Simplified Serum Phosphorus Analyses by Continuous-Flow Ultraviolet Spectrophotometry

KEL-198

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Serum phosphorus can be measured by continuous-flow uv spectrophotometry without reduction of the phosphomolybdate complex. The dilute sample is dialyzed into dilute (1 ml/100 ml) sulfuric acid, then mixed with an ammonium molybdate–sulfuric acid–"Tween 80" solution. The absorbance of the sample peaks is measured at 340 nm with a linear-absorbance spectrophotometer. Peak heights are directly proportional to concentration, because logarithmic conversion is performed within the spectrophotometer. The method obeys Beer's law up to 10 mg of P per deciliter, and results correlate closely with those for standard methods based on reduced phosphomolybdate blue. The miniature manifold uses a 12-in. dialyzer. The sampling rate can be 60 to 120 samples per hour when a Gilford one-piece debubbler flow cell is used.

Additional Keyphrases *AutoAnalyzer • linear absorbance spectrophotometry • nonreduction of phosphomolybdate complex*

Serum inorganic phosphorus concentration is measured by continuous-flow systems (AutoAnalyzer) as the reduced phosphomolybdate complex (*1–3*). However, this blue complex readily precipitates, and may produce a significant sample interaction or carryover. Daly and Ertingshausen (*4*) found that the unreduced phosphomolybdate complex absorbs ultraviolet light. By using an acidified ammonium molybdate–"Tween 80" reagent and a centrifugal analyzer ("CentrifiChem"), they developed a direct end-point method. We have found that by using the molybdate–Tween 80 reagent, serum inorganic phosphate can be measured at 340 nm with a miniaturized AutoAnalyzer manifold and a linear absorbance spectrophotometer. The results obey Beer's law at concentrations of 2 to 10 mg of P per 100 ml of serum, and correlate closely with conventional phosphomolybdate blue methods.

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Materials and Methods

Instrumentation

An AutoAnalyzer Sampler II, Proportioning Pump II, and 12-inch dialyzer were used.[1] A Model 4016 electronic sampler timer was used to avoid the inaccuracies produced by inaccuracies in multispoke mechanical cams (*5*), and linear absorbance was read with a Model 3019-A one-piece debubbler flow cell made of Kel-F (all from Gilford Instrument Laboratories, Oberlin, Ohio 44074). The output was recorded on a 10-inch servo chart recorder (Gilford Model 242). The spectrophotometer's signal to the recorder was dampened with a low-pass filter consisting of a 4 kΩ resistor placed in series on the positive line, followed by a 100-mF 6-V capacitor connecting the recorder inputs. The recorder was set to full-scale deflection with an absorbance of 1.0.

Reagents

1. *Sulfuric acid, 0.18 mol/liter, diluent and recipient.* Concentrated sulfuric acid, 10 ml, is added to about 900 ml of demineralized water, the mixture diluted to 1 liter, and 1 ml of wetting agent "A" (ultra wet 60-L, Technicon) is added.

2. *Ammonium molybdate, 2 g/liter in 0.3 molar sulfuric acid.* Concentrated sulfuric acid, 16.6 ml, is added to about 800 ml of water contained in a 1-liter volumetric flask. Ammonium molybdate tetrahydrate (Mallinckrodt), 2.0 g, is dissolved into this solution, the final volume is adjusted to 1 liter, and 3 ml of "Tween-80" (Difco Labs., Detroit, Mich. 48232) is added.

Manifold

In the manifold (Figure 1) standard-type Technicon Tygon proportioning pump tubing is used. The

[1] Technicon Instruments Corp., Tarrytown, N.Y. 10591. The 12-inch U-groove dialyzer was obtained as subassemblies Nos. 177-B078 and 771-B010 for the dialyzer plates, No. 177-0043 for the dialyzer bracket, and No. 177-0027 for the torque screws. Premounted Cuprophane membranes were obtained as No. 170-0406-03. The 12-inch dialyzers are also available as item No. 70131-2 from Acculab Supplies, Inc., P.O. Box 83, Orange, N.Y. 10962.

