Enzymatic Colorimetric Method for the Determination of Inorganic Phosphorus in Serum and Urine

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Summary: The performance of an enzymatic colorimetric method for the determination of inorganic phosphorus in serum and urine is described. Phosphate ions react with inosine in the presence of purine nucleoside phosphorylase to form hypoxanthine; this is oxidized by xanthine oxidase to uric acid with production of hydrogen peroxide. The latter is determined with the aid of the chromogen system peroxidase/4-aminophenazone/N-ethyl-N-(3-methylphenyl)-N'-acetylethylenediamine, the coloured product being measured at 555 nm. This series of reactions is completed in 5 min at 37 °C. The test is linear up to 240 mg/l. Analytical recovery in serum averaged $101.2 \pm 1.2\%$ and in urine $101.9 \pm 3.2\%$. Within-run and between-run precision studies in serum and urine samples gave CVs $\leq 4.54\%$ (at 22.0 mg/l). Results obtained by this method agree ($r = \geq 0.983$) with the molybdate UV and molybdenum blue methods. Interference by endogenous substances, including organic phosphate, was negligible.

Introduction

Most techniques for the determination of inorganic phosphorus in serum or other biological fluids are based on phosphomolybdate complex formation by reaction of inorganic phosphate with molybdate in acid solution.

This colourless to pale yellow compound can be determined directly by UV absorption, or by colorimetric measurement after reduction to molybdenum blue or complexing with dyes such as malachite green (1).

These methods have several drawbacks: the direct UV method is affected by serum turbidity or pigmentation; the molybdenum blue method is influenced in its specificity, sensitivity and colour stability by the type of reducing agent used; and the strongly acidic conditions of the malachite green method may result in hydrolysis of organic phosphate.

All these methods are used nevertheless in the clinical laboratory, especially those based on direct procedures without deproteinization. Many attempts have been made to develop enzymatic methods: phosphorylase, phosphoglucomutase and glucose-6-phosphate dehydrogenase have been used to generate NADPH from inorganic phosphate and glycogen (2, 3). Glyceraldehyde-3-phosphate dehydrogenase was used as a key enzyme in an alternative enzymatic route leading to NADH (4). *Hwang* (5) reacts inosine with phosphate in the presence of purine nucleoside phosphorylase:[1] the resulting hypoxanthine, in the presence of milk xanthine oxidase,[1] produces uric acid, which is determined from its absorbance at 293 nm.

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[1] Enzymes:
Purine nucleoside phosphorylase (EC 2.4.2.1)
Xanthine oxidase (EC 1.1.3.22)
Peroxidase (EC 1.11.1.7)

All these enzymatic methods, however, have various limitations such as the need for reading in the UV range; poor stability of substrates or enzymes; unfavourable reaction equilibrium; and laborious procedures. Significant developments have been reported by *Fossati* (6) and *Machida* (7), both using the *Hwang* enzymatic sequence. More precisely, *Fossati* assayed the superoxide ion (formed as hydrogen peroxide precursor during hypoxanthine oxidation) by reducing a tetrazolium salt to formazane; *Machida* assayed phosphate, likewise in the visible region, using the peroxidase/phenol/4-aminophenazone chromogen system with an appropriate microbial xanthine oxidase.

A fully enzymatic colorimetric method based upon the *Machida* enzymatic sequence has been recently described (8, 9).

The reaction is illustrated in figure 1: inorganic phosphate reacts with inosine in the presence of purine nucleoside phosphorylase (EC 2.4.2.1) to form hypoxanthine and ribose-1-phosphate. Hypoxanthine is then oxidized by xanthine oxidase (EC 1.1.3.22) to hydrogen peroxide and xanthine, which is in turn oxidized to uric acid with further production of hydrogen peroxide. The hydrogen peroxide thus formed reacts with the chromogen system 4-aminophenazone/ N-ethyl-N-(3-methylphenyl)-N'-acetylethylenediamine, in the presence of peroxidase (EC 1.11.1.7), to produce a purple-red dye.

