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Research Article

STABILITY STUDIES OF RETESTING OF RETAINED AND ALIQUOTED SAMPLES IN 21 BIOCHEMICAL ANALYTES

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ABSTRACT

Introduction: There is a definite sample retention policy for clinical biochemistry labs for retesting as well as testing for additional analytes. Minimum of 24 hours retention period is needed as per ISO 15189:2012 guidelines. The blood sample is collected and transported to the laboratory for analysis. During this time, the samples bear many extra-analytical factors prior to analysis. Contact time of plasma or serum with the blood cells is one of these factors because of ongoing cell metabolism as well as active and passive movement of analytes in cellular compartments. In a tertiary care hospital with a sample load of around 2500 samples per day, it is practically impossible to aliquot the separated serum after centrifugation from the primary collection tube for storage for 24 hours.

Aim and Objectives: The aim of this study is to determine the stability of twenty-one different biochemical analytes in primary collection tube (without separation of serum/plasma) and secondary collection tube (with separation of serum/plasma in fresh aliquot).

Materials and Methods: Venous blood sample of 50 patients were collected in plain vacutainer and fluoride vacutainer (primary collection tube). A set of twenty-one biochemical analytes including sodium, potassium, urea, creatinine, total bilirubin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, glucose, total protein, albumin, calcium, phosphorus, uric acid, amylase, lactate dehydrogenase, creatine kinase, triglycerides, total cholesterol, LDL-cholesterol and HDL-cholesterol were analyzed immediately after separation from clot/red cells to obtain the baseline values. Consequently, the samples were divided into two tubes, one being the primary collection tube (without separation of serum/plasma) and secondary collection tube (with separation of serum/plasma in fresh aliquot).

All tubes were stored at 4°C ± 2°C. After 24 hours, same set of tests were done on the same equipment and statistical analysis was done.

Results: Using the SCL approach to determine the relevant clinical changes in concentration of these 21 biochemical analytes, the present study observed that concentration of serum potassium, phosphorous, lactate dehydrogenase and plasma glucose changed significantly in the primary collection tube as compared to the secondary collection tube

Conclusion: Most routine biochemical analytes are stable and can tolerate a delay in separation of serum from clot for upto 24 hours when stored at 4°C ± 2°C except potassium, glucose, phosphorus and LDH.

INTRODUCTION

Changes in test results can be induced by preanalytical, analytical or normal biological variations in addition to pathophysiologic processes. For accurate results of patients investigations, preanalytical, and analytical variations must be reduced to acceptable levels at which they cause no impact on clinical interpretation of the results.[1]

Most common problem that is faced by the clinical laboratories is maintaining the stability of serum/plasma analytes during sample storage. According to the guideline of Indian Laboratory Accreditation Body, NABL 112 [2] and CLSI [3], the retention period of a sample after collection is 24 hours.

The optimum time interval between sample collection and separation of serum from the clot should be long enough to allow complete clot formation but shorter than the time in

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which a significant change in test result is induced by serum-clot contact.

In a busy routine clinical biochemistry laboratory with approximate test sample load of 4500 tests per day, preservation of samples for 24 hours in separate aliquots is a tedious and cumbersome process when done for all the samples coming to the lab. However, it is mandatory to store the samples for reanalysis or performing additional tests as per the advice of clinician.

It is recommended that serum or plasma should be physically separated from contact with cells as soon as possible, unless conclusive evidence indicates that longer contact times do not contribute to result inaccuracy [4].

Discrepency in laboratory results due to prolonged contact between cells and serum may be attributed to sustained metabolic activities of blood cells, alterations in cell membrane integrity resulting in continuous release of metabolites, or release of degradation products from clots.

In the present study, we tried to find out, if there is any deviation in keeping the samples in primary containers itself instead of preserving in aliquots for 24 hours at 4°C ± 2°C and also if the deviation is present, whether it is in the acceptable limits.

However information about test stability after prolonged contact with clot is available in literature for 41 chemistry tests [5-9]. All studies except Chu *et al* (1986) reported 24-h stability at room temperature[7]. In addition, Zhang *et al* (1998) studied the effect of serum-clot contact time on 63 analytes at different time intervals [10].