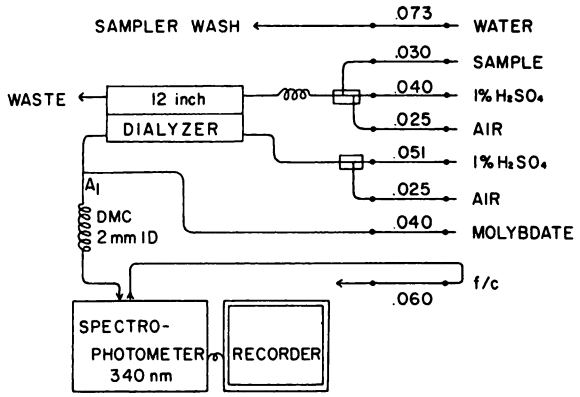

Fig. 1. Manifold diagram for serum phosphorus analyses

28-turn mixing coil is 2 mm i.d. Technicon connector assembly No. 177-B004-02 was used to merge sample with diluent, and No. 177-B004-01 to merge the recipient and air strains. These connectors produce a short and regular bubble pattern, and do not require the air bar that is part of the Technicon Proportioning Pump III. Alternatively, the bubble regulator we have described previously (*6*) may be used. The miniature (12 inch) dialyzer[1] is screwed onto the manifold platter. A Cuprophane ("C") membrane is used. The sample and recipient streams should flow through the dialyzer at the same speed to obtain best sample separation. The sampling rate is 60 samples/hour at a sample to wash ratio of 1:1. The reagent baseline has to be set electronically to 0.2 absorbance units, by using the absorbance display control, because the tungsten bulb does not emit sufficient light to overcome the rather high baseline absorbance of reagent.

Results

Absorption spectrum. Unreduced phosphomolybdate absorbs ultraviolet light with a peak absorbance at 320 to 325 nm; at 340 nm this absorbance is 82% as great. The tungsten lamp of the linear absorbance spectrophotometer we used emits sufficient light at 340 nm, but not at 325 nm, to allow easy measurement of the unreduced phosphomolybdate complex.

Color reagent. The molybdate reagent proposed by Daley and Ertingshausen (*4*) includes Tween 80 as the wetting agent. When Tween 80 was omitted, or substituted by wetting agent A, the sample peaks did not appear. We have no explanation for this detergent effect. The concentration of ammonium molybdate was varied from 0.1 to 2.0 g/liter, and the largest sample peaks were found with 0.2 g/liter. The sulfuric acid normality was varied from 0.1 to 2.0 N, and the optimum was found to be 0.6 N.

Beer's law. A rectilinear relationship between inorganic phosphorus concentration and absorbance was found up to a concentration of 10 mg/dl, with a correlation coefficient of $r = 0.999$ (Figure 2). The aqueous standards we use are 2, 4, 6, and 8 mg of phosphorus per 100 ml, which encompass the most frequently encountered range of clinical specimens.

Sensitivity. The molar absorptivity, ϵ, of the unreduced phosphomolybdate complex was 1.94×10^4 at 325 nm and 1.44×10^4 at 340 nm. In contrast, for the reduced complex it was 0.59×10^4 at 660 nm. The peak heights were 1.7 times greater with the proposed manifold at 340 nm than those measured at 660 nm after reduction. The discrepancy between the molar absorptivity ratios and the peak heights at 660/340 is the result of different dilution ratios used in the 340- vs. the 660-nm manifolds.

Accuracy. The stated P_i concentrations of seven different commercial sera were used as the reference points to measure the relative accuracy of the proposed method (Table 1). There was a mean deviation of 0.1 mg/dl or less between the measured and the stated values at sampling rates of 60, 80, 100, and 120 per hour, a negligible systematic error.

