Continuous-Flow Assay with Immobilized Enzymes for Determining of Inorganic Phosphate in Serum

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An automated method for the determination of inorganic phosphate based on flow-injection analysis and the use of immobilized enzymes is reported. The method features a linear range between 0.1 and 20 μmol/L with a CV <2.1% and 3.4% for the within-run and between-run studies, respectively, and a sampling throughput of 40 h⁻¹. The sensitivity of the method makes a 1:250 dilution of the serum samples feasible, thus making undetectable the interferences from analytes commonly present in serum. The method shows an excellent correlation with conventional automated analyzers based on the same enzymatic reaction (Hitachi, r = 0.986) but with the catalyst in solution, and with the Kodak Ektachem method (r = 0.974) based on the use of dry reagents and formation of the phosphomolybdo heteropolyacid.

Indexing Terms: flow-injection analysis/intermethod comparison

Most of the procedures for the colorimetric determination of inorganic phosphate are based on the formation of molybdenum blue (1-3), on direct measurements of molybdo- and vanadomolybdophosphoric acid (4, 5), or on complex formation between molybdophosphoric acid and basic dyes (6). These chemical methods have serious shortcomings, however: Molybdate reduction is affected by slight changes in pH, the rate of complex formation is markedly influenced by protein concentration, and the acid pH required leads to hydrolysis of organic phosphate, which results in overestimates of P1 concentration (7).

A number of enzymatic methods that overcome these limitations have been reported. They are based on the ability of phosphate to activate some enzymatic reactions catalyzed by glyceraldehyde-3-phosphate dehydrogenase (8), phosphorylase a (9), maltose phosphorylase (10), sucrose phosphorylase (11), and nucleoside phosphorylase (NP; EC 2.4.2.1) (12, 13).4

Flow injection (FI) systems including immobilized enzymes for the determination of electrolytes have recently been reported (14, 15). They have several advantages over the use of dissolved enzymes in batch assays, such as lower analytical cost, higher selectivity and stability, and long life span. Male and Luong (16) developed the first FI method for the determination of phosphate with immobilized NP and xanthine oxidase (XOD; EC 1.1.3.22) and amperometric detection.

A new FI method for the determination of phosphate based on its effect on the enzymatic activity of NP is reported here. The reaction sequence is as follows:

\[
\text{NP} \quad \text{Inosine} + P_{1} \xrightleftharpoons[\text{HPOX}]{\text{XOD}} \text{hypoxanthine} + \text{ribose-5-P} \quad (1)
\]

\[
\text{HPOX} \quad \text{Hypoxanthine} + 2 \text{H}_{2}\text{O} + 2\text{O}_{2} \xrightarrow{\text{uric acid} + 2 \text{H}_{2}\text{O}_{2}} \quad (2)
\]

\[
\text{H}_{2}\text{O} + \text{bi(p-hydroxyphenylacetic acid)} \quad (3)
\]

where HPOX refers to peroxidase (EC 1.11.1.7).

The species monitored fluorometrically is the dimer of p-hydroxyphenylacetic acid (p-HPA), which exhibits maximum excitation at 325 nm and maximum emission at 415 nm.

Materials and Methods

Reagents and Chemicals

Inosine (cat. no. I-4125), p-HPA (cat. no. H-4377), NP (cat. no. N-8264), XOD (cat. no. X-4875), and HPOX (cat. no. P-8375) were purchased from Sigma (St. Louis, MO). Tris and all other reagents were supplied by Merck (Darmstadt, Germany). The reagents used for the determination of phosphate with the FI system were: Reagent A, 100 mmol/L Tri-HCl, pH 8.5; reagent B, 4.75 mmol/L inosine in reagent A; and reagent C, 10 mmol/L p-HPA and 8 U/L HPOX in 10 mmol/L Tri-HCl, pH 7.0. Reagents B and C were stable for at least 24 h. Phosphate standard solution (cat. no. 807575) and calibrator (cat. no. 759350) were purchased from Boehringer Mannheim (Mannheim, Germany).

All solutions were prepared daily with doubly distilled water purified with a Millipore (Bedford, MA) Milli-RO system.

