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Case Report

Stability of analytes related to clinical chemistry and bone metabolism in blood specimens after delayed processing☆

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Abstract

Objectives: We investigated the stability of 36 analytes related to clinical chemistry in a controlled storage study.

Design and methods: Blood was collected from 11 subjects and was maintained for 45 min, 2.5 h, 5 h, or 24 h after phlebotomy before centrifugation.

Results: Statistically significant changes were observed only for parathyroid hormone, osteocalcin, zinc, pyridoxal 5′-phosphate, and homocysteine.

Conclusions: These studies indicate that many analytes in clinical chemistry are stable for 24 h before centrifugation.

Keywords: Centrifugation; Clinical chemistry; Nutrition assessment; Nutritional status; Space flight

Introduction

As part of a study designed to assess the nutritional status of astronauts during long-duration space flight, in-flight phlebotomy sessions are performed on the International Space Station (ISS). Blood specimens are nominally centrifuged within 45 min of phlebotomy, and then stored at −80 °C. Because of the nature of the space missions, blood processing can unavoidably be delayed beyond the standard recommended 30- to 120-min timeframe. This recommended timeframe is based on data showing that changes in glucose, potassium, and lactate dehydrogenase occurred after 120 min of storage of whole blood at room temperature.

Prompt centrifugation is critical to preserve the integrity of blood samples when measuring certain analytes, including cholesterol, creatinine, potassium, calcium, and chloride [1–4]. Other analytes, such as triglycerides, plasma parathyroid hormone (PTH), sodium, and ferritin [5–7], have been shown to be unaffected by increased storage time (up to 48 h) before centrifugation. Prolonged contact of serum or plasma with red blood cells can result in an exchange of substances between serum and the cells, which can increase or decrease analyte concentration in serum. Hemolysis can result in altered values for similar reasons.

To our knowledge, no studies have been done to investigate the effects of delayed centrifugation on other markers of bone metabolism and clinical chemistry, including bone-specific alkaline phosphatase (BSAP), osteocalcin, and vitamin D.

In this study, we sought to determine the effects of delayed centrifugation on the concentrations of 36 analytes nominally measured during space flight as part of the nutritional status assessment experiment. The results presented here are data from a ground-based study designed to parallel the conditions on the

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ISS that could lead to delayed centrifugation. These data establish information about the integrity of samples that undergo delayed processing up to 24 h after phlebotomy.

Subjects and methods

Subjects

Eleven healthy subjects were recruited for the study. Five were recruited for the initial study and then 6 subjects were added to the study 1 y later. The findings from the initial study with 5 subjects indicated that meaningful results could be obtained by having more subjects but analyzing at fewer time points for some of the tests. The following analytes were measured 45–60 min and 24 h after phlebotomy: 25-hydroxyvitamin D, calcium, bone-specific alkaline phosphatase, retinol-binding protein, ceruloplasmin, cortisol, total antioxidant capacity, zinc, copper, iron, selenium, pyridoxal 5′-phosphate, homocysteine, folate, transferrin receptors, transthyretin, and methylmalonic acid. All others were analyzed after 45–60 min, 2.5 h, 5 h, and 24 h. All subjects fasted for 8 h before the blood collection. The protocol for this study was approved by the Johnson Space Center Committee for the Protection of Human Subjects.

Sample collection and processing

Blood samples (5 tubes per person) were collected into plastic BD Vacutainer® SST™ Plus Blood Collection Tubes (reference #: 367986, 5 mL, BD, Franklin Lakes, NJ) and stored at room temperature, shielded from light. Samples were centrifuged using a swinging bucket rotor at 1850 ×*g* for 15 min at different time points after the blood draw: 45–60 min, 2.5 h, 5 h, and 24 h after blood collection (some analytes were intentionally analyzed only at the first and last time points). The serum was removed and divided into aliquots that were frozen in cryogenic polypropylene vials (Corning, Lowell, MA) at −80 °C for subsequent analysis. Enough aliquots were made so that only one analyte was measured from each aliquot, thus minimizing the number of freeze–thaws for the sample.

Biochemical analyses

For most tests, analyses were performed at the Johnson Space Center. Most analyses were performed by standard commercial techniques, and all have been previously described in detail [8,9].

