Clinical Evaluation of a Modified “Oxford T₄-by-Column” Method for Serum Thyroxine

Marcia Lee,¹ Norbert W. Tietz, and Charles J. Martinez

Three commercially available kits for determining serum thyroxine (T₄) by column were evaluated. Results with all were less than satisfactory, but a series of changes in the Oxford method resulted in increased reproducibility, substantially improved recovery, and extended linearity of the standard curve. Results compared well with the thyrometabolic status of patients and normal individuals. The 95% confidence limits for 219 “normal” hospital employees were 2.9-6.2 μg/100 ml. The suggested normal range for 241 clinically euthyroid patients is 2.5-6.0 μg/100 ml. The coefficient of correlation between results of the proposed method and of the PBI method was 0.30; for the T₄ by the Murphy–Pattee method it was 0.75.

Additional Keyphrases three commercial kits for thyroxine evaluated • normal range • PBI method • Murphy–Pattee method

Since the early 1960's several column chromatographic (“T₄-by-column”)¹ methods for determination of T₄ have been made available. These methods have proven to be superior to the PBI method, because they offer a greater degree of specificity for T₄I and thus minimize interference from exogenous iodine.

The various methods in which the principle of competitive protein binding is used (Murphy–Pattee) provide further specificity for thyroxine than does the T₄-by-column method, but to use these methods isotope equipment is required, and thus they cannot be utilized by most smaller institutions. In addition, reagent cost is higher and total experimental time longer. Thus there appears to be a continued need for a reliable T₄-by-column method.

Despite its advantages, determination of T₄-by-column did not become popular until a number of commercial supply houses such as BioRad Laboratories (1), Curtis Nuclear Corporation (2), Oxford Laboratories (3), and others marketed prepacked columns and reagents and until a number of modifications of Pileggi’s method (4) had been published (5-8) [see also Bittner, D. L., Young, D. P., Maffe, M. R., and Grechman, R. J., An automated method for the bromination and quantitation of thyroxine in resin column eluates. Thyroxine Round Table, Annual Meeting, American Society of Clinical Pathologists, 1968.]

We investigated some of these commercial procedures (BioRad, Curtis, and Oxford) and found them to be less than satisfactory in regard to accuracy, reproducibility, and dynamic range. We thought that the Oxford T₄-by-column method offered the greatest potential and so we have optimized this method in respect to sample volume, pH for dissociation of thyroxine from the protein carrier, volume of column priming and elution solvent, pH of the elution solvent, and concentration of bromate–bromide reagent.

Changes in these parameters as detailed under “Methods” resulted in increased reproducibility (sd, ± 0.1 μg/100 ml), improved recovery (92-105%, mean 98%), extension of dynamic range (up to 12.0 μg/100 ml), and improvement in the linearity of the standard curve.

Materials and Methods
Reagents

All reagents used are of AR grade.

Serum diluent. Stock solution: Dissolve 80.0 g of NaOH in de-ionized water and dilute to 2 liters. Adjust the concentration to 1.00 mol/liter by titration with standardized HCl (1.00 mol/liter), with a few drops of phenolphthalein solution (1 g/100 ml) as indicator.

¹From the Mount Sinai Hospital Medical Center and The University of Health Sciences/The Chicago Medical School, California Ave. at 15th St., Chicago, Ill. 60608.
²Nonstandard abbreviations used: T₄, thyroxine; T₄I thyroxine iodine; PBI, protein-bound iodine; T₄, transmission.
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**Working diluent (0.50 mol/liter):** Dilute 500 ml of the preceding solution with de-ionized water to 1 liter. Adjust the concentration to 0.50 mol/liter.

**Working diluent (0.10 mol/liter):** Dilute 100 ml of the stock diluent (1.00 molar NaOH) to one liter with de-ionized water. Adjust the concentration to 0.10 mol/liter.

**Alkaline acetate wash (pH 8.0).** To 500 ml of anhydrous isopropanol, add 400 ml of de-ionized water. Add 11.8 ml of glacial acetic acid, and mix well. Adjust the pH to 8.0 with 10.0 molar NaOH. Dilute to 1 liter and recheck the pH.

**Acid acetate wash (pH 2.2).** Add 150 ml of glacial acetic acid to 850 ml of de-ionized water, and do not dilute to the 1-liter mark. Adjust the pH to 2.2.

