Direct Method for Determining Inorganic Phosphate in Serum with the “CentrifiiChem”

John A. Daly and Gerhard Ertingshausen

A direct method was developed for determining inorganic phosphate in serum, which requires only a single reagent addition. The method quantitates the unreduced phosphomolybdate heteropolyacid at 340 nm and is linear to at least 10 mg of phosphate per 100 ml. Only 10 μl of serum is required. The unique blanking capabilities of centrifugal analyzers permit the “on run” elimination of serum and reagent background absorbances, which are automatically subtracted. Data on precision, correlation, and recovery are presented. Kinetics of the reaction were studied, and theoretical limits of automatic blanking when applied to a first-order reaction are discussed.

Additional Keyphrases  direct w method  single reagent  automatic blanking  kinetics

Inorganic phosphate in biological samples is conventionally determined photometrically by use of the molybdenum blue reaction (1). Various reducing agents such as FeSO₄, SnCl₂, ascorbic acid, and aminonaphtholsulfonic acid have been used. These vary with respect to the stability and color intensity of the reduced phosphomolybdate heteropolyacid they all produce (2, 3). Measurement of inorganic phosphate via the yellow molybdovanadophosphate heteropolyacid has been suggested but has not yet been accepted as a routine procedure (4).

Even less effort has been made to quantitate the phosphomolybdate complex before it is reduced. This heteropolyacid complex absorbs maximally in the ultraviolet, and high sample and reagent blanks have to be eliminated.

The optical system of the “CentrifiiChem,” which permits absorbance to be measured in a linear range from 0 to 2.5, enabled us to use the unreduced form of the phosphomolybdate complex to determine inorganic phosphate. In addition, the unique blanking capabilties of centrifugal analyzers provide blank measurements in less than 3 s after sample and reagent are mixed. Individual blank measurements for all cuvets are automatically subtracted from the final absorbance reading, thus eliminating reagent and sample blanks simultaneously. If small amounts of “Tween-80,” a polyoxyethylene sorbitan monoleate, are added to the molybdate reagent, protein need not be removed from serum samples, and a single-step reagent for the determination was developed. The reaction is linear to at least 10 mg of inorganic phosphate per 100 ml, and is complete within 12 min. Only 10 μl of serum is required per test.

Materials and Methods

Reagents

\( H_2SO_4, 0.60 \text{ mol/liter} \). Pipet 33 ml of reagent-grade \( H_2SO_4 \) into 500 ml of distilled water. Cool to room temperature and dilute to 1 liter.

Molybdate solution. Dissolve 2.0 g of ammonium molybdate \( [(NH_4)\_2Mo_7O_{24}\cdot 4 \text{ H}_2\text{O}] \) in 1 liter of \( H_2SO_4, 0.60 \text{ mol/liter} \). Stable indefinitely.

Tween 80. Mix one volume of Tween 80 (Fisher Scientific Co., Springfield, N. J. 07081) with two volumes of water.

Working reagent. Mix 100 ml of molybdate solution with 0.9 ml of the diluted Tween 80. Use after 30 min.

Stock phosphate standard. 100 mg/100 ml. Dissolve 439 mg of \( KH_2PO_4 \) in water and dilute to 100

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ml. Add a few drops of chloroform as a preservative.

Working phosphate standard. Dilute 5.0 ml of the stock standard to 100 ml with water.

Instrumentation

A "CentrifChem" (Union Carbide Corp., Tarrytown, N. Y. 10591) as previously described [Daly, J. A., Fabiny, D. L., and Ertingshausen, G., CentrifChem, a high speed analyzer for the clinical laboratory, CLIN CHEM. 16, 530 (1970), abstract], is used as supplied by the manufacturer. A 10-µl serum sample is pipetted into the CentrifChem transfer disk, while 400 µl of working reagent is pipetted into the reagent cavity. An initial reading (T₀) is taken at 2.0 s and a final reading 12 min later at 340 nm. Standards are included in assigned positions to determine the appropriate factor for relating change in absorbance to concentration of inorganic phosphate.