In this paper we report in full the performance characteristics of this method for both serum and urine.

Materials and Methods

Apparatus

A double-beam spectrophotometer with temperature-controlled cuvette holder (Model Lambda 5, Perkin Elmer Corp., Norwalk, CT 06856) was used for all the spectrophotometric studies.

A CentrifiChem® System 400 (Baker Instruments Corp., Allentown, PA) was used for all the other studies.

Reagents

The reagent kit for the inorganic phosphate assay (SERA-PAK® enzymatic colorimetric method, code 6683) was from Miles Italiana S.p.A., Ames Division, Cavenago Brianza (Italy).

The kit contains reagents to make two solutions in Pipes buffer (20 mmol/l, pH 6.25). Solution 1 contains 1.8 mmol of N-ethyl-N-(3-methylphenyl)-N'-acetylethylenediamine, $\geq$ 50 U of xanthine oxidase, and $\geq$ 2400 U of peroxidase per liter of Pipes. Solution 2 contains 1.3 mmol of inosine, $\geq$ 50 U of purine nucleoside phosphorylase, $\geq$ 70 U of xanthine oxidase, $\geq$ 1200 U of peroxidase, and 0.7 mmol of 4-aminophenazone per liter of Pipes. Both solutions are stable for four weeks at 2–8 °C.

A "single working solution" can be prepared by mixing equal volumes of Solution 1 and 2. Stored in a dark container protected from direct light, this solution will keep for three days at 2–8 °C and for one day at 15–25 °C.

The kit also contains a calibration standard of potassium dihydrogen phosphate equivalent to 50 mg/l (1.61 mmol/l) of inorganic phosphorus.

Procedures

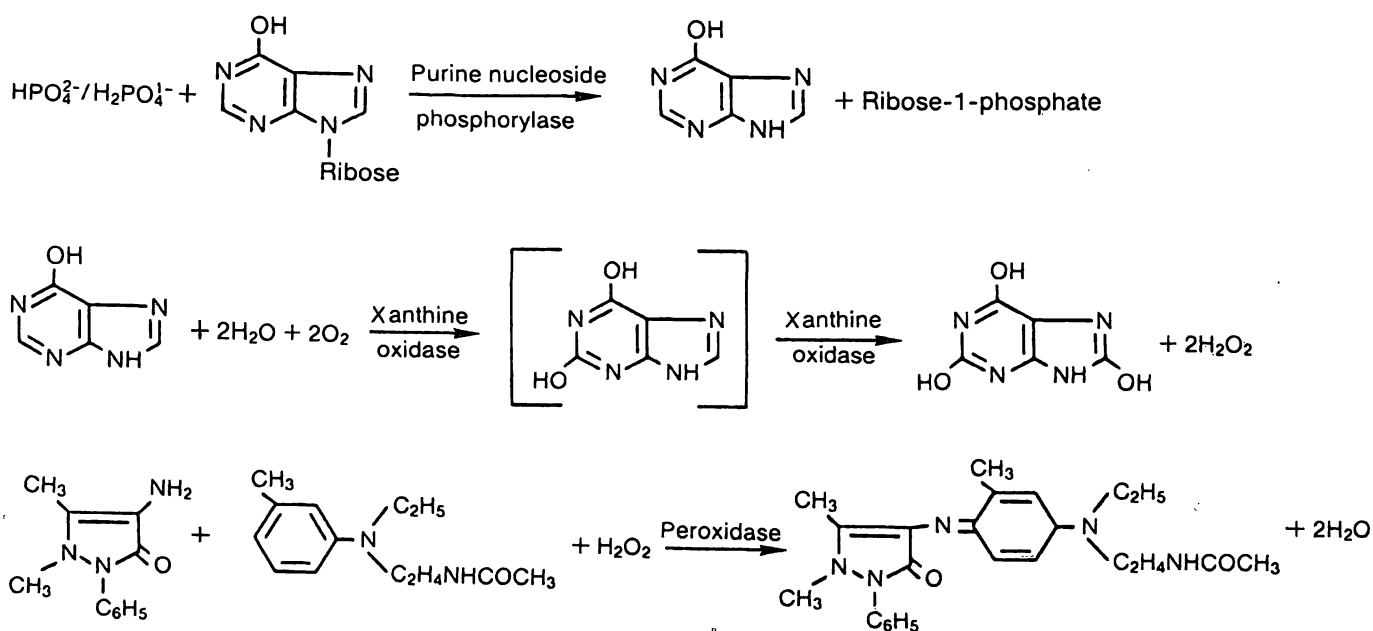Serum and diluted urine (1:10 with distilled water) were used as samples.

