Error Introduced by Specimen Handling before Determination of Inorganic Phosphate Concentrations in Plasma and Serum

James E. Carothers, Neil M. Kurtz, and Jacob Lemann, Jr.

Values for serum inorganic phosphate (P1) concentrations in groups of healthy adults vary widely, the coefficient of variation ranging from 10 to 15%. We undertook to determine in 23 healthy adults whether part of this variation could be accounted for by (a) drawing blood in syringes vs. evacuated tubes, (b) the time between blood sampling and separation of serum or plasma, and (c) the prevention of clotting. Values were unaffected by a, decreased significantly with time at room temperature between blood sampling and separation of cells in both serum and plasma, and were significantly lower in plasma than in serum. The group coefficient of variation for P1 averaged 13% and was uninfluenced by the blood-processing technique.

Additional Keyphrases: variation, source of • normal values • pre-instrumental error • sample preparation • biological vs. analytical variation

The concentration of inorganic phosphate (P1) in serum varies widely in healthy humans (1-5). For 68 healthy adults fasted overnight, the group coefficient of variation (CV) in one study (5) was 10.9%. In contrast, the CV for serum calcium was only 2.8%. Similarly, in our own experience, the intersubject CV in healthy adults for serum P1 is 14.3%, while that for Ca is only 3.7% (6). Part of this group variance for serum P1 may have been accounted for by the fact that serum P1 values vary with the age of the individual (1-4), sex (5-4), time of the last meal (7), dietary phosphorus intake (8-10), activity (11), and as a consequence of the action of various hormones (12). In addition, Williams et al. (5) observed that when serum P1 concentration was measured weekly for 10 to 12 weeks after overnight fasting in the same 68 subjects, whose diets had not been restricted, the individual CV was 7.5% whereas the individual CV for Ca was only 1.7%. We have also examined the individual variation in serum P1 and Ca during metabolic balance studies in eight apparently healthy adults who were eating constant diets (i.e., the same amounts of the same foods each day) (10, 13). Blood was drawn into evacuated tubes after overnight fasting on six to 15 different days from each subject, and the serum was separated 2 to 3 h after blood sampling and frozen. All samples from the same subjects were then analyzed at the conclusion of that subject’s study. The intra-individual CV for P1 ranged from 2.7 to 16% and averaged 7.4%; that for Ca ranged from 1.4 to 3.2% and averaged 2.2%. These variations for subjects eating constant normal diets are very similar to those observed by Williams et al. in subjects eating unrestricted normal diets, suggesting that small day-to-day fluctuations in dietary phosphorus intake are not a major factor in the variation of serum P1.

Although part of this variation in serum P1 within the same subject might have been related to the time of their last meal and the extent of physical activity immediately preceding the sampling, as well as the variance of the chemical measurement, we questioned whether the variation might not also be contributed to by the technique for drawing the blood specimen or the elapsed time from venipuncture to separation of plasma or serum from the blood cells, or both. We also re-examined the effect of preventing blood clotting, because Lum and Gambino (14) observed that P1 concentrations were slightly but significantly lower in plasma than in serum.

Materials and Methods

With their informed consent, we studied 11 women and 12 men, all apparently in good health, who were eating their usual self-chosen diets. They were asked to avoid vigorous exercise on arising and the blood was drawn before breakfast. Immediately after the subjects were supine, blood was aspirated from an antecubital venipuncture through a 21-gauge needle modified to serve both a syringe and an evacuated tube. A

| Table 1. Within-Day and Day-to-Day Analytical Variation of Inorganic Phosphate in Serum and in Urine |
|--------------------------------------------------|---|---|---|---|
|                  | P1 mmol/liter |
| **Within-day**    |        |     |     |     |
| Serum pool        | 1.69   | 0.02| 1.2 |
| Urine pool        | 1.44   | 0.01| 0.7 |
| **Day-to-day**    |        |     |     |     |
| Serum pool        | 1.74   | 0.06| 3.4 |
| Urine pool        | 1.46   | 0.02| 1.4 |

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tourniquet was used only for initial venous distention to insert the needle and thereafter was removed. Each subject donated a total of 70 ml of whole blood. The blood was drawn in seven separate containers that were processed as follows:

1. 10 ml into a heparinized syringe; sample rapidly transferred to test tube; plasma separated immediately;
2. 10 ml into a heparinized evacuated tube; plasma separated immediately;
3. 10 ml into a plain syringe; sample immediately transferred to test tube; serum separated 1 h later;
4. 10 ml into a plain syringe; sample immediately transferred to test tube; serum separated 2 h later;
5. 10 ml into a plain evacuated tube; serum separated 2 h later;
6. 10 ml into a heparinized syringe; sample immediately transferred to test tube; plasma separated 2 h later;
7. 10 ml into a plain syringe; sample transferred to test tube; serum separated 3 h later.

