ejpmr, 2017,4(01), 550-556

SJIF Impact Factor 3.628

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article
ISSN 2394-3211
EJPMR

THE EFFECT OF ANTICOAGULANTS, STORAGE TIME AND TEMPERATURE ON LIPOPROTEIN CONCENTRATIONS OF APPARENTLY HEALTHY SUBJECTS IN PORT HARCOURT, NIGERIA.

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Article Received on 18/11/2016 **Article Revised on 09/12/2016** **Article Accepted on 30/12/2016**

ABSTRACT

Background: This study was designed to investigate the effect of anticoagulants (lithium heparin and fluoride oxalate) on lipoprotein levels obtained from samples which were analyzed within 2h after collection (control), stored at -4^{0}C for one week (168h), 25^{0}C for one week (168h), -4^{0}C for two weeks (336h) and 25^{0}C for two weeks (336h). 30 apparently healthy subjects ranging between the ages of 23 to 75 years participated in the study. **Materials and Methods**: Standard analytical procedures were used to determine the levels of the lipoproteins in the sample. **Results**: The results of the means ± SD of the plasma samples in the different anticoagulants stored for both 1 week and 2weeks at -4°C and 25°C for TC, TG, HDL-C and LDL-C showed significant differences ($p< 0.05$) decrease and deterioration when compared with the controls. There was no significant difference ($p>0.05$) in the lipoprotein concentrations between lithium heparin and fluoride oxalate controls suggesting that lipoprotein determinations can be performed using fluoride oxalate as anticoagulant if the samples are to be analyzed immediately after collection. **Conclusion**: Anticoagulants, length and storage temperature, affect dramatically the measurement of total cholesterol, triglycerides, high density lipoprotein and low density lipoproteins.

KEYWORDS: Lipoprotein, lithium heparin, fluoride oxalate, anticoagulants, storage, temperature.

INTRODUCTION

Estimation of cardiovascular risk has become the cornerstone of cardiovascular prevention and management and this is achieved by the measurement of body lipids and lipoproteins.[1] Lipoproteins are complex aggregates of lipids and proteins that render the lipids compatible with the aqueous environment of body fluids and enable their transport throughout the body to tissues where they are required.[2] There are different types of lipoprotein molecules namely; low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, total cholesterol and chylomicrons. Serum or plasma, free of haemolysis is the ideal specimen for the analysis of lipoproteins.[3]

An anticoagulant is a substance that prevents the clotting of blood. Anticoagulants can be used endogenously or/and exogenously. The exogenous anticoagulants are compounds that have been developed using several mechanisms of action.[4] Anticoagulants exert osmotic effects in which water leaves the cells and enters the plasma, thus diluting the plasma and lowering the concentrations of non-diffusible components. The magnitude of this effect depends on the anticoagulant used and its concentration. Heparin, ethylenediamine tetra-acetate (EDTA) and fluoride oxalate are the mostly used anticoagulants in clinical chemistry.[5,6] The heparin contained in the heparin tubes serves as blood anticoagulant by deterring the conversion of fibrinogen to fibrin through acceleration of the thrombin neutralizer antithrombin 111. Due to calcium's integral role in the blood coagulation process, oxalate contained in the fluoride oxalate tubes function as anticoagulant by sequestering calcium through the formation of insoluble complexes and chelates of calcium.[7,8]

It has been reported that improper use of anticoagulant sample tubes, improper storage time and temperature, such as inadequate refrigeration of samples can lead to inaccurate test results.[9] According to the WHO, MONICA project[10], isolation of HDL-C should be done on fresh aliquots on the day of blood collection. A study by Shih et al.[11], recommended that the total cholesterol

and high density lipoprotein-cholesterol levels should be assessed on the day of sample collection.

As part of the laboratory accreditation process and to address the pre-analytical requirements of ISO 15189[12] it was necessary to investigate the effect of different pre-analytical storage conditions of blood samples. In literature, the stability of numerous analytes following prolonged contact of serum with cells has been described.[13,14] To our knowledge, no studies have been conducted on the investigation of the stability of lipoproteins at different laboratory temperatures, storage times and in different anticoagulant tubes in Nigeria. This study was therefore designed to provide information on:

A. The effect of storage times (delays before analysis) (2h, 1 week-168hr), and 2 weeks- 336hr),
B. The effect of different storage temperatures (4^0C, +/- 2 or room temperatures (RT) 25^0C, +/-2),
C. The effect of choice of anticoagulant tubes (lithium heparin, and fluoride oxalate) and
D. The effect of analysis within 2hr of storage versus delays before analysis on the levels of total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) in blood samples collected from apparently normal subjects in Port Harcourt, Nigeria.