Fig. 1. Reaction sequence: enzymatic colorimetric assay of inorganic phosphate.

Manual two-step procedure

Add 20 µl of sample (or standard) to 1.5 ml of Solution 1, mix and incubate at 37 °C for 2—5 minutes. Add 1.5 ml of Solution 2; mix and incubate 5 minutes at 37 °C, then read at 555 nm against the reagent blank. The results reported for the manual procedure were obtained by this method.

Manual one-step procedure

The assay can also be carried out as follows: add 20 µl of sample (or standard) to 3.0 ml of "single working solution", mix and incubate 5 minutes at 37 °C. Read at 555 nm vs the reagent blank.

Automated procedure

The following instrument setting was used for CentrifiChem: temperature 37 °C; wavelength 550 nm; sample volume 5 µl, wash volume 55 µl; single working solution 350 µl; 1st reading time 3 s; time interval 300 s; control standard 50 mg/l.

This procedure was validated by assaying 60 human sera by the automated procedure (y) and by the manual one-step procedure (x). Calculated linear regression was: $y = 0.990x + 0.3$ mg/l, $r = 0.993$, $S_{yx} = 1.4$, $\bar{x} = 43.7$, $\bar{y} = 43.6$, range 20—80 mg/l.

Comparison methods

For the UV method without deproteinization (10) we used the Inorganic Phosphorus Reagent kit from F. Hoffmann-La Roche & Co. Assays were made by the fixed-time technique on a CentrifiChem 400 system at 37 °C with reading at 340 nm.

For the molybdenum blue method with *p*-methylaminophenol sulphate as reducing agent (11) without deproteinization we used the Phosphorus Auto/Stat® Diagnostic kit from Lancer Division of Sherwood Medical (Ireland). Assays were made manually.

Reference intervals

Serum samples were taken from 152 adult blood donors (75 men and 77 women). The reference limits were determined by a non-parametric technique (12).

Twenty-four hour urine samples were collected from 48 healthy adults (19 men and 29 women). Because of the small number of samples, the reference limits were determined by parametric technique, after checking the Gaussian distribution.

Control sera

We used the following commercially available control sera: Monitrol (Merz & Dade); Precinorm, Precilip (Boehringer Mannheim); Validate (General Diagnostics); Wellcomtrol (Wellcome); Ortho (Ortho); Roche (Roche); Kontrollogen (Behringwerke); Seronorm (Nyegaard); Biotrol (Biotrol); Q-Pak (Hyland).

