A Rapid Semimicro Method for Determining Protein-Bound Iodine

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An improved 4 hr. semimicro method for the determination of PBI that has an S.D. of 0.30 is presented. The advantageous features include the use of a 0.1-ml. sample size, the removal of inorganic iodide by an anion resin obviating the protein-precipitation step, and the utilization of an ashing time of 35 min. The suitability of this method for the small laboratory becomes apparent in terms of the time saved as well as in the expense of equipment and reagents.

Clinical laboratories handling a large number of analyses for protein-bound iodine (PBI), have found the automated technic, which has been increasingly utilized (1, 2), more convenient than the manual methods which employ the classic Barker (3) alkaline-ash procedure. However, for smaller laboratories, with limited facilities that are called upon to perform an infrequent PBI analysis, there exists a need for a rapid, accurate procedure. This communication describes a procedure incorporating several features that have not previously appeared together in any single method in the extant literature. A strong anion-exchange resin is used to remove inorganic iodide (4, 5), and in conjunction with the utilization of a 0.1-ml. serum sample, precipitation of the protein material is eliminated. By determining the appropriate ceric-arsenite ratio, measurements were obtained in the 20–80% transmittance region using 19 X 105 mm. cuvets. Normally, 12 specimens, in duplicate, can be analyzed in a period of 4 hr.

Materials

Reagents

Sodium arsenite, 0.035 N Dissolve 2.27 gm. of reagent-grade sodium arsenite (NaAsO₂) in approximately 500 ml. water, add 46 ml. concentrated H₂SO₄, and dilute to 1 L.

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Ceric ammonium sulfate, 0.005 N  Dissolve 3.17 gm. of ceric ammonium sulfate (purified for PBI determinations; G. F. Smith) in approximately 500 ml water, add 57 ml concentrated H₂SO₄, and dilute to 1 L.

Amberlite IRA-401, 20–50 mesh (Cl⁻) form  Rebottle the resin in small batches in clean containers to avoid any accidental contamination. Initially, the resin is checked for possible iodine contamination, otherwise no further treatment is necessary.

Potassium carbonate, 2.5 N  Dissolve 17.3 gm. anhydrous potassium carbonate in a small amount of water and dilute to 100 ml. Store in small polyethylene bottle.

Stock iodine solution, 100 μg. I⁻/ml.  Dissolve 84.3 mg. potassium iodate (KIO₃) in water and dilute to 500 ml in a volumetric flask.

Working standard, 1 μg. I⁻/ml.  Dilute 1.0 ml of the stock iodine solution to 100 ml. Store in a small polyethylene bottle in the refrigerator. Discard when large deviations from the established range of values for the calibration graph appear.

Standard for calibration graph, 0.01 μg. I⁻/ml.  Dilute 0.1 ml of the working standard with arsenite reagent to 10 ml in a volumetric flask. Prepare fresh and discard after using.

All reagents and solutions should be poured out in small quantities and discarded after use to eliminate possibilities of contamination.

Equipment

Glassware

All pipets were cleaned by soaking and rinsing in the following: (1) potassium dichromate-sulfuric acid cleaning solution, (2) tap water, (3) 10% (v/v) methanol, and (4) distilled water. To facilitate the cleaning of the pipets, several polyethylene pipet jars and baskets were used. The pipets were placed in the baskets with the constrictions pointing up to allow complete drainage of the viscous dichromate cleaning solution. Other satisfactory cleaning procedures may be used, but the above procedure has been found to effectively minimize contamination. A device using disposable plastic tips, for measuring the 0.1 ml volumes, may be used in place of the pipets. The technic for operating this device* was slightly more exacting, but following sufficient practice, excellent results were obtained.

The test tubes used for ashing (16 × 100 mm. Pyrex) were washed with detergent, rinsed with distilled water, and dried. These tubes were used once and then discarded.

The syringes for drawing the blood samples must also be appropri-

* Baltimore Biological Laboratories, Baltimore, Md.
ately cleaned. The use of Vacutainers (Becton, Dickinson and Co.) for obtaining specimens is highly recommended. The serum was transferred to clean 12 × 75 mm. tubes which were discarded after use. This tube was also a convenient size for use during resin deionization of the serum. A polyethylene or similar-type film should be used to seal the tubes; the use of rubber stoppers should be avoided.

Cuvets were rinsed with tap water, soaked in 1% (W/V) sodium thiosulfate, and washed.