MATERIALS AND METHODS

Apparently 30 healthy male volunteers at the University of Port Harcourt teaching Hospital (UPTH), Port Harcourt were recruited into the after approval by the University of Port Harcourt Teaching Hospital (UPTH) Local Ethics Committee. Whole blood samples (10ml each) were collected through clean venepuncture after a 12 hour fast into well-sealed screw-capped sample bottles which helped to prevent oxidation of the unsaturated lipids. 2.5ml of this were dispensed into commonly used fluoride oxalate and lithium heparin anticoagulant sample bottles in Nigeria and centrifuged at 3000rpm for 5 minutes immediately after collection. Fresh plasma supernatants were analyzed at room temperature (25^0C+/-2) within 2hr after centrifugation and the samples were then stored at room (25°C -/+2) for I week (168hr) and 2 weeks (336hr) and freezer temperatures (-4°C -/+2) for I week (168hr) and 2 weeks (336hr) respectively before being analyzed for total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) respectively.

The enzymatic procedure for total cholesterol determination in serum based upon the Trinder[15] method as modified by the Centers for Disease Control and Prevention was used. The method of Lopes-Virella et al[16] for the determination of high-density cholesterol in serum was employed. The Friedewald et al.[17] equation was used to calculate the LDL-cholesterol in mmol/L.

LDL-C (mmol/L) = Total Cholesterol - TG/2.2 - HDL-C

The data obtained from the study were analyzed using the GraphPad Instat Version 3.10, 12 bit for Windows. The analyses performed included the computation of the means and standard deviation. Comparison of the means was done using the one- way analysis of variance (ANOVA). The Dunnett's comparison test was used to verify significant differences between the means of the parameters at the at 2hr storage at 25^0C and those stored at the various storage temperatures and times. The differences in mean were considered significant at $p<0.05$.

RESULTS

The means ± SD (mmol/L) of the total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL) and low density lipoprotein (LDL) of the samples collected into lithium heparin anticoagulant and analyzed within 2hr (control), stored at -4°C for 168 hours (1 week), 25°C for 168 hours (1 week), -4°C for 366 hours (2 weeks) and 25° for 366 hours (2 weeks) is shown in table 1. The mean of the total cholesterol concentration decreased significantly ($p<0.05$, F=54.00) from 4.78 ±1.13mmol/L at within 2hr at 25^0C of storage time and temperature to 1.88 ± 0.86 mmol/L at 336hr (2 weeks) at 25^0C. Similarly, significant decrease in means were also observed in the concentrations of triglycerides ($p<0.05$, F=98.41) and high density lipoprotein ($p<0.05$, F=9.33) from 1.41±0.28 mmol/L and 1.69±0.70 mmol/L at within 2hr at 25^0C of storage time and temperature to 0.53 ±0.15mmol/L and 1.11 ±0.59mmol/L at 336hr (2 weeks) at 25^0C respectively. The LDL concentration also showed similar significant ($p<0.05$, F=49.74) decrease from 2.45 ±0.61 mmol/L at within 2hr at 25^0C of storage time and temperature to 0.51 ±0.47 mmol/L at 336hr (2 weeks) at 25^0C respectively.

The effect of storage temperatures and time on the total cholesterol, triglycerides, high density lipoprotein and low density lipoprotein concentrations in the samples collected in lithium heparin was investigated using the Dunnett's multiple comparison test. This involved the comparison of the lipoprotein means from the experimental temperatures and time of storage against the mean of the control samples. The result is shown in table 2. The result showed that significant differences ($p<0.05$) exist between the means of samples stored at 25^0C for 2hr (control) and those stored at the other experimental storage temperatures and times for total cholesterol, triglycerides, high density lipoprotein and low density lipoprotein. However, for the data obtained for the high-density lipoprotein and low density lipoprotein, the comparison of the means of the control sample against the means of the samples stored at -4^0C for 168hr did not show significant difference ($p>0.05$). Graphical representation of these is shown in fig. 1,2,3,4.

