A Micromethod for Rapid Quantitative Determination of Phosphonate Phosphorus


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A rapid method for initial quantitative estimation of the phosphate present in compound containing a carbon-phosphorus bond is described.

Two phosphorus assays are employed. One assay is for total phosphorus, which can be determined by digesting with perchloric acid and the other assay is for total non phosphonate phosphorus which can be determined by digesting with sulfuric acid simultaneously. The difference between total phosphorus and the non phosphonate phosphorus determined represents the amount of phosphorus present in a carbon-phosphorus linkage in a crude phospholipid sample.

Introduction

During the last two decades it was progressively established that aminoalkylphosphonic acids, as building stones of lipid and protein molecules, are much more widespread in nature than previously recognized. In addition, accumulating evidence has revealed a multilateral connection between their natural occurrence and biological significance [1].

So far, the qualitative and quantitative determination of phosphono compounds in the presence of phospho-derivatives is based on the chemical stability of the C–P bond, which cannot be broken down by prolonged heating with mineral acids [2, 3]. Aalbers and Bieber [4] have shown that even prolonged treatment at 160°C with concentrated sulphuric acid in conjunction with H₂O₂ according to Bartlett [5], is not effective in digesting phosphonates to orthophosphate. Accordingly, the phosphonate content may be calculated as the difference between total phosphorus and the non phosphonate phosphorus determined represents the amount of phosphorus present in a carbon-phosphorus linkage in a crude phospholipid sample.

Experimental

Lipid samples

Chromatographically pure egg phosphatidylcholine was prepared by extraction of egg yolk according to Bligh and Dyer [9], removal of neutral lipids according Galanos and Kapoulas [10] and...
silicic-acid column chromatographic fractionation of the phospholipid fraction. Pure sphingo-phosphonolipids of *Pelagia noctiluca* \(^{ab}\) were isolated by extraction (9), followed by silicic-acid column chromatographic fractionation (11, 16) and further purification via alkaline hydrolysis (12). AEP \(^{a}\) was purchased from Sigma.

**Reagents**

Deionized distilled water is used throughout to bring solutions to volume. All glassware is cleaned with nitric acid to eliminate contaminative phosphates [7]. Anhydrous analytical grade KH\(_2\)PO\(_4\) is used to prepare a stock phosphate standard with a phosphorus content 4 µg/ml.

The ammonium molybdate reagent (0.4%) is prepared with ammonium paramolybdate, \((\text{NH}_4)_2\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}\).

Stock ANSA solution is prepared by dissolving 0.5 g of purified 1-amino-2-naphthol-4-sulfonic acid in 200 ml of 12% NaHSO\(_3\) plus 2.4% Na\(_2\)SO\(_3\). The solution is filtered into a dark bottle and, if stored in the refrigerator, it may be used for 2–3 weeks. A portion of this solution diluted at hoc with 1.5 volume of water is used as the working ANSA reagent.

**Procedure**

1. Transfer duplicate aliquots A and B (0.4–4.0 µg P; in each one) of the lipid solution into Pyrex test tubes (15 to 18 x 180 mm) and remove all solvent by heating in a water bath or under a stream of nitrogen or air. Add 0.5 ml 72% HClO\(_4\) to sample A and 0.5 ml 10 \(\times\) H\(_2\)SO\(_4\) to the sample B.

2. Prepare two reagent blanks with 0.5 ml 72% HClO\(_4\) the first and 0.5 ml 10 \(\times\) H\(_2\)SO\(_4\) the second. Prepare also two sets of tubes for standards, set (I), set (II), add 0.5 ml 72% HClO\(_4\) in each tube of set I and 0.5 ml 10 \(\times\) H\(_2\)SO\(_4\) in each tube of set II.

3. Heat all test tubes at 160°C–170°C (sand bath) for 3 h, then let them cool to ambient temperature.

4. Add to each one of the standard tubes 1 ml of the appropriate phosphate solution, containing 1–4 µg P and to all the other tubes 1 ml of water. Mix well the contents of all tubes, add 3 ml 0.4% ammonium molybdate and mix again.

5. Add to all tubes 0.5 ml of the working ANSA reagent, mix well and place in a boiling water bath for 15 min.

6. Cool, add 5 ml ethyl acetate and mix well by vortexing for 30 s. Allow 15 min for phase equilibration and measure the optical densities of the clear blue upper layers at 780 nm, against the corresponding reagent blank. (Very dense extracts may be diluted with ethyl acetate.)

7. Calculations: The content of “phosphonate-P% of total-P” is equal to 100 \((M_A - M_B)/M_A\) where \(M_A\) and \(M_B\) are the amounts of phosphorus of the samples A and B respectively calculated by the corresponding calibration curves.

**Results and Discussion**

The novel modifications in the devised present method may be summarized as follows:

1. Conversion of non-phosphonate P to inorganic phosphate is quantitatively effected by heating at 160–170°C with concentrated sulphuric acid alone, without further treatment with additional hydrogen peroxide to decolorize the digest.

2. The phosphomolybdenum blue color at the final step is extracted with ethyl acetate for measuring its optical density.

3. Separate calibration curves are used for the sulphuric and perchloric acid digests respectively.

By applying the method described above on purified egg phosphatidyl choline it was shown that 3-h digestion at 160–170°C with sulphuric acid alone is effective for the quantitative conversion of phospholipid-P to inorganic phosphate.

Extraction of the blue color in an organic solvent overcomes the problem of incomplete decolorisation. It has been used in the past for other reasons in conjunction with isobutanol [13], isobutanol-benzene 1:1 [14] or butyl acetate [15]. Ethyl acetate was preferred in the present method because it is less toxic (compared with the isobutanol–benzene mixture), less expensive than butyl acetate and more common as a laboratory reagent. As shown in Fig. 1, the maximum wavelength of the phosphomolybdenum blue in ethyl acetate is translocated to 770–780 nm.

**Note**

To facilitate clearance of upper layers, 0.1–0.2 ml methanol may be added and mixed gently with the upper-layer prior to its transfer to the cuvette.
Finally, long experience had indicated that the calibration curves obtained according to the Bartlett procedure and to its modifications using perchloric instead sulphuric acid (at the same final normality) are different. This difference was found even greater by extracting the blue color in organic solvents. The separate calibration curves described in the present method overcome effectively this problem.

The method described above were checked on pure phosphatidylcholine (egg), sphingo-phospholipids (P. noctiluca) and AEP containing phosphonate-P 0%, 100% and 100% of total-P respectively. These samples, as well as their artificial mixtures with phosphate-P content in the range 10–90%, were analyzed by the method of Aalbers and Bieber [4] and by the present procedure. The latter yielded always satisfactory results, whereas values obtained by the Aalbers–Bieber method were less reproducible (especially in samples of no or low phosphate content), for reasons already mentioned.

In conclusion, the methods proposed herein overcome effectively most of common unforeseen sources of inaccuracy.