Results

Absorption spectrum

The dye has an absorption peak at 555 nm. The absorption spectrum is broad enough to allow measurements in the range of 540—560 nm without significantly affecting sensitivity.
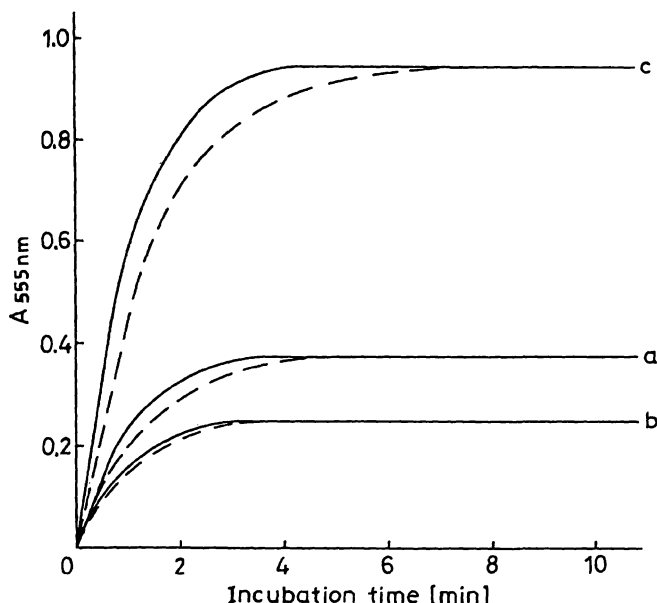
Fig. 2. Colour development curves at 20 °C (---) and 37 °C (—) for inorganic phosphorus standard at 50 mg/l (a), normal human serum (b), and abnormal human serum (c).

Colour development

We tested human sera and urines both normal and pathological for colour development rates. Complete colour development occurred within 5 min at 37 °C or less than 10 min at room temperature (18—25 °C). Examples of colour development for aqueous inorganic phosphorus standard and human sera are shown in figure 2.

After the recommended incubation time, no significant colour changes were seen at room temperature for at least 2 hours.

Linearity range and sensitivity

We assessed (sample/reagent ratio of 1/150) method linearity and sensitivity with aqueous phosphate solutions. When net absorbance was plotted versus inorganic phosphorus concentration, the response proved linear up to 240 mg/l (regression equation $y = 0.0758 x + 0.007$; $r = 0.9999$).

Absorbance per mg of phosphorus (1 cm light path) is 0.076, representing dye molar lineic absorbance of 1780 m² · mol⁻¹ at 555 nm. For the CentrifiChem instrument, this requires a sample/reagent ratio of 1/80; the linear range extends to 120 mg/l.

Recovery

Recovery tests, made by spiking serum and urine pools with NBS potassium dihydrogen phosphate, gave (tab. 1) an average recovery of 101.2 ± 1.2% (SD) for serum and 101.9 ± 3.2% (SD) for urine.

Tab. 1. Analytical recovery

Sample	Added	Found	Recovery (%)
	Inorganic phosphorus (mg/l)		
Serum pool	—	37.6	—
	25.0	62.4	99.2
	50.0	88.2	101.2
	100.0	139.8	102.2
	150.0	191.4	102.5
	170.0	209.3	101.0
	200.0	240.0	101.2
Urine pool	—	300	—
	240	544	101.7
	275	586	104.0
	315	607	97.5
	504	826	104.4

Tab. 2. Precision data

Serum			Urine		
Mean (mg/l)	SD (mg/l)	CV (%)	Mean (mg/l)	SD (mg/l)	CV (%)
Within-run (n = 10)			Within-run (n = 12)		
22.0	1.00	4.54	903.6	19.1	2.11
38.8	0.60	1.55	1424.6	21.9	1.54
90.0	1.50	1.67			
Between-run (n = 12)[a]			Between-run (n = 12)[a]		
40.7	1.00	2.45	216.0	5.5	2.55
51.5	1.70	3.30	508.1	15.1	2.97
80.0	2.20	2.75	630.0	10.5	1.67

[a] over a 15-day period

Precision

We assessed assay precision by replicate analysis of various human serum and urine pools at different phosphate concentrations. Table 2 shows the results obtained.

Method comparison

We explored the correlation between the enzymatic colorimetric method and two chemical methods, the phosphomolybdate UV method and the molybdenum blue method, in routine serum and urine assays. Results were processed by least-square regression analysis (13); correlation plots and statistical parameters are shown in figures 3 and 4.

Interferences

We explored the effects of known potential interferents in Trinder-type reactions, either endogenous or exogenous (bilirubin, haemoglobin, ascorbic acid, drugs such as L-dopa and its metabolite 3,4-dihydroxyphenylacetate, and α-methyldopa), by spiking pooled human sera at normal inorganic phosphorus concentration with known amounts of these substances (for haemoglobin we used washed and lysed erythrocytes). We also tested the effect of hypoxanthine and xanthine, since these occur in the reaction.

