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Assessing the stability of uncentrifuged serum and plasma analytes at various post-collection intervals

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Abstract

Objectives: Our study aimed to assess the stability of 26 biochemistry analytes in serum or plasma samples separated from blood samples centrifuged at different time intervals after collection, simulating sample transport via despatch delivery systems.

Methods: Blood from 41 volunteers was collected using five serum separator tubes (SST) and five fluoride oxalate tubes (FOT) for each volunteer following written informed consent. Each of the five tubes in both groups of SST and FOT was centrifuged at one of the time intervals: 0.5 h, 4 h, 8 h, 12 and 24 h after collection. These samples were left standing prior to centrifugation at room temperature. We calculated the percentage difference for each analyte between the 0.5 h and other time intervals to assess analyte stability. The percentage difference was compared to the desirable specification for bias and reference change value (RCV).

Results: Mean concentration of serum potassium showed a significant increase in the percentage RCV after 8 h, while CKMB showed an increase after 12 h of delayed centrifugation compared to the baseline (0.5 h). There were no significant percentage RCV for the other analytes at all timelines.

Conclusions: Serum potassium and CKMB were stable up to 8 and 12 h of delayed centrifugation respectively, whilst all other analytes appear stable up to 24 h, suggesting that sample transport delay of up to 8 h, with the condition that room temperature is maintained, may not have a significant impact on accuracy of the biochemistry/immunochemistry test results.

Keywords: biochemistry; biological variation; delayed sample processing; stability; preanalytics; sample transport

Introduction

Medical laboratories play an essential, complementary role in assisting clinicians with the diagnosis and management of patients. The use of plasma and serum samples in diagnosing diseases and prognostication has been instrumental in providing optimal patient care [1–4]. These samples are obtained by centrifugation of blood samples collected in tubes containing anticoagulants [4]. It is generally known that an SST or FOT is to be centrifuged within 1 h of collection and no longer than 2 h after collection. This is to avoid hemolysis or red cell leakage resulting from the release of hemoglobin and other intracellular components from red blood cells [5]. Delayed separation can influence the concentrations of circulating biomarkers due to prolonged contact with cells [6–11]. This can pose the risk of misinterpreting results that could consequently have a negative impact on patient care. Therefore, plasma and serum should be separated from whole blood at the earliest time to ensure that accuracy of the results are not jeopardized by preanalytical errors such as this [7].

Part of maintaining quality in a medical laboratory is to monitor quality indicators which serve to identify any issues within the laboratory that can impact patient results and provide solutions to those risks. Specimen rejection rate is among the quality indicators monitored by a medical laboratory and *in vitro* hemolysis has been one of the highest percentages of sample rejection reported [12]. One of the causes of *in vitro* hemolysis is delayed serum/plasma centrifugation.

Certain laboratories in Malaysia receive blood samples from multiple collection sites in various locations daily.

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These laboratories typically utilize a despatch delivery system in which employed riders are deployed to different areas in a state or district to transport samples from multiple collection sites in one assigned route [13]. Completing tube centrifugation within the specified time interval following collection is a common issue at such laboratories due to multiplicity of the collection sites assigned for a single route, non-harmonized timings of sample collection at each site, and unpredictable traffic flow for a given route, consequently affecting the stability of biochemistry analytes and quality of results [14]. However, the stability of biochemistry analytes in blood samples centrifuged at varied time intervals after collection is still poorly understood.

In a large private medical lab servicing well over 10,000 samples per day, the major causes of serum separation delay would be the prolonged time taken for samples to arrive at the laboratory due to the distance of the collection site from the laboratory or any unexpected transport deviations. Bottlenecks at the preanalytical section of the laboratory (sample sorting and data entry processes) due to a surge in samples arriving at the laboratory may also pose a risk to serum separation delays [15].

Given the risk of result inaccuracies that could potentially arise from delayed sample separation, it is necessary to determine if any unavoidable deficiencies in the process can have a major impact on test results. In this case, to determine if any prolonged delay in serum/plasma separation can lead to significant changes to test results, an exercise to determine the impact of time delay for sample processing on test results was conducted. The knowledge of this will be able to help laboratories plan transportation more effectively and understand the maximum transportation time limit and conditions they can work with to ensure sample integrity is maintained. Therefore, the aim of this study is to determine the stability of 26 biochemistry analytes in serum or plasma which were separated from blood samples centrifuged at several time intervals after collection, to understand the potential impact of delayed centrifugation on accuracy of test results.

