Modification of the Fiske and SubbaRow Method for Total Phospholipid in Serum

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Many of the difficulties encountered in the original Fiske and SubbaRow method for estimating serum phospholipid were corrected through the use of vanadium pentoxide, a catalyst used in sample digestion, and through other minor modifications. Results obtained by the modified method agree well with those obtained by three other established phospholipid procedures. The greatly improved speed, simplicity, accuracy, and precision now makes the modified Fiske and SubbaRow method ideal for clinical or research use.

Over the years, a multitude of phospholipid methods have evolved, that of Fiske and SubbaRow (1) being by far the most frequently used (2), in spite of the fact that it has been criticized for lack of reproducibility (3), narrow range of acid tolerance (4), poor sensitivity (5, 6), lack of linearity (7), instability of the final color (8), turbidity formation (8), and for being a time-consuming procedure. I would like to describe a modification of the Fiske and SubbaRow method (1) in which most of the problems listed above are eliminated.

Materials and Methods

Reagents

Distilled deionized water was used throughout.

Digestion mixture. Dissolve 0.25 g of vanadium pentoxide (Baker, reagent grade; J. T. Baker Chemical Co., Phillipsburg, N. J. 08865) in 900 ml of concentrated perchloric acid (Fisher, reagent ACS grade, Fisher Scientific Co., Cleveland, Ohio 44128). Gentle heat is necessary to completely dissolve the vanadium pentoxide, and 24 h is usually required. Dilute to 1000 ml with concentrated perchloric acid. Store the reagent in a brown bottle. The reagent is stable for one to two months.

Ammonium molybdate, 25 g/liter. Dissolve 25 g of \((NH_4)_6Mo_7O_{24}\cdot4H_2O\) (Baker, reagent grade) in water, add no more than 1.0 ml of concentrated sulfuric acid to promote solution, and dilute to 1 liter with water. Discard the reagent if a precipitate develops. The shelf life of this reagent is about one to two weeks.

1-Amino-2-naphthol-4-sulfonic acid, digestion mixture. Weigh out 1.587 g of Fiske and SubbaRow Reducer (a formulation of 1-amino-2-naphthol-4-sulfonic acid, sodium sulfite, and sodium bisulfite; Sigma Chemical Co., St. Louis, Mo. 63178) and dilute with water to 100 ml. Be sure that the solute is completely dissolved before using. The reagent is stable for about two weeks. When the solution becomes dark yellow, discard the reagent.

Stock trichloroacetic acid, 1000 g/liter. Dissolve the contents of a one-pound bottle of trichloroacetic acid (Fisher, reagent grade) in water and dilute to 454 ml with water. Store the reagent in a brown bottle and keep away from bright light.

Trichloroacetic acid, 100 g/liter. Dilute 50 ml of stock trichloroacetic acid to 500 ml with water and store the reagent in a brown bottle.

Phosphorus stock standard, 1.0 g/liter. Dissolve 439.38 mg of potassium phosphate, monobasic (primary standard; Fisher, certified) in 100 ml of 2.5 mol/liter sulfuric acid.

Phosphorus working standards, 5, 10, and 20 mg/liter. Dilute the stock standard appropriately with water.

Methods

I. Digestion procedure A: Direct method—phosphorus determination on TCA precipitate. Into disposable 15 × 125 mm glass tubes, place 0.1 ml of
serum or plasma, add 3.0 ml of 100 g/liter trichloroacetic acid (TCA) solution, and carefully mix the contents without allowing the precipitate to adhere to the walls of the tube above the solution, let stand for 10 min at room temperature, then centrifuge the samples for 15 minutes at 2000 × g. Carefully discard the supernatant fluid and wash the precipitate with 5 ml of water by resuspending it and then recentrifuging. Carefully discard the supernatant fluid. It is very important that every drop of supernatant is emptied before you add the digestion mixture. To each tube add 0.5 ml of digestion mixture and heat for about 15 min at 240 °C on a microKjeldahl digestion unit (cat. No. 21-131-5; Fisher Scientific Co.). The higher the phosphorus concentration, the longer the digestion time required. It is very important that after digestion, the digestion mixture be allowed to mix with the contents on the walls of the tube by placing the tube in a horizontal plane and rolling the tube to allow the digestion mixture to wash the side of the tube. If the digestion mixture becomes light orange, digestion is incomplete; digest for another 5 min. The digestion mixture should be clear and colorless if digestion is complete. Allow the mixture to cool to room temperature and add 4.0 ml of water.