As another check on relative accuracy, the P_i concentration of 24 sera was measured by the proposed method and by a manual phosphomolybdenum blue method. The two groups of results (Table 2) correlated closely. The correlation coefficient was $r = 0.996$, and the linear regression equation was $y = 0.974\ x + 0.192$ mg/dl. The systematic mean bias of the proposed method was 0.1 ± 0.094 mg/dl (paired $t = 5.2$, $P < 0.001$).

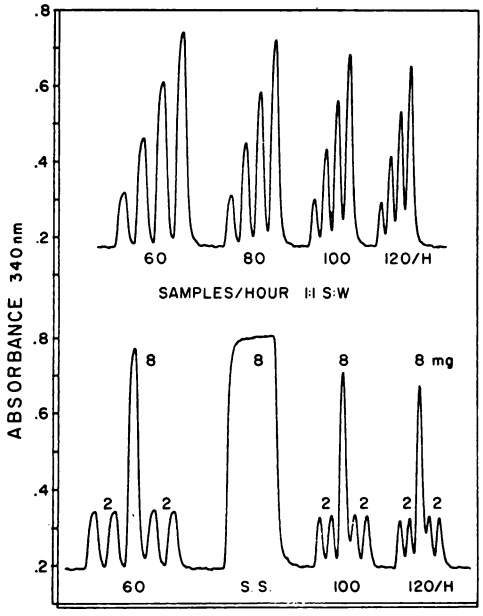

Fig. 2. Beer's law is obeyed at sampling rates of 60 to 120 samples/hour. Sample carryover is negligible, as shown by the sequences of low-high-low peaks. Steady state (s.s.) is readily attainable

Table 1. Accuracy of Proposed Phosphorus Method[a]

Reference serum	Label value, mg/dl	Sampling frequency 60	80	100	120
Fisher	6.5	6.5	6.5	6.4	6.4
Hyland	5.7	5.7	5.7	5.8	5.8
Monitrol	3.6	3.6	3.6	3.7	3.7
Technicon SMA 12	5.7	5.6	5.6	5.6	5.7
Validate	3.5	3.5	3.5	3.5	3.5
Validate A	6.3	6.2	6.3	6.3	6.4
Versatol A	8.0	8.1	8.1	8.0	8.1
Mean deviation		0.014	0	0	−0.042
±SD		0.063	0.054	0.077	0.077

[a] Accuracy is defined as "the extent to which a measured value agrees with the assumed or accepted value" (*7*).

Table 2. Correlation between Results of the Automated uv Method and a Manual Phosphomolybdenum Blue Method

Serum no.	Automated uv method, mg/dl	Manual method, mg/dl
1	2.7	2.7
2	4.0	3.9
3	4.4	4.2
4	2.7	2.5
5	3.9	3.8
6	2.8	2.8
7	4.0	4.0
8	3.9	3.7
9	3.6	3.4
10	1.3	1.1
11	4.6	4.7
12	2.8	2.6
13	2.7	2.5
14	2.7	2.6
15	3.4	3.3
16	4.0	4.0
17	4.6	4.4
18	2.5	2.4
19	2.4	2.3
20	3.6	3.4
21	5.1	5.0
22	6.5	6.4
23	5.8	5.8
24	3.6	3.7

When sera were assayed in the absence of ammonium molybdate, no peaks appeared, indicating a zero blank value.

Repeatability. The repeatability (*6*) of the proposed method was determined by 20 replicate determinations performed on pooled serum at 60, 80, 100, and 120 samples per hour. The coefficients of variation (Table 3) reflect the excellent repeatability regardless of sampling rates.

Table 3. Repeatability of the Proposed Method (n = 20)[a]

Samples/h[b]	$\bar{X}$, mg/dl	±SD, mg/dl	CV, %
60	5.7	0.06	0.5
80	5.9	0.10	0.9
100	5.8	0.15	1.5
120	5.9	0.14	1.3

[a] Repeatability is defined as the "deviation of test results from their mean value, all determinations being performed by one operator and without change of apparatus" (*7*).
[b] Sample-to-wash ratio, 1:1.