Materials and methods

Study subjects

This study was a collaborative effort between the Faculty of Medicine, Universiti Teknologi MARA, and Innoquest Pathology Laboratory, Petaling Jaya. This was a retrospective study extracting 26 biochemistry and immunochemistry test results of 41 Innoquest Pathology Laboratory staff during a quality exercise aimed at addressing the issue of delayed sample separation at Innoquest Pathology Laboratory in 2021. Prior informed consent was taken before the quality exercise and all subject-specific information was thoroughly anonymized and treated with utmost confidentiality.

Prior institution ethical approval for this study was obtained [Ref. No: REC/02/2023 (ST/MR/42)]. Additionally, written informed consent was obtained from all participants before the commencement of the quality exercise. It is important to note that only willing participants who voluntarily enrolled in the quality exercise were included. The exercise was conducted over the period from 1st to 3rd March 2021.

Study methods

This study was conducted following the recommendations for the design of stability studies on clinical specimens by the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) Working Group Preanalytical Phase (WG-PRE) [16]. To minimize common variables that contribute to analyte instability in clinical samples, we implemented the following procedures:

1) Variability Due to the Collection Process: Twenty milliliters of blood was drawn from each volunteer by a trained, designated phlebotomist. The blood was collected into five BD SST™ II Advance tubes with clot activator containing silica particles (3 mL in each tube) and five BD Vacutainer Fluoride/Oxalate tubes (1 mL in each tube for glucose tests) (BD Vacutainer, Becton, Dickinson and Company, New Jersey, US).
2) Sample Grouping and Handling: The ten tubes (five with clot activator and five with fluoride/oxalate) were assigned into five groups based on the duration before centrifugation: 0.5 h (baseline), 4 h, 8 h, 12 h, and 24 h. The blood samples in the baseline group were centrifuged at 2000 rpm for 10 min immediately after clotting occurred, which is defined by the manufacturer as 30 min after blood draw. Blood samples assigned to the 4-h, 8-h, 12-h, and 24-h groups were kept standing in an upright position at room temperature (18–21 °C) for their respective times before centrifugation.
3) Preservatives: We used specific tubes for glucose tests that contain preservatives to ensure the stability of the analytes.
4) Temperature Control: Samples were kept standing in an upright position at room temperature (18–21 °C) throughout the study period to maintain consistent conditions.
5) Contact with Air; Diffusion and Evaporation: The collection tubes were decapped using a Roche P512 pre-

analytical system (LCP1) (Roche Diagnostics, Rotkreuz, Switzerland) only after centrifugation to avoid evaporation and the subsequent concentration changes of analytes.

6) Exposure to Light: Samples were left unprotected from light since the analytes analyzed were not reported to be clinically significant when exposed to light.
7) Cellular Metabolism and Cell Lysis: Blood samples were centrifuged, and serum or plasma was separated from the cells to halt the impact of cellular metabolism on the composition of serum or plasma.
8) Tube Orientation and Mixing: Blood samples were kept standing in an upright position allowing for complete coagulation and reduced mixing of components.
9) Analytical Method: Serum and plasma samples were analyzed for twenty-six biochemistry tests using an automated Cobas 8000 Modular Analyzer Series (Roche Diagnostics, Rotkreuz, Switzerland).

The following tests were measured from the serum/plasma samples in all the groups:

1) Serum liver function test: aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT) and total bilirubin.
2) Serum renal function test: sodium (Na^+), potassium (K^+), chloride (Cl^-), calcium (Ca), creatinine, phosphate, urea, uric acid, and total protein.
3) Serum lipid profile: total cholesterol (TC), high-density lipoprotein (HDL-c) and triglyceride (TG).
4) Serum cardiac enzymes: creatine kinase (CK) and creatine kinase-MB (CKMB).
5) Serum thyroid function test: thyroid stimulating hormone (TSH) and free thyroxine (FT4).
6) Serum fertility hormones: follicle stimulating hormone (FSH), luteinizing hormone (LH) and estradiol.
7) Plasma glucose.
8) Serum/plasma hemolysis index (HI); used for monitoring the degree of potential interference due to hemolysis. The analyser automatically performed this index.

Statistical analysis

Sample size was calculated using the Raosoft sample size calculator with the prevalence of hemolysis taken as 3.3 % based on Azman et al. [17]. Taking a 90 % confidence interval and a 5 % margin of error, the minimum sample size was determined at 35 samples.