Statistical analyses

Markers of bone metabolism and clinical chemistry analytes were analyzed using a 1-way repeated-measures analysis of variance, with time as the dependent variable. For some analytes, such as homocysteine, 25-hydroxyvitamin D, and folate, statistical analyses were only performed to compare the 45–60-min and 24-h time points because of the larger sample size ($n=11$ compared to $n=5$ at the 2.5- and 5-h time points). For those 3 analytes with data from 5 subjects at 4 time points, the data are included in the results but statistical analyses were not done. For all analytes, the sample size included in the

Table 1
Serum concentrations of analytes from blood that was centrifuged and processed at different times after phlebotomy [a, b]

	45–60 min	2.5 h	5 h	24 h
25-hydroxyvitamin D, nmol/L	51±28	#	#	49±28
Alanine aminotransferase, U/L	16±7	16±7	15±5	16±6
Albumin, g/L	39±2	39±3	41±3	39±3
Alkaline phosphatase, U/L	57±14	58±14	57±18	56±16
Aspartate aminotransferase, U/L	20±4	19±4	19±3	20±4
Alpha 1 globulin, g/L	2±0	2±1	2±0	2±1
Alpha 2 globulin, g/L	7±1	7±1	7±1	7±1
Beta globulin, g/L	13±2	13±2	13±2	13±2
Bone-specific alkaline phosphatase (BSAP), U/L	25±11	#	#	25±12
Calcium, mmol/L	2.2±0.1	#	#	2.2±0.0
Ceruloplasmin, mg/L	290±100	#	#	300±120
Chloride, mmol/L	107±6	104±2	105±6	102±3
Cholesterol, mmol/L	5.2±0.9	5.3±0.7	5.2±0.9	5.2±0.9
Copper, μmol/L	18±7	#	#	19±7
Cortisol, nmol/L	569±244	#	#	570±222
Creatinine, μmol/L	80±9	75±8	76±10	77±11
Ferritin, pmol/L	148±127	201±184	200±167	207±185
Folate, nmol/L	42±36	#	#	47±38
Gamma globulin, g/L	11±3	11±2	11±2	11±3
Homocysteine, μmol/L	8±2	#	#	12±2**
Iron, μmol/L	19±7	#	#	18±5
Methylmalonic acid, mmol/L	172±38	#	#	181±40
Osteocalcin, nmol/L	2.5±0.7	2.4±0.8	2.3±0.7	1.6±0.5**
Parathyroid hormone (PTH), ng/L	71±27	68±24	73±22	60±19*
Potassium, mmol/L	4.3±0.4	4.2±0.4	4.3±0.4	4.2±0.3
Pyridoxal 5′-phosphate (PLP), nmol/L	97±56	#	#	113±72*
Retinol-binding protein, mg/L	55±14	#	#	55±7
Selenium, μmol/L	2.9±0.3	#	#	2.9±0.4
Sodium, mmol/L	143±7	140±2	142±6	140±3
Total protein, g/L	73±4	72±4	74±6	72±4
Transferrin, g/L	3.24±0.72	3.15±0.77	3.17±0.71	3.21±0.87
Transferrin receptors, mg/L	7±4	#	#	7±3
Transthyretin, g/L	0.28±0.06	#	#	0.27±0.05
Total antioxidant capacity, mmol/L	1.8±0.1	#	#	1.8±0.1
Triglyceride, mmol/L	1.9±1.7	2.0±1.8	1.9±1.7	2.2±1.7
Zinc, μmol/L	16±2	#	#	18±2**

[a] Values are means±SD of all subjects at each time point. $n=11$ for 25-hydroxyvitamin D and bone-specific alkaline phosphatase; $n=9$ for calcium, retinol-binding protein, ceruloplasmin, total antioxidant capacity, homocysteine, methylmalonic acid, transthyretin; $n=8$ for pyridoxal 5′-phosphate, and $n=10$ for all other analytes. The sample size was constant across time points.

[b] 45–60 min, and 2.5, 5, and 24 h are the times following phlebotomy at which blood was centrifuged. Samples remained at ambient temperature in the dark until they were centrifuged and processed. *$P<0.05$, **$P<0.001$. [#]Intentionally not analyzed at this time point because no samples were collected at those time points.

statistical analysis is indicated in Table 1. The Bonferroni *t* test was used *post hoc* to determine main-effect differences between the first time point (45–60 min) and each of the other time points. Differences were considered to be statistically significant when P was <0.05, and significance is noted in the table. Statistical analyses were performed using SigmaStat 3.1 (SYSTAT Software, Richmond, CA).