Table 1: Mean ±SD of lipoprotein concentrations stored lithium heparin anticoagulant at various storage times and temperatures

Storage Times/Temperatures	Lipoproteins			
	TC (mmol/L)	TG (mmol/L)	HDL (mmol/L)	LDL (mmol/L)
2hr at 25^0C (Control)	4.78±1.13	1.41±0.28	1.69±0.70	2.45±0.61
168hr at -4^0C	3.99±0.67	1.29±0.29	1.45±0.46	1.93±0.48
168hr at 25^0C	3.03±0.84	1.41±0.30	0.86±0.57	1.54±0.78
336hr at -4^0C	2.80±0.57	0.59±0.17	1.32±0.49	1.26±0.41
336hr at 25^0C	1.88±0.86	0.53±0.15	1.11±0.59	0.51±0.47
P-value	p<0.05, F=54.00	p<0.05, F=98.41	p<0.05, F=9.33	p<0.05, F=49.74

Note: TC represents total cholesterol, TG = triglycerides, HDL = high density lipoprotein and LDL = low density lipoprotein.

Table 2: Results of Dunnett's multiple comparison test for means of lipoproteins in lithium heparin anticoagulant

Storage Times/Temperatures	Lipoproteins							
	TC		TG		HDL		LDL	
	p-value	q-value	p-value	q-value	p-value	q-value	p-value	q-value
Control. vs storage for 168hr at -4^0C	p<0.05	3.87	p<0.05	7.69	NS	1.65	NS	2.32
Control vs. storage for 168hr at 25^0C	p<0.05	6.46	p<0.05	9.72	p<0.05	5.65	p<0.05	3.05
Control vs. storage for 336hr at -4^0C	p<0.05	9.76	p<0.05	10.31	p<0.05	2.57	p<0.05	7.33
Control vs storage for 336hr at 25^0C	p<0.05	12.87	p<0.05	11.90	p<0.05	3.96	p<0.05	10.47

NS represents not significant

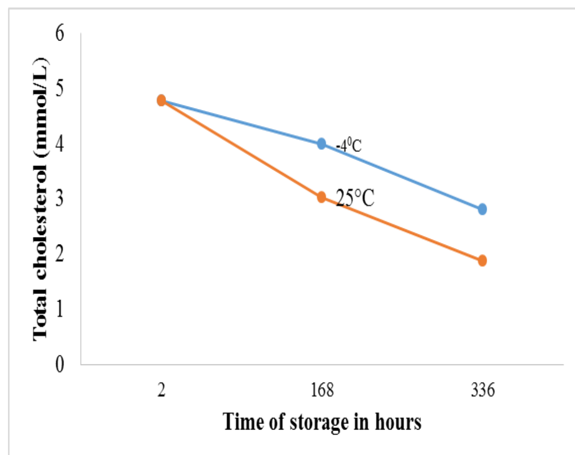

Fig. 1: Effect of time of storage and temperature on TC concentration in lithium heparin

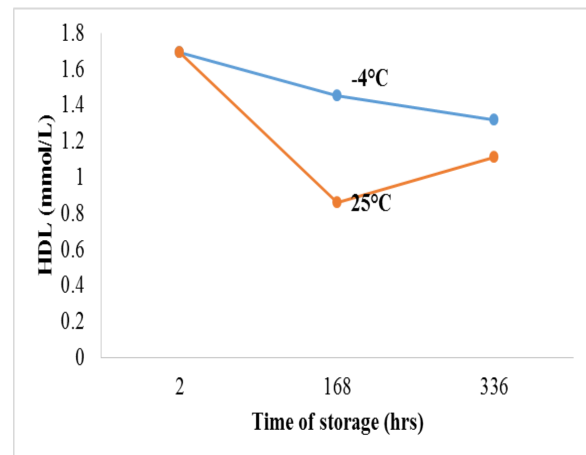

Figure 3: Effect of time of storage and temperature on HDL concentration in lithium heparin