Statistical analysis to determine significant differences between timelines were done in two methods. The first method utilizes the one-way repeated measures ANOVA to assess significant difference between baseline and the other groups by using a statistical software (SPSS Statistics for Windows, version 27.0), where time intervals were assigned as within subject and set to five levels (0.5, 4, 8, 12, and 24 h). A p-value less than 0.05 was set as significant data.

The second method employed the reference change value (RCV) method to compare the 4, 8, 12 and 24 h results with baseline (0.5 h), considering the analytical and biological variations. To assess RCV, mean result change between baseline and other timeline groups were established and the resulting from the mean results change between the baseline and other groups, we considered the coefficient of variation (CV_a) and the biological coefficient of variation (CV_b), which represent the analytical and biological variation of each analyte within subjects. In this study, the CV_a for each analyte was calculated from the quality control samples assayed over a month and was reported in Table 1. The CV_b for each analyte was obtained from the list of the European

Table 1: Analytical coefficient of variation and within subject biological coefficient of variation of different analytes.

Analyte	CV_a%	CV_b%	H index
AST	2.9	9.6	40
ALT	4.5	10.1	90
Albumin	3.2	2.5	1,000
ALP	3.5	5.4	200
GGT	3.4	9.1	200
Total bilirubin	4.0	20.0	800
Na	0.9	0.5	90
Cl	1.1	1.1	90
K	1.3	4.1	90
Ca	1.4	1.8	1,000
Creatinine	4.5	4.5	1,000
Phosphate	3.0	7.8	300
Urea	2.8	13.9	1,000
Uric acid	3.3	8.3	1,000
Total protein	1.3	2.6	500
TC	2.4	5.3	700
HDL	1.6	5.8	1,200
TG	2.3	19.9	700
CK	1.7	15.0	100
CKMB	4.6	5.6	20
Glucose	1.5	5.0	1,000
TSH	3.5	17.7	Not applicable
FT4	3.9	4.9	Not applicable
FSH	6.7	12.4	Not applicable
LH	4.1	22.8	Not applicable
Estradiol	3.2	15.0	Not applicable

CV_a, coefficient of variation calculated from the quality control samples assayed over a month; CV_b, biological coefficient of variation (within-subject biological variation) obtained from https://biologicalvariation.eu/ [18]; H index, above value indicated measurement considered as hemolyse samples according to the analytes.

Federation of Clinical Chemistry and Laboratory Medicine Biological Variation [18] (Table 1). The RCV was calculated using the Reference Change Value calculator provided by https://biologicalvariation.eu/ [18] which utilizes the asymmetrical RCV formula by Fokkema et al. [19], which yields different percentage RCVs for increases and decreases with 95 % confidence intervals (CI). If the percentage change value showed a rise greater than the one-sided percentage RCV Increase or a fall of more than the percentage RCV Decrease, then the change is greater than what is attributable to the combined analytical and biological variation, at the probability of 0.05 (z-value of 1.64).

Results

Demographic characteristic of study subject

Data extracted consisted of 41 laboratory staff [9 males and 32 females; mean age (29.5 ± 6.0 years)] who volunteered in this exercise.

One-way repeated measures ANOVA

A one-way repeated measures ANOVA revealed significant main effects for multiple analytes. Among the 26 analytes tested, 21 showed a significant main effect of delayed centrifugation time on analyte concentration (AST: $F(df)=4.77$, $p<0.001$, $\eta2=0.11$; Albumin: $F(df)=7.3$, $p<0.001$, $\eta2=0.15$; ALP: $F(df)=5.95$, $p<0.001$, $\eta2=0.13$; GGT: $F(df)=3.6$, $p=0.01$, $\eta2=0.08$; Sodium: $F(df)=8.38$, $p<0.001$, $\eta2=0.17$; Chloride: $F(df)=24.57$, $p<0.001$, $\eta2=0.38$; Potassium: $F(df)=141.92$, $p<0.001$, $\eta2=0.78$; Calcium: $F(df)=14.32$, $p<0.001$, $\eta2=0.26$; Creatinine: $F(df)=2.84$, $p=0.03$, $\eta2=0.07$; Phosphate: $F(df)=12.62$, $p<0.001$, $\eta2=0.24$; Urea: $F(df)=19.3$, $p<0.001$, $\eta2=0.33$; Uric Acid: $F(df)=19.02$, $p<0.001$, $\eta2=0.32$; Total Protein: $F(df)=9.21$, $p<0.001$, $\eta2=0.19$; TC: $F(df)=22.8$, $p<0.001$, $\eta2=0.36$; HDL: $F(df)=12.27$, $p<0.001$, $\eta2=0.24$; Triglyceride: $F(df)=19.82$, $p<0.001$, $\eta2=0.33$; CK: $F(df)=8.37$, $p<0.001$, $\eta2=0.17$; CKMB: $F(df)=14.97$, $p<0.001$, $\eta2=0.27$; Glucose: $F(df)=16.22$, $p<0.001$, $\eta2=0.29$; FSH: $F(df)=21.36$, $p<0.001$, $\eta2=0.35$; and Estradiol: $F(df)=9.29$, $p<0.001$, $\eta2=0.19$) as shown in Table 2.