We used 0.1 ml of sodium heparin (5000 units/ml) in the heparinized tubes or syringes. All samples were stored at room temperature until centrifuged.

Serum and plasma from each subject were refrigerated immediately after separation from the cells. It was not necessary to eliminate any samples because of visible hemolysis, nor were any of the sera lipemic.

We determined P1 in each subject’s serum and plasma simultaneously, the same day the samples were obtained. All analyses were done by continuous-flow analysis based on the colorimetric method of Fiske and SubbaRow (15). We ran two standard curves, one at the beginning and one at the end, together with duplicate samples of a pooled human serum and a pooled human urine, in conjunction with each subject’s serum and plasma. The standard curves were derived from dilutions of a stock KH2PO4 solution to the following concentrations of P1: 5, 10, 20, 30, 50, 70, 100, and 125 mg/liter. All controls and unknown values were calculated from both standard curves, read to the nearest 0.5 mg/liter, and the two results were averaged. Except for one replacement of the dialyzing membrane, no changes were made in the analyzer during the study. Water-bath and heating-bath temperatures were maintained at 37 and 55 °C, respectively, and checked daily. The linearity of the recorder was checked before each day’s analyses. Reagents, standards, and controls were prepared before the study and the same solutions were used throughout.

Results for P1 in the pooled serum and control urine samples during each of 30 days were analyzed to evaluate day-to-day variation. In addition, we repetitively analyzed the same serum and urine pools on the same day to similarly evaluate the within-day variation.

We compared results for samples drawn by syringe or evacuated tube, for serum or plasma derived from the same blood sample (drawn by syringe), and for serum or plasma samples separated at different times. We evaluated differences between means by Student’s t-test for paired data.

### Table 2. Comparison of P1 Concentrations (mmol/liter) in Serum and in Plasma Drawn by Syringe and by Evacuated Tube

<table>
<thead>
<tr>
<th></th>
<th>Syringe</th>
<th>Evac. tube</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>X</td>
</tr>
<tr>
<td>Plasma separated immediately after blood drawn</td>
<td>23</td>
<td>1.06</td>
</tr>
<tr>
<td>Serum separated 2 h after blood drawn</td>
<td>23</td>
<td>1.10</td>
</tr>
</tbody>
</table>

### Table 3. Effect on P1 Concentrations of Time between Blood-Drawing by Syringe and Separation of Plasma or Serum

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>X</th>
<th>SD</th>
<th>CV, %</th>
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</thead>
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<td>Plasma 0 time</td>
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<td>1.06</td>
<td>0.15</td>
<td>14.1</td>
</tr>
<tr>
<td>Plasma 2 h</td>
<td>23</td>
<td>1.00</td>
<td>0.14</td>
<td>14.0</td>
</tr>
<tr>
<td>Serum 1 h</td>
<td>23</td>
<td>1.11</td>
<td>0.13</td>
<td>11.7</td>
</tr>
<tr>
<td>Serum 2 h</td>
<td>23</td>
<td>1.10</td>
<td>0.13</td>
<td>11.8</td>
</tr>
<tr>
<td>Serum 3 h</td>
<td>23</td>
<td>1.09</td>
<td>0.13</td>
<td>11.9</td>
</tr>
</tbody>
</table>

**Results**

**Within-day variation.** As shown in Table 1, when the same pool of serum or urine was repetitively analyzed on the same day, the CV for the measurement of P1 was about 1% or less.

**Day-to-day variation.** Also as shown in Table 1, when a serum pool and a urine pool were analyzed repeatedly on different days during 10-months, the CV for P1 averaged 3.4 and 1.4%, respectively.

Blood withdrawal with syringe vs. withdrawal with evacuated tube. To evaluate the possibility that the initial jet of blood entering an evacuated tube might mechanically damage blood cells and release P1, we compared paired P1 values for plasma drawn with a syringe and with an evacuated tube and separated immediately after blood sampling and in serum drawn with a syringe and with an evacuated tube, the samples being centrifuged and serum separated 2 h after blood sampling. As shown in Table 2, mean values in the 23 subjects for P1 were not different when the blood was drawn by the two techniques.

