Determination of serum thyroxine using a resin sponge technique

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SYNOPSIS A method is described for the determination of serum thyroxine using ion-exchange resin sponges. The principle is based on the use of protein-binding sites and part of the technique is similar to the Triomet resin uptake test. An isotope extraction recovery procedure is incorporated in the method. Studies of factors influencing the analysis are reported and the results of tests of accuracy, precision, and specificity are presented. Serum thyroxine iodine values are compared with protein-bound iodine estimations. The values found in health and disease are similar to the results of previous workers.

The specificity of the analysis represents a considerable advantage over protein-bound iodine determinations since no interference is caused by iodides, iodine-containing contrast media, mercurial diuretics, mono-iodotyrosine, di-iodotyrosine, di-iodothyronine, and also tri-iodothyronine in physiological amounts. Therapy with diphendylhydantoin and tri-iodothyronine will tend to depress protein-bound iodine levels and thyroxine values are similarly influenced.

Methods have previously been described for the estimation of serum thyroxine utilizing the binding properties of serum proteins (Ekins, 1960; Murphy and Pattee, 1964). An improvement resulted from the use of ion-exchange resin granules (Murphy, 1965) and the further convenience of resin sponges has been reported (Nakajima, Kuramochi, Horiguchi, and Kubo, 1966). As in previous techniques, this method is a form of saturation analysis. The principle is illustrated diagrammatically in Figure 1. The labelled compound is distributed between the thyroxine-binding sites in aliquots of pooled serum and the resin sponge according to the amount of standard thyroxine added to the aliquot. The thyroxine content of the ethanolic extract of an unknown serum is read off the standard curve. An isotope extraction recovery procedure is included in the method presented.

MATERIALS AND METHODS

A 0·1M, pH 8·60 BARBITAL BUFFER 0·2M Barbital1, 500 ml., 0·2N HCl, 60 ml., made up to 1,000 ml. with distilled water. Check pH and adjust to 8·60.

B STANDARD THYROXINE Sodium L-thyroxine penta-hydrate2, 17·9 mg., chromatographically pure, is dissolved in a few millilitres of 1N-NaOH and made up to 250 ml. with distilled water. Two millilitres of this solution are made up to 250 ml. with buffer. One millilitre of this solution contains 0·5 μg. thyroxine (free acid). A fresh solution is used in the preparation of standard curve solutions and in recovery experiments.

C 0·01% ALBUMIN SOLUTION 0·3 ml. 30% bovine albumin, fraction V solution3, is made up to 1,000 ml. with distilled water. This solution is kept at 4°C.

D RADIOACTIVE THYROXINE Thyroxine-131I, 200 μc. per ml., in 50% propylene glycol-specific activity approximately 30 mc per mg.4 Lower specific activities will tend to reduce the slope of the standard curve slightly.

1 Thyroxine-131 solution for the standard curve solutions Thyroxine-131I, 0·2 ml., is diluted to 15 ml. with buffer (solution A) and aliquots are immediately taken for the standard curve solutions.

2 Thyroxine-131I solution for the unknown samples Thyroxine-131I, 0·25 ml., is diluted to 100 ml. with 0·01% aqueous albumin. Albumin is used to prevent adsorption of the hormone to glass. This solution is kept in the dark at 4°C. when not in use.

E and F SOLUTIONS CONTAINING POOLED SERUM FOR THE STANDARD CURVE AND FOR THE UNKNOWN SAMPLES, RESPECTIVELY The methods of preparing the standard

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2Mann Research Laboratories, U.S.A.

3Abbott Pharmaceuticals, U.S.A.
curve solutions and the solution for unknown samples are shown in Table I. Bulb pipettes and 50 ml. volumetric flasks are used. Several flasks of solution F for the unknown samples are prepared as required. Pooled serum collected from clinical laboratories is filtered and stored frozen in convenient small amounts. These solutions are prepared at intervals of two weeks on the receipt of fresh labelled thyroxine. They are kept at 4°C. and used at room temperature.

G RESIN SPONGES, PLASTIC PLUNGERS, AND ASPIRATORS

H ABSOLUTE ETHANOL

I The accuracy of the method can be checked as required by analysing samples of pooled serum with and without added thyroxine.

Pooled serum + 5 μg. thyroxine per 100 ml. is conveniently prepared by the addition of 100 μl. of the standard thyroxine solution to 1-0 ml. pooled serum in a conical centrifuge tube. The sample is then processed in the usual manner.