Further analysis using post-hoc Bonferroni tests as shown in Table 3 revealed significant differences between the

Table 2: One-way repeated measures of ANOVA of analytes.

Analytes	Mean ± SD					F-Value	p-Value	Effect size (η2)
	0.5 h	4 h	8 h	12 h	24 h			
AST	17.48 ± 4.57	18.06 ± 4.46	18.11 ± 4.35	18.52 ± 4.54	18.03 ± 4.47	4.77	[a]<0.001	0.11
ALT	17.06 ± 10.47	17.33 ± 10.54	17.33 ± 10.38	17.47 ± 10.39	17.20 ± 10.46	1.21	0.31	0.03
Albumin	44.28 ± 3.00	44.68 ± 2.91	44.96 ± 2.88	44.69 ± 2.48	45.25 ± 2.52	7.30	[a]<0.001	0.15
ALP	63.63 ± 16.00	63.81 ± 16.28	63.93 ± 16.27	63.71 ± 15.96	64.46 ± 16.12	5.95	[a]<0.001	0.13
GGT	17.78 ± 10.38	17.88 ± 10.28	18.00 ± 10.21	18.17 ± 10.12	18.34 ± 10.38	3.60	[a]0.01	0.08
Total bilirubin	8.36 ± 5.27	8.36 ± 5.29	8.41 ± 5.25	8.38 ± 5.29	8.31 ± 5.22	0.67	0.61	0.02
Na	138.68 ± 1.78	137.95 ± 1.92	137.32 ± 1.90	139.00 ± 1.55	138.27 ± 1.98	8.38	[a]<0.001	0.17
Cl	101.50 ± 2.23	100.81 ± 2.25	100.38 ± 1.78	101.54 ± 1.70	98.84 ± 2.52	24.57	[a]<0.001	0.38
K	4.21 ± 0.30	4.43 ± 0.36	4.67 ± 0.39	4.90 ± 0.42	5.09 ± 0.50	141.92	[a]<0.001	0.78
Ca	2.35 ± 0.08	2.34 ± 0.074	2.33 ± 0.078	2.32 ± 0.077	2.35 ± 0.08	14.32	[a]<0.001	0.26
Creatinine	62.95 ± 13.72	62.17 ± 13.39	60.93 ± 13.17	63.05 ± 12.69	63.93 ± 13.78	2.84	[a]0.03	0.07
Phosphate	1.15 ± 0.14	1.15 ± 0.15	1.14 ± 0.15	1.10 ± 0.15	1.11 ± 0.18	12.62	[a]<0.001	0.24
Urea	3.73 ± 1.18	3.77 ± 1.18	3.78 ± 1.17	3.82 ± 1.18	3.89 ± 1.19	19.30	[a]<0.001	0.33
Uric acid	0.29 ± 0.08	0.29 ± 0.08	0.29 ± 0.08	0.29 ± 0.07	0.29 ± 0.08	19.02	[a]<0.001	0.32
Total protein	76.51 ± 4.51	76.94 ± 4.39	76.72 ± 4.35	76.62 ± 4.36	77.51 ± 4.17	9.21	[a]<0.001	0.19
Total cholesterol	5.15 ± 0.95	5.19 ± 0.96	5.23 ± 0.97	5.23 ± 0.98	5.28 ± 0.98	22.8	[a]<0.001	0.36
HDL	1.61 ± 0.37	1.60 ± 0.37	1.60 ± 0.37	1.59 ± 0.37	1.58 ± 0.37	12.27	[a]<0.001	0.24
TG	1.03 ± 0.61	1.03 ± 0.61	1.04 ± 0.61	1.04 ± 0.61	1.06 ± 0.62	19.82	[a]<0.001	0.33
CK	118.05 ± 75.97	118.73 ± 75.75	119.85 ± 76.19	120.22 ± 76.77	120.68 ± 75.92	8.37	[a]<0.001	0.17
CKMB	12.62 ± 2.44	13.77 ± 4.20	14.82 ± 3.99	15.28 ± 3.39	16.4 ± 3.04	14.97	[a]<0.001	0.27
Glucose	5.08 ± 0.87	4.95 ± 0.83	4.90 ± 0.80	4.92 ± 0.86	5.01 ± 0.84	16.22	[a]<0.001	0.29
TSH	2.93 ± 4.31	2.98 ± 4.60	3.00 ± 4.52	3.01 ± 4.57	3.00 ± 4.46	2.24	0.07	0.05
FT4	15.56 ± 2.08	15.74 ± 2.09	15.54 ± 2.16	15.54 ± 2.14	15.60 ± 2.14	1.65	0.16	0.04
FSH	5.49 ± 3.11	5.22 ± 3.13	5.59 ± 3.18	5.59 ± 3.16	5.65 ± 3.21	21.36	[a]<0.001	0.35
LH	9.73 ± 10.50	9.78 ± 10.68	9.83 ± 10.71	9.70 ± 10.61	9.80 ± 10.71	4.38	0.054	0.10
Estradiol	396.45 ± 521.63	401.29 ± 514.59	385.16 ± 498.82	389.19 ± 515.61	386.32 ± 508.18	9.29	[a]<0.001	0.19