**Column priming solution:** Glacial acetic acid.

**Elution solvent.** Add 530 ml of glacial acetic acid, to 470 ml of de-ionized water. Do not dilute to the 1-liter mark.

**Sulfuric acid (2.0 mol/liter).** Add 48.5 ml of concentrated H2SO4 to about 300 ml of de-ionized water. Cool and dilute to 500 ml.

**Alkaline bromate–bromide reagent.** Dissolve 0.30 g of KBrO3 and 0.975 g of KBr in 500 ml of de-ionized water. Adjust the pH to 10.5 with NaOH.

**Arsenious acid.** Dissolve 5.0 g of As2O3 in 200 ml of boiling water containing 1.50 g of NaOH. Add 500 ml of water, cool. Add 27.5 ml of concentrated H2SO4 slowly, while stirring. Cool and dilute to 1 liter.

**Ceric ammonium sulfate.** Add 48.6 ml of concentrated H2SO4 to about 800 ml of water. Cool and add 20.00 g of ceric ammonium sulfate. Dissolve and dilute to 1 liter.

**Standards**

**Stock standard.** Dissolve 42.1 mg of sodium L-thyroxine pentahydrate (Sigma Chemical Co., St. Louis, Mo. 63178) in 200 ml of absolute ethanol. This is the equivalent of 12 mg of T4I per 100 ml, and is stable indefinitely if stored frozen.

**Working standard (in protein diluent).** Dilute 1 ml of stock standard to 200 ml with absolute ethanol. Refrigerated, this is stable for two weeks. Add 20, 50, 70, and 100 μl, respectively, of this diluted stock standard to 0.50 ml of protein diluent (duplicates). This corresponds to 2.4, 6.0, 8.4, and 12.0 μg of T4I per 100 ml.

**Protein diluent.** “Bovine Albumin Solution” (35 g/100 ml; Sigma, cat. No. A-5128) diluted sixfold with physiological saline (9 g NaCl/liter).

**Controls**

Each run should include one normal and one abnormal serum control. “Monitrol I” (Lot LTD 107D), “Monitrol II” (Lot PTD 24A), “Metrix” (Lot 5422), and Hyland “Abnormal Clinical Chemistry” (Lot 0368H016A1) were successfully used in this study. It is not recommended that the volume of a normal control that is used be simply doubled to get a value in the abnormal range, because linearity of response is lost (possibly because there is more protein in the mixture).

**Ion-Exchange Columns**

The first part of the study was performed with the type of columns that were commercially available from Oxford Laboratories during 1970. However, those columns were discontinued and new columns (Oxford cat. No. 0971) were made available to us during December, 1970. Results with both columns are comparable if the method is modified as described here.

**Method**

**A. Column preparation.** The column consists of two units: the disposable column element containing the ion-exchange resin and the reusable column funnel.

1. Resuspend the column element by shaking. Remove the cap.
2. Place the column in a rack which has been positioned over a waste tray for draining.
3. Insert the small tip of the funnel into the top of the column and press together.
4. Cut off the tip of the column squarely against the shoulder about 5 min before the end of the incubation period in step B, 1 (below).
5. After the resin packing fluid has drained from the column, the column is ready for chromatography.

*(NOTE: Do not use columns of different lots in the same run.)*

**B. Chromatography.** 1. Dilute 0.50 ml of protein diluent (blank), standards, and serum, respectively, with 2.5 ml of 0.50 molar NaOH (duplicates). Mix by swirling the test tube (avoid frothing) and allow to stand at room temperature for 15 min (not longer). (Reduction in sample size from 1.0 ml to 0.5 ml results in extension of dynamic range. The larger amount of sample may have overloaded the column and thus decreased its efficiency. The increased concentration of NaOH provides a pH of 13.2, resulting in a more complete dissociation of T4 from its protein carrier.)

2. Deliver the diluted blank, standard, and serum onto previously prepared columns followed by a test-tube rinse with 3 ml of 0.10 mol/liter NaOH. Make all additions slowly; avoid disturbing the resin bed. (The test-tube rinse provides for a quantitative transfer of T4I from the original container.)*

*(NOTE: Column effluents from steps 2, 3, 4, and

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*The rationale for the most important changes are included in parentheses at the respective steps of the procedure.*
5 are all discarded. Each subsequent addition of reagent to the column is made after flow from the previous addition has stopped.