**Furnace**

Any furnace with heating elements on all sides, top, and bottom is suitable. A temperature control is required. The chamber must have sufficient height to accommodate the 16 × 100 mm. tubes.

**Procedure**

**Specimen Preparation**

1. Add Amberlite 401 resin (amount that a spatula-tip holds) to each of several numbered 12 × 75 mm. test tubes.
2. Pour approximately 0.5 ml. of sample or control serum into the correspondingly labelled tube. Mix and allow to stand at least 5 min. Centrifuge for several minutes to pack the resin.

**Analysis**

1. Pipet duplicate 0.1-ml. samples of each serum specimen into clean 16 × 100 mm. Pyrex test tubes which have been labelled. Check for any resin beads, and if present, repeat pipetting of the sample.
2. Label 5 empty tubes for the calibration graph (0, 5, 10, 15, 20).
3. Add 0.3 ml. of 2.5 N potassium carbonate to all tubes and mix.
4. Place the tubes in the rack for ashing and dry at 130–140°; 25–45 min. will be required.
5. Place the rack in the ashing furnace which is at 600–650°. Allow to ash for 35 min., remove rack from the furnace, and let cool.
6. Pipet exactly 0.1 ml. of the 1 μg. I^-/ml. solution into a 10-ml. volumetric flask and dilute to volume with 0.035 N sodium arsenite. Mix well.
7. Add 3.0, 2.5, 2.0, 1.5, and 1.0 ml. of sodium arsenite reagent to the ashed 0, 5, 10, 15, and 20 standard tubes, respectively. Using volumetric pipets, add 0, 0.5, 1.0, 1.5, and 2.0 ml. of the diluted iodide standard in the same sequence. All tubes should have a final volume of 3.0 ml.
8. Add 3.0 ml.* sodium arsenite reagent to specimen and control

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*Dispensers of 3.0 ml. volumes (HyCel, Inc., Houston, Texas) were found to be convenient for this addition.
serum tubes, mix, and allow to stand until bubbling stops. Centrifuge all tubes for 10 min.

9. Decant all of the supernatant from each tube into a labelled 19 × 105 mm. cuvet.

10. Place the cuvets into 56° water bath for at least 5 min.

11. Add 3.0 ml. of ceric ammonium sulfate at 30-sec. intervals (shorter intervals may be used) to each tube, using a stopwatch (start the first addition at the 20-sec. mark). Mix and replace the tube in the water bath.

12. Exactly 30 min. after the first addition of ceric ammonium sulfate to the first tube, measure the % T at 420 mµ against a water blank set at 100% T. Remove the tube, wipe, and replace in the colorimeter, beginning the removal of the tube at the 20-sec. mark of each 30-sec. interval.

Calculations

A calibration graph is obtained by plotting the % T versus µg. I⁻/100 ml. on semi-log paper for the iodide standards (0, 0.5, 1.0, 1.5, and 2.0 ml. of diluted standard) used. One milliliter of the diluted standard, 0.1 ml. of the 1 µg. I⁻/ml. to 10 ml., will be equivalent to 10 µg. I⁻/100 ml. Results for the serum samples are read from this graph.

Discussion and Results

The circumvention of protein precipitation using either trichloracetic acid or zinc hydroxide is advantageous in time saved and the avoidance of an additional manipulation. Complete removal of the inorganic iodine normally found in serum, without any concomitant loss of thyroid hormone material, is, therefore, critical. This removal was achieved by the use of a strong anion-exchange resin. Although Scott and Reilly used a treated Dowex resin (5), Amberlite IRA-400 (4) and IRA-401 were found to be as suitable. To test the effectiveness of the resin, PBI determinations were performed on the same specimen to which amounts of inorganic iodide were added—up to a concentration of 1000 µg./100 ml. No breakthrough of iodide could be detected by these means, corroborating the results of Farrell and Richmond who added NaI¹³¹ and found 98.6–99.3% removal. Thus, a considerable margin of safety for removal of inorganic iodide is obtained by resin-deionization treatment.

Additions of 4, 8, and 10 µg. of thyroxine to several serum specimens, which were analyzed in duplicate, gave the recoveries shown in Table 1. From the evidence presented here and from that of other investigators,
resin-deionization treatment of serum appears to be a satisfactory means of avoiding the protein-precipitation step in PBI determinations.