Allopurinol was tested as a known inhibitor of xanthine oxidase. The effect on test results of some representative organic phosphates, as well as of the more commonly used anticoagulants, was evaluated simi-
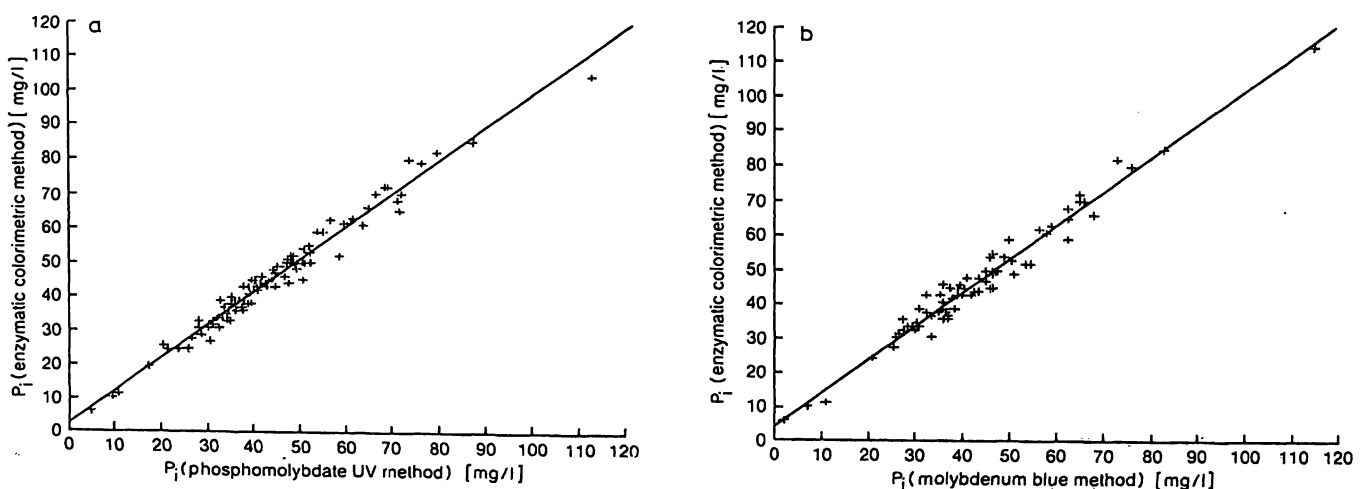

