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ORIGINAL ARTICLE

Inorganic Phosphorus and Potassium Are Putative Indicators of Delayed Separation of Whole Blood

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Abstract
Objectives: The delayed separation of whole blood can influence the concentrations of circulating blood components, including metabolites and cytokines. The aim of this study was to determine whether clinical-biochemistry analytes can be used to assess the delayed separation of whole blood.
Methods: We investigated the plasma and serum concentrations of five clinical-biochemistry analytes and free hemoglobin when the centrifugation of whole blood stored at 4°C or room temperature was delayed for 4 hours, 6 hours, 24 hours, or 48 hours, and compared the values with those of matched samples that had been centrifuged within 2 hours after whole-blood collection.
Results: The inorganic phosphorus (IP) levels in the plasma and serum samples were elevated $\geq$ 1.5-fold when whole-blood centrifugation was delayed at room temperature for 48 hours. Furthermore, the IP levels in the plasma samples showed excellent assessment accuracy [area under the receiver-operating-characteristic curve (AUC) $>$ 0.9] after a 48-hour delay in whole-blood separation, and high sensitivity (100%) and specificity (95%) at an optimal cutoff point. The IP levels in the serum samples also exhibited good assessment accuracy (AUC $>$ 0.8), and high sensitivity (81%) and specificity (100%). The potassium (K^+) levels were elevated 1.4-fold in the serum samples following a 48-hour delay in whole-blood separation. The K^+ levels showed excellent assessment accuracy (AUC $>$ 0.9) following a 48-hour delay in whole-blood separation, and high sensitivity (95%) and specificity (91%) at an optimal cutoff point.
Conclusion: Our study showed that the IP and K^+ levels in the plasma or serum samples could be considered as putative indicators to determine whether whole-blood separation had been delayed for extended periods.

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1. Introduction

Plasma and serum samples have been used to diagnose a variety of diseases, predict prognoses, or identify disease-related biomarkers [1−4]. These samples are obtained by centrifugation after anticoagulation (using an anticoagulant, such as EDTA or heparin) and coagulation of whole blood, respectively [4]. Currently, the delay between collection and whole-blood centrifugation can influence the concentrations of circulating blood components, including metabolites and cytokines, due to prolonged contact with cells [5−10]. Hemolysis, which results in the release of hemoglobin and other intracellular components from red blood cells, might also occur when whole-blood processing is delayed [11]. Therefore, plasma and serum samples should be prepared as soon as possible after whole-blood collection by venipuncture [6]. However, whole-blood separation can be delayed for various reasons.

In laboratory tests (such as clinical biochemistry and hematology) routinely performed for diagnosis or prediction of prognosis in a clinical laboratory, the main causes of laboratory errors are preanalytical variables involved with biospecimen collection, processing, and storage conditions [12−14]. Several studies demonstrated that delayed whole-blood separation affects the plasma and serum concentrations of biochemical analytes [e.g., alanine aminotransferase (ALT), aspartate aminotransferase (AST), inorganic phosphorus (IP), potassium (K^+), and lactate dehydrogenase (LDH)] [6−9]. In this study, we investigated whether clinical-biochemistry analytes can be used to assess the delayed whole-blood separation. Moreover, to determine whether hemolysis influences variations in the tested analytes, we also investigated the variation of free plasma and serum hemoglobin concentrations induced by delayed whole-blood separation.

2. Materials and methods

2.1. Sample preparation

Whole-blood samples were collected from 135 participants into Vacutainer serum-separator tubes (Becton Dickinson, East Rutherford, NJ, USA) and plasma-separator tubes (Becton Dickinson). Each sample was divided into several 1.5-mL tubes (300 μL × 5 tubes), and stored at 4°C or room temperature until centrifugation for 15 minutes at 1,600*g*. Among the samples, one aliquot was centrifuged within 2 hours after whole-blood collection, and other aliquots were centrifuged at 4 hours, 6 hours, 24 hours, or 48 hours after the first centrifugation. (These represent "delay times" in the separation of plasma and serum.) The plasma and serum samples were separated, and then stored at −70°C before being used for clinical-biochemistry or enzyme-linked immunosorbent assay experiments.