Instruments and Apparatus

A Perkin-Elmer (Norwalk, CT) 204 spectrofluorometer furnished with a Hellma 178.12QS flow cell (18 μL inner volume), a Perkin-Elmer 56 recorder, and a Perkin-Elmer UDR-3 multimeter were used. A Julabo-5 (Juchheim Labortechnik KG, D-7633 Seelbach, Germany) recirculating thermostat, a Gilson Minipuls-2 (Gilson Medical Electronics S.A., BP 45-95400, Villier Le Bel, France) four-channel peristaltic pump provided...
with a rate selector, a Rheodyne (Cotati, CA) Model 5041 injection valve, and Teflon tubing [0.5 mm (i.d.)] were used to build the hydrodynamic manifold. A Model 072 pH meter (Beckman Instruments, Fullerton, CA), a Hitachi 717 analyzer (Boehringer Mannheim), and an Ektachem 700XR automated analyzer (Kodak, Rochester, NY) were also used.

Enzyme Immobilization

NP and XOD were immobilized on controlled-pore glass (CPG 120-200 mesh; Electronucleons, Fairfield, MA) by using Masoom and Townshend's procedure (17). Pump tubes of different lengths [1.5 mm (i.d.)] were then packed with each support–enzyme conjugate and stored at 4°C in the following solutions: 100 mmol/L Tris-HCl, pH 7.0, for the NP immobilized enzyme reactor (IMER) and 1 mol/L ammonium sulfate + 0.5 mmol/L sodium salicylate in 100 mmol/L Tris-HCl, pH 7.0, for the XOD IMER. Under these conditions both enzyme reactors kept their activity for at least 3 weeks.

Samples

Serum samples from a routine clinical laboratory were diluted 250-fold (100 μL of sample diluted to 25 mL) with reagent A before being injected into the FI system.

Both the FI system and the Hitachi 717 analyzer were calibrated with a calibrator for automated methods purchased from Boehringer Mannheim (cat. no. 759350). The Ektachem 700XR analyzer was calibrated according to the instructions of the Kodak manual.

FI Manifold and Procedure

The hydrodynamic system used (Fig. 1) consists of a peristaltic pump that propels the reagent streams through the channels. The sample, diluted appropriately, is injected into a stream of reagent A, which merges with a stream of reagent B; reagent B contains inosine, the substrate for NP biocatalysis. The first two enzymatic reactions take place along the NP and XOD IMERs. An additional merging point located after the IMERs allows the main stream to be mixed with reagent C (which contains p-HPA and HPOX), which reacts and catalyzes, respectively, the derivatizing reaction of the hydrogen peroxide produced in the previous step. The derivatizing reaction is developed along the reactor. Finally, the sample reaches the flow cell and provides the analytical signal. The enzyme reactor and the open reactor are thermostated at 37°C.

Results and Discussion

The variables affecting the analytical process and hence the signal it provided were classified as chemical, physical, and hydrodynamic (Table 1), and then studied by univariate analysis.

Physical variables. Increased temperature caused a sharp increase of the FI peak up to 37°C; the signal remained constant at 40°C, above which it decreased, probably because of denaturation of the biocatalyst.

Chemical variables. The pH optima for NP and XOD were similar (8.5 and 8.0, respectively), whereas the monitored fluorescent product yielded a maximal signal at pH 9.0. A pH of 8.5 was adopted as a compromise for further experiments.

The analytical signal increased with the concentration of inosine between 1.00 and 7.5 mmol/L, plateauing at >5 mmol/L; we thus chose to use 4.75 mmol/L. A similar effect was observed with the p-HPA concentration between 2 and 15 mmol/L, and we adopted 10 mmol/L as optimum. A concentration of 8 U/L of HPOX was best: Higher concentrations provided a strong fluorescent baseline, and lower concentrations decreased the analytical signal.

FI analysis variables. High flow rates (2.32 mL/min) decreased the analytical signal, but low flow rates (0.58 mL/min) decreased the sampling frequency and resulted in increased dispersion. A flow rate of 1.60 mL/min was selected as a compromise.

A sample volume of 300 μL was chosen to obtain the best analytical signal, since at greater volumes the signal remained almost constant.

