation from bound and free radioactivity is done by centrifugation and appropriate corrections are made for the nonspecific binding or trapping of labeled thyroxine in the precipitate (9–10). The standard curve is plotted using the logit transformation (13).

All reagents are of the analytical grade. Statistical results are presented as mean ± SE.

Subjects under investigation. Normal sera were obtained from a total of 79 clinically euthyroid subjects including 51 healthy students and 28 patients seen at the outpatient clinic and free from any endocrine disorder. The 30 hyperthyroid and 21 myxedematous patients had their diagnoses confirmed by the classical procedures including PBI and radioiodine studies. A group of eight euthyroid pregnant women was also investigated.

METHODOLOGICAL EXPERIMENTS

Production of antibodies. After 8 weeks of immunization, T₄-antibodies were detectable in the 11 rabbits used in this study. After three courses of immunization, all the animals showed high titers of T₄ antibodies. The mean ¹²⁵I-T₄ bound precipitated was 62 ± 4.9% at a final dilution of 1:500. The antibodies remain present for months at high levels in the animals injected with thyroglobulin. The specificity of the antiserum for T₄ was checked by constructing displacement curves in the presence of various concentrations of stable T₄ or T₃ in the conditions used for the RIA. Other thyroid analogs were not tested here (10).

Blockage of TBG-binding. Because complete blocking of TBG and T₄ displacement from TBG is essential in this RIA, different concentrations of ANS and preincubation time were used. A group of 14 different sera with low, normal, and high values as measured by CPBA was assayed at various concentrations of ANS (3, 6, and 9 mg/ml) and at different preincubation times (30, 60, and 120 min). The best agreement between the RIA and CPBA data was obtained when using 6 mg/ml of ANS and a preincubation time of 60 min, particularly when measuring samples in the hyperthyroid range. It should, however, be stressed that the amount of ANS and the preincubation time needed to block the TBG can be different for different T₄-antisera.

Calibration curve. The mean standard curve obtained from six different assays performed in 3 months of experiments is presented in Fig. 1. It may be noted that concentrations of T₄ up to 10 ng are correctly read on the calibration curve especially when using the logit transformation of the (B/B₀) value (11). Because the measurements are performed with 25 μl of serum, such a calibration curve makes it possible to measure in one step a range of T₄ concentrations up to 40 μg/ml without a preliminary dilution of the sample.

Serial dilutions of a given serum sample give an excellent agreement between the various T₄ con-
centrations when appropriate corrections are made for their respective dilutions. In a given sample, the results of \( T_4 \) concentration were, respectively, 6.2 (1:1); 6.0 (1:2); 5.8 (1:4); and 6.3 (1:8) \( \mu g/100 \) ml. Recovery experiments with known amounts of stable \( T_4 \) added to a given sample are also completely satisfactory. The addition of 2.5 and 5 ng of \( T_4 \) to 25 \( \mu l \) of serum used for the measurement gives a recovery of 96–100%. The reproducibility of estimates of serum \( T_4 \) by the radioimmunological procedure was checked by comparison of the values obtained in 10 sera measured in duplicate in different assays. The coefficient variation of duplicates from their mean was 10.5 \( \pm \) 1.0.

Comparison of the values obtained by the CPBA and RIA methods. A group of 79 subjects with \( T_4 \) values in the normal, hypothyroid, and hyperthyroid range was simultaneously studied using the CPBA and RIA methods. The values for I-\( T_4 \), obtained either by CPBA or RIA are in excellent agreement with a correlation coefficient of 0.931, \( p < 0.001 \) (Fig. 2). The calculated regression line is \( y \) (CPBA) = \( 1.172 + 0.616 \times (RIAX) \).

CLINICAL EVALUATION

Normal euthyroid subjects. In a group of 79 euthyroid subjects, the I-\( T_4 \), value was 6.22 \( \pm \) 0.145 \( \mu g/100 \) ml by RIA. In a subgroup of 28 of these patients, the I-\( T_4 \), concentration was 6.41 \( \pm \) 0.241 \( \mu g/100 \) ml by RIA and 5.68 \( \pm \) 0.226 \( \mu g/100 \) ml by CPBA. A group of eight euthyroid pregnant women gave a I-\( T_4 \), value of 8.55 \( \pm \) 0.968 \( \mu g/100 \) ml by RIA and 8.29 \( \pm \) 0.737 \( \mu g/100 \) ml by CPBA (Fig. 3).