2.2. Sample analysis

Concentrations of ALT (U/L), AST (U/L), IP (mg/dL), K^+ (mmol/L), and LDH (U/L) in the plasma and serum samples were determined using an automated chemistry analyzer (Roche Modular P800 autoanalyzer; Roche Diagnostics GmbH, Mannheim, Germany) with the appropriate reagents (Roche Diagnostics) according to the recommended protocol. Free-hemoglobin concentrations (mg/dL) in the plasma and serum samples were measured using the QuantiChrom Hemoglobin Assay Kit (Bioassay Systems, Haywood, CA, USA) according to the manufacturer's recommendations. All samples were measured in duplicate.

2.3. Statistical analysis

The analyte concentrations were expressed as means ± standard deviation. To identify analytes whose concentrations increased or decreased with statistical significance in plasma and serum samples following delayed whole-blood separation, we performed a paired two-tailed *t* test and a repeated-measures analysis of variance using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

To determine whether the plasma or serum concentrations of clinical-biochemistry analytes can be used for assessing delayed whole-blood separation, we carried out receiver-operating-characteristic (ROC) curve analysis using MedCalc software for Windows (MedCalc Software, Ostend, Belgium). The area under the ROC curve (AUC) values < 0.7, between 0.7 and 0.8, between 0.8 and 0.9, and > 0.9 were considered as poor, fair, good, and excellent, respectively. Statistical significance was determined as $p < 0.05$.

3. Results

3.1. Effect of delayed whole-blood separation on the stability of clinical-biochemistry analytes

We evaluated the concentration changes of ALT, AST, IP, K^+, and LDH in the plasma and serum samples when the centrifugation of whole blood stored at 4°C or room temperature was delayed for 4 hours, 6 hours, or 24 hours as compared to the separation being performed within 2 hours after whole-blood collection (baseline; Tables 1 and 2). The concentrations of AST, K^+, and LDH increased slightly (3.7−21.2%), but significantly ($p < 0.05$), in the plasma samples when whole-blood centrifugation was delayed at 4°C for 24 hours. Additionally, the AST and LDH concentrations changed more markedly at room temperature as compared to those at 4°C. The IP and LDH levels in the plasma were elevated $> 20\%$ relative to baseline when separation was delayed for 24 hours at room temperature. In the serum samples, the K^+ and LDH concentrations increased when whole-blood centrifugation was delayed

Table 1. Concentration variations of five clinical-biochemistry analytes in the plasma samples after delayed whole-blood separation.

	Delay time at 4°C (n = 57)					Delay time at room temperature (n = 62)				
Analyte	0 h (baseline)	4 h	6 h	24 h	p [a]	0 h (baseline)	4 h	6 h	24 h	p [a]
ALT [b]	23.67 ± 18.74	23.47 ± 18.40	23.26 ± 18.53	23.54 ± 18.30	0.917	24.40 ± 13.57	23.94 ± 13.68	24.03 ± 13.73	24.21 ± 13.66	0.865
(U/L)		(−0.8)	(−1.7)	(−0.5)			(−1.9)	(−1.5)	(−0.8)	
AST [b]	26.54 ± 18.95	26.35 ± 17.83	26.39 ± 18.16	27.53 ± 17.79 *	0.716	24.27 ± 14.17	24.13 ± 14.08	24.37 ± 14.02	25.47 ± 14.54 *	0.639
(U/L)		(−0.7)	(−0.6)	(+3.7)			(−0.6)	(+0.4)	(+4.9)	
IP [b]	3.33 ± 0.66	3.28 ± 0.67 *	3.25 ± 0.64 *	3.37 ± 0.71	0.780	3.25 ± 0.77	3.16 ± 0.77 *	3.17 ± 0.77 *	4.10 ± 1.15 *	< 0.001
(mg/dL)		(−1.5)	(−2.6)	(+1.1)			(−2.8)	(−2.6)	(+26.3)	
K^+ [b]	22.09 ± 2.32	22.42 ± 2.23 *	22.79 ± 1.96 *	26.78 ± 3.34 *	< 0.001	25.35 ± 3.15	24.13 ± 3.05 *	23.77 ± 3.66 *	24.37 ± 3.56 *	0.060
(mmol/L)		(+1.5)	(+3.2)	(+21.2)			(−4.8)	(−6.2)	(−3.9)	
LDH [b]	336.74 ± 100.80	336.26 ± 98.15	337.82 ± 98.65	396.61 ± 145.78 *	0.008	311.29 ± 82.07	313.58 ± 81.74 *	314.81 ± 81.03 *	382.34 ± 95.58 *	< 0.001
(U/L)		(−0.1)	(+0.3)	(+17.8)			(+0.7)	(+1.1)	(+22.8)	