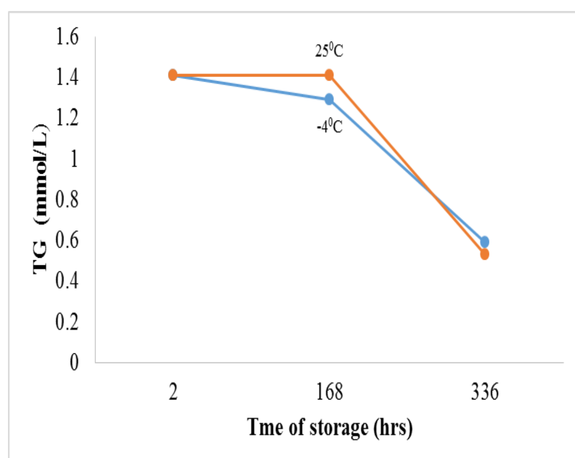

Figure 2: Effect of time of storage and temperature on TG concentration in lithium heparin

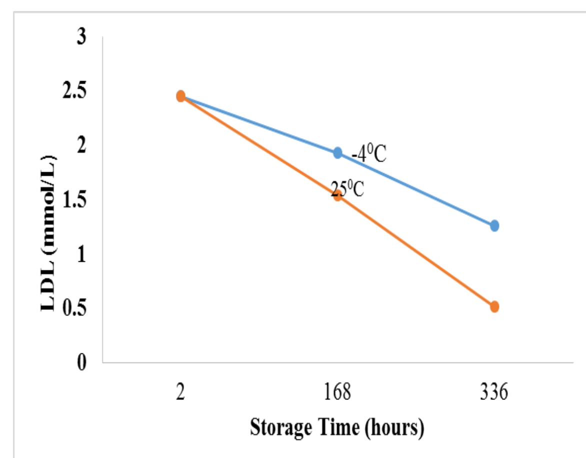

Figure 4: Effect of time of storage and temperature on HDL concentration in lithium heparin

Similarly, the mean ±SD (mmol/L) of the total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL) and low density lipoprotein (LDL) of the samples collected and stored in fluoride oxalate anticoagulant and analyzed within 2hr (control), stored at -4°C for 168 hours (1 week), 25°C for 168 hours (1 week), -4°C for 366 hours (2 weeks) and 25° for 366 hours (2 weeks) before analysis is shown in table 3. The table also showed significant ($p<0.05$) progressive decrease in the means of total cholesterol, triglycerides, high density lipoprotein and low density lipoproteins from 2hr storage time to 338hr and 25^0C and -4^0C respectively. The effect of storage temperatures and time on the total cholesterol, triglycerides, high density lipoprotein and low density lipoprotein concentrations on the samples collected in fluoride oxalate was also investigated using the Dunnett's multiple comparison test (table 4). The result showed that significant differences ($p<0.05$) exist between the means of samples stored at 25^0C for 2hr (control) in fluoride oxalate and those stored at the other experimental storage temperatures and times for total cholesterol, triglycerides, high density lipoprotein and low density lipoprotein. However, with respect to high-density lipoprotein and low density lipoprotein, the comparison of the mean of the control sample against the mean of the samples stored at -4^0C for 168hr did not show significant difference ($p>0.05$). Graphical representation of these is shown in fig. 5,6,7,8. The effect of the anticoagulants (lithium heparin and fluoride oxalate) on lipoprotein concentrations in the control samples which were stored for 2 hours at 25^0C did not show any significant difference ($p>0.05$) (table 5) although the means of the samples stored in lithium heparin anticoagulant were higher that than obtained for samples stored in fluoride oxalate.

DISCUSSION

Laboratory evaluation of patients is an integral part of diagnostic procedure. Although serum is used for most assays, plasma is a useful alternative due to its rapid processing time.[18] Plasma which was obtained with an appropriate anticoagulant has been reported to be an equally valid sample and in certain conditions preferable to serum.[19] Anticoagulants used to preserve analytes may interfere with other analyte determinations when using plasma[5] and changes as little as possible in the concentration of substances to be measured has been reported to occur before the analytical processes.[20] Results obtained from this study show that storage temperature and duration affects the concentration of TC, TG, HDL and LDL in the samples stored in both lithium heparin and fluoride oxalate anticoagulants. The length of time of storage also has effects on the levels of the analytes obtained in the study. The concentration of the analytes deteriorated considerably as the duration of storage was increased. The samples stored in lithium heparin show a percentage decrease of 60.67% for TC, 62.41% for TG, 34.32% for HDL and 79.18% for LDL while the percentage decrease in analyte concentration stored in fluoride oxalate show a percentage decrease of 65.97% for TC, 60.00% for TG, 36.42% for HDL and 86.30% for LDL respectively (table 1and 3). The finding in this study does agree with the result of Ignatius et al[21] in respect of TC and HDL who observed increase in these parameters even after 16 days (384 hours) of storage. Prolonged power failure, a very common experience in the developing countries has been reported to affect sample storage at various temperatures (for example samples stored at refrigerator and freezer temperatures).[21] In a related study, storage time and temperature were also reported to affect measurable concentration of progesterone in the blood of zebu cows.[22] In effect, storage time considerably affected the concentration of the lipoproteins in the sample.