The validation of such a policy would save time of pouring samples in aliquots which will further save the laboratories from the personnel error during the process of pouring and also the unnecessary procurement of micro centrifuge tubes needed to preserve the serum/plasma in aliquots.

MATERIAL AND METHODS

Study Design

This hospital-based study was conducted at Vardhman Mahavir Medical College and Safdarjung Hospital, New Delhi, India, from September 2017 to November 2017 andincluded 50 random samples from outpatients being treated at the hospital clinics. The samples collected from each patient were for physician-ordered laboratory testing; no additional blood was taken from the subjects. The investigational proposal was approved by the institutional ethical committee. All volunteers were informed of the rationale for the study and consent was obtained. All procedures were conducted in accordance with the guidelines of the Helsinki declaration on human experimentation.

Instrumentation

Sodium, Potassium, Urea, Creatinine, Total Bilirubin, Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), Glucose, Total Protein, Albumin, Calcium, Phosphorus, Uric Acid, Amylase, Lactate dehydrogenase (LDH), Creatine kinase (CK), Triglycerides (TG), Cholesterol, Low density lipoprotein (LDL), High density lipoprotein (HDL) were analyzed in Advia 2400, Germany, fully automated biochemistry analyzer. Standard reagent kits from Siemens Pvt. Limited were used for analysis of samples (Table1)

Specimen collection and Analysis

Venous blood was collected from each of 50, fasting volunteers after antecubital venipuncture using a using a Vacuette® standard tube holder and Vacuette® 22GA × 1" (0.7 mm × 25 mm) multisample needle (Becton, Dickinson and Company, USA). The blood specimens were drawn into 6.0 mL plastic serum tubes (BD Vacutainer® serum; BD, Franklin Lakes NJ, USA, ref 367815) and into 2.0 mL sodium fluoride/ Na_2 EDTA 3mg/6mg (BD Vacutainer® serum; BD, Franklin Lakes NJ, USA, ref 365069). Tubes were filled completely. Ambulatory volunteers were seated in an upright posture for approximately 1 min to prevent hem concentration and the production of metabolic byproducts. Total collection time per volunteer was 10-15 min.

After collection, the sample tubes for plasma preparation, were gently rocked to mix the anticoagulant. The sample tubes for serum separation, were left in an upright position for 30 min at room temperature. Both the tubes were then centrifuged at 3500 rpm for 10 min. The samples were examined for hemolysis and lipemia to prevent possible interference.
The serum and plasma samples from each patient were analysed immediately after separation from clot/ red cells to obtain the baseline fresh values (0.5hour value).

Once the baseline value was obtained, the specimen was divided into two tubes, first is the primary collection tube (without separation of serum from clot) and another is secondary collection tube (where serum is aliquoted in another tube to separate it from cell contact). Both these sample tubes were stored at 4°C ± 2°C in refrigerator for 24 hours. Direct exposure to sunlight was avoided for the stored specimens. Refrigerated samples were analyzed once the room temperature was obtained.

Inclusion Criteria

Samples were randomly selected from samples received for analysis in biochemistry laboratory.

Exclusion Criteria

1. Hemolysed sample as hemolysis may interfere with result.
2. Sample inadequate to perform all twenty-one parameters.
3. Lipemic samples

Statistical Analysis

The mean of all 50 specimen for all twenty one analytes were obtained for 0.5 hour and 24 hours to establish time-dependent changes in each analyte. Statistical analysis of result was done by Students' t-test. P-value of 0.05 or less was considered to be statistically significant. Statistical analyses were performed with SPSS Version 21. Clinically significant changes were determined using the significant change limit (SCL) approach[as] described by Passey *et al* (1996) [11], which is defined as: SCL = Initial value ± 2.8 usual standard deviation (USD). This is based on the assumption that the usual USD is representative of the inherent day-to-day variability of the method. In our study, the calculated mean for each analyteat0.5hr represented the

initial value. For simplicity, the SCL was computed for each analyte by establishing the range (±3.0 USD) from the subject meanat0.5h.