[a]p-Value of <0.05 considered as significant value calculated using SPSS Statistics for Windows, version 27.0.

Table 3: Post-hoc Bonferroni test of analytes.

Analytes	Post-hoc analysis Mean ± SD (p-value)				
	0.5 h	4 h	8 h	12 h	24 h
AST	17.48 ± 4.57	18.06 ± 4.46 (0.40)	18.11 ± 4.35 ([a]0.07)	18.52 ± 4.54 ([a]<0.001)	18.03 ± 4.47 (0.09)
ALT	17.06 ± 10.47	17.33 ± 10.54 (1.00)	17.33 ± 10.38 (1.00)	17.47 ± 10.39 (0.35)	17.20 ± 10.46 (1.00)
Albumin	44.28 ± 3.00	44.68 ± 2.91 ([a]0.06)	44.96 ± 2.88 ([a]<0.001)	44.69 ± 2.48 (0.85)	45.25 ± 2.52 ([a]0.00)
ALP	63.63 ± 16.00	63.81 ± 16.28 (1.00)	63.93 ± 16.27 (0.90)	63.71 ± 15.96 (1.00)	64.46 ± 16.12 ([a]0.00)
GGT	17.78 ± 10.38	17.88 ± 10.28 (1.00)	18.00 ± 10.21 (1.00)	18.17 ± 10.12 ([a]0.03)	18.34 ± 10.38 ([a]0.01)
Total bilirubin	8.36 ± 5.27	8.36 ± 5.29 (1.00)	8.41 ± 5.25 (1.00)	8.38 ± 5.29 (1.00)	8.31 ± 5.22 (1.00)
Na	138.68 ± 1.78	137.95 ± 1.92 (0.12)	137.32 ± 1.90 ([a]<0.001)	139.00 ± 1.55 (1.00)	138.27 ± 1.98 (1.00)
Cl	101.50 ± 2.23	100.81 ± 2.25 (0.15)	100.38 ± 1.78 ([a]<0.001)	101.54 ± 1.70 (1.00)	98.84 ± 2.52 ([a]<0.001)
K	4.21 ± 0.30	4.43 ± 0.36 ([a]<0.001)	4.67 ± 0.39 ([a]<0.001)	4.90 ± 0.42 ([a]<0.001)	5.09 ± 0.50 ([a]<0.001)
Ca	2.35 ± 0.08	2.34 ± 0.074 (0.17)	2.33 ± 0.078 ([a]0.01)	2.32 ± 0.077 ([a]<0.001)	2.35 ± 0.08 (1.00)
Creatinine	62.95 ± 13.72	62.17 ± 13.39 (0.15)	60.93 ± 13.17 ([a]<0.001)	63.05 ± 12.69 (1.00)	63.93 ± 13.78 (1.00)
Phosphate	1.15 ± 0.14	1.15 ± 0.15 (1.00)	1.14 ± 0.15 (1.00)	1.10 ± 0.15 ([a]<0.001)	1.11 ± 0.18 (0.12)
Urea	3.73 ± 1.18	3.77 ± 1.18 (0.31)	3.78 ± 1.17 ([a]0.02)	3.82 ± 1.18 ([a]<0.01)	3.89 ± 1.19 ([a]<0.001)
Uric acid	0.29 ± 0.08	0.29 ± 0.08 (0.33)	0.29 ± 0.08 ([a]<0.001)	0.29 ± 0.07 ([a]<0.001)	0.29 ± 0.08 (0.08)
Total protein	76.51 ± 4.51	76.94 ± 4.39 ([a]0.04)	76.72 ± 4.35 (1.00)	76.62 ± 4.36 (1.00)	77.51 ± 4.17 ([a]<0.001)
TC	5.15 ± 0.95	5.19 ± 0.96 ([a]0.00)	5.23 ± 0.97 ([a]<0.001)	5.23 ± 0.98 ([a]<0.001)	5.28 ± 0.98 ([a]<0.001)
HDL	1.61 ± 0.37	1.60 ± 0.37 ([a]0.02)	1.60 ± 0.37 ([a]<0.001)	1.59 ± 0.37 ([a]<0.001)	1.58 ± 0.37 ([a]<0.001)
TG	1.03 ± 0.61	1.03 ± 0.61 (0.29)	1.04 ± 0.61 (1.00)	1.04 ± 0.61 (1.00)	1.06 ± 0.62 ([a]<0.001)