[a]Significance measured using repeated-measures analysis of variance; [b]Numbers in brackets represent percentage change, with a "+" for an increase and a "−" for a decrease relative to baseline. * Indicates $p < 0.05$, calculated using a paired two-tailed t test. ALT = alanine aminotransferase; AST = aspartate aminotransferase; IP = inorganic phosphorous; K^+ = potassium; LDH = lactate dehydrogenase.

Table 2. Concentration variations of five clinical-biochemistry analytes in the serum samples after delayed whole-blood separation.

	Delay time at 4°C (n = 55)					Delay time at room temperature (n = 58)				
Analyte	0 h (baseline)	4 h	6 h	24 h	p [a]	0 h (baseline)	4 h	6 h	24 h	p [a]
ALT [b]	22.64 ± 18.19	22.29 ± 18.06 *	22.29 ± 17.96	22.89 ± 17.97	0.876	22.41 ± 14.14	22.34 ± 13.91	22.16 ± 13.89	22.81 ± 14.39	0.823
(U/L)		(−1.5)	(−1.5)	(+1.1)			(−0.3)	(−1.2)	(+1.8)	
AST [b]	25.13 ± 15.80	24.45 ± 15.18 *	24.58 ± 15.24 *	24.91 ± 15.02	0.837	23.40 ± 11.50	23.36 ± 11.22	23.88 ± 11.16	23.36 ± 9.55	0.819
(U/L)		(−2.7)	(−2.2)	(−0.9)			(−0.1)	(+2.1)	(−0.1)	
IP [b]	3.45 ± 0.62	3.43 ± 0.62 *	3.43 ± 0.61	3.49 ± 0.60 *	0.647	3.48 ± 0.82	3.26 ± 0.81 *	3.15 ± 0.81 *	3.71 ± 1.23	0.007
(mg/dL)		(−0.7)	(−0.6)	(+1.0)			(−6.5)	(−9.4)	(+6.6)	
K^+ [b]	4.42 ± 0.51	5.01 ± 0.52 *	5.39 ± 0.58 *	9.60 ± 2.00 *	< 0.001	4.28 ± 0.46	4.24 ± 0.49	4.27 ± 0.52	4.47 ± 0.60 *	0.079
(mmol/L)		(+13.4)	(+22.1)	(+117.3)			(−0.8)	(−0.2)	(+4.5)	
LDH [b]	336.93 ± 99.78	338.56 ± 97.07	339.02 ± 97.32	360.60 ± 109.54 *	0.269	318.55 ± 88.11	333.03 ± 85.03 *	335.97 ± 85.17 *	340.36 ± 81.55 *	0.213
(U/L)		(+0.5)	(+0.6)	(+7.0)			(+4.5)	(+5.5)	(+6.8)	

[a]Significance measured using repeated-measures analysis of variance; [b]Numbers in brackets represent percentage change, with a "+" for an increase and a "−" for a decrease relative to baseline. * Indicates $p < 0.05$, calculated using a paired two-tailed t test. ALT = alanine aminotransferase; AST = aspartate aminotransferase; IP = inorganic phosphorous; K^+ = potassium; LDH = lactate dehydrogenase.

at 4°C or room temperature for 24 hours, with LDH levels increasing with delay times at both 4°C and room temperature. Moreover, the extent of the increase in K^+ concentration was greater at 4°C as compared to room temperature in both the plasma and serum samples.

Next, we assessed how the plasma and serum concentrations of five clinical-biochemistry analytes varied after whole-blood centrifugation was delayed at room temperature for 48 hours as compared to the concentrations in the samples separated within 2 hours after whole-blood collection (using samples distinct from those used in the aforementioned experiments). The concentrations of all analytes increased with the 48-hour delay (Figure 1). Among these, the IP level increased nearly twofold in both the plasma and serum samples. The LDH and K^+ increased by 1.4-fold in the plasma and serum samples, respectively.