II. Digestion procedure B. Indirect method—phosphorus determination on organic solvent extract of total lipid. Use 1 ml of serum or plasma for the extraction of the total lipids by the method of Sperry and Brand (9) and wash the extract with 0.5 g/liter CaCl2 solution. Evaporate the extract to dryness on a rotary evaporator. Redissolve the contents in chloroform and dilute to 10.0 ml with chloroform. Carefully transfer a 1.0-ml aliquot (equivalent to 0.1 ml of serum) to a 15 × 125 mm glass tube and evaporate under a stream of nitrogen. Add 0.5 ml of digestion mixture, mix, and digest as described above in Procedure A.

Analysis. After the digestion step in either Procedure A or Procedure B, set up reagent blank and standards as follows:

<table>
<thead>
<tr>
<th>Blanks</th>
<th>Standards</th>
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<tbody>
<tr>
<td>ml</td>
<td></td>
</tr>
<tr>
<td>Digestion mixture</td>
<td>0.5</td>
</tr>
<tr>
<td>Working standards</td>
<td>—</td>
</tr>
<tr>
<td>Water</td>
<td>4.0</td>
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</tbody>
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To all tubes, add 1.0 ml of ammonium molybdate solution and mix. Add 1.0 ml of 1-amino-2-naphthol-4-sulfonic acid reagent, mix, and place in boiling water for 10 min. Cool to room temperature and read at 660 nm. Zero the spectrophotometer with the reagent blank.

Calculation:

\[ \text{Phospholipid, mg/liter} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times 100(\text{mg/liter}) \]

Results and Discussion

The following results are based on the direct method (Procedure A). However, extensive data on the comparison of Procedure A to Procedure B indicate that phospholipid estimation by either method gives comparable results.

1. After adding trichloroacetic acid to the serum, the sample can be kept in the refrigerator for a maximum of two days. Keeping samples for five days resulted in a 98% decrease in phosphorus values, for 7 days in a 16% decrease, and for 30 days a 27% decrease.

2. Omission of this water-wash step causes 17% higher values and greater variance in values, probably because of incomplete removal of inorganic phosphate from the suspension.

3. We used a Coleman Junior II, Model 6/20 (Fisher Scientific Co., Cleveland, Ohio 44128).

4. If a three point standard curve is not used, the phosphorus and phospholipid concentrations can be calculated by running a 10 mg/liter phosphorus standard and using the value obtained in the formula shown above. This applies to both Procedures A and B.

With use of primary standard phosphorus solutions, the curve (Figure 1) is linearly related to concentration to 1000 mg/100 ml (10 g/liter). Concentrations as low as 250 m/liter can be measured. The slope of the line is 1.246, with a y-intercept of .094. The correlation coefficient is .98.

For a within-run reproducibility test, 12 samples from a reference-sera pool were run on the same day. The CV was 1.9%.

For a between-run reproducibility test, 25 samples from a reference-sera pool were run, five samples each day for five days. The CV was 2.5%.

The proposed method was compared with the well-accepted Bartlett (4) and Youngburg and Youngburg (10) methods. A recently published method of Baginski et al. (2) was also compared to the proposed method. The results are shown in Table 1. By Student’s t-test the mean values obtained from the four procedures were not different statistically. The data indicates that the proposed method is as accurate as the two well-accepted phospholipid methods (4, 10), and within acceptable limits of the method of Baginski et al. (2).

This study shows that the use of vanadium pentoxide not only greatly enhances the digestion process, but also prevents any possible pH changes of the mixture. The resulting color developed with 1-amino-2-naphthol-4-sulfonic acid is stable for several hours.

These improvements make this procedure for phospholipid determination simple, fast, sensitive, precise, and accurate.

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References