Pooled serum + 10 μg. thyroxine per 100 ml. is similarly prepared by the addition of 200 μl. of the standard solution.

PROCEDURE

EXTRACTION One millilitre of the thyroxine-131I solution for unknown samples (solution D2) is pipetted into a 15 ml. stoppered conical centrifuge tube and also into each of two 100 × 15 mm. test tubes (labelled I and II). These test tubes are used in the determination of extraction recovery, a resin sponge being added to each to maintain counting geometry.

One millilitre of the unknown serum is added to the centrifuge tube and shaken gently. Ten millilitres of absolute ethanol are then added. If multiple samples are being extracted, it is convenient to add the ethanol with an automatic syringe. The centrifuge tube is subsequently stoppered and the contents mixed. After centrifugation at 3,000 r.p.m. for five minutes, the supernatant is decanted into a 300 ml. round-bottomed flask and taken.
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To dryness rapidly in a rotary flash evaporator at approximately 60°C.

To the residue in the flask is added 4.0 ml of the pooled serum solution for unknown samples (solution F) and the residue is taken up by swirling.

Duplicate 1.0 ml aliquots are now taken into 100 x 15 mm. test tubes for thyroxine analysis. Duplicate 1.0 ml aliquots are also taken from each standard curve solution (solution E) into test tubes.

DETERMINATION OF PERCENTAGE RESIN-BOUND RADIOACTIVITY Plastic plungers and aspirators of the type supplied in the Triomet kits are employed.

A resin sponge is added to each standard and unknown tube in turn at timed one-minute intervals. Using a well type scintillation counter the count rate for each tube is determined and corrected for background if indicated. A preset time of 0.5 to 1 minute is adequate. When the initial count rate has been determined, the tube is placed in a water bath at just above ambient temperature for 90 minutes.

Incubation is terminated by aspirating excess fluid and washing the sponge with three changes of approximately 5 ml distilled water. This procedure is carried out at one-minute intervals in the same order in which the sponges were added. The tubes are now counted a second time and this final count expressed as a percentage of the initial count.

The standard curve is obtained by plotting the percentage resin-bound radioactivity of the standard tubes against their content of added thyroxine (μg per ml). The thyroxine content of the unknown tubes is read off this curve. This value is then corrected by the extraction recovery figure for that tube and the thyroxine content of the unknown sample expressed in μg per 100 ml.

EXTRACTION RECOVERY The extraction recovery value for each unknown tube is determined by expressing the initial count rate for that tube as a percentage of the counts added before extraction (mean of I and II).

Example

Mean of I and II = 200,000 c.p.m.
Sample no. 1 initial count = 40,000 c.p.m.
Extraction recovery = 20%

That is, this tube contains 20% of the thyroxine present in 1.0 ml of serum before extraction. This figure is used to correct the thyroxine content read for that tube. Since this tube only contains one fourth of the total residue in the flask, the actual extraction recovery is 80.0%.

Thyroxine content
T4 read = 100 x 100 μg per 100 ml.

% Recovery = 100 x 100 μg. per 100 ml.

RESULTS

FACTORS AFFECTING THE STANDARD CURVE A study was made of factors influencing the appearance of the standard curve.

From these studies, conditions were selected to yield the most suitable standard curve with regard to slope, shape, and reproducibility.

FIG. 2. Effect of different buffers on standard curve.

TYPE OF BUFFER The difference in the standard curve produced by changing from barbital to tris-maleate buffer is shown in Figure 2. It will be seen that at the same pH the slope achieved with barbital buffer is significantly greater. This finding could be related to the inhibition of thyroxine binding by pre-albumin which has been observed in electrophoretic studies when barbital buffer has been used (Antoniades, 1960).

FIG. 3. Effect of pH on standard curve.

pH OF BUFFER It is recognized that protein-binding activity increases with higher pH values and that the specificity of binding sites tends to fall. The activity of the ion-exchange resin is also influenced by changes of pH. Figure 3 shows decreased protein binding of thyroxine at pH 7.4 compared with pH 8.6 using 0.1 M barbital buffer. Further, since the procedure for the unknown samples involves the addition of an alkaline residue to diluted plasma, it was felt that the use of buffer at the higher pH would minimize changes in pH.

Finally, barbital buffer at pH 7.4 tends to crystallise out in the cold.
Standard curves using tris-maleate buffer were similarly influenced by pH.

**DEGREE OF DILUTION OF THE STANDARD POOLED SERUM** The effects of varying dilution is shown in Figure 4. Although the slope of the standard curve using a dilution of 1 in 25 was rather greater than with 1 in 10, the latter dilution appeared to yield more consistent results.

