Radioimmunoassay of Thyroxine in Unextracted Serum, by a Single-Antibody Technique

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A radioimmunoassay procedure for thyroxine (T\textsubscript{4}) is described, for which only 25 \(\mu\)l of serum is required. The antibody, commercially available, is produced in rabbits in response to injections of a T\textsubscript{4}-albumin conjugate. Native T\textsubscript{4}-binding proteins are denatured by heating 25 \(\mu\)l of serum in 0.2 ml of a glutamate buffer in a boiling water bath. After a 1-h incubation of heat-treated serum with 1\textsuperscript{25}T\textsubscript{4} and T\textsubscript{4} antibody, "free" and "bound" T\textsubscript{4} are separated by use of hemoglobin-coated charcoal. The method has excellent sensitivity, is rapid, and possesses significant advantages over a standard competitive protein binding method with which it is compared.

Additional Keyphrases: results for T\textsubscript{4} by competitive protein binding compared • assessment of thyroid function • T\textsubscript{4}-albumin conjugate as antigen • normal range

We report here a method that uses a commercially available T\textsubscript{4} antibody produced in rabbits in response to an albumin-T\textsubscript{4} conjugate (7). In the procedure 25 \(\mu\)l of serum is used, with no extraction or adsorption before analysis. "Free" and "bound" T\textsubscript{4} are conveniently separated with hemoglobin-coated charcoal and 25 specimens can be processed completely in less than 2 h. The method is shown to yield slightly higher results than a popular CPB method, but this bias might be expected because in this RIA procedure recovery is complete in contrast to the somewhat variable recovery in any CPB method. The improved recovery and the specificity imparted by the use of a T\textsubscript{4}-antibody, constitute important advantages in favor of the RIA method.

Materials and Methods

Reagents

1. Phosphate buffer, pH 7.6, 0.1 mol/liter: Dissolve 13.92 g of potassium phosphate, dibasic (K\textsubscript{2}HPO\textsubscript{4}); 2.76 g of sodium phosphate, monobasic, hydrate (NaH\textsubscript{2}PO\textsubscript{4}-H\textsubscript{2}O); and 8.76 g of NaCl in about 900 ml of water. Adjust the pH to 7.6 ± 0.05 with phosphoric acid (33 mmol/liter) or KOH (0.1 mol/liter). Dilute to 1000 ml with water and mix well. Recheck the pH and adjust if necessary. Stable for at least six months at 4 \(^\circ\)C.

2. Standard diluent: Dilute 100 ml of reagent No. 1 with 800 ml of water. Add 7.9 g of NaCl and 10 g of bovine serum albumin (Plasma Fraction V). Adjust pH to 7.4 ± 0.05 with phosphoric acid (33 mmol/liter) or KOH (0.1 mol/liter). Dilute to 1000 ml with water. Recheck the pH and adjust if necessary. Stable for at least three months at 4 \(^\circ\)C.

3. Phosphate-buffered albumin saline, pH 7.4 (P-BAS): Dilute 100 ml of reagent No. 1 with 800 ml of water in a 1-liter volumetric flask. Add 7.9 g of NaCl, 5 g of bovine serum albumin (Fraction V), and 3.8 g of EDTA-Na\textsubscript{2}, and dissolve. Adjust the pH to 7.4 ± 0.05 as above. Dilute to volume with water. Recheck the pH and adjust if necessary. Stable for at least three months at 4 \(^\circ\)C.

4. Glutamate buffer, pH 3.3: Dissolve 1.2 g of L-glutamic acid in 200 ml of water. Add 1.0 ml of KCN

\begin{quote}
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\(^1\) Nonstandard abbreviations used: CPB, competitive protein binding; T\textsubscript{4}, thyroxine; RIA, radioimmunoassay; P-BAS, phosphate-buffered albumin saline solution; and EDTA, ethylenediaminetetraacetate.

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\end{quote}
solution (4 g/liter). Check the pH and adjust to 3.3 with molar HCl or NaOH if necessary. Stable for six months at room temperature.


6. **Working isotope solution, 700 pg/ml:** Stock [125I]thyroxine is diluted with reagent No. 1 to yield a final concentration of 700 pg/ml. Stable one week when stored at 4 °C in a P-BAS prerinsed glass container.

7. **T₄ antibody (Wien Laboratories, Inc., Succasunna, N. J. 07876):** Stable for six months at 4 °C. Dissolve the contents of 1 vial in sufficient reagent No. 1, such that 100 μl of antibody is able to bind about 560 pg of [125I]thyroxine. Stable two to three weeks at 4 °C.