P1 in serum and plasma. As shown in Table 3, when paired P1 values were compared for serum and for plasma separated from the blood cells at 2 h, the values in serum were significantly higher than in plasma by about 10% (Δ, plasma – serum = 0.10 ± 0.03 mmol/liter; P < 0.001).

**Effect of time between blood withdrawal and separation of plasma or serum.** Table 3 also shows that paired P1 concentrations in plasma declined by about 6% in 2 h when plasma separated at 2 h after blood withdrawal was compared to plasma separated immediately after blood sampling (Δ, plasma0 – plasma2h = −0.06 ± 0.03 mmol/liter; P < 0.001). Serum paired P1 concentrations also declined, but by only about 2% over 2 h when serum separated at 1 h after blood withdrawal was compared to serum separated at 3 h (Δ, serum1h – serum3h = −0.02 ± 0.02 mmol/liter; P < 0.001).

As shown in Tables 2 and 3, the CV for P1 in our 23 subjects ranged from 11.7 to 14.1% regardless of the technique of blood withdrawal, the use of plasma or serum, or the elapsed time.
between blood withdrawal and separation of serum or plasma from cells.

**Discussion**

First of all, the results show that neither plasma nor serum P1 concentrations are affected by the technique used to withdraw the blood specimens. Presumably, mechanical damage to blood cells sufficient to cause phosphate release, if any, is equivalent when blood is drawn by syringe or by evacuated tube.

Second, the results confirm the observation that plasma P1 concentrations are slightly but significantly lower than those in serum from the same individual at the same time (14). We speculate that the higher values in serum could be the result of the hydrolysis of platelet ADP during the clotting process. In quantitative terms, the 10% lower P1 values in plasma could contribute to the wide variation of P1 data in population studies if data for P1 obtained by different laboratories were based on serum in some subjects and on plasma in others. However, the CV for P1 in plasma and in serum are similar (Table 3: plasma 14.0%, serum 11.8%) so that other factors must account for the wide variation in P1 concentrations within a population when all samples are either plasma or serum.

Third, P1 values decline with time as either plasma or serum are allowed to remain in contact with blood cells before centrifugation and separation. This effect appears to be greater early after blood is withdrawn, because the relative decrement in P1 in plasma was greater over the initial 2 h after withdrawal of blood (6%, Table 3) than it was over the second and third hours after blood sampling in serum when P1 in serum separated at 1 and 3 h were compared (2%, Table 3). Presumably, the decrease in plasma or serum P1 reflects ongoing entry and phosphorylation of glucose in blood cells (17). Such an effect would be expected to be more marked initially and would decrease with time as substrate availability diminishes.

In most laboratories P1 concentrations are generally measured in serum, an hour or so being required for collection of blood specimens, delivery of the blood to the laboratory, and clot retraction. Most sera can be expected to be separated from blood cells within 3 h. As a practical matter, our data indicate that this technique does not contribute significantly to the overall variation in serum P1 concentrations, since serum P1 fell only by about 2% between 1 and 3 h. Thus, this practice should be continued.

In terms of the overall intersubject or population variation in serum or plasma P1 levels, our data indicate that the techniques used for blood withdrawal and processing do not contribute significantly to this variation. Day-to-day variation in the chemical analysis could account for approximately one-third to one-half of the variation, but biological factors are clearly much more important. The day-to-day standard deviation (s_m) of the analytical method for P1 averaged 0.06 mmol/liter (Table 1), while the total observed standard deviation (s_o) averaged 0.14 mmol/liter (Tables 2 and 3). Thus the biological variation (s_b), which equals \( \sqrt{s_o^2 - s_m^2} \), is about 0.13 mmol/liter and is the most important factor in the total variation of serum or plasma in our study group (18). Age affects mean serum P1 values but does not appear to influence the variation (3, 4). Sex may be a much more important factor because population studies demonstrate a distinct decline in mean serum P1 with advancing age in males but not in females (3, 4). By contrast, dietary phosphate deprivation results in an immediate and progressive fall in serum P1 in females but not in males (10). Thus, in population studies both sex and prior diet undoubtedly play an important role in the broad variation in serum P1. However, even within the same subject on the same diet, serum P1 varies considerably. This observation suggests that the mechanisms that ultimately regulate serum P1 are not so precise as those that regulate certain other serum constituents—for example, calcium.

We conclude that biological factors are of prime importance in determining the wide variation in serum or plasma P1 and that plasma separated immediately after blood withdrawal or serum separated between 1 and 3 h after blood sampling provide nearly comparable P1 values.

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**References**