Simplified Technic for the Determination of Serum Protein-Bound Iodine

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A simplified technic for the determination of serum protein-bound iodine (PBI) is presented. Endogenously labeled $^{131}$I PBI is completely recovered. The technic is based on the method of Barker et al. (1) and includes the following modifications: The number of washes of the precipitated serum proteins are decreased. Unashed iodide standards are used in a sodium carbonate solution rather than an internal standard. A temperature-correction formula is employed to allow the ceric-arsenite-iodide reaction to be carried out at room temperature, and a stable cerate color is produced with brucine sulfate so that the colorimetry is carried out with greater convenience. The serum PBI concentrations are read directly from a graph, which simplifies the calculations.

Interest in the technic of determining serum protein-bound iodine (PBI) has continued to increase since this test was recognized as a valuable tool in the diagnosis and management of thyroid disease. Although most of the commonly employed methods for this determination are considered to be accurate and reproducible, they are not completely satisfactory for busy clinical and research laboratories because they are time consuming and require constant attention by the analyst.

In this laboratory, several published methods were tried. The method of Barker et al. (1) employed the internal standard which not only limited the number of samples that could be completed at one time, but involved tedious calculations. Grossman and Grossman (2) presented a method for the production of a stable cerate color with brucine sulfate. Their modification increased the number of

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tests that could be carried out at one time. It also improved the accuracy by preventing color drift during the colorimetric stage of analysis. Their technic, however, required cumbersome temperature controls in order to achieve an apparent adherence to Beer’s law.

In order to overcome some of these undesirable features, the original alkaline ashing technic of Barker and co-workers (1) was modified by incorporating the published suggestions of others with those developed by the authors. Unashed external iodide standards containing sodium carbonate were substituted for internal standards. Brucine sulfate was used to terminate the ceric-arsenite-iodide reaction at room temperature. This allowed the reaction to proceed at room temperature, thus avoiding the need for controlled temperatures at different levels. The concentration of the PBI was read directly from a semi-logarithmic plot of the colorimetric data, eliminating the need for mathematical calculations.

The present communication describes the technic which was developed and indicates its accuracy, reproducibility, and clinical results.

Materials

Reagents

All solutions were prepared with deionized water. All chemicals were reagent grade, but were tested for their iodine concentration. Only lots containing minimum concentrations of iodine were used.

- **Zinc sulfate, 10%**. 100 gm. of ZnSO₄·7H₂O per liter of solution
- **Sodium hydroxide, 0.5 N**. 20 gm. of NaOH per liter of solution
(A permanent pink color should be produced when 10.8–11.2 ml. of this solution is titrated with 10 ml. of the 10% zinc sulfate in 50 ml. of water, using phenolphthalein as the indicator.)
- **Sodium carbonate, 4.0 N**. 212 gm. of anhydrous Na₂SO₃ per liter of solution
- **Hydrochloric acid, 2 N**. 167 ml. of HCl, sp. gr. 1.19, per liter of solution
- **Hydrochloric acid, 4 N**. 334 ml. of HCl, sp. gr. 1.19, per liter of solution
- **Sulfuric acid, 3 N**. 84 ml. of H₂SO₄, sp. gr. 1.84, per liter of solution
- **Ceric ammonium sulfate solution.** Five grams of Ce(SO₄)₂·2(NH₄)₂SO₄·4H₂O were added to 400 ml. of water and 500 ml. of 3N sulfuric acid. The material was dissolved and then diluted to 1 L. with water. Because different brands of ceric ammonium sulfate
have different moisture contents, an adjustment was made between the salt and the sulfuric acid. This was accomplished by placing 1.0 ml. of 4N sodium carbonate in a tube with 1.0 ml. of the standard containing 0.12 μg./ml. and treating both as standards. The colorimeter reading of the blank should be between 850 and 900, and that of the standard should be between 200 and 250. If the readings exceeded these ranges, the ceric sulfate solution was diluted with 3N sulfuric acid, and if the readings were below the prescribed range, more ceric sulfate was added.

**Brucine sulfate, 1%**. Ten grams of brucine sulfate were dissolved in water by warming and diluted to 1 L.

**Sodium arsenite, 0.1 N**. After 4.95 gm. of AS₂O₃ was dissolved in 25 ml. of 4% sodium hydroxide by warming, the resultant solution was diluted with 300 ml. of water and 7N sulfuric acid was added until the solution was acid to litmus paper; about 4 ml. usually sufficed. This solution was then diluted to 1 L.

