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[54] **AUTOMATED DIRECT METHOD FOR THE DETERMINATION OF INORGANIC PHOSPHATE IN SERUM**


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[58] **Field of Search** .. 23/230 B, 230 R, 253 R, 259; 250/218; 356/197, 246, 196, 204



[56] **References Cited**

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[57] **ABSTRACT**

A direct method is provided for the spectrophotometric determination of inorganic phosphate in fluids, particularly body fluids, such as blood serum. The process requires only a single reagent addition and comprises reacting the phosphate-containing fluid with an ammonium molybdate solution and thereafter measuring the absorbance within a specified time interval before the reaction has measurably proceeded and at the end of the reaction by means of a centrifugal analytical photometer. Inasmuch as a linear relationship exists between the phosphate concentration and the change of absorbance, the concentration is an unknown sample can be conveniently calculated by comparison with the results obtained from the simultaneous measurement of a sample of known concentration.


**5 Claims, No Drawings**

# AUTOMATED DIRECT METHOD FOR THE DETERMINATION OF INORGANIC PHOSPHATE IN SERUM

This invention relates in general to a process for the determination of inorganic phosphate in fluids, particularly body fluids. In one aspect, the invention relates to a process for the determination of inorganic phosphate in blood serum. In a further aspect the invention relates to a process for the determination of phosphate using a centrifugal analytical photometer.

In recent years the need for more sophisticated quantitative analytical methods has increased markedly due to the numerous microanalytical studies in biochemical research, routine clinical testing for physicians and hospitals, and the like. In addition to the increased demand for new methods of analysis, in certain fields it is often highly desirable that the method be simple to perform, be rapid and yet provide consistently reliable results. This is particularly important for clinical testing of body fluids where a proper diagnosis of treatment often depends upon the information provided by analyses. However, few methods are available which can rapidly and accurately handle the increasing number and varied test desired by clinicians.

For example, the determination of inorganic phosphate in body fluids, such as blood serum, is assuming a steadily growing share of the clinical laboratory's work load. The determination of phosphate in serum is important in several diseases in particular uremia and chronic renal diseases were phosphate retention occurs. Only the so-called inorganic phosphate is estimated since the significance of changes in phospholipids, phosphate esters and nucleotide phosphate is not easily related to clinical problems. However, in spite of the advent of many new chemical methods, the photometric determination of inorganic phosphate in biological samples is conventionally performed by the use of the molybdenum blue reaction (I.M. Kolthoff and P.D. Elving, Eds., Part II, Vol. 5 pages 317–402, 1961).

It has now been found that inorganic phosphate can be determined conveniently and accurately by a method which utilizes a centrifugal automatic analyzer. Analytical photometers which utilize a centrifugal field have recently become available for the rapid microanalysis of a wide variety of liquids such as body fluids, e.g., blood serum, food products, and the like. Since numerous analyses can be performed rapidly and simultaneously these devices are of particular interest wherein a large number of samples is involved or a variety of tests on one sample is desired. Moreover, since these devices allow the use of relatively small volumes of reagents, i.e., in the microliter range, the use of expensive reagents can be minimized.

One such device which utilizes a centrifugal field in microanalytical studies is described in U.S. Pat. No. 3,555,284. This device employs the principle of double-beam spectrophotometry wherein absorbances of a liquid sample and a reference solution are intercompared. The system is basically a series of cuvets arranged around the periphery of a rotor so that when it is spun, centrifugal force transfers reagents and samples to the cuvets where the concentration is measured spectrophotometrically. A sample loading disk is provided which consists of rows of cavities arranged concentrically. Reagents are placed in the inner-most cavity and serum samples in the center cavity of the sample loading disk which is then indexed and positioned in the rotor with each reagent and serum sample having its respective cuvet. As the rotor is accelerated, centrifugal force moves the reagents and sample to the outermost cavity where they are transferred through a small channel to the cuvet. During the transfer, the reagent and sample mix. The filled cuvets rapidly spin past the fixed light beam and the transmission of light is measured.



As previously indicated, most of the approaches for the determination of inorganic phosphate involve the use of the molybdenum blue reaction. This reaction involves the formation of a phosphate molybdate complex which is subsequently reduced by means of stannous chloride, phenylhydrazine, ascorbic acid, amino naphtholsulfonic acid or other reducing agents. A blue colored complex of the reduce heteropolyacid is formed and the absorbance of the complex measured at around 700nm (nanometers). The preparation of a protein free serum sample is required for this test, which makes the test cumbersome to perform. Additionally, the sensitivity of the test is low. Moreover, in order to perform the test properly, at least two sequential additions of reagents are required.