3. Add 10 ml of alkaline acetate wash to each column.
4. Add 10 ml of acid acetate wash to each column.
5. Add 0.60 ml of column prime to each column. (0.6 ml of column prime is adequate and prevents loss of T\textsubscript{4}, as observed with the use of larger volumes of column prime).
6. After the column flow has stopped, place the columns so that they will discharge into a 19 × 150 mm cuvet and add 5.00 ml of elution solvent. Label this collected effluent “first elution.” (Use of a larger volume of elution solvent and change of composition of the solvent results in a more quantitative elution of T\textsubscript{4}I from the column.)
7. After the first elution has been collected, place the columns over a second set of cuvets and add 5.00 ml of elution solvent. Label this collection “second elution.”

C. Colorimetry. 1. Add 0.50 ml of 2.0 molar H\textsubscript{2}SO\textsubscript{4} to each eluate, mix.
2. Add 0.50 ml of alkaline bromate–bromide solution to each eluate. Mix well on a vortex-type mixer and let stand at room temperature for 10 min. (The increased concentration of bromide and bromate increases the sensitivity and facilitates the T\textsubscript{4}I ceric–arsenite reaction).
3. Add 1.00 ml of arsenious acid to each eluate and mix well.
4. Place all tubes for 5 min in a water bath maintained at 37°C.
5. Deliver 1.00 ml of the ceric ammonium sulfate reagent to each eluate in turn at 20-s intervals, beginning with the first elution of the column blank. Mix well after each addition. Allow the mixture to incubate for 20 min in the water bath.
6. Set the spectrophotometer to 420 nm and adjust to 100% T vs. de-ionized water.
7. When the first tube (the first elution of the column blank) has incubated for 19.5 min, place it into the spectrophotometer. Adjust the reading to 10% T by increasing the wavelength. Readjust at 20 min if necessary.
8. The % T of the other eluates is measured in the same order and at the same interval at which the ceric ammonium sulfate was added. (If desired, absorbance readings instead of % T readings may be taken).
9. Plot the curve for % T values against blank and standard concentrations on semilog paper.\textsuperscript{4}

D. Second elution. 1. Follow the same procedure as for first elution. The second elution of the column blank must also be set to read 10% T by adjusting the wavelength.
2. If for any unknown the T\textsubscript{4}I of the second elution exceeds 25% of the value of the first elution, or 1.0 μg/100 ml, contaminating iodine may be suspected.

Results

Patient Material

All 293 hospitalized patients used in this study were clinically evaluated with respect to thyrometabolic status under the supervision of one of us (C.J.M.), the clinical evaluation being based on the patient’s history, physical examination, and personal interview. Patients were also questioned with regard to intake of drugs, estrogenic preparations, iodine-containing preparations, previous x-rays, etc. The clinical evaluation was supplemented by performing T\textsubscript{4} by-column and R\textsubscript{31} determinations on all 293 patients. In 165 of these 293 cases the T\textsubscript{4} by Murphy–Pattee test (9) was also performed. In 129 cases a resin T\textsubscript{4} test was performed and in 53 cases I\textsuperscript{131} uptake was also measured. These results were solely used to confirm the clinical evaluation.

Studies Related to Precision, Accuracy, and Linearity

The precision of the method was demonstrated by analyzing 10 samples of a commercial control (Metrix, Lot 5422) in replicate. The results of this analysis were 3.6 ± 0.1 μg/100 ml. The value listed on the analysis sheet of the control package was 3.6 μg/100 ml.

Recovery studies were performed by assaying a serum pool before and after a known amount of thyroxine was added. Results are summarized in Table 1.

Recovery studies of this type performed with the original method as supplied with the Oxford test kit and with other methods tested showed extremely poor results, especially in the higher range, as shown in Table 2.

The standard curve for the proposed method

\textsuperscript{4} In a recent article [Schales, O., CLIN. CHEM. 17, 670 (1971)] it was recommended that the standard curve be prepared by plotting absorbance on semilog paper vs. iodine concentration. We have plotted the standard curve as recommended by Schales and found no substantial difference in values.
shows an essentially linear response to 8.0 μg/100 ml and deviates only slightly from linearity between 8.0 and 12.0 μg/100 ml. If necessary, the curve can be extended beyond 12.0 μg/100 ml. In contrast, the calibration curve with the original Oxford method is linear to only 3.0 μg/100 ml, and deviates grossly from linearity above 9.0 μg/100 ml, which makes this part of the curve unusable.