Results

After 24 h, the blood tubes looked as if the serum and cells had separated somewhat. No hemolysis was visible after 2.5, 5, or 24 h.

Table 1 lists the mean concentration (for all subjects) and standard deviation of each analyte at each time point after phlebotomy. Five of the analytes tested – PTH, osteocalcin, zinc, pyridoxal 5′-phosphate (PLP), and homocysteine – showed statistically significant changes in mean concentration after delayed centrifugation. Fig. 1 shows the changes in mean concentration of the 5 analytes that changed statistically after delayed centrifugation, expressed as percentage change from the control (centrifugation at 45–60 min after phlebotomy). The remaining 31 analytes were not affected up to 24 h after sample collection.

Data for 25-hydroxyvitamin D from 5 subjects, analyzed at all 4 time points, were 31±11, 34±13, 36±14, and 33±16 nmol/L at 45–60 min, 2.5 h, 5 h, and 24 h respectively. Homocysteine data from those same 5 subjects showed a gradual increase over time (9±2, 9±2, 10±2, and 13±2 μmol/L for 45–60 min, 2.5 h, 5 h, and 24 h respectively). Serum folate was also measured in 5 subjects at all time points, and the values were 16±10, 16±10, 15±9, and 14±6 nmol/L for 45–60 min,

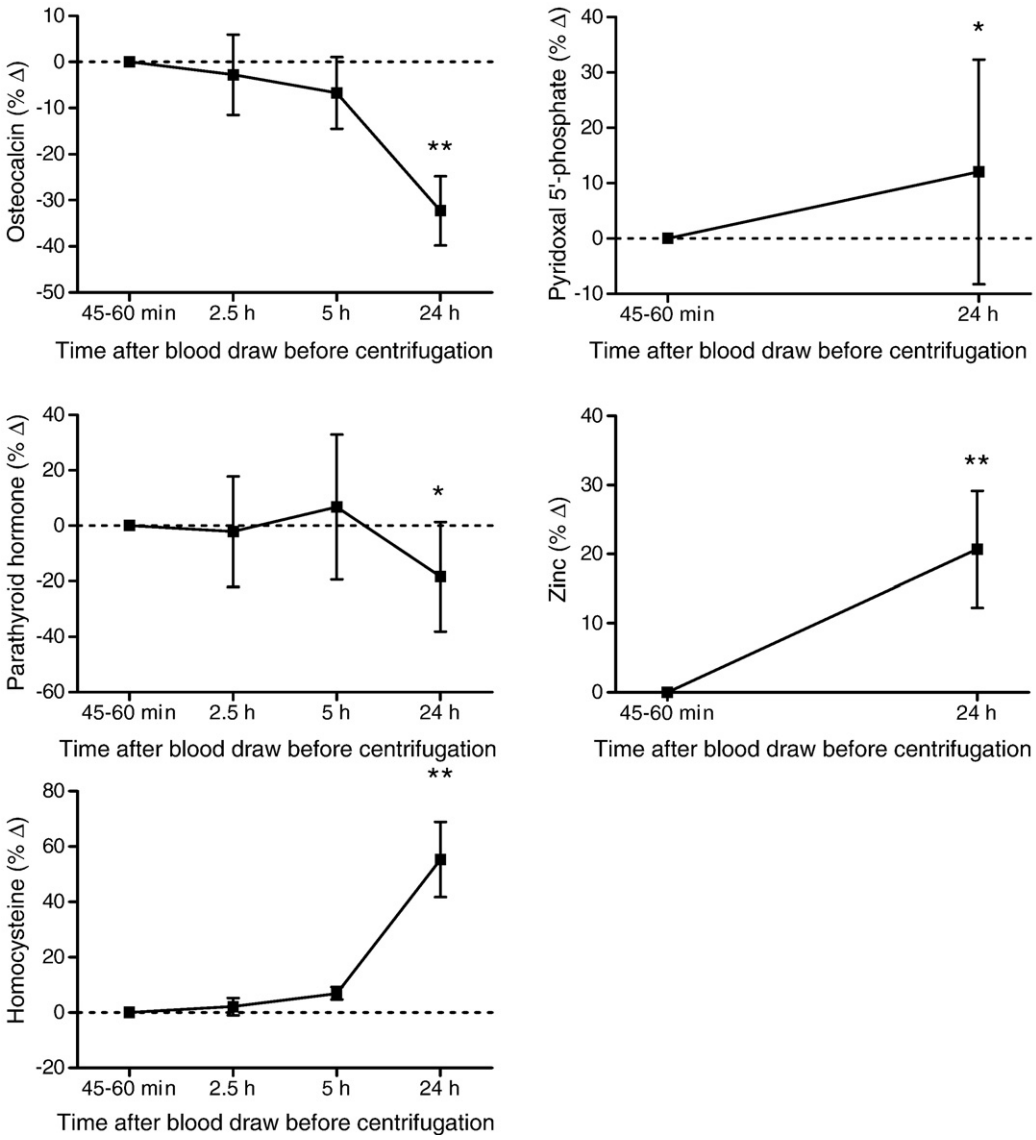

Fig. 1. Mean percentage change (±SD) from control mean value (45–60 min after phlebotomy) of osteocalcin ($n=10$), parathyroid hormone ($n=10$), homocysteine ($n=9$ for the first and last time points and $n=5$ for the 2.5- and 5-h time points), pyridoxal 5′-phosphate ($n=8$), and zinc ($n=10$) in blood samples when centrifugation occurred at different amounts of time after phlebotomy. All samples remained at ambient temperature and were protected from light until centrifugation. *$P<0.05$, **$P<0.001$.

2.5 h, 5 h, and 24 h. The data from 5 subjects were not analyzed statistically but are included here so that information can be gained about what happens to these analytes at time points less than 24 h.

Discussion

The results from this study not only affect the use of blood samples in nutritional status assessment of astronauts on the International Space Station, but also have wide application to ground-based situations. In a hospital or field location, a blood sample may not be processed immediately because of the logistics of the situation.

The present study demonstrates that 25-hydroxyvitamin D, calcium, alkaline phosphatase, BSAP, retinol-binding protein, ceruloplasmin, cortisol, total antioxidant capacity, transferrin receptors, copper, iron, selenium, chloride, sodium, potassium, folate, methylmalonic acid, alanine aminotransferase, aspartate aminotransferase, triglycerides, cholesterol, creatinine, ferritin, transferrin, total protein, transthyretin, albumin, and alpha 1, alpha 2, beta, and gamma globulin can be measured reliably in whole-blood samples kept at room temperature and protected from light for at least 24 h before serum separation.