Attempts to measure inorganic phosphate by means of the yellow molybdovanadophosphate heteropolyacid have been suggested but have failed to date to become accepted as a routine procedure. Even less effort has been made to quantitate the unreduced phosphomolybdate complex prior to its reduction. The absorbance maximum of this heteropolyacid complex lies in the ultraviolet and high sample and reagent blanks have to be eliminated.

It is therefore an object of this invention to provide a process wherein many of the disadvantages indicated above are eliminated or minimized. A further object is to provide a process for the determination of inorganic phosphate which is accurate and requires only a single reagent addition. Another object of the invention is to provide a process for the determination of inorganic phosphate in which the background absorbances of sample and reagent are automatically eliminated. A further object is to provide a process which utilizes a centrifugal analytical photometer. These and other objects will readily become apparent to those skilled in the art in the light of the teachings herein set forth.

In its broad aspect the invention is directed to a process for the determination of inorganic phosphate in body fluids. The process comprises the steps of:

a. forming a mixture of the phosphate containing fluid and an ammonium molybdate solution;
b. measuring by means of a centrifugal analytical photometer a first absorbance reading at 340nm within two seconds after said mixture is formed,
c. measuring a second absorbance reading at 340nm within ten minutes after said mixture is formed
d. comparing the absorbance differential with at least one other differential obtained simultaneously under the same conditions from a fluid containing a known concentration of inorganic phosphate, and
e. determining the amount of inorganic phosphate in the phosphate-containing fluid.

The process of this invention provides an accurate and rapid method for the quantitative determination of inorganic phosphate in body fluids. In view of the fact that a linear relationship exists between the change of

absorbance and the phosphate concentration, up to at least 10 milligram per 100 milliliters phosphate can be measured. Results of precision studies and correlation with a known method indicated that this method was of equal accuracy.

Moreover, the process simplifies the diagnostic reagent by measuring the color formed in the first reaction alone. The absorption of the unreduced phosphomolybdate complex occurs in the ultraviolet and can be quantitatively measured at 340nm. In addition protein does not have to be removed from the sample and is kept in solution.

As previously indicated the process comprises the determination of inorganic phosphate by measurement of changes in absorbance due to the interaction of inorganic phosphate and ammonium molybdate.

For the analysis of inorganic phosphate by the process of this invention a centrifugal rotary photometer supplied by Union Carbide Corporation under the trademark "CentrifiChem" was utilized. In this instrument, a Teflon disk containing samples and reagents is inserted into a rotor with 30 radially arranged cuvets. When the rotor starts spinning, the reagent rises from every individual reagent well up to individual sample cavities, and the sample reagent mixture is transferred into the single cuvets within 1.5 seconds. One cuvet containing water is used as a reference when the cuvets spin past the stationary light beam of a spectrophotometer which measures the absorbance and displays it on an oscilloscope. Two sets of digitized absorbance readings of each cuvet can be stored simultaneously and the difference between them processed in a computer. A reading is taken of the first absorbance after start and the time interval after which the second measurement occurs also noted. A number of readings can be taken after the initial one. In the rate mode the absorbance change per interval is expressed in $\Delta A$/min. In the end point mode the absorbance differences between initial and every individual subsequent set of readings is measured. This approach allows compensation for cuvet to cuvet variations and for serum and reagent blanks if readings are taken before the reaction has started to a measurable degree. A blank reading recorded in a preceding run can be used as initial reading in a subsequent run. After a selected time interval has elapsed, the readings are printed out. The last set of data stored in the memory can be multiplied by means of the computer which permits direct print-out in concentration units. The rotor is thermostated within $\pm 0.1$°C. in an air bath.

A particular advantage of the process of this invention is that removal of protein which normally would interfere with the reaction is avoided. It was observed that serum protein could be kept in solution and turbidity minimized by the addition of a small amount of a surface active agent. Although a wide variety of surface active agents can be employed, it has been found that the non-ionic surface active agents are preferred. Illustrative agents include among others, the sorbitan monooleates, sold by Atlas Powder Company under the trademark "tween". "Tween–80" is particularly effective in eliminating protein interference. The amount of surface active agent employed need only be such as will prevent precipitation and turbidity. In practice it has been found that if the molybdate solution contains from about 0.1 to about 1.0 per cent of the surface active agent that protein interference is suppressed.