3.2. Accuracy analysis of clinical-biochemistry-analyte levels in the assessment of delayed whole-blood separation

To determine whether the plasma and serum concentrations of clinical-biochemistry analytes can be used for assessing delay in whole-blood separation, we carried out ROC-curve analysis on the clinical-biochemistry analytes that increased significantly in the plasma and serum samples following a 48-hour delay in whole-blood separation (Table 3).

Among the tested analytes, the plasma concentration of IP exhibited excellent assessment accuracy (AUC = 0.999) for delayed whole-blood separation. Additionally, the sensitivity (100%) and specificity (95%) were close to 100% at an optimal cutoff point of 4.3 mg/dL. The serum concentration of IP also showed good assessment accuracy (AUC = 0.856), and high sensitivity (81%) and specificity (100%) at an optimal cutoff point of 4.5 mg/dL. The K^+ levels showed excellent assessment accuracy (AUC = 0.965), and high sensitivity (95%) and specificity (91%) at an optimal cutoff point of 4.7 mmol/L in the serum samples.

3.3. Change in free-hemoglobin concentration following delayed whole-blood separation

We measured the free-hemoglobin concentrations in the plasma and serum samples after whole-blood centrifugation was delayed at 4°C or room temperature for 4 hours, 6 hours, or 24 hours, and compared these values with the corresponding concentrations in samples separated within 2 hours after whole-blood collection (Table 4). The free-hemoglobin concentrations increased from 6.9% to 21.2% in both the plasma and serum samples when whole-blood separation was delayed at 4°C and room temperature for 24 hours (except in the case of the serum samples at room temperature), and tended to increase relative to the delay time.

We also compared the free-hemoglobin concentrations in the plasma and serum samples (samples distinct from those used previously) obtained after whole-blood centrifugation was either delayed at room temperature for 48 hours or performed within 2 hours after whole-blood collection. The concentration of free hemoglobin increased ~1.2-fold in the plasma, but not in the serum samples. At baseline and after a 48-hour delay, the free-hemoglobin concentrations in the plasma were 46.05 ± 22.57 mg/dL and 56.39 ± 28.78 mg/dL, and in the serum were 58.90 ± 36.41 mg/dL and 57.75 ± 32.08 mg/dL, respectively (data not shown).

4. Discussion

We investigated how the plasma and serum concentrations of clinical-biochemistry analytes were changed by delayed whole-blood separation. Previous studies showed that the plasma or serum concentrations of ALT, AST, IP, K^+, and LDH were slightly, but significantly, changed by delayed whole-blood separation [6–9]. The ALT concentration was elevated by 1.5% in serum samples obtained from whole blood

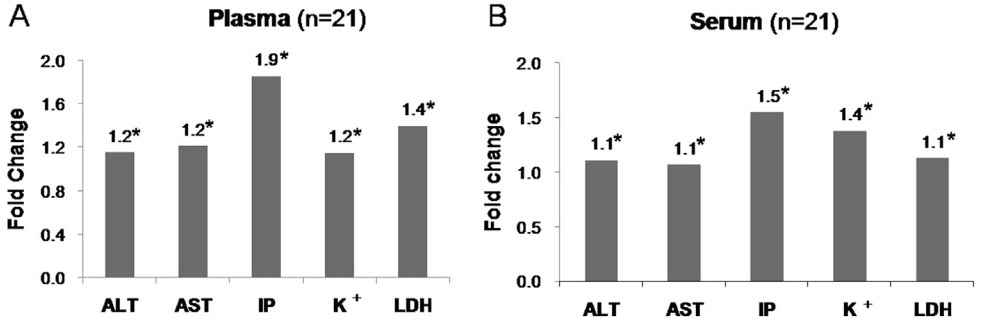

Figure 1. Concentration changes of five clinical-biochemistry analytes in (A) plasma and (B) serum samples when whole-blood separation was delayed at room temperature for 48 hours as compared to separation within 2 hours of whole-blood collection. * Indicates $p < 0.05$, calculated using a paired two-tailed t test. ALT = alanine aminotransferase; AST = aspartate aminotransferase; IP = inorganic phosphorous; K^+ = potassium; LDH = lactate dehydrogenase.

Table 3. Accuracy of clinical-biochemistry-analyte levels in the assessment of delayed whole-blood separation at room temperature for 48 hours as compared with levels in samples separated within 2 hours of whole-blood collection.