CK	118.05 ± 75.97	118.73 ± 75.75 (1.00)	119.85 ± 76.19 (0.12)	120.22 ± 76.77 ([a]0.01)	120.68 ± 75.92 ([a]0.00)
CKMB	12.62 ± 2.44	13.77 ± 4.20 (0.30)	14.82 ± 3.99 ([a]0.00)	15.28 ± 3.39 ([a]<0.001)	16.4 ± 3.04 ([a]<0.001)
Glucose	5.08 ± 0.87	4.95 ± 0.83 ([a]<0.001)	4.90 ± 0.80 ([a]<0.001)	4.92 ± 0.86 ([a]<0.001)	5.01 ± 0.84 (0.45)
TSH	2.93 ± 4.31	2.98 ± 4.60 (1.00)	3.00 ± 4.52 (0.74)	3.01 ± 4.57 (0.85)	3.00 ± 4.46 (0.39)
FT4	15.56 ± 2.08	15.74 ± 2.09 (0.45)	15.54 ± 2.16 (1.00)	15.54 ± 2.14 (1.00)	15.60 ± 2.14 (1.00)
FSH	5.49 ± 3.11	5.22 ± 3.13 (0.46)	5.59 ± 3.18 ([a]0.00)	5.59 ± 3.16 ([a]<0.001)	5.65 ± 3.21 ([a]<0.001)
LH	9.73 ± 10.50	9.78 ± 10.68 (1.00)	9.83 ± 10.71 (0.34)	9.70 ± 10.61 (1.00)	9.80 ± 10.71 (1.00)
Estradiol	396.45 ± 521.63	401.29 ± 514.59 (1.00)	385.16 ± 498.82 (0.21)	389.19 ± 515.61 (0.11)	386.32 ± 508.18 ([a]0.02)

[a]p-Value of <0.05 considered as significant value calculated using SPSS Statistics for Windows, version 27.0.

baseline and 4 h of delayed centrifugation for potassium (p<0.001), TC (p=0.001), total protein (p=0.041), and glucose (p<0.001). Similarly, significant differences were observed between the baseline and 8 h of delayed centrifugation for sodium (p<0.001), chloride (p<0.001), potassium (p<0.001), albumin (p<0.001), calcium (p=0.005), CKMB (p=0.004), creatinine (p<0.001), FSH (p=0.001), TC (p<0.001), urea (p=0.018), uric acid (p<0.001), and glucose (p<0.001). Additionally, significant differences were observed between the baseline and 12 h of delayed centrifugation for potassium (p<0.001), AST (p<0.001), calcium (p<0.001), creatinine kinase (p=0.007), CKMB (p<0.001), FSH (p<0.001), GGT (p=0.034), HDL (p<0.001), phosphate (p<0.001), TC (p<0.001), urea (p<0.001), uric acid (p<0.001), and glucose (p<0.001). Finally, significant differences were observed between the baseline and 24 h of delayed centrifugation for chloride (p<0.001), potassium (p<0.001), albumin (p=0.002), ALP (p=0.002), creatinine kinase (p=0.001), CKMB (p<0.001), estradiol (p=0.024), FSH (p<0.001), GGT (p=0.005), HDL (p<0.001), TC (p<0.001), triglyceride (p<0.001), total protein (p<0.001), and urea (p<0.001). No significant differences were found for the remaining analytes.