**Iodide standards**. Reference solution: 130.8 mg. of potassium iodide, previously dried to constant weight was dissolved in water, and diluted to 1 L., yielding a solution containing 100 μg. iodide per milliliter. Stock solution: 2.0 ml. of the above solution were diluted to 1 L. with 4N sodium carbonate. Working standards: 20.0, 40.0, and 60.0 ml. of the stock solution were diluted with 4N sodium carbonate solution to 100.0 ml., yielding standards containing .04, .08, and .12 μg. of iodide per milliliter, respectively.

**Methods**

**Care of Glassware**

Precautions were taken at all times to prevent iodine contamination of the room and glassware. All glassware was soaked overnight in a sulfuric acid-dichromate cleaning solution and then thoroughly rinsed with deionized water. Incineration tubes were used only once.

**Technic of Determination**

All determinations were performed in duplicate. A control serum of known PBI content and a reagent blank without serum were included with each series of unknown samples.

**Precipitation and Washing of Serum Proteins**

To 1 ml. of serum in a 15 X 125-mm. Pyrex test tube were added 7 ml. of water, 1 ml. of 10% zinc sulfate, and 1 ml. of 0.5N sodium hydroxide. The contents were thoroughly mixed by inversion and
then centrifuged for 10 min. The supernatant liquid was decanted, and 10 ml of water was added. The precipitate was broken into a fine suspension with a glass stirring rod. The suspension was centrifuged for 5 min. The supernatant fluid was then decanted, and the precipitate was washed once more.

The same stirring rod was put into the tube and 1.0 ml of 4N sodium carbonate was added in two portions. Approximately one-half of the volume was allowed to flow down the sides of the tube, and the precipitate was broken into a homogeneous suspension. The remaining one-half of the carbonate was then used to wash the sludge from the stirring rod.

In order to determine the optimum number of washes in the technique for removing the inorganic iodide from the precipitated serum proteins, both labeled and unlabeled iodide were added to serum. One milliliter of a solution containing 0.10 µg of iodide with tracer amounts of I\(^{125}\) iodide was added to duplicate 1.0-ml aliquots of a serum before precipitation of the proteins. The radioactivities of the supernatant fluid from the precipitated proteins, from four washes, from the precipitates, and from the ashed precipitates were measured to determine the location and extent of inorganic iodide removed during the analysis. The PBI was also measured to determine the effect of the added iodide on it.

Table 1 shows that approximately 33 per cent of the iodide remained in the unwashed precipitate and that, after two washes, 15 per cent was still present. Two additional washes, however, removed not more than 1 per cent of the I\(^{125}\) per wash. Loss of the residual iodide during the remainder of the analysis was insignificant. Thus,

### Table 1. Efficiency of Removal of Iodide from Serum by Washing

<table>
<thead>
<tr>
<th>Serum sample* (µg/100 ml)</th>
<th>I(^{125}) added</th>
<th>% (\text{I}^{125}) found</th>
<th>% error due to added iodide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Precipitate</td>
<td>Ashed</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pre-cipitate</td>
<td>Precipitate</td>
<td>Precipitate</td>
</tr>
<tr>
<td></td>
<td>unwashed</td>
<td>washed (1X)</td>
<td>washed (2X)</td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>35.4</td>
<td>20.0</td>
</tr>
<tr>
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<td>10</td>
<td>35.2</td>
<td>20.0</td>
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<td>30.9</td>
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<td>19.0</td>
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<tr>
<td><strong>Mean</strong></td>
<td>33.4</td>
<td>19.4</td>
<td>15.8</td>
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</table>

*All serum samples were 1.0-ml aliquots of the same pooled serum to which tracer I\(^{125}\) iodide was added.
even after four washes and incineration, approximately 14 per cent of the tracer iodide still remained, representing an amount of stable iodine which would raise the PBI by 0.5 μg./100 ml.