RESULT

The statistical analysis of prolonged contact with serum/plasma for 24 hours is presented in Table 2, indicating the mean value, standard error, usual standard deviation and significant change limit. 0.5 hours mean values of all analytes are within analytes reference interval. All the parameters except potassium, glucose, phosphorus and LDH in primary and secondary tubes at 24 hours are within the significant change limit.

Table 3 shows relative percentage bias and P value. Positive % bias is maximum in potassium (58.4%) followed by LDH (39.06%) in the primary tubes. Similarly, maximum negative % bias is seen in glucose in the primary tube after 24 hours.

Significant differences in the mean values were observed for potassium, total bilirubin, glucose, calcium, phosphorus, uric acid, amylase, LDH, triglycerides and LDL-cholesterol between 0.5 hours and 24 hours in the primary tubes when determined by two tailed paired t-test ($P \leq 0.05$). However, all these analytes except glucose, potassium, phosphorus and LDH were within significant change limit.

Table 1 Methods used for measuring biochemical parameters:

Analyte	Method
Sodium, mmol/L	ISE Indirect
Potassium, mmol/L	ISE Indirect
Serum Urea, mg/dL	Urease, UV
Creatinine, mg/dL	Jaffe's Kinetic
Total bilirubin, mg/dL	Vanadate oxidation
Aspartate transaminase (SGOT), U/L	UV w/o P5P
Alanine transaminase (SGPT), U/L	UV w/o P5P
Alkaline phosphatase(ALP), U/L	PNPP, AMP Buffer
Glucose	GOD POD
Total protein, g/dL	Biuret method
Albumin, g/Dl	Bromocresol green method
Total calcium, mg/dL	Arsenazo III
Phosphorus, mg/Dl	Phosphomolybdate-UV
Uric acid, mg/Dl	Uricase method
Amylase, U/L	Ethylidene blocked-pNPG7
Lactate dehydrogenase(LDH), U/L	Lactate/NAD IFCC
Total creatine kinase(CK), U/L	NAC activated
Triglycerides, mg/dL	Enzymatic Endpoint
Total cholesterol, mg/dL	CHOD- Esterase Peroxide
LDL cholesterol, mg/dL	Direct measure
HDL cholesterol, mg/dL	Direct measure

Table 2 Mean (Standard Error)

Biochemical parameter			Primary collection tube (without separation of serum/plasma	Secondary collection tube (with separation of serum/plasma	USD Value	SCL Range
	No. of samples	0.5 hour Mean (SE)	24 hour Mean	24 hour Mean		
Sodium	50	141.5 (0.67)	139.6 (0.81)	144.5 (1.2)	1.8	136.1-146.9
Potassium	50	4.43 (0.11)	7.02 (0.91)	4.47 (0.18)	0.11	4.1-4.76
Urea	50	25.6 (1.31)	26.7 (1.41)	25.8 (1.24)	1.14	22.18-29.02
Creatinine	50	0.65 (0.03)	0.65 (0.03)	0.66 (0.03)	0.06	0.47-0.83
T.Bilirubin	50	0.48 (0.07)	0.45 (0.06)	0.47 (0.07)	0.12	0.12-0.84
AST	50	24.8 (2.52)	26.2 (2.7)	25.2 (2.61)	0.83	22.31-27.29
ALT	50	29.9 (2.55)	29 (2.5)	28 (2.4)	0.62	28.04-31.76
ALP	50	86.3 (4.87)	87.3 (4.83)	87.7 (4.85)	8.62	60.44-112.84
Glucose	50	130.2 (8.5)	90.2 (8.1)	126.1 (7.5)	12.88	91.56-168.84
Total Protein	50	7.52 (0.14)	7.53 (0.15)	7.59 (0.14)	0.25	6.77-8.27
Albumin	50	4.23 (0.06)	4.20 (0.07)	4.23 (0.07)	0.15	3.78-4.68
Calcium	50	8.61 (0.13)	8.9 (0.16)	8.46 (0.10)	0.19	8.04-9.18
Phosphorous	50	3.74 (0.13)	4.9 (0.57)	3.8 (0.12)	0.21	3.11-4.37
Uric Acid	50	4.56 (0.24)	4.31 (0.23)	4.58 (0.25)	0.36	3.48-5.64
Amylase	50	58.7 (4.47)	57.7 (4.41)	59.1 (4.34)	10.11	28.37-89.03
LDH	50	156.4 (6.05)	217.5 (10.93)	160.3 (8.35)	14.13	114.01-198.79
Creatine Kinase	50	105.1 (39.92)	107.5 (39.29)	100.3 (40.19)	25.26	29.32-180.88
Triglycerides	50	110.6 (13.23)	113.2 (13.48)	110.6 (13.22)	12.1	74.3-146.9
Cholesterol	50	162.01 (7.73)	162.59 (7.42)	161.05 (7.55)	8.3	137.11-186.02
HDL-c	50	40.66 (2.49)	41.6 (2.82)	41.6 (2.56)	2.4	33.46-47.86
LDL-c	50	96.59 (9.49)	94.29 (9.26)	95.64 (9.27)	9.01	69.56-123.62