![Figure 4](image1.png)

**FIG. 4. Effect of dilution on standard curve.**

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**FIG. 4. Effect of dilution on standard curve.**

**WORKING VOLUME FOR RESIN SPONGE UPTAKE** It was found that a volume of 2·0 ml. overloaded the sponge and gave less reproducible results than 1·0 ml.

**DURATION OF INCUBATION** The slope of the standard curve after 90 minutes' incubation was significantly higher than after 60 minutes (Fig. 5).

![Figure 5](image2.png)

**FIG. 5. Effect of length of incubation time on standard curve.**

**TEMPERATURE OF INCUBATION** Temperature probably influences the binding of thyroxine to both the resin and the serum proteins. The lower the incubation temperature the lower the standard curve, although the slopes remained almost parallel (Fig. 6). The height of the curve changes by approximately 2% per 1°C. In view of this the incubation should be carried out at a constant temperature, preferably in a waterbath at just above ambient temperature.

![Figure 6](image3.png)

**FIG. 6. Effect of incubation temperature on standard curve.**

**POOLED SERUM** With some lots of pooled serum, a tendency to a sigmoidal standard curve was noted. A curve of significantly reduced initial slope could have an adverse influence on thyroxine determinations in the lower ranges but this was rarely found.

This appearance has been noted previously (Murphy, 1965) and was attributed to the use of pooled serum with a significant content of hypothyroid serum. The addition of a small calculated amount of thyroxine to the pooled serum was recommended in these circumstances. In the present study, however, the phenomenon could not be attributed to this cause as subsequent analysis of the pooled serum yielded normal thyroxine values. No other studies of hormone binding were undertaken.

**RECOVERY OF ADDED THYROXINE131-I** When 90 serum samples processed on different days were considered, the mean recovery of radioactivity was $83.2 \pm 5.7$ (S.D.)%.

**ACCURACY** This was tested by analysing samples of pooled serum before and after the addition of known amounts of exogenous thyroxine. The recovery
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figures at two incremental levels on five different days are shown in Figure 7. Mean overall recovery was 100.6%.

![Graph showing recovery of exogenous thyroxine added to pooled serum.]

**FIG. 7.** Recovery of exogenous thyroxine added to pooled serum.

**TABLE II**

<table>
<thead>
<tr>
<th>Compound Added</th>
<th>Amount (µg.%)</th>
<th>Thyroxine Content (µg.%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>—</td>
<td>7.8 ± 0.7 (S.D.)</td>
</tr>
<tr>
<td>Triiodothyronine</td>
<td>40</td>
<td>11.5</td>
</tr>
<tr>
<td>Diiodothyronine</td>
<td>40</td>
<td>7.4</td>
</tr>
<tr>
<td>Diiodotyrosine</td>
<td>40</td>
<td>8.2</td>
</tr>
<tr>
<td>Monoiodotyrosine</td>
<td>40</td>
<td>7.5</td>
</tr>
<tr>
<td>Potassium iodide</td>
<td>100,000</td>
<td>7.5</td>
</tr>
<tr>
<td>Diphenylhydantoin</td>
<td>600,000</td>
<td>14.1</td>
</tr>
<tr>
<td>Biligrafin</td>
<td>250,000</td>
<td>7.5</td>
</tr>
<tr>
<td>Urografin</td>
<td>300,000</td>
<td>7.6</td>
</tr>
</tbody>
</table>

**TABLE III**

<table>
<thead>
<tr>
<th>COMPARISON OF PROTEIN-BOUND IODINE AND THYROXINE IODINE BEFORE AND AFTER ADMINISTRATION OF POSSIBLE INTERFERING COMPOUNDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein-bound Iodine (µg. per 100 ml.)</td>
</tr>
<tr>
<td>Before</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>Intravenous pyelogram</td>
</tr>
<tr>
<td>Cholecystogram</td>
</tr>
<tr>
<td>Intravenous cholangiogram</td>
</tr>
<tr>
<td>Mersalyl (i.m.)</td>
</tr>
</tbody>
</table>

**PRECISION** Repeated analysis of a single pool of serum over a period of one month gave a thyroxine concentration of 7.4 ± 0.9 (S.D.) µg. per 100 ml.

In order to test precision in different ranges of thyroxine concentration, duplicate estimations were carried out in 17 normal subjects, 12 patients with hypothyroidism, and 19 patients with hyperthyroidism. Standard deviations \( \sqrt{\frac{\sum d^2}{2N}} \) were 0.9, 0.5, and 1.1 µg. per 100 ml. respectively.