Fig. 3. Correlation plots for serum samples. Regression parameters are for
a) enzymatic colorimetric method (y) vs phosphomolybdate UV method (x):
$y = 0.949x + 2.6$; $r = 0.985$; $S_{yx} = 2.8$; $\bar{x} = 44.2$; $\bar{y} = 44.5$; n = 98;
b) enzymatic colorimetric method (y) vs molybdenum blue method (x):
$y = 0.969x + 3.8$; $r = 0.983$; $S_{yx} = 3.1$; $\bar{x} = 43.0$; $\bar{y} = 45.5$; n = 75.
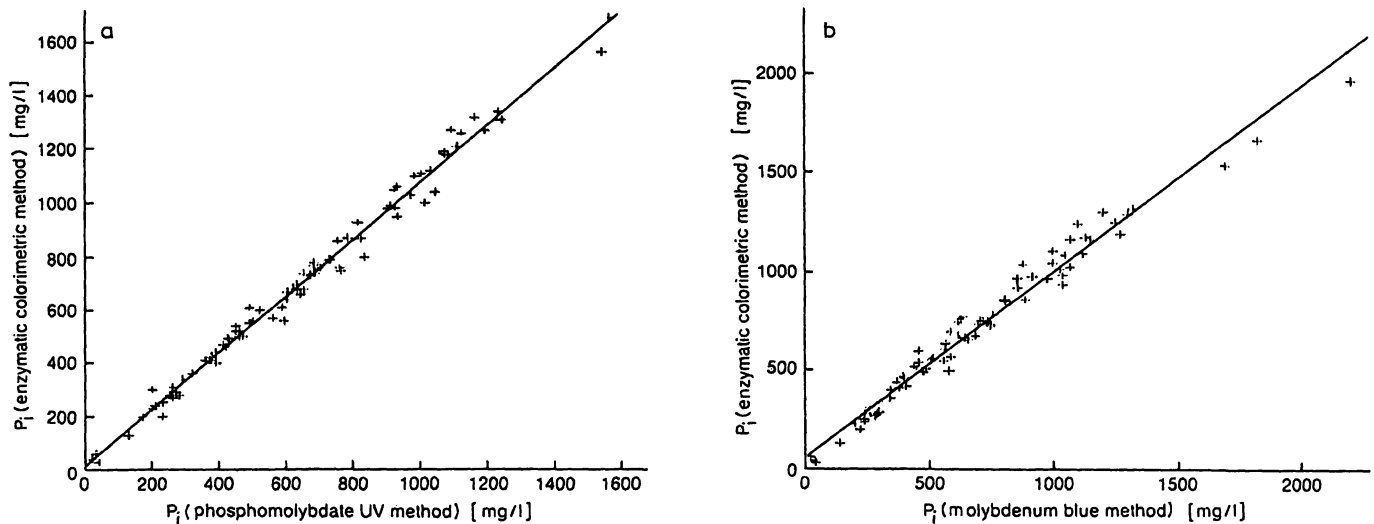
Fig. 4. Correlation plots for urine samples. Regression parameters are for
  a) enzymatic colorimetric method (y) vs phosphomolybdate UV method (x):
     $y = 1.06x + 17.3$; $r = 0.995$; $S_{yx} = 35.9$; $\bar{x} = 660.8$; $\bar{y} = 719.2$; $n = 123$;
  b) enzymatic colorimetric method (y) vs molybdenum blue method (x):
     $y = 0.958x + 53.9$; $r = 0.989$; $S_{yx} = 58.6$; $\bar{x} = 709.4$; $\bar{y} = 733.6$; $n = 121$.

larly. Results were assessed by analysing aliquots of pools with and without the interferent in ten replicates; means ± 3 SD were calculated and values outside this range were taken to indicate interference. No interference was found for concentrations as great as those shown in table 3.

Reference intervals

The reference limits, 2.5th−97.5th percentiles (0.90 confidence intervals) for serum inorganic phosphorus were: 24 (22−26)−45 (43−46) mg/l. For urine spec-

imens the reference limits (x ± 2 SD) were 134−1320 mg/24 h, with a mean value of 727 mg/24 h.

Values for the two sexes did not show a statistically significant difference (t-test, $p > 0.05$).

Control sera

We ran tests on several control sera from different sources. The values obtained and the claimed control sera values are shown in table 4. It should be pointed out that large differences exist among the claimed values by the different chemical methods; therefore the two extreme assay values (and the overall acceptable ranges) are given in the table.

Tab. 3. Results of interference tests

Substance tested	No interference up to
Metabolites	
Bilirubin	150 mg/l
Haemoglobin	1000 mg/l
Hypoxanthine	1 mmol/l
Xanthine	2 mmol/l
Adenosine-5-triphosphate	1 mmol/l
Creatine phosphate	1 mmol/l
Fructose-1,6-bisphosphate	1 mmol/l
Glycerol-2,3-bisphosphate	1 mmol/l
Phosphoenolpyruvate	1 mmol/l
Drugs	
Ascorbic acid	100 mg/l
L-Dopa	20 mg/l
3,4-Dihydroxyphenylacetate	80 mg/l
α-Methyldopa	40 mg/l
Allopurinol	2000 mg/l
Anticoagulants	
Disodium EDTA	2000 mg/l
Sodium heparinate	500 kilo-USP units/l
Sodium citrate	4000 mg/l
Sodium fluoride	4000 mg/l
Sodium oxalate	4000 mg/l