8. **Hemoglobin, 100 g/liter:** Centrifuge 1 unit of outdated blood and aspirate the plasma. Wash three times with NaCl solution (9 g/liter), aspirating the supernatant fluid after each wash. Add an equal volume of water to the packed cells. Mix well and allow the cells to lyse for 5 min while shaking on a mechanical shaker. Add ¼ volume of toluene to the lysed cell solution and shake for 5 min on a mechanical shaker. Centrifuge, aspirate the toluene layer, and filter the hemoglobin solution through Whatman No. 1 filter paper. Determine the hemoglobin concentration of the filtered solution and dilute to a final concentration of 100 g/liter. Place 5-ml aliquots into plastic screw-capped tubes and freeze. Stable one year in the frozen state.

9. **Hemoglobin-coated charcoal:** Suspend 10 g of Norit A (neutral) charcoal in 200 ml of water. Prepare a separation solution containing 5 ml of the hemoglobin solution and 195 ml of water. Mix the two solutions together. (Note: The charcoal should be mixed continuously while the hemoglobin solution is being added. Continue mixing for an additional 20 min.) Stable three months at 4 °C.

10. **Thyroxine stock standard, 1 mg/ml:** Add 28.5 mg of L-thyroxine (Na salt, pentahydrate; Sigma Chemical Co., St. Louis, Mo. 63178) and 2.5 ml of propylene glycol to a 25-ml volumetric flask. Dissolve by adding NaOH (0.1 mol/liter) in 2-ml fractions with mixing until the solution is clear. Dilute to volume with water and mix. Transfer 2.5-ml aliquots to screw-capped polystyrene tubes and store frozen. Stable for six months.

11. **Thyroxine dilute stock standard, 1 mg/dl:** Dilute 1 ml of thyroxine stock standard to volume with standard diluent (reagent No. 2) in a 100-ml volumetric flask. Aliquot 7-ml portions into screw-capped polystyrene tubes and store frozen. Stable for six months. Thyroxine working standards (5, 10, 20, and 30 μg/dl) are prepared by appropriate dilution of the thyroxine dilute stock standard with standard diluent. Stable for three months at 4 °C when stored in brown glass bottles.

### Procedure

**Note:** All glass tubes used in this procedure must be pre-rinsed in P-BAS buffer (reagent No. 3) before use.

To appropriately labeled 13 × 100 mm pre-rinsed glass tubes, add 0.2 ml of glutamate buffer. Add 25 μl of standard diluent (for blank), serum, or standards directly into the buffer and mix well. Add all tubes in a vigorously boiling water bath for 15 min. Cool the tubes, add 1.0 ml of T₄ isotope solution to each tube, mix well, add 0.1 ml of T₄ antibody solution to each tube, and again mix well. Incubate at room temperature for 1 h.

Add 0.5 ml of cold hemoglobin-coated charcoal solution to each tube, mix, and allow to stand for 5 min. (The charcoal solution must be continuously mixed while being pipetted.) Centrifuge for 5 min and decant the supernatant fluid into appropriately labeled plastic counting vials and count each tube in a well-type gamma counter. Sufficient counts (10,000) accumulate in less than 1 min. Calculations are made from a plot of concentration (abscissa) vs. the ratio Counts zero standard/Counts standard (or unknown).

Glass tubes used in preparing standard dilutions and in the test procedure must be pre-rinsed with P-BAS buffer (reagent No. 3), to prevent adsorption of the sample onto the glass walls. P-BAS must be allowed to coat the entire surface of the tubes. Rinsed tubes should be inverted and allowed to drain for at least 1 h before use.

In our experience, coated glass tubes as described above are preferable to any plastic tube, including polystyrene. Because antibodies and binding agents can become enmeshed in certain plastics, polyethylene and polypropylene have been used in solid-phase RIA methods. Although we present no data here, we have had similar experiences with polystyrene tubes. Such undesirable losses of antibody into polystyrene can lead to erroneous “bound-free” information.

### Results and Discussion

Figure 1 shows a typical calibration curve. The data are nearly linear when one plots the ratio Counts, zero standard/Counts, standard or unknown vs. concentration. The data shown include calibration curves prepared from T₄ standards made up in reagent No. 3, which were diluted with glutamate buffer and then heated in a boiling water bath for 15 min, and from fresh T₄ standards made up in phosphate buffer (no albumin) and which were not boiled but simply diluted with glutamate buffer. All standards, heated and unheated, then were processed identically as indicated in the procedure. Since the standard curves are identical within the usual RIA analytical limits, we conclude that T₄ is stable for at least 15 min when boiled in glutamate buffer.