**SPECIFICITY** The specificity of the method for thyroxine was tested by the addition to pooled serum in vitro of known amounts of possible interfering compounds and by analysing patients' sera before and after therapy with mercurial diuretics or ingestion of iodine-containing radio-opaque dyes.

Table II shows that some interference is produced by tri-iodothyronine, but only in large, unphysiological concentrations. Diphenylhydantoin also showed interference in vitro.

Therapy with diphenylhydantoin is known to depress the protein-bound iodine level and serum thyroxine is similarly affected.

The results of the studies in vivo are shown in Table III. The thyroxine iodine content calculated from the serum thyroxine value is compared with protein-bound iodine values (Farrell and Richmond, 1961) and it is seen that under the conditions studied the values for protein-bound iodine gave misleading results, while the serum thyroxine remained normal. The correlation between serum thyroxine iodine (expressed as 65.3% of the serum thyroxine concentration) and the protein-bound iodine done on the same sample, is shown in Figure 8.
VALUES IN NORMAL SUBJECTS AND PATIENTS WITH HYPOTHYROIDISM AND HYPERTHYROIDISM

The diagnosis of hypothyroidism and hyperthyroidism was made on clinical grounds with supporting laboratory dates, including isotope studies. The normal subjects were healthy volunteers. The mean values and ranges for serum thyroxine and calculated serum thyroxine iodine in a limited study are shown in Table IV.

**Table IV**

<table>
<thead>
<tr>
<th>Serum Thyroxine Concentration (μg per 100 mL)</th>
<th>Hypothyroid</th>
<th>Normal</th>
<th>Hyperthyroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroxine</td>
<td>0-1:4-8</td>
<td>5-5-10:2</td>
<td>10-3-26:7</td>
</tr>
<tr>
<td>(1-9)*</td>
<td>(8-0)</td>
<td>(17-3)</td>
<td></td>
</tr>
<tr>
<td>Thyroxine iodine</td>
<td>0-1:3-0</td>
<td>3-5-6-5</td>
<td>6-6-17-0</td>
</tr>
<tr>
<td>(1-2)</td>
<td>(5-2)</td>
<td>(11-3)</td>
<td></td>
</tr>
<tr>
<td>No. of patients</td>
<td>12</td>
<td>17</td>
<td>19</td>
</tr>
</tbody>
</table>

*Mean values in parenthesis.

**DISCUSSION**

The principle of this method is similar to previous methods (Ekins, 1960; Murphy and Pattee, 1964; Murphy, 1965; Nakajima et al., 1966). The main difference is the incorporation of an isotope extraction recovery procedure with the added convenience of ion exchange resin sponges and techniques similar to those used in the conventional resin uptake test.

Tests of accuracy, precision, reproducibility, and specificity were carried out and satisfactory results obtained. Although not as precise as the protein-bound iodine method this test has the advantage of specificity since iodides, iodine-containing contrast media, iodo-amino acids, and mercurial diuretics cause no interference. It is possible for one technician to analyse about 12 samples in a run and this is completed in less than six hours. Convenient use is readily made of a well type scintillation counter with automatic sample changing.

The method has been found useful in differentiating euthyroid subjects from those with hypothyroidism and hyperthyroidism. The thyroxine values found in these groups are similar to previous reports (Ekins, 1960; Murphy and Pattee, 1964; Murphy, 1965; Nakajima et al., 1966), and the calculated thyroxine iodine values in the normal subjects correspond to accepted values for protein-bound iodine. The correlation with estimations of protein-bound iodine on a range of samples was good. A diagnostic dose of radioactive iodine does not interfere with the method though a recent therapeutic dose of radioactive iodine may necessitate delay in processing the serum sample. Therapy with triiodothyrone will depress the protein-bound iodine and the serum thyroxine values. Diphenylhydantoin therapy is also known to depress the protein-bound iodine level (Oppenheimer, Fisher, Nelson, and Jailer, 1961) by competing for protein-binding sites, resulting in a normal free thyroxine level in the serum maintained by a reduced level of protein-bound hormone.

Part of the facilities and materials used in this study were provided by the Royal Infirmary and the University of Glasgow, and the invaluable assistance of Professor E. M. McGirr and Dr. J. H. Wright is greatly appreciated. Much of the material used was provided by Abbott Laboratories, Chicago, through the kindness of Dr. Howard Glenn.

**REFERENCES**


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