The optimal lengths of the enzyme reactors were 1 cm each. Using a longer NP IMER provided a sharp in-

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Table 1. Assay variables.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Range studied</th>
<th>Optimal value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature, °C</td>
<td>20–45</td>
<td>37</td>
</tr>
<tr>
<td>Chemical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tris-HCl buffer, mmol/L</td>
<td>50–500</td>
<td>100</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td>6–9</td>
</tr>
<tr>
<td>Inosine, mmol/L</td>
<td>1.0–7.5</td>
<td>4.75</td>
</tr>
<tr>
<td>p-HPA, mmol/L</td>
<td>2–15</td>
<td>10</td>
</tr>
<tr>
<td>HPOX, U/L</td>
<td>4–12</td>
<td>8</td>
</tr>
<tr>
<td>FI analysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flow rate, mL/min</td>
<td>0.3–2.5</td>
<td>1.60</td>
</tr>
<tr>
<td>Injected volume, μL</td>
<td>50–500</td>
<td>300</td>
</tr>
<tr>
<td>Length of L₁, cm</td>
<td>50–400</td>
<td>250</td>
</tr>
<tr>
<td>Length of NP-IMER, cm</td>
<td>0.5–3</td>
<td>1</td>
</tr>
<tr>
<td>Length of XOD-IMER, cm</td>
<td>0.5–3</td>
<td>1</td>
</tr>
</tbody>
</table>

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crease in the baseline and a decreased analytical signal. Increasing the XOD IMER did not improve the analytical signal.

A length of 250 cm for the open reactor was enough to achieve a reproducible mixture of reagent C and the main stream, thus providing optimal analytical signal.

Features of the Method

A series of eight standard solutions with concentrations between 0.1 and 20.0 μmol/L were prepared from the phosphate standard solution described in Materials and Methods. The equation of the analytical signal obtained by triplicate injection of these standards into the FI manifold was as follows: fluorescence intensity = 27.5 + 49.2 [P] (μmol/L); intercept standard error, 4.59; slope standard error, 0.44; residual standard deviation, 12.41; r, 0.9994; sampling frequency, 40 h⁻¹.

Validation of the Method

The linearity of the method was assessed by means of Kroll and Emancipator's procedure (18, 19) recently adopted by the College of American Pathologists (20). Both the dimensional nonlinearity and the relative nonlinearity were calculated by using serum sampling in the range 0.15–5.00 mmol/L with a 1:250 dilution: the dimensional nonlinearity was 0.063 mmol/L; the relative nonlinearity, 1.29%.

These reflect an excellent linearity for the proposed method, since according to Kroll and Emancipator (19) the acceptable value for relative nonlinearity must be <2.5%. The relative nonlinearity of the analyzers used for comparison with the method reported here was also checked, and both had relative nonlinearity <2%.

The precision and recovery of the method was checked by assaying three serum pool samples from a clinical laboratory that contained low, medium, and high concentrations of phosphate (~0.900, 1.070, and 1.700 mmol/L, respectively). Aliquots of the three samples were analyzed after a 1:250 dilution, both in a single run and during 11 days for within- and between-run studies, respectively. The results obtained in both instances are summarized in Table 2.

Two aliquots of six samples were subjected to additions of standards (0.5 and 1.7 mmol/L) to establish the recovery of the method. The results obtained (Table 3) ranged from 96% to 104%, which represent a good recovery for the supplemented samples.

The performance of the proposed method and that of conventional automated analyzers were compared by determining the concentration of phosphate in 50 serum samples (concentration range 0.5–2.5 mmol/L) by the FI method and then by both the Hitachi method based on the same enzymatic reactions but with the biocatalyst in solution, and by the Ektachem 700RX method based on formation of heteropolyacid with molybdate (dry reagents technology). The reported method (γ) correlated well with both automated methods (x and x'), the correlation equations being γ = 0.968x + 3.37 × 10⁻² (r² = 0.976) and y = x' + 2.20 × 10⁻² (r² = 0.949).