Table 3 Percentage difference and P value

Biochemical parameter	Primary collection tube (without separation of serum/plasma)	Secondary collection tube (with separation of serum/plasma)
	24 hours % diff (P value)	24 hours % diff (P value)
Sodium	- 1.34(0.07)	2.12(0.01)
Potassium	58.4(0.01)	0.90(0.68)
Urea	4.29(0.15)	0.78(0.59)
Creatinine	0.00(1.0)	0.61(0.79)
T.Bilirubin	-5.62(0.02)	-1.87(0.32)
AST	5.64(0.13)	1.61(0.63)
ALT	3.34(0.12)	-6.35(0.06)
ALP	1.16(0.32)	1.62(0.10)
Glucose	-40.57(0.002)	-5.07(0.08)
Total Protein	0.13(0.75)	0.93(0.01)
Albumin	-0.70(0.19)	0.00(1.0)
Calcium	3.37(0.03)	-1.74(0.12)
Phosphorous	31.01(0.03)	1.60(0.14)
Uric Acid	-5.48(0.01)	0.44(0.50)
Amylase	-2.38(0.008)	0.68(0.10)
LDH	39.06(0.01)	2.49(0.39)
Creatine Kinase	2.28(0.15)	-4.56(0.01)
Triglycerides	2.35(0.009)	1.22(0.00)
Cholesterol	0.65(0.19)	0.39(0.62)
HDL-c	2.31(0.12)	2.31(0.08)
LDL-c	-2.38(0.04)	-0.98(0.45)

DISCUSSION

Considering the patient load in a tertiary care center as well as academic institute, it is not uncommon to receive add on test request and re-do test request if there occurs any discrepancy in the clinical findings and the test results. Moreover, chances of delay in receiving the samples from the patients either due to misplacement of samples or shuffling of samples or due to any pre-analytical error cannot be ruled out completely.

Although adverse effect of prolonged contact between cells and serum on the laboratory parameters has been recognized since long. Laessig RH *et al* (1976) described changes in serum biochemical values as a result of prolonged contact with the clot extending for 48 hours[12]. Similarly a study by Kirankumar *et al* (2018) showed the stability of seventeen biochemical analytes on storage of the samples for 24 hours [13]. Zhang *et al* (1998) determined that serum should be separated from the clot within three hours for glucose, potassium and phosphorous, and within six hours for albumin, bicarbonate,

chloride, C-peptide, HDL-cholesterol, iron, LDL-cholesterol and total protein[10].

Immediate separation of serum from cells has always been considered essential to accurate laboratory test results. The current recommendation of an acceptable time interval between blood drawing and serum separation is 2 hours [14].However, it is observed that for most of the Indian laboratories, it is difficult to separate out serum or plasma from contact with cells within 2 hours. Apart from this, it is also mandatory to retain the samples for 24 hours as per standard guidelines to do ad-on test or re-testing from stored samples with acceptable variation in test result.