Ferritin appears to have increased; however, the increase is because of one outlier. After that subject was removed, the means were 163 ± 125, 156 ± 131, 162 ± 122, and 161 ± 132 pmol/L for the 1, 2.5, 5, and 24 h time points, respectively. A similar situation was true for chloride, which appears to have decreased. Without the one outlier, the means were 105 ± 3, 104 ± 2, 105 ± 6, and 102 ± 3 mmol/L for the 1, 2.5, 5, and 24 h time points, respectively. Ferritin and chloride were not changed significantly over time with or without the outliers.

Data from this study show that PTH, osteocalcin, zinc, PLP, and homocysteine are not stable 24 h after phlebotomy in unseparated blood samples. The changes in these analytes after 24 h were not clinically significant enough to cause a change in the mean from within to outside of the reference range. Nevertheless, results must be interpreted with caution. The mean homocysteine concentration was not higher than 14 μmol/L after a 24-h delay in processing, but 4 out of 11 subjects did have concentrations >14 μmol/L, which could falsely be interpreted as a risk factor for cardiovascular disease [10].

Data pertaining to the stability of PTH in whole blood containing EDTA have been reported, and unlike data from our study, they seem to show that PTH is stable for at least 24 h before centrifugation [5]. One important difference is that our results apply only to blood without anticoagulant; EDTA is known to protect some peptides from proteolysis through its chelating properties, and this may have protected PTH in the study that found it to be stable for 24 h [5]. Degradation of proteins by blood enzymes is one probable cause of instability in PTH and osteocalcin, but it appears that some proteins are more stable (or protected) than others. The increase in PLP could also be due to instability of enzymes with a PLP cofactor. Hemolysis (even if not visible) could explain increases in serum zinc and homocysteine, although there was no visible hemolysis in this study.

We have demonstrated that significant changes in the concentration of PTH, osteocalcin, zinc, PLP, and homocysteine occurred in the serum fraction derived from normal human blood that was held at 25 °C for up to 24 h before centrifugation. When blood samples are not processed immediately, the implied stability of these analytes in serum should be interpreted with caution. Although the shortest possible time between phlebotomy and processing is always the best lab practice, we have shown that many analytes do remain stable for up to 24 h in the dark.

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