Paraprotein Interference with Colorimetry of Phosphate in Serum of Some Patients with Multiple Myeloma

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In untreated serum of three patients with multiple myeloma, concentrations of inorganic phosphate ranged from 130 to 270 mg/L as measured with a chromogenic assay based on the interaction of phosphate ion with ammonium molybdate in the presence of ferrous sulfate. There were no clinical features of hyperphosphatemia, and values for total calcium concentration in serum remained within normal limits throughout. Subsequent investigations demonstrated that this hyperphosphatemia was spurious and was caused by high concentrations of the paraprotein interfering with the chromogenic assay. Because this type of assay, adapted for automated systems, is now widely used in clinical laboratories, we call attention to this limitation to avoid confusion in clinical evaluations of patients with multiple myeloma.

Additional Keyphrases: "kit" methods • analytical error

Azotemia, hypercalcemia, hyperuricemia, and hypernatremia are well-known features of multiple myeloma (I-4); hyperphosphatemia is not. We recently have encountered three myeloma patients with very high phosphate concentrations in their serum as measured with the Monoreactive Phosphate diagnostic kit (Biotrol Labs, Paris, France). Further investigation showed this spurious hyperphosphatemia to be the result of interference with the chromogenic assay by high concentrations of paraprotein. Here we describe the clinical features of the three patients and the in vitro studies aimed at clarifying the nature of this spurious hyperphosphatemia.

Case Descriptions

Patient no. 1: An 84-year-old man was admitted with bilateral bronchopneumonia and progressive dysphagia. Physical examination yielded no additional diagnostic information. His hemoglobin concentration was 93 g/L and serum creatinine 11 mg/L. Total serum protein concentration was 94 g/L, that of the globulin fractions 64 g/L. A bone-marrow aspirate showed massive infiltration by plasma cells, and a radiological survey of the skeleton indicated multiple osteolytic lesions. Immunoelectrophoresis showed IgG lambda paraprotein. The value for total serum calcium was 89 mg/L, and values for serum phosphorus, as determined with the Biotrol Monoreactive Phosphate diagnostic kit, ranged from 280 to 300 mg/L. There were no clinical manifestations of secondary hypocalcemia associated with hyperphosphatemia, and oral therapy with aluminum hydroxide had no effect on the concentrations of serum phosphorus measured. The patient was treated with the antineoplastic drug melphalan for six months without affecting serum protein concentrations and without any change in serum phosphorus.

Patient no. 2: This 69-year-old man was admitted because of diffuse skeletal pains. The diagnosis of multiple myeloma was based on plasma cell infiltration of the bone-marrow aspirate, hyperglobulinemia of 79 g/L, and the demonstration of IgG kappa paraprotein on serum immunoelectrophoresis. The calcium concentration in serum was 85 mg/L; that of serum phosphorus, determined by the Biotrol kit, was 240 mg/L. There were no clinical manifestations of hyperphosphatemia. This patient was treated with a combination of cyclophosphamide, vincristine, prednisone, lomustine, and melphalan, with excellent clinical response. Within six weeks the value for serum globulin had decreased to 30 g/L, with a simultaneous decrease in serum phosphorus to 34 mg/L.

Patient no. 3: This 81-year-old man was admitted for evaluation of anemia. A bone-marrow aspirate showed massive plasma cell infiltration, and IgG kappa paraprotein was demonstrated on immunoelectrophoresis of serum. There were no osteolytic lesions on skeletal survey. His hemoglobin concentration was 95 g/L, serum creatinine 12 mg/L, calcium 91 mg/L, and phosphorus (Biotrol) 130 mg/L. During the subsequent year, treatment with melphalan and prednisone had no demonstrable effect. The concentration of paraprotein in serum remained unchanged, but phosphorus remained high, ranging from 100 to 130 mg/L. There were no clinical symptoms of hyperphosphatemia.

Materials and Methods

We assayed serum samples simultaneously by three independent methods for phosphorus.

Method I: The Biotrol Monoreactive Phosphate diagnostic kit, introduced in recent years as the standard procedure for quantifying serum phosphorus at our clinical laboratory, is based on the use of ferrous sulfate as the reducing agent, with reaction with ammonium molybdate to produce a green-blue color, which is measured at 690 nm. The reaction is performed in unprocessed serum, i.e., in the presence of all serum proteins. Samples are assayed in a

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discrete analyzer (Model 203-S; Gilford Instrument Labs., Oberlin, OH). The procedure is a modification of the method described originally by Tausky and Shorr (5).