Significant variations in analyte concentrations over time were observed when comparing baseline (0.5 h) to subsequent time points using one-way repeated measures ANOVA and post-hoc Bonferroni tests. Out of the 26 analytes analysed, significant differences were found for 4 analytes at 4 h, 13 at 8 and 12 h, and 14 at 24 h. Notably, consistent significant differences were found for potassium at 4, 8, 12, and 24 h, calcium at 8 and 12 h, creatinine kinase at 12 and 24 h, CKMB at 8, 12, and 24 h, FSH at 8, 12, and 24 h, GGT at 12 and 24 h, HDL-c at 8, 12, and 24 h, TC at 4, 8, 12, and 24 h, urea at 8, 12, and 24 h, uric acid at 8 and 12 h, and glucose at 4, 8, and 12 h.

Reference change value of biochemistry analytes.

We further assessed the probability of significant differences by reference change value (RCV) in the serial results of the 21 analytes in blood that were significantly different based on ANOVA. RCV is a concept used in clinical chemistry to assess

Table 4: Mean percentage deviation of different analytes.

Analytes	Percentage change, %				Reference change value,%	
	4 h	8 h	12 h	24 h	Increase	Decrease
AST	3.4	3.6	5.9	3.1	26.1	−20.7
Albumin	0.9	1.5	0.9	2.2	9.9	−9.0
ALP	0.3	0.5	0.1	1.3	16.0	−13.8
GGT	0.6	1.2	2.2	3.1	25.3	−20.2
Na	−0.5	−1.0	0.2	−0.3	2.5	−2.4
Cl	−0.7	−1.1	0.0	−2.6	3.6	−3.5
K	5.2	[a]10.9	[a]16.4	[a]20.9	10.4	−9.4
Ca	−0.4	−0.9	−1.3	0.0	5.4	−5.1
Creatinine	−1.2	−3.2	0.2	1.6	15.8	−13.7
Phosphate	0.0	−0.9	−4.3	−3.5	21.4	−17.6
Urea	1.1	1.3	2.4	4.3	38.8	−28.0
Uric acid	0.0	0.0	0.0	−3.4	22.9	−18.6
Total protein	0.6	0.3	0.1	1.3	7.0	−6.5
TC	0.8	1.6	1.6	2.5	14.5	−12.6
HDL	−0.6	−0.6	−1.9	−1.9	14.8	−12.9
TG	1.0	1.0	1.0	2.9	58.5	−36.9
CK	0.6	1.5	1.8	2.2	41.7	−29.4
CKMB	8.9	17.0	[a]20.7	[a]29.3	18.3	−15.5
Glucose	−2.6	−3.5	−3.1	−1.6	12.8	−11.3
FSH	0.5	1.6	1.8	2.9	38.4	−27.7
Estradiol	1.2	−2.8	−1.8	−2.6	42.4	−29.8

[a]Indicated mean percentage change of the analytes exceeded percentage reference change value calculated using the Reference Change Value calculator tool provided by https://biologicalvariation.eu/ [18].

whether a change in a patient's laboratory test results is significant when considering three inherent sources of variation, namely, preanalytical variation, analytical imprecision, and within-subject biological variation.

In this current study, of the 21 analytes, all results were within the RCV at all time points of delayed centrifugation except for serum potassium [exceeding the increase percentage RCV=10.4 % (percentage change after 8 h=10.9 %, 12 h=16.4 % and 24 h=20.9 %)] and CKMB [exceeding the increase percentage RCV=18.3 % (percentage change after 12 h=20.7 % and 24 h=29.3 %)] as shown in Table 4. The mean serum or plasma HI of each sample did not exceed the maximum allowable interference of hemolysis provided by the manufacturer Table 2.