Tab. 4. Results with control sera

Control serum and Lot No.	Inorganic phosphorus (mg/l)	
	Claimed (range)	Present method
Monitrol I-E, LTD-205	39.2−42.1 (36.2−45.0)	40.3
Monitrol II-E, PTD-103	74.5−77.8 (68.5−83.2)	79.1
Precinorm U, 1-588	52.6−57.2 (45.2−65.2)	54.3
Precilip, 1-375	33.4−41.2 (28.7−43.7)	35.8
Validate, 3268022	34.4−36.5 (29.4−40.3)	40.3
Validate, 4333112	29.0−33.0 (27.0−35.0)	32.2
Validate A, 3725102A	61.0−68.0 (52.0−87.0)	69.0
Wellcomtrol N, K7475	38.4 (37.2−40.3)	40.5
Ortho Normal, 020A01	35.0−44.0 (25.0−48.0)	38.0
Ortho Abnormal, 025A01	66.0−75.0 (59.0−83.0)	79.3
Roche N, P1833	33.5−34.0 (30.5−37.0)	36.3
Kontrollogen L, 623122K	40.5−45.0 (36.0−51.0)	44.8
Kontrollogen LP, 623208	70.5−72.0 (60.0−81.0)	79.5
Seronorm, 159	34.4−37.2 (32.5−39.3)	38.3
Biotrol N, 559	48.0−56.0 (45.0−59.0)	56.1
Biotrol P, 707	78.0−84.0 (74.0−90.0)	93.1
Q-Pak I, N41B	30.0−35.0 (27.0−38.0)	28.1
Q-Pak II, P32B	66.0−71.0 (61.0−77.0)	72.0

Discussion

Existing chemical methods for assaying inorganic phosphorus in biological fluids generally lack sufficient specificity and ease of execution — two main considerations for routine use. The method at issue is highly specific and easy to perform, being based on an appropriate enzymatic reaction coupled with a sensitive colorimetric measurement.

Method linearity (up to at least 240 mg/l) is broad enough to make reassays unnecessary. Owing to the high sensitivity, precision is very good at the various phosphate levels tested, including the low ones. Comparative studies versus the phosphomolybdate UV method and the molybdenum blue method showed good agreement for both serum and urine.

High bilirubin and ascorbic acid concentrations, as well as moderate haemolysis do not affect results; haemoglobin above 1 g/l causes increased absorption and leads to phosphorus overassay.

Endogenous hypoxanthine and xanthine might interfere by generating stoichiometric amounts of hydrogen peroxide; but they are neutralized in the two-step procedure by reaction with N-ethyl-N-(3-methylphenyl)-N'-acetylethylenediamine during preincubation, forming a colourless product in the absence of inosine and 4-aminophenazone.

No interference from these oxypurines was seen with concentrations in excess of values ever found in serum (14). It should, however, be noted that even in the one-step procedure the results are affected only at very high values, such as those of patients receiving allopurinol (15). Allopurinol showed no inhibitory effect of its own. For L-dopa and α-methyldopa, a borderline negative interference may occur; no interference from 3,4-dihydroxyphenylacetate was noted. Organic phosphates with relatively high free energy of hydrolysis (≥ 21 kJ/mol $\triangleq \geq 5.0$ kcal/mol) are easily degraded in an acidic medium and are reported to interfere more or less significantly in chemical assay methods for inorganic phosphate (1). We tested a variety of phosphate links (ester, acetal, phosphoric acid anhydride, and amidophosphate) and found that none would interfere in the present method, even at concentrations much higher than those found in serum (16) or likely to be released by moderate haemolysis. Anticoagulants did not interfere.

In conclusion the method discussed in this paper shows good sensitivity, linearity and precision. With respect to accuracy, the method offers the advantage over chemical procedures of being free of interference from organic phosphate. Added advantages are the use of noncorrosive reagents, ease of handling, and adaptability to clinical laboratory instruments.

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