Results and Discussion

Precision and Accuracy

Within-run precision, as determined by assaying 27 aliquots of a serum pool on a single rotor was 1.5% (cv) at a concentration of 3.0 mg/100 ml.

Day-to-day precision was studied for 10 days; the coefficient of variation was 2.7% for a commercial control containing 8.2 mg of phosphate per 100 ml. Results of our method were compared to those obtained with the molybdenum blue method used with the SMA 12/60 (Technicon Corp., Tarrytown, N. Y. 10591). Ninety-five samples were analyzed, with the results shown in Figure 1. Recovery experiments (Table 1) showed the accuracy of the method.

Kinetics

The kinetics of the overall molybdenum blue reaction were studied by Crouch and Malmstadt (5). The reaction rate with respect to phosphate was first order. They also found that the velocity is highly sensitive to changes in pH and molybdate concentration. The relationships depended very much on the kind of acid used and on the absolute ranges of pH and molybdate concentration in the reaction mixture. It was, therefore, important to investigate the reaction rate with respect to all those variables in the new reagent.

The possibility of an "initial rate" reaction was eliminated when it was discovered that serum protein had an accelerating effect on the reaction. This phenomenon varied from serum to serum without affecting the final absorbance difference between the blank and the reaction product.

<table>
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<th>Table 1. Percentage Recovery of Phosphate Added to Two Sera</th>
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Fig. 1. Correlation between Technicon SMA 12/60 molybdenum blue method and CentrifChem phosphomolybdate method (n = 95)

The dependence of the reaction rate on the pH was studied at three different sulfuric acid concentrations and it was confirmed that the speed of reaction was inversely proportional to the 9th power of the H⁺ concentration. This relationship was determined by plotting initial reaction rate vs. the H⁺ concentration on log-log paper and measuring the slope of the curve (Figure 2).

The test is also highly sensitive to molybdate concentration (Figure 3). The plots of the initial rate of the reaction vs. the molybdate concentration on log-log paper yielded a positive slope of 5, indicating the reaction velocity to follow Equation 1:

\[
\frac{d[PM]}{dt} = k \frac{[PO₄^{3-}][Mo^{3+}]^{5}}{[H^{+}]^{9}}
\]

(PM is phosphomolybdate)

This expression is very similar to that found by Malmstadt and Crouch.

The high dependence of the reaction rate on the pH provided a convenient parameter to use in designing a test such that, at the time of the blank readings at 2 s, the reaction was less than 2% complete.

The overall reaction when applied to this test was assumed to be first order because of the high
Fig. 2. Dependence of reaction rate of phosphomolybdate method on acidity, as measured at three phosphate concentrations and three acidities: \( \cdots \), 0.50; \(-\), 0.55; and \(-\), \(-\), 0.6 molar H\(_2\)SO\(_4\) 

Fig. 3. Dependence of reaction rate of phosphomolybdate method on molybdate concentration, as measured at two phosphate concentrations and three molybdate concentrations: \( \cdots \), 2.0; \(-\), 1.6; and \(-\), \(-\), 1.2 mmol/liter 

Fig. 4. Illustration of automatic elimination of different serum blanks for samples with identical phosphate concentration (3.8 mg/100 ml phosphate)

Based standards have half-life times of about 100 s, and have reacted to 99\% completion after 12 min.

Other Analytical Considerations

The limit of the molybdate concentration was determined by the absorbance of the reagent blank at 340 nm, which is considerable, but combined reagent and sample blanks having absorbance values near 0.8 could be tolerated because the optical system of the instrument is linear to well beyond an absorbance of 2.0.

Many organic polymers were checked as to their properties to keep serum protein in solution: Tween 80 most effectively prevented protein precipitation and turbidity.

Figure 4 illustrates the blanking capabilities of the CentriflChem. Two sera with equal phosphate concentration but different serum blanks were run simultaneously. Readings of blanks, which ordinarily are subtracted from the final absorbance, were retrieved and the absolute absorbance was measured every 2 min throughout the test. Figure 4 makes it obvious that the two absorbance changes are the same, even though the initial blank values differ considerably.

References