**Method II**: For this procedure reagents from Sigma Chemical Co., St. Louis, MO, are used for the vanadate method for phosphate (6), with assay in a continuous-flow analyzer (AutoAnalyzer II; Technicon Instruments Corp., Tarrytown, NY). In contrast to the previous method, proteins and other macromolecules are separated from the soluble salts in serum by dialysis, and phosphates are then measured in the protein-free filtrate. The method depends on photometry at 420 nm of the stable yellow color produced when an acidified solution of phosphate is added to a solution of excess ammonium molybdate and ammonium metavanadate.

**Method III**: This procedure is based on atomic emission spectroscopy with inductively coupled plasma. Serum samples are wet-ashed in nitric acid and perchloric acid to destroy all organic material. The clear final solution is diluted to a constant volume in distilled water and assayed with a Plasmatherm RF Generator Model JY 48 (Jovin Yvon, Long Jumeeau, France) operated at 1.25 kW at a frequency of 27 MHz (7).

**Results**

Table 1 lists the creatinine, calcium, and phosphate concentrations measured in the sera of the three patients. Phosphate concentrations determined by Methods II and III were within or slightly above our normal reference interval. In contrast, values determined by Method I, in which the color reaction is developed in the presence of serum proteins, were very high for all three patients. These high readings were not the result of increased turbidity.

To examine in more detail the relation between phosphate readings by Method I and the globulin concentrations in the serum, we plotted these two variables collectively for all three patients (Figure 1). As shown, there was a close linear correlation ($r = 0.876$) between the serum globulins and results by the colorimetric reaction used in Method I.

Consequently, we reviewed the results for more than 4000 phosphate measurements performed in recent years by the above method, excluding patients with renal failure or hyperparathyroidism, in whom high serum phosphate might be expected. In none of these patients did serum phosphorus exceed 70 mg/L, even in five additional patients with hyperglobulinemias caused by multiple myeloma, one with macroglobulinemia, and two with hyperglobulinemia secondary to active cirrhosis.

**Discussion**

Because hyperphosphatemia is not a recognized feature of myeloma, the discovery of three myeloma patients with extremely high concentrations of serum phosphate within a period of less than one year was highly unexpected and raised the possibility of a methodological problem. Several considerations suggested spurious hyperphosphatemia in these patients. First, none of them exhibited clinical problems associated with hyperphosphatemia such as tetany, or soft-tissue calcifications caused by reduced calcium solubility. Secondly, their values for serum calcium were within the normal range, which was incompatible with hyperphosphatemia of such magnitude. Lastly, therapeutic interventions aimed at decreasing serum phosphates, such as administration of aluminum hydroxide, had no effect on serum phosphate measurements. Thus the need for alternative methods for confirming the above measurements was considered justified.

The vanadate method (Method II) differs from the Biotrol Monoreactive Phosphate diagnostic kit (Method I) in that serum proteins and other macromolecules are removed from the sample before the chromogenic reagents are added. It is therefore a true measure of inorganic phosphate, the concentration of which was found to be normal in all three patients. This finding did not, however, exclude the possibility that phosphate, when in high concentrations, might be bound to the non-dialyzable paraprotein. Such a possibility was explored by the next method (Method III), atomic emission spectroscopy by inductively coupled plasma. These measurements showed total phosphates to be within the normal reference interval for all three patients. The fact that these values were about 60% higher than values obtained by the vanadate method is in keeping with the ability of the procedure to measure phosphates bound to phospholipids and proteins in addition to the inorganic phosphate in serum that is measured by the vanadate method.

Clearly, serum phosphate concentrations in our three patients were within normal limits, and the very high values obtained with the kit represent spurious hyperphosphatemia caused by a methodological error inherent to this procedure. The Biotrol Monoreactive Phosphate diagnostic kit is representative of a new generation of simplified commercial kits such as the SKI reagent (SmithKline diagnostic kit) and others in which phosphate is measured.