The time and conditions required for complete ashing of samples were studied and the results are shown in Table 2. A more comprehensive study of the ashing conditions has been done by Foss et al. (6). Additions of small quantities of potassium oxalate or potassium chlorate (as oxygen source) to the potassium carbonate was found to be unnecessary for this purpose. Using a commercial control (Iodotrol—Dade Reagents, Inc.), which appeared to contain an iodinated-protein material quite different from that encountered in human sera, concentrations of less than 0.2% (w/v) potassium oxalate resulted in the formation of carbon using an ashing time of 60 min. While the amount of potassium chlorate was less (0.1% w/v), interference with the ceric-

<table>
<thead>
<tr>
<th>Sample</th>
<th>PBI (µg./100 ml.)</th>
<th>Added (µg./100 ml.)</th>
<th>Found (µg.)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.6</td>
<td>10.0</td>
<td>16.4</td>
<td>98</td>
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<td>2</td>
<td>5.5</td>
<td>10.0</td>
<td>15.2</td>
<td>97</td>
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<td>3</td>
<td>5.4</td>
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</tr>
<tr>
<td>4</td>
<td>5.4</td>
<td>4.0</td>
<td>9.3</td>
<td>98</td>
</tr>
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Table 2. A Study of Conditions for Ashing with Different Solutions

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time (min.)</th>
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<tbody>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>2.5 N K₂CO₃</td>
<td></td>
</tr>
<tr>
<td>Iodotrol</td>
<td></td>
</tr>
<tr>
<td>Hyland abnormal</td>
<td></td>
</tr>
<tr>
<td>Subject A</td>
<td></td>
</tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>Iodotrol</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2% K₂CO₃</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
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</table>

Results are average of duplicates.  
*Formation of carbon.  
†0.2% (w/v) K₂CO₃ in 2.5 N K₂CO₃.
arsenite reaction was found. In a comparison with the potassium carbonate alone, employing ashing times of less than 60 min., the oxalate-carbonate formed carbon from human sera or commercial control material. Our studies indicate that the oxalate accelerated the drying of the deionized serum prior to ashing by precipitation of calcium oxalate. With the carbonate, the drying process required approximately, 10–15 min. more, but this was amply compensated for by a decrease in the ashing time of from 60 to 35 min. Clearly, from the data in Table 2, the inclusion of potassium oxalate is superfluous, and in fact, lengthened the time required for ashing.

In varying the concentration ratio (ceric:arsenite) during the determination of the calibration graph we found that the ratio of 1:7 yielded the most practical working range with 19 × 105 mm. cuvets. The zero concentration of iodide for this ratio consistently gave readings of approximately 20% T. With the particular colorimeter used, measurements were made in % T rather than absorbance to increase the readability. Obviously, the concentration ratio of ceric-arsenite, cuvet size, and dimensions of measurements are relative values. Thus, they need not be adhered to rigorously but may be accordingly adjusted to other working parameters.

Two commercial control sera were assayed in duplicate for several days, and Table 3 depicts the data obtained.

A statistical analysis of the results of 50 samples, done in duplicate over a period of time, yielded a standard deviation of 0.30.

Glassware contamination and the screening of samples continues to be a problem. Maintaining strict control over handling of glassware and using disposable items wherever possible minimized the former problem. As for the latter problem, work is now progressing in this laboratory in the development of a simple, rapid procedure for screening samples which contain large amounts of artifactual iodine (iodine from sources other than the thyroid hormones). Such high iodine-content specimens cause gross crosscontamination during the ashing process so that the ashing must be repeated minus these contaminated specimens.

<table>
<thead>
<tr>
<th>Sample</th>
<th>PBI (μg/100 mL)</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Versatol</td>
<td>3.6</td>
<td>3.4</td>
<td>3.6</td>
<td>3.5</td>
<td>3.9</td>
<td>4.0</td>
</tr>
<tr>
<td>Monitrol</td>
<td>5.4</td>
<td>5.2</td>
<td>5.6</td>
<td>5.7</td>
<td>5.5</td>
<td>5.3</td>
</tr>
</tbody>
</table>

Results are average of duplicates.
An effective screening procedure would naturally save much time and effort.

**Addendum**

If traces of heavy metals, such as mercury from drugs or other sources are encountered, the addition of 1 gm./L. of sodium chloride to the 0.035 N sodium arsenite may overcome this inhibitory interference. The amount of sodium chloride necessary must be determined empirically, otherwise variations from one laboratory to another will be found.

**References**