Drying and Incineration

The tubes were dried overnight by inclining them at a 10-degree angle in an oven at 80 to 90°. They were then removed from the oven and placed in a slanting position in a cold muffle furnace. The furnace was gradually heated over a period of an hour until it reached 610°. It was maintained within 10° of this temperature for 3 hours. The rack of tubes was removed from the furnace and covered with aluminum foil. The furnace temperature was kept at 900° for 4 hours to remove any iodine contamination that may have occurred.

Dissolving the Iodide from the Ash

Two milliliters of 2N hydrochloric acid were run down the sides of each tube to loosen the ashed material. The tubes were allowed to stand until there was no longer an odor of hydrogen sulfide (about 10 min.).

Standards

Duplicate 1.0-ml. portions of each working standard and the sodium carbonate solution were added to 15 × 125-mm. test tubes. One milliliter of 4N hydrochloric acid was then added to each.

Production of Stable Cerate Color

From this stage all tubes—i.e., blanks, standards, controls, and unknowns—were treated alike. Three milliliters of 3N sulfuric acid was added to each tube by allowing it to flow down the sides. The tubes were thoroughly mixed by the "flicking" technic, which consists of grasping the tube near the rim and flicking the bottom with several fingers in rapid succession. The tubes were centrifuged for 5 min. Four milliliters of supernatant fluid were removed from each tube and placed in 18 ×150-mm. tubes. One-half milliliter of 0.1N sodium arsenite was added, and the contents were mixed by the flicking technic.

A "blow-out" pipet was used to add 1 ml. of ceric ammonium sulfate to each tube at 30-sec. intervals. Each tube was mixed immediately with the flicking technic. The temperature of the laboratory had previously been noted. If the temperature was 24°, an 18-min. reaction was used, starting with the addition of the ceric solution to
the first tube and ending with the addition of the brucine sulfate to the same tube. This time interval was increased or decreased by 30 sec. for each 1.0° decrease or increase in laboratory temperature, respectively. After the reaction period, 0.5 ml. of brucine sulfate was added from a blow-out pipet in the same time sequence in which the ceric reagent was added, and the reagents were mixed promptly by the flicking technic. The samples were read within 24 hours in a Klett-Summerson photoelectric colorimeter using a No. 42 blue filter and water as a reference solution.

Calculations

A graph was made on semi-logarithmic paper with the colorimeter readings on the vertical axis, and the serum PBI equivalents of the standards on the horizontal axis (Fig. 1). The colorimeter readings of the unknown samples were then converted directly to micrograms of PBI per 100 ml. of serum. The incinerated reagent blank should check within 0.2 μg./100 ml. with the tube containing 1.0 ml. of sodium carbonate solution.

Efficiency of Protein Precipitation

Skanse and Hedenskog (3) have recommended a waiting period of several hours between the precipitation of the serum proteins and the subsequent washing of the precipitate in order to avoid loss of iodine in the washing. Because such a delay is inconvenient, its necessity was examined. Serum containing labeled endogenous thyroid hormone was obtained from a euthyroid patient 72 hours after a therapeutic dose of I¹³¹ was given as treatment for heart disease.
This serum was dialyzed overnight to remove all of the nonprotein-bound radioactivity. It was precipitated by the routine technic and promptly subjected to the usual washing procedure. The radioactivity was measured. All of it remained in the precipitated serum proteins and none was present in the supernatant fluid or washes. Loss of radioactivity from the precipitate during the remainder of the procedure was negligible. Precipitated serum proteins may therefore be washed immediately without apparent loss of PBI.

Results

Recovery of Inorganic Iodide from Ashed Standards

When the iodide standards were incinerated, as much as one-half of the iodide was lost. Because the loss from the inorganic I\(^-\) standards is greater than that from the protein-bound fraction, unashed standards were used.

Reproducibility of Colorimetry

The temperature correction formula contributes greatly to the simplicity and accuracy of the PBI determination. It is based on the observed relationship between temperature and the rate of the first order ceric-arsenite-iodide reaction. Colorimeter readings were reproducible from day to day, as long as the laboratory temperature remained between 20 and 28\(^\circ\).  

Recovery of 1-thyroxine and \(^{131}\)I-1-thyroxine

The effectiveness of the technic was shown by the recovery of added 1-thyroxine and \(^{131}\)I-1-thyroxine from serum. To 1-ml. aliquots of serum were added 0.1-ml quantities of 1-thyroxine representing 0.02, 0.04, and 0.08 \(\mu\)g. of iodine per milliliter, containing tracer amounts of chromatographically purified \(^{131}\)I-1-thyroxine.* These mixtures were incubated at 37\(^\circ\) for 2 hours. The PBI content of the serums and their radioactivities were measured.