With heat stability thus indicated, we elected to utilize heat denaturation to free T₄ from the native serum binders before radioimmunoassay. Murphy (8)
studied various methods of deproteinization to free hormones from their native serum binders, but, in the case of cortisol, concluded that heating in a boiling water bath for 1 min was inadequate. We presume that such experiences led her and others to pursue the use of organic extraction techniques in subsequent work in this field. However, Wide and Killander (9) found that vitamin B₁₂ could be effectively released from serum by heating at 100 °C for 15 min. Thus, it appears that heat treatment can be used to release heat-stable molecules from native serum binding proteins, but that such treatment may require more than just a few minutes. In preliminary experiments we found that 15 min in a vigorously boiling water bath was necessary to free T₄ from its serum binders.

Table 1 illustrates that recovery is good when a pooled serum sample is supplemented with increasing concentrations of thyroxine, and that when serum is serially diluted with the albumin-phosphate buffer, the results for T₄ compare well with those obtained for serum alone (after correction for the dilution). Thus, one can reasonably infer from such data that recovery is complete and that native serum binding proteins do not constitute a source of error.

Table 2 indicates the within-run and between-run reproducibility. Each result was calculated from a single processed specimen and not from duplicates. We are not advocating the use of single-tube assays for RIA, but the reproducibility shown does suggest that a single-tube assay yields acceptable data with this procedure.

Figure 2 indicates the comparisons from T₄ values for 72 patients' sera, chosen without conscious bias and tested by the proposed RIA method and by a commercial CPB method (2). The 54 patients who were clinically normal and had normal T₄ values by the CPB method (45–115 μg/liter) yielded RIA results ranging 42 to 118 μg/liter. Based on a normal curve distribution and the regression curve (Figure 2) we have derived a tentative normal range by the RIA method of 45–118 μg/liter.

The slightly higher mean value for T₄ by RIA as compared with the CPB method (83 vs. 81 μg/liter) agrees with the similar bias found by Chopra et al. (5, 6), who compared a double-antibody RIA method for T₄ with a CPB technique. Furthermore, they presented evidence that the discrepancy between the results of RIA and CPB methods, especially in the case of sera from hyperthyroid patients, was caused by incomplete extraction of T₄ into a butanol–ethanol mixture before CPB assay. In the case of the CPB

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**Table 1. Recovery of Thyroxine from Heat-Treated Sera**

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Found</th>
<th>Expected</th>
<th>Recovery, %</th>
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<tbody>
<tr>
<td>None</td>
<td>64</td>
<td>64</td>
<td>102</td>
</tr>
<tr>
<td>1:2</td>
<td>128</td>
<td>114</td>
<td>109</td>
</tr>
<tr>
<td>1:4</td>
<td>67</td>
<td>57</td>
<td>105</td>
</tr>
<tr>
<td>1:8</td>
<td>25</td>
<td>28</td>
<td></td>
</tr>
</tbody>
</table>

*All data are single determinations.*

**Table 2. Reproducibility of the RIA Procedure for Thyroxine**

<table>
<thead>
<tr>
<th>Level</th>
<th>Within run</th>
<th>Between run</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tube No.</td>
<td>Result μg/liter</td>
</tr>
<tr>
<td>I</td>
<td>1</td>
<td>66</td>
</tr>
<tr>
<td>II</td>
<td>2</td>
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<td>7</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>66</td>
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</tbody>
</table>

*Unassayed lyophilized quality control sera (General Diagnostics, Morris Plains, N.J.) were assayed by six technologists during 23 separate analytical runs.*
assay we used (2), the recovery of T4 by adsorption is about 80%, and should the percent recovery increase during storage of serum or vary from sample to sample, then the results would be slightly in error. No such effect is possible with the proposed RIA procedure, because the denatured serum proteins can no longer bind T4. To demonstrate this, we incubated heat-denatured sera for 1 h with 125I-T4 and subsequently treated with hemoglobin-coated charcoal. Table 3 shows that the counts for the supernate are not different from those obtained with buffer alone, indicating the inability of the serum proteins to bind added T4.

The specificity of the method is good, as would be expected when an antibody produced to a T4-albumin conjugate is used. The manufacturer has tested a number of closely related iodinated compounds for interaction with the antibody. Triiodothyronine, 10,000 pg, was equivalent to only 410 pg of thyroxine (7). The specificity appears related to the tetra-iod ring system because the closely related compounds represented by deletion of the amino function (3,3',5,5'-tetraiodo desamino thyronine) or replacement of the aminated propionic acid residue with acetic acid (3,3',5,5'-tetraiodo thyroacetic acid) were 75% and 50% as reactive, respectively, as thyroxine (7).

The present method has many advantages: it is rapid, sensitive (only 25 μl of serum required), and reproducible. The antibody to T4 renders specificity, and heat denaturation completely prevents interference from native serum protein binders of T4.

References
2. "Tetra-Tab T4 Diagnostic Test"; Nuclear-Medical Laboratories, Inc., Dallas, Texas.
7. Thyroxine Antibody (Product Insert); Wien Laboratories, Inc., Succasunna, N. J.