At room temperature (25^0C) and at 2-hour storage, the concentration of the analytes in the fluoride oxalate anticoagulants decreased appreciably when compared with the levels in the lithium heparin anticoagulants (table 5). Lithium heparin has been generally recommended as the most suitable anticoagulant for plasma biochemical measurements.[23,6] Although serum and heparinized plasma specimens are considered equivalent for many assays, differences in results between these two sample types have been reported for several chemistry analytics.[18] Oxalate inhibit several enzymes, such as amylase, lactate dehydrogenase, and acid and alkaline phosphatase.[24] Fluoride may also interfere with electrolyte measurements by altering cell permeability and promoting haemolysis by red blood cell ATP with subsequent potassium efflux.[24] Results of our study showed that all the means of the measured parameters were not significantly different ($p>0.05$) among the sample types stored at 25^0C for 2hours in the lithium heparin and fluoride although the values obtained were reduced in the fluoride oxalate samples. The study by Nwangwu et al.[25] reported that lithium heparin has a stabilizing effect on blood sample stored for 2 hours and may not likely cause alteration in the concentrations of most biochemical parameters measured in humans. The results of this study on lipoprotein concentrations as the measured parameters in human plasma stored for 2 h in lithium heparin thus agrees with the findings of Nwangwu et al.[25]

In conclusion, anticoagulants, length and storage temperature, affect dramatically the measurement of total cholesterol, triglycerides, high density lipoprotein and low density lipoproteins. In experimental planning, it is advisable to take into account the effect of these factors, in addition to sample analysis and reported interpretations of these data in literature.

Table 3: Mean ±SD of Lipoprotein Concentrations Stored Fluoride Oxalate Anticoagulant at Various Storage Times and Temperatures

Storage Times/Temperatures	Lipoproteins			
	TC (mmol/L)	TG (mmol/L)	HDL (mmol/L)	LDL (mmol/L)
2hr at 25^{0}C	4.32±1.04	1.30±0.30	1.51±0.77	2.19±0.62
168hr at -4^{0}C	3.46±0.70	0.67±0.30	0.94±0.60	1.68±0.71
168hr at 25^{0}C	2.89±0.92	0.67±0.30	0.94±0.60	1.62±0.71
336hr at -4^{0}C	2.16±0.68	0.63±0.20	1.01±0.51	0.87±0.63
336hr at 25^{0}C	1.47±0.89	0.52±0.20	0.96±0.51	0.30±0.82
P-value	$p<0.05$	$p<0.05$	$p<0.05$	$p<0.05$

Note: TC represents total cholesterol, TG = triglycerides, HDL = high density lipoprotein and LDL = low density lipoprotein.

Table 4: Results of Dunnett's multiple comparison test for means of lipoproteins in fluoride oxalate anticoagulant

Storage Times/Temperatures	Lipoproteins							
	TC		TG		HDL		LDL	
	p-value	q-value	p-value	q-value	p-value	q-value	p-value	q-value
Control. vs storage for 168hr at -4^{0}C	$p<0.05$	3.86	$p<0.05$	7.70	NS	1.70	NS	2.32
Control vs. storage for 168hr at 25^{0}C	$p<0.05$	6.46	$p<0.05$	9.72	$p<0.05$	3.56	$p<0.05$	4.05
Control vs. storage for 336hr at -4^{0}C	$p<0.05$	9.75	$p<0.05$	10.31	$p<0.05$	3.15	$p<0.05$	6.33
Control vs storage for 336hr at 25^{0}C	$p<0.05$	12.80	$p<0.05$	11.90	$p<0.05$	3.45	$p<0.05$	9.47