From the data in Table 2, it can be seen that 90.1 per cent of the \(^{131}\)I-1-thyroxine was recovered as PBI. The 9.4 per cent of the radioactivity in the washes accounted for virtually all of the loss in the measurement. The recovery of the unlabeled 1-thyroxine showed good agreement with the \(^{131}\)I-1-thyroxine when more than 2.0 \(\mu\)g./100 ml. of 1-thyroxine was added.

*Abbott Laboratories, Oak Ridge, Tenn.
Table 2. Recovery of I\textsuperscript{131} 1-thyroxine and I\textsuperscript{127} 1-thyroxine from Serum

<table>
<thead>
<tr>
<th>Serum sample*</th>
<th>(1^\text{st} 1\text{-thyroxine added} (\mu g./100 \text{ ml.}))</th>
<th>% recovery of (1^\text{st} 1\text{-thyroxine})</th>
<th>In pooled washes</th>
<th>In precipitate after washes</th>
<th>In ashed sample</th>
<th>PBI found (\mu g./100 \text{ ml.})</th>
<th>% recovery of added (1^\text{st} 1\text{-thyroxine})</th>
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<td>Mean</td>
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<td>91.9</td>
<td>90.1</td>
<td></td>
<td>85.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*All serum samples were 1.0-ml aliquots of the same pooled serum to which tracer amounts of I\textsuperscript{131} 1-thyroxine were added.

Accuracy of Method

Three serum samples representing the low, the medium, and the high ranges of PBI were each analyzed in duplicate on 5 different days. These determinations showed that the means were 3.7, 6.5, and 11.9 \(\mu g./100 \text{ ml.}\) (S.D.: 0.07, 0.3, and 0.7, respectively).

There was excellent agreement between duplicate PBI determinations on 800 consecutive specimens examined. The majority showed a difference of less than 0.5 \(\mu g./100 \text{ ml.}\) while less than 1 per cent showed a difference greater than 1 \(\mu g./100 \text{ ml.}\).

Clinical Findings

PBI levels in 65 euthyroid subjects showed a mean of 5.0 \(\mu g./100 \text{ ml.}\), with a range of 3.7–7.2 \(\mu g./100 \text{ ml.}\).

Discussion

Incineration at 610 ± 10° for 3 hours avoided loss of iodine from incomplete ashing and through volatilization. The effectiveness of the incineration was demonstrated by the recovery of virtually all of the added 1-thyroxine and I\textsuperscript{131} 1-thyroxine from serum.

The concentration of the hydrochloric acid was found to be optimal in dissolving and loosening the ash from the tubes without loss of iodide.

In this laboratory the technic for the production of the stable cerate color recommended by Grossman (2) was modified to take place in an acid medium at room temperature. The standards, however, were made up in 4N sodium carbonate. External unashed
standards treated in the same manner as the unknowns in a given series, permitted the duplication of results from day to day when the temperature corrections were applied. This is consistent with the observations of Connor and his co-workers (4) who stated that at 29°, a difference of 1° influences the velocity of the ceric-arsenite-reaction about 3 per cent.

Friis (5) advised that the precipitated serum proteins be washed 4 times to remove the unbound iodide, and Danowski and co-workers (6) recommended a routine of 6 washes. Our results indicate that two washes are sufficient to remove unbound iodide in concentrations up to 10 μg./100 ml. of serum. This number is sufficient except in grossly contaminated serums.

Acland (7) showed that 76.7 per cent of the thyroxine was recovered when it was added to the washed precipitates and then ashed. Our work, while demonstrating a 90 per cent recovery of the thyroxine when added to serum, also revealed that 10 per cent was removed by the washing process. These findings confirm the work of Tong and associates (8) who observed that when zinc hydroxide was used in the precipitation of the proteins, a 10 per cent loss of the added I¹³¹ thyroxine occurred. Our study showed, however, that endogenously labeled I¹³¹ 1-thyroxine was completely recovered as PBI, and that none was lost in the washes.

**References**