Therefore, it is felt significant to perform sample stability study at local laboratory set up.As we observed that, in our set up, even after separation of serum from clot within 2 hours and stored at 4°C ± 2°C for 24 hours had statistical significant difference, therefore we applied the concept of significant change limit (SCL), according to which even if statistical significant differences exist, how much analytical system contribute to this difference.

Although this study included electrolytes but as per accreditation guidelines electrolytes are preferred to be retested from freshly collected sample. However, this study observed an increase in sodium concentration in the secondary tube and decrease in primary collection tube. The variation is within SCL and our observation is consistent with similar studies investigating serum with prolonged contact with cells reported by Ono *et al* (1981), Zhang *et al* (1998) and Kirankumar *et al* (2018) respectively[6,10,13].

Serum potassium increases drastically if the serum is not separated from the clot within 2 hours and the result is out of SCL when kept for 24 hours. This increase can be attributed to the malfunctioning of the Na^+/K^+ ATPase pump, resulting in diffusion of K^+ from the erythrocytes driven by the intracellualar-extracellular concentration gradient[15].

Glucose concentration decreases with increasing serum-clot contact time in plain tube. Concentration of glucose is lower at 24 hours compared to concentration at 0.5 hour which is statistically significant and in accordance with the reports of Chan *et al* (1989) and Weisman *et al* (1986) respectively [16,17]. It is attributed to glycolytic action of erythrocytes and leucocytes.

Increase in concentration of phosphorus has been observed in primary tube where contact with the cells is maintained and is statistically significant ($P<0.05$). The increase is attributed to the presence of organic phosphates in erythrocytes which are susceptible to hydrolysis to produce inorganic phosphorous that gets leaked from the cells and increases its concentration in serum [18].

Our study did not observe significant difference in concentration of urea, creatinine, total protein and albumin when stored at 4°C ± 2°C for 24 hours which suggests that these parameters are stable in both with or without serum-clot separation tube. The results are similar when compared to the previous studies reported by Rehak *et al* (1988) and Kirankumar *etal* (2018) [8,13].

We observed an increase in concentration of cholesterolin primary collection tube after 24 hours which is also consistent with the reports of Bobby *et al* (2002) [19]. The proposed mechanism is that cholesterol gets continuously removed from blood cells via lecithin: cholesterol acyl transferase. We also noticed a significant upward trend in triglycerides and downward trend in LDL-cholesterol but both were within significant change limit. This is in contrast to the study done by Heins *et al* (1995) which suggests that triglyceride was stable over the 56-hours period [9].

LDH concentration increased in both primary and secondary collection tubes over 24 hour interval even when stored at 4°C ± 2°C with SCL surpassed in the primary tube. The increase in concentration of LDH was attributed to changes in the membrane integrity of cells. Heins *et al* (1995) and Zhang *et al* (1998) reported similar results [9,10]. An upward trend in the value of creatine kinase was observed in samples stored for 24 hours in contact with cells in contrast to the aliquoted sample. This observation was similar to study done by Clark *et al* (2003) [20] though increase is not significant ($P>0.05$)

This study also observed significant decrease in serum amylase levels in the primary collection tube after 24 hours at 4°C ± 2°C, though falling within SCL. Only one previous study reported by Kachhawa *et al* (2017) showed similar result with amylase levels[21].

Though total bilirubin levels showed significant decrease ($P<0.05$) in the primary tube but lies within the significant change limit alongwith AST, ALT and ALP levels at 24 hours interval. The result of this study for AST and ALP is similar to other studies done by Ono *et al* (1981), Chu *et al* (1986) and Zhang *et al* (1998) respectively[6,7,10].

CONCLUSION

In conclusion, most of the routine biochemical analytes are stable and may be stored in the primary container for upto 24 hours at 4°C ± 2°C for retesting as per the standard guidelines. However few analytes that require stringent control before serum-clot separation are glucose, potassium, phosphorus and LDH.

This validation study may be helpful in sample preservation but the validity is also dependent on certain other factors like laboratory environment, instrument, reagents, competency of laboratory personnel, time lag between collection and receipt by the laboratory. Any deviation from such factor/factors needs re-evaluation.

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