The single active reagent employed in the process of this invention is an ammonium molybdate solution. The solution is prepared by dissolving 2.0 grams of ammonium molybdate ( $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$) in one liter of 1.2N $H_2SO_4$. The solution is stable indefinitely. The surface active agent can be added to the molybdate solution. For example, 0.9 milliliters of a solution containing 1 part of the surface active agent to two parts of water can be added to each 100 milliliter of the molybdate solution.

A standard phosphorous reference solution can be prepared by dissolving 439 milligrams of $KH_2PO_4$ in 100 milliliters of water. A few drops of chloroform can be added as a preservative. 5.0 milliliters of this stock solution is then diluted to 100 milliliters with water.

In practice, 400 microliters of the molybdate reagent are pipetted into the innermost reagent cavity of the sample holder disk and 10 microliters of serum sample is pipetted into the sample cavity. With the analyzer in operation an initial absorbance reading is taken at 2.0 seconds and a final reading at 10 minutes. The instrument is equipped with a filter permitting readings to be taken at 340 nanometers.

The optical system of the analyzer permits the measurement of absorbance in a linear dynamic range from 0 to 2.5 at 340 nm. Since the reaction is linear to above 10 milligram per 100 milliliter phosphate, and the unreduced form of the phosphomolybdate complex can be employed, the inorganic phosphate can be determined rapidly and accurately. By measuring the difference in the two absorbance readings and comparing it with a solution containing a known quantity of phosphorous, the concentration can easily be determined. Comparison of the method of this invention with conventional literature methods confirmed its accuracy. The following example is illustrative:

## EXAMPLE I

The majority of the sample cavities of a Teflon disk sample holder of a CentrifiChem Automatic Analyzer were loaded with 10 microliters of human blood sera. The reference position contained 400 microliters of distilled water in the innermost cavity. The remaining cavities were loaded with 10 microliter aliquots of various phosphorous standards ranging from 2 to 10 milligrams per hundred milliliters. An ammonium molybdate solution was prepared containing 2 grams of $(NH_4)_6MO_7O_{24}\text{–}4H_2O$ and 3 milliliters of a non-ionic surface active agent, sold by Atlas Powder Company under the trademark "tween-80". In one liter of 1.2N $H_2SO_4$·400 microliters of this solution was then placed in the innermost reagent cavity of the sample holder.

The initial reading of all 30 cuvettes was taken two seconds after starting the rotor spin. The second reading was taken after ten minutes. The absorbance change was used to determine phosphorus concentrations based upon the standards. The test was run with a 340nm interference filter.

According to Beer's Law, the absorbance of a solution is proportional to the concentration of the solution's chromophore as long as the concentration is low. Accordingly, the concentration of phosphomolybdate formed in the reaction is at any time proportional to the absorbance it generates.

Absorbance X F = Concentration

In order to convert absorbance into concentration, the absorbance has to be multiplied with a factor F. In

the analyzer employed this is achieved by setting the appropriate factor on a digital switch which automatically multiplies the absorbance whereupon concentration units will be printed out directly.

During the analytical run, a standard of known phosphorus concentration is always run with the samples. If the results for the standard show slight variations from the correct concentrations, a setting of a digital switch is changed until the correct value is printed out. The same correction is then automatically applied to the results from the unknown sample.

Although the invention has been illustrated by the preceding disclosure, it is not to be construed as being limited to the particular embodiments or materials disclosed therein. Rather, the invention encompasses the generic area hereinbefore disclosed. Various modifications and embodiments thereof can be made without departing from the spirit and scope thereof.

What is claimed is:

1. A process for the determination of inorganic phosphate in a phosphate-containing fluid which comprises the steps of:

a. forming a mixture of said phosphate containing fluid and an ammonium molybdate solution;

b. measuring by means of a centrifugal analytical photometer a first absorbance reading at 340 nanometers within two seconds after said mixture is formed,

c. measuring a second absorbance reading at 340 nanometers within 10 minutes after said mixture is formed,

d. comparing the absorbance differential with at least one other differential obtained simultaneously under the same conditions from a fluid containing a known concentration of inorganic phosphate, and

e. determining the amount of inorganic phosphate in said phosphate-containing fluid.

2. The process of claim 1 wherein said fluid is a body fluid.

3. The process of claim 1 wherein said fluid is blood serum.

4. The process of claim 1 wherein said mixture contains a surface active agent.

5. The process of claim 4 wherein said surface active agent is a sorbitan monooleate.

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