Study of Interferences

The effects of bilirubin and hemoglobin as potential optical interferences and of uric acid, acetic acid, xanthine, hypoxanthine, and glucose as possible chemical interferences were studied. Each compound was added to a pool of serum (phosphate concentration 1.25 mmol/L) and its influence established. No interferences were detected from bilirubin for concentrations <600 mg/L, hemoglobin <20 g/L, uric acid <150 mg/L, acetic acid <75 mg/L, and glucose <200 mmol/L. Xanthine and hypoxanthine, substrates of the second enzymatic step, were also studied as possible interferents in the determination of phosphate, but gave no special problem, being intermediate metabolites and present in serum very infrequently. Nevertheless, this interference can be easily avoided by using a blank signal obtained by injecting the serum into the system in which inosine has been removed from reagent B; this repeat analysis increases slightly the determination cost. The presence of allopurinol <200 mg/L in serum did not cause interference; it did cause an error of ~4% at 400 mg/L.

Assay Advantages

FI analysis has proved to be a useful tool for clinical analysis because of its simplicity, repeatability, absence of carryover, low reagent consumption, and high sample throughput. In addition, the affordability of incorporating biocatalysts immobilized on suitable supports dramatically decreases the cost of analyses with respect to batch methods involving enzymes in solution. All these advantages make the method reported here a valid al-

### Table 2. Assay precision.

<table>
<thead>
<tr>
<th>Phosphate, mmol/L</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
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<tbody>
<tr>
<td><strong>Within-run (n = 22)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level I</td>
<td>0.890</td>
<td>0.018</td>
<td>2.03</td>
</tr>
<tr>
<td>Level II</td>
<td>1.064</td>
<td>0.016</td>
<td>1.50</td>
</tr>
<tr>
<td>Level III</td>
<td>1.700</td>
<td>0.012</td>
<td>0.70</td>
</tr>
<tr>
<td><strong>Between-run (n = 22)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level I</td>
<td>0.909</td>
<td>0.031</td>
<td>3.3</td>
</tr>
<tr>
<td>Level II</td>
<td>1.073</td>
<td>0.021</td>
<td>2.0</td>
</tr>
<tr>
<td>Level III</td>
<td>1.669</td>
<td>0.017</td>
<td>1.7</td>
</tr>
</tbody>
</table>

### Table 3. Assay recovery.

<table>
<thead>
<tr>
<th>Addition 1*</th>
<th>Addition 2*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Phosphate, mmol/L</td>
</tr>
<tr>
<td>1</td>
<td>1.28</td>
</tr>
<tr>
<td>2</td>
<td>1.79</td>
</tr>
<tr>
<td>3</td>
<td>1.56</td>
</tr>
<tr>
<td>4</td>
<td>0.45</td>
</tr>
<tr>
<td>5</td>
<td>1.20</td>
</tr>
<tr>
<td>6</td>
<td>1.02</td>
</tr>
</tbody>
</table>

*0.5 and 1.7 mmol/L for additions 1 and 2, respectively.
ternative for the determination of phosphate in serum.

Both the proposed assay and the Hitachi method have a similar sensitivity and linear range. The reagent cost per analysis with the proposed method is one-fifth that of the enzymatic Hitachi method: At least 500 determinations were performed with the reactors packed with 5 U of immobilized XOD and 10 U of immobilized NP vs 990 and 66 U, respectively, required in the in-solution enzyme Hitachi method for a similar number of analyses; only 8 kU/L HPOX is required in the proposed method vs 11 kU/L in the Hitachi method; and laboratory-prepared reagent costs less than commercial kits.

Our method also surpasses other FI analysis methods for this analyte (16) in terms of stability of the enzyme reactor (loss of 30% of its original activity in 3 weeks when stored in the appropriate phosphate buffer vs full working stability at least during the same time interval), sensitivity (detection limit of 1.25 μmol/L vs a determination limit of 0.1 μmol/L), sampling frequency (10–12 h⁻¹ vs 40 h⁻¹), and documented freedom from interferences.

A noticeable feature of the FI–immobilized enzymes method is the fast development of the reactions involved and also the rapid fluorometric monitoring, thus making possible it record the FI peak 75 s after injection.

The proposed method is not subject to interferences from bilirubin, hemoglobin, uric acid, ascorbic acid, allopurinol, and glucose. Only substances capable of reducing hydrogen peroxide cause interference. This is possible with uric acid, but the concentration at which it causes interference is found only in serum samples with pathological concentrations of this acid.

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References