**Normal Values**

The normal values for the revised T₄-by-column method were calculated from values obtained on 219 presumably normal hospital employees and 241 clinically euthyroid hospitalized patients. Five additional normal employees who were taking contraceptives were excluded from this calculation, but are included in the data shown in Figure 1.

The cumulative distribution of T₄I values obtained for the normal employees was plotted on normal (gaussian) probability paper and showed a fairly good fit except for a slight deviation at the upper end, suggesting a slightly skewed rather than an exactly gaussian distribution of values. This type of distribution was also seen when values were plotted in histogram form (Figure 1).

Assuming a gaussian distribution of values, the 95% confidence limits are 2.5 and 6.1 μg/100 ml (mean, 4.29 ± 0.9 μg/100 ml). The observed (empirical) 95% limits are 2.9 and 6.2 μg/100 ml.

The same values were plotted on log (gaussian) probability paper but showed consistently poor fit at the upper end, indicating that a log transformation is unsuitable.

The corresponding values for the 241 clinically euthyroid patients are shown in Figure 2. The 95% confidence limits were 2.1 to 6.0 μg/100 ml (mean, 4.06; standard deviation, 0.97).⁴

By the Becktel procedure (10), the mean was 4.0, the standard deviation 0.98, and the 95% confidence limits were 2.0 to 6.0 μg/100 ml. Inspection of the histogram (Figure 2) suggests that the distribution of data is slightly skewed and thus we think that the normal values of 2.5 to 6.0 μg/100 ml may represent a clinically more useful normal range for patients at bed rest.

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Table 2. Recovery Studies with the Original Oxford Method

<table>
<thead>
<tr>
<th>Pool no.</th>
<th>Assayed value</th>
<th>Assayed value after addition of 3.0 μg T₄I</th>
<th>Recovery of added T₄I, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>7.5/7.7</td>
<td>9.7/9.7</td>
<td>67-73</td>
</tr>
<tr>
<td>II</td>
<td>2.8/2.9</td>
<td>5.4/5.4</td>
<td>84-87</td>
</tr>
<tr>
<td>III</td>
<td>2.8/2.9</td>
<td>7.3/7.3</td>
<td>73-75</td>
</tr>
<tr>
<td>IV</td>
<td>4.5/4.8</td>
<td>9.0/9.0</td>
<td>70-75</td>
</tr>
<tr>
<td></td>
<td>4.1/4.1</td>
<td>9.0/9.5</td>
<td>82-90</td>
</tr>
</tbody>
</table>

⁴ Among the values obtained on the 241 clinically euthyroid patients, there were three values which exceeded three standard deviations. These results were eliminated and the 95% confidence limits recalculated.
Fig. 3. Values obtained with the modified T₄-by-column compared with those by the PBI method

Comparison of T₄-by-Column vs. PBI Methods

In previous years a good correlation was thought to exist between results by the T₄-by-column method and by the PBI procedure. Our recent experience indicates, however, that the PBI procedure is no longer a reliable test for thyroid function because of the increased use of iodine-containing x-ray contrast media, medications, and food additives; about a fourth of specimens are contaminated. Figure 3 shows the relatively poor agreement observed when T₄-by-column and PBI determinations were performed on the same sera from 293 hospitalized patients. The coefficient of correlation for the two methods was found to be 0.80. PBI values also correlated poorly with thyrometabolic status, probably also a result of the large number of contaminated specimens.

Comparison of T₄-by-Column vs. T₄ by Murphy–Pattee Methods

The interference in the T₄-by-column method by some x-ray contrast media sets certain limits for this method. In this respect, the T₄ procedure by Murphy–Pattee offers distinct advantages. In our study of 293 patients, 165 specimens were analyzed by both the T₄-by-column and the T₄ by Murphy–Pattee (9) techniques (Figure 4). The coefficient of correlation for these two procedures was calculated to be 0.75.

All patients with known iodine contamination were excluded from the comparative study of T₄-by-column and T₄ by the Murphy–Pattee method.

Fig. 4. Values obtained with the T₄ by the Murphy–Pattee (9) method compared with those by the modified T₄-by-column method

In general, the results of both methods agreed well with the clinical status of the patients. In each of the clinically hypothyroid and hyperthyroid groups, however, two results obtained with the T₄-by-column agreed with the clinical picture, while results obtained with the T₄ by the Murphy–Pattee method fell within the normal range. About 5% discrepancies were found between the two methods. This proportion of disagreement is to be expected because normal values for both techniques were based on the 95% confidence limits.

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References