Patients with thyroid disorders. In 21 hypothyroid patients, the I-\( T_4 \), concentration was, respectively, 1.70 \( \pm \) 0.215 \( \mu g/100 \) ml by RIA and 1.43 \( \pm \) 0.248 \( \mu g/100 \) ml by CPBA (Fig. 3). These results were significantly lower than in the controls (\( p < 0.001 \)).

In 30 patients suffering from thyrotoxicosis, the I-\( T_4 \), by RIA was 16.40 \( \pm \) 0.954 \( \mu g/100 \) ml and 11.17 \( \pm \) 0.613 \( \mu g/100 \) ml by CPBA. Both values were significantly higher than the ones found in the group of normal subjects (\( p < 0.001 \)). However, it should also be stressed that the absolute amount of circulating \( T_4 \) appeared to be very significantly higher when measured by RIA instead of by CPBA (\( p < 0.001 \)).

DISCUSSION

The present RIA procedure opens a new way for measuring serum thyroxine within a wide range of concentrations. From the methodological point of view, the technique, if properly used, offers all the advantages of the radioimmunological procedure, particularly enabling the processing of a large number of samples at the same time and lowering the final cost of this type of measurement. Moreover, the standard curve is linear in a large range of \( T_4 \) concentrations, allowing accurate measurements of 1–40 \( \mu g/100 \) ml of \( T_4 \) in one attempt. The results are obtained within 24 hr and eventually in a shorter period of time if less diluted antibodies are used. Compared to most of the CPBA methods, no extraction procedure is necessary; the calibration curve is based on several dilutions of the stable \( T_4 \), and only
25 μl of serum are necessary. Undoubtedly, such a sensitive method will be most useful not only in pediatrics in which there is always the problem of collecting enough serum for the various laboratory studies, but also in all the conditions in which repeated blood determinations have to be made in the same individual. The T₄, RIA can be easily adapted for automation.

The pioneer work of Chopra, et al has shown how easily T₄-binding antibodies can be raised in rabbits injected with thyroglobulin (8–12). Some of these antisera may have a significant affinity for serum triiodothyronine (T₃), but if the cross-reactivity is carefully checked in order to keep for RIA the only adequate antisera, no particular problem is to be expected.

Two main conditions for correct radioimmuno-
logical measurement of serum T₄, are met in the present procedure, i.e., the total displacement of T₄ from its binding proteins and the complete blockage of their binding capacities. Our data confirm those previously reported by Chopra (10) and show the excellent correlation of the T₄ values measured either by CPBA and RIA. Using adequate amounts of ANS, any extraction procedure is avoided, this step being a source of difficulty in the reproducibility in many CPBA for T₄, as previously noted by Chopra (10).

In principle, drugs like salicylate can replace ANS for blocking thyroxine-binding globulin (TBG) but it remains unsettled if a complete displacement of T₄ from TBG by salicylates can be obtained as easily as with ANS (5). Insignificant cross-reaction with different thyroid analogs has been previously shown by Chopra (8,10,14,15).

The diagnostic efficacy of RIA or CPBA is not different and the results agree quite well except in the cases of thyrotoxicosis. In the hyperthyroid sera, it should be stressed that the differences in the T₄ concentrations as obtained by CPBA and RIA are more related to the thyroid disease itself than to the enhanced concentration of the circulating T₄. Indeed, other elevations of circulating T₄, as in pregnant women, do not show any significant difference in the T₄ levels as measured either by CPBA or RIA. The reasons for these discrepancies observed only in the group of thyrotoxic patients remain unclear and could be related to the release in the blood stream of iodioproteins from highly hyperactive thyroid follicles (10). Whatever the significance of this finding for the thyroid physiologist, this artificial enhancement of the serum T₄ concentration in thyrotoxic patients will prove very useful for separating cases of true hyperthyroidism from those presenting a high T₄ level because of thyroid or estrogenic medications.

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