Sample	Analyte	Optimal cutoff point	Area under the ROC curve	Sensitivity (%)	Specificity (%)	*p*
Plasma	ALT	> 19.0 U/L	0.629	52.4	76.2	0.141
	AST	> 23.0 U/L	0.706	52.4	90.5	0.013
	IP	> 4.3 mg/dL	0.999	100.0	95.2	< 0.001
	K^+	> 22.3 mmol/L	0.765	95.2	57.1	< 0.001
	LDH	> 409.0 U/L	0.863	76.2	85.7	< 0.001
Serum	ALT	> 13.0 U/L	0.576	71.4	42.9	0.398
	AST	> 16.0 U/L	0.583	81.0	33.3	0.355
	IP	> 4.5 mg/dL	0.856	81.0	100.0	< 0.001
	K^+	> 4.7 mmol/L	0.965	95.2	90.5	< 0.001
	LDH	> 328.0 U/L	0.686	81.0	52.4	0.025

ALT = alanine aminotransferase; AST = aspartate aminotransferase; IP = inorganic phosphorous; K^+ = potassium; LDH = lactate dehydrogenase; ROC = receiver operating characteristic.

Table 4. Free-hemoglobin concentrations after delayed whole-blood separation.

		Delayed time				
Storage temperature	Sample	0 h (baseline)	4 h	6 h	24 h	*p* [a]
4°C	Plasma (mg/dL) $n = 29$ [b]	43.89 ± 18.68	48.07 ± 19.07 * (+9.5)	49.76 ± 21.40 * (+13.4)	51.05 ± 20.48 * (+16.3)	0.856
	Serum (mg/dL) $n = 27$ [b]	46.62 ± 21.06	47.71 ± 17.84 (+2.3)	49.20 ± 19.30 (+5.5)	56.48 ± 20.32 * (+21.2)	0.108
Room temperature	Plasma (mg/dL) $n = 68$ [b]	47.12 ± 31.35	47.33 ± 29.33 (+0.5)	48.05 ± 28.31 (+2.0)	50.39 ± 35.15 * (+6.9)	0.835
	Serum (mg/dL) $n = 68$ [b]	51.14 ± 33.62	47.67 ± 31.56 (−6.8)	47.66 ± 33.84 (−6.8)	45.90 ± 33.73 (−10.2)	0.938

[a]Significance measured using repeated-measures analysis of variance; [b]Numbers in brackets represent percent change, with a "+" for an increase and a "−" for a decrease relative to baseline. * Indicates $p < 0.05$, calculated using a paired two-tailed t test.

stored at room temperature for 24 hours as compared to 30 minutes [8]. By contrast, the ALT concentrations in plasma samples separated from whole blood stored at 4°C or room temperature for up to 24 hours did not differ significantly from that observed in samples separated immediately after collection [6,7]. The concentrations of AST (3.0%) and LDH (8.9%) were slightly higher in serum samples prepared from whole blood stored at room temperature for 24 hours as compared to that observed in samples obtained from whole blood stored for 30 minutes [8]. The K^+ concentration increased in plasma and serum samples obtained from whole blood stored at room temperature for up to 56 hours [6]. The IP concentrations changed in plasma (7.0%) and serum samples (3.8%) after whole-blood separation was delayed at room temperature for 24 hours as compared with the concentrations measured after immediate separation [9]. Our results were generally consistent with those reported previously. Additionally, we demonstrated that the IP concentration was elevated ∼2-fold in the plasma and serum samples following a 48-hour delay in separation at room temperature, and displayed high assessment accuracy, sensitivity, and specificity for a 48-hour delay in separation by ROC-curve analysis. The K^+ concentration also displayed high assessment accuracy, sensitivity, and specificity in the serum samples. These findings suggest that the IP and K^+ concentrations can serve as potential indicators to determine whether whole-blood centrifugation had been delayed for extended periods.

The ALT, AST, K^+, and LDH concentrations are elevated in hemolytic plasma [15] or serum samples [14]. These analytes and free hemoglobin were elevated slightly, but significantly, in the plasma or serum samples when whole-blood centrifugation was delayed at 4°C or room temperature for up to 48 hours. Thus, the ALT, AST, K^+, and LDH concentrations may be affected by hemolysis.

In conclusion, our findings indicate that the IP and K^+ concentrations in the plasma or serum samples could be used to predict whether or not whole-blood separation was delayed for extended periods.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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