NS represents not significan

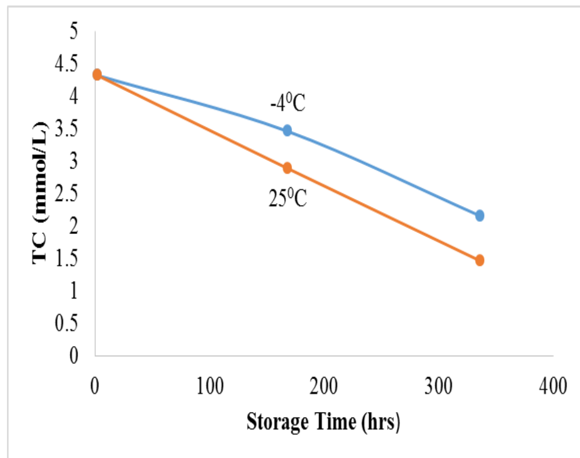

Figure 5: Effect of Time of Storage and Temperature on TC Concentration in Fluoride Oxalate

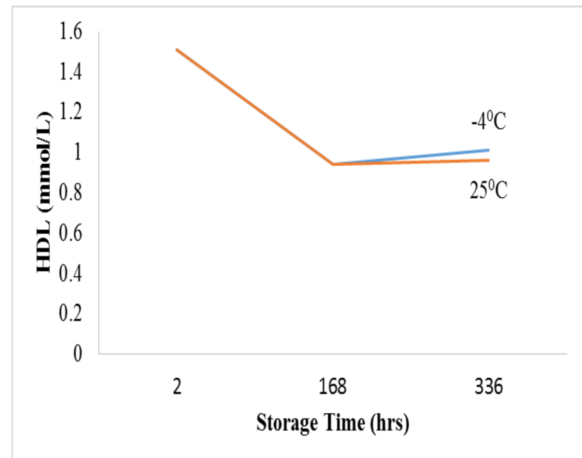

Figure 7: Effect of Time of Storage and Temperature on HDL Concentration in Fluoride Oxalate

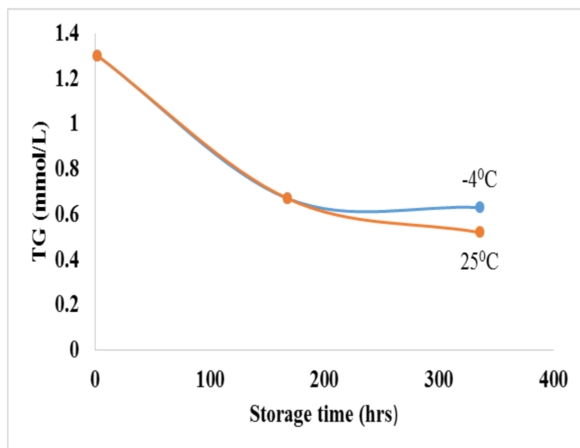

Figure 6: Effect of Time of Storage and Temperature on TG Concentration in Fluoride Oxalate

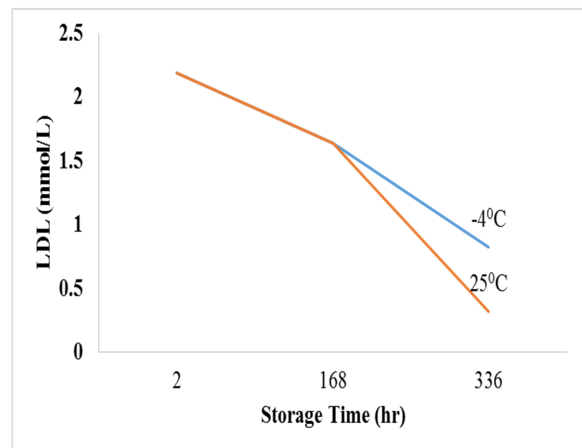

Figure 8: Effect of time of storage and Temperature on LDL Concentration in Fluoride Oxalate

Table 5: Mean ±SD of lipoprotein concentrations in plasma stored for 2hr at 25^{0}C in lithium heparin and fluoride oxalate anticoagulants

Anticoagulants	Lipoproteins			
	TC (mmol/L)	TG (mmol/L)	HDL (mmol/L)	LDL (mmol/L)
Lithium heparin	4.78 ±1.13	1.42 ±0.28	1.69 ± 0.70	2.45 ±0.61
Fluoride oxalate	4.32 ±1.04	1.30 ± 0.31	1.51 ±0.77	2.19 ±0.62
p-value	ns	ns	ns	ns

Note: TC represents total cholesterol, TG= triglycerides, HDL= high density lipoprotein, LDL= low density lipoprotein.

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