**Table 1. Creatinine, Calcium, and Phosphorus Concentrations (mg/L) in Serum from Patients with Multiple Myeloma**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Creatinine</th>
<th>Calcium</th>
<th>Phosphorus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Method I (Biotrol)</td>
<td>Method II (Vanadate)</td>
<td>Method III (ICP)</td>
</tr>
<tr>
<td>1</td>
<td>11</td>
<td>89</td>
<td>270</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>85</td>
<td>240</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>91</td>
<td>130</td>
</tr>
<tr>
<td>Normal ref. interval</td>
<td>8–15</td>
<td>87–106</td>
<td>25–42</td>
</tr>
</tbody>
</table>

ICP, inductively coupled plasma atomic absorption spectroscopy. Normal range shown is for healthy Israeli adults.
in unprocessed serum, in contrast to previous procedures such as the vanadate method in which serum proteins and other macromolecules are first removed by dialysis before colorimetry of inorganic serum phosphates. That proteins in general do not interfere with the Biotrol determination of serum phosphates is illustrated by the fact that in over 4000 phosphate determinations by that method in a general hospital population, including most of the patients with myeloma, we encountered no unexpectedly high phosphate values. Problems were encountered only in our three patients with monoclonal gammapathy. That the paraprotein was directly responsible for the spurious hyperphosphatemia in these patients is illustrated by the direct linear correlation between serum proteins and phosphates (Figure 1) in all three patients and the normalization of serum phosphates in patient no. 2 after serum protein concentration decreased in response to chemotherapy.

Color production in the Biotrol and similar procedures depends on the interaction of phosphate ion with ammonium molybdate in the presence of a reducing agent (8). Although the mechanism whereby some paraproteins may be responsible for abnormally high readings with this assay is at present unclear, its potential implications are obvious. In view of the widespread use in Europe and elsewhere of the Biotrol and similar kits adapted for automatic systems in clinical laboratories, this limitation of the method should be recognized in order to avoid confusion in the clinical evaluation of patients with multiple myeloma.

References

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Determination of Amylase Activity in Serum by Using a Wheat Germ Inhibitor with the Du Pont aca

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A rapid procedure for determining salivary- and pancreatic-type amylase (EC 3.2.1.1) in serum by incorporating a wheat germ inhibitor (from Triticum aestivum) was developed for the Du Pont aca IV analyzer. Under optimal assay conditions, activities of salivary and pancreatic amylase were inhibited by 93% and 19%, respectively. The 95% central reference interval for the percentage of inhibition of serum amylase was 38-84%. Patients with acute pancreatitis showed less than 26% inhibition of amylase after addition of the wheat germ extract, reflecting the prevalence of pancreatic-type amylase in this disorder.

Additional Keyphrases: isoenzymes · acute pancreatitis

In humans, \( \alpha \)-amylase (EC 3.2.1.1) exists in several isoenzyme forms. The salivary (S-type) amylase fraction is found in the salivary, lacrimal, parotid, mammary, and sweat glands, and in lungs, fallopian tubes, leukocytes, and thrombocytes. The pancreatic (P-type) amylase is found in the acinar cells of the pancreas, the intestine, and semen. In addition, an amylase isoenzyme has been isolated from serous ovarian tumors (1). The S-type and P-type fractions can be further separated into several major and minor isoenzymes (2).

Hyperamylasemia occurs in many diseases, and numerous drugs have been reported to increase amylase in vitro and in vivo (3). The P-type amylase is increased in acute pancreatitis, chronic relapsing pancreatitis, pancreatic cysts, glomerulonephritis, and hyperparathyroidism and is decreased in pancreatic insufficiency. S-type amylase is increased in serum from patients with mumps, Sjögren's syndrome, cholelithiasis, chronic pancreatitis, lung cancer, chronic renal failure, and other diseases. The concentration of S-type in serum is approximately equal to that of P-type amylase (4). Newborns and infants show a relative decrease in the P-type fraction (5).

The well-known lack of specificity of measurements of serum amylase for the diagnosis of acute pancreatitis is partly due to the difficulty in making a clinical diagnosis of acute pancreatitis. For this diagnosis serial measurements of serum amylase are more useful than a single amylase determination. Isoenzymes of amylase can specifically confirm the diagnosis of pancreatic disease (6).

Methods for separating amylase isoenzymes include ion-exchange chromatography, electrophoresis (7), isoelectric focusing (8), radioimmunoassay (9), and monoclonal antibody inhibition (10, 11). After O'Donnell and McGee and (12)