Day-to-day precision was measured by assaying four different reference sera at four different sampling rates (Table 4). Most of the standard deviations were less than 0.1 mg/dl. The coefficients of variation ranged from 0.87% to 3.02%, and evidently were not influenced by sampling rate.

Kinetic parameters. The proposed system has a lag phase of 4.2 s and half-wash time of 5 s, which compares favorably with these values for the SMA 12/60 phosphorus method, which are, respectively, 7 s and 7.8 s.

Percent carryover and percent steady state were measured at several sampling rates (Table 5). A small increase in carryover occurred as the sampling rate increased, but carryover was still quite low (1.8%), even at 120 samples/hour.

Discussion

The simplified method described here, based on Daly and Ertingshausen's reagent (*4*), has several advantages over the conventional automated measurement of reduced phosphomolybdate blue. These are: (*a*) carryover is only 1%, (*b*) the manifold is simplified, (*c*) the unstable hydrazine sulfate reagent is eliminated, (*d*) a sampling rate of up to 120 samples/hour can be used, and (*e*) sensitivity is enhanced. Moreover, in this simplified method the same basic chemistry is used as in conventional methods, namely the formation of phosphomolybdate complex, so that discrepant results as compared with traditional methods should be negligible. We attribute the low carryover to the absence of reducing agent, because when it was added to the molybdate stream, the carryover at 60 samples/hour was 3%, as contrasted with 0.7% for the proposed method. The use of an all-glass manifold should reduce carryover further. The use of wetting agent A for the sample diluent instead of the conventional Brij-35 simplifies reagent preparation, and eliminates a potential source of error from the phosphorus content of the Brij-35.

Table 4. Day-to-Day Precision of Proposed Method

Serum	Sampling rate/hour			
	60	80	100	120
	mg P_i/dl			
Monitrol	3.43 ± 0.082[a]	3.45 ± 0.071	3.46 ± 0.051	3.42 ± 0.079
	2.38%	2.04%	1.47%	2.3%
Ledernorm	3.09 ± 0.073	3.13 ± 0.095	3.16 ± 0.051	3.17 ± 0.067
	2.37%	3.02%	1.61%	2.11%
Ledertrol	7.57 ± 0.067	7.61 ± 0.110	7.60 ± 0.066	7.62 ± 0.123
	0.88%	1.44%	0.87%	1.61%
Hyland	5.67 ± 0.095	5.71 ± 0.137	5.74 ± 0.084	5.79 ± 0.087
	1.67%	2.39%	1.46%	1.5%

[a] Mean ±SD; (n = 10); CV, %.

Table 5. System Efficiency of Proposed Phosphorus Method

Samples/h[a]	Carryover,[b] %	Steady state, %
60	0.7	99
80	0.7	96
100	1.0	93
120	1.8	88

[a] Sample-to-wash ratio, 1:1.

[b] Measured by running a sample of low concentration (2 mg/dl), a sample of high concentration (8 mg/dl), and again a sample of low concentration.

The instrumental advantages of using a Gilford Model 300-N spectrophotometer for continuous-flow analyses are that: (*a*) ultraviolet light can be used without having to purchase a specialized uv colorimeter, (*b*) the linear-absorbance feature gives chart peaks that are directly proportional to concentration and follow Beer's law, (*c*) sample peak heights can be electronically converted into printed concentration results, (*d*) the wavelength is continuously variable between 340 and 800 nm, (*e*) the working absorbance range is greater than 2.0 *A* units, (*f*) the instrument is operable in a manual or in a continuous-flow mode by simply changing cuvets, and (*g*) any common strip-chart recorder, including the inexpensive Heath Kit IR-18M recorder, can be used.

The matrix in which the standards are prepared can affect the rate of continuous-flow dialysis for serum calcium, magnesium, and iron (*8–10*)—greater dialysis and higher peaks occur in the presence of a nondialyzable polymer such as albumin or polyvinylpyrrolidone (PVP). No matrix effects were observed for the proposed manifold, as aqueous standards had the same peak absorbances as standards prepared in PVP (8 g/100 ml), and the automated results from aqueous standards were the same as those obtained manually.

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