Discussion

This study highlights the blood sample stability of 24 analytes except for serum potassium and CKMB when samples are stored for up to 24 h at room temperature. In a systematic review by Hedayati et al. [20], several conditions can affect the analytes and therefore, it is difficult to approach a congruous conclusion on sample stability with delayed separation. Type of samples, blood collection tubes, temperature and storage timeline can all influence the stability of samples prior to analysis and can potentially lead to inaccuracies of results. In this present study and to the best of our ability, we standardized the blood collection tubes, ambient temperature and centrifugation speed and time, to determine whether different serum separation time delays could affect the accuracy of routine biochemistry tests.

A report by Lee et al. [21], concluded that inorganic phosphorus is one of the putative indicators of delayed separation of whole blood as the concentrations elevated ~2-fold in serum samples following a 48-h delay in separation at room temperature. However, this is not observed during this quality exercise. This may be due to differences in the study design, in which the serum samples were separated, and then stored at −70 °C prior to analysis. Another report by Oddoze et al. [10] highlighted that the inorganic phosphorus concentrations changed in serum samples (3.8 %) after whole-blood separation was delayed at room temperature for 24 h as compared with the concentrations measured after immediate separation. It was noted that the study had used plain glass serum tubes which differed from this current study that used SSTTM II Advance with clot activator tubes. The type of blood collection tubes and the storage timeline may impact the results of the analytes being analysed. Given the observed differences in sample handling procedures, including the type of blood collection tubes used and the storage timeline, it is plausible that our study yielded different results compared to those reported by Lee et al. [21] and Oddoze et al. [10]. As a precaution, we conducted the research as close as possible to the sample analysis process, mimicking the routine laboratory preanalytical procedures of this laboratory.

This present study highlighted that serum potassium was affected by sample processing delays. Serum potassium has been reported to be unstable in most related studies. Tanner et al. [22] reported that the concentration of serum potassium exceeded total change limit in serum gel separator tubes kept in 15 °C, 25 °C, and 35 °C, immediate testing after delaying centrifuges at 4 h, 8 and 24 h respectively compared to the baseline value. A study by Daves et al. [23] reported that potassium concentration exceeded the acceptable limits after 3 h of storage before centrifuge. Findings from these studies are in line with this current study. However, a study conducted by Lee et al. [21] partially agreed with this current study where they reported that serum potassium concentration was significantly greater after delayed whole-blood separation only after 24 h at room temperature. Potassium concentration in plasma or serum samples could be considered as putative indicators to

determine whether whole-blood separation had been delayed for extended periods. This could be due to prolonged contact with cellular components and release of intracellular components [24]. Furthermore, cells remaining in plasma, even after centrifugation, can also lead to the release of potassium, resulting in increased concentration of potassium over time [25]. Therefore, potassium appears to be one of the analytes most influenced by delayed centrifugation, by virtue that they are the most abundant ion within cells [26].

To the best of our knowledge, this current study is the first to report CKMB stability which was shown to be unstable with 12 h delayed centrifugation. According to Hedayati et al. [20], there were twelve reports of either slight or prominent changes in creatine kinase (CK) activity and the maximum stability reported was 7 days delayed centrifugation when stored at 4 °C [8]. Unlike CK, CKMB is its isoenzyme. The difference in their structure may be attributed to the difference in the stability of both analytes. Further study is warranted to justify the significant differences in serial results of CKMB after delaying centrifugation.

It is worth noting that our study employed a different statistical analysis approach compared to previous research on delayed centrifugation of samples. While most studies typically utilize ANOVA for comparison, our study implemented Reference Change Value (RCV) calculation to further investigate differences in combination with ANOVA [7, 15, 22, 27]. This allowed us to not only assess statistical significance but also consider biological and clinical variances using RCV, providing a more comprehensive analysis of the impact of delayed centrifugation on the analytes studied.

Conclusions

In conclusion, it is advisable to centrifuge the whole blood specimens within 8 h at room temperature before analysis for biochemistry test, especially renal function, and cardiac enzyme tests to ensure accurate results for serum potassium and CKMB concentrations. Further delay in separating serum may cause incorrect interpretation of results as shown by this study for those analytes, which may consequently impair the clinical decision making.

This current study also provides some reassurance that samples arriving beyond the 4-h mark and up to 24 h would still be stable for analysis, given that the temperature during transport and handling is maintained and not exceeding 24 h which this current study has established. Further studies to determine stability of these analytes beyond the 24 h at room temperature and to also assess the effects of variability in sample transport on sample integrity would further help large laboratories implement good sample handling guidelines to ensure that samples are fit for analysis with minimal effects on the accuracy of test results.

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