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Influence of Freeze-Thawing and Storage on Human Serum Lipid Analytes

P.C. Ugwuezumba[1], P. Nwankpa[2*], F.C. Emengaha[2], C.N. Ekweogu[2], C.C. Etteh[2] and O.G. Chukwuemeka[3]

[1]Department of Medical Physiology, [2]Department of Medical Biochemistry, Imo State University, Owerri
[3]Department of Biochemistry, Michael Okpara University of Agriculture, Umudike Nigeria

**Corresponding author*

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A B S T R A C T

Temperature and storage are important determinants of sample stability which is essential for getting credible concentrations of analytes including those of the lipids. This study examined the influence of freeze-thawing and storage on the concentration human of lipid analytes. The study was conducted using samples from One hundred (50 males and 50 females) randomly selected outpatient participants between the ages of 20 – 60 years. Collection of blood samples was done using the antecubital venipuncture. All procedures were in accordance with the guidelines of the Helsinki declaration on human experimentation. Serum sample of each subject was divided into five (5) aliquots. Three (3) aliquots were used for analysis of 7 days storage at -4^0 C, -20^0 C, and -70^0 C. One (1) aliquot was used for freeze-thawing analysis at -4^0 C, -20^0 C, and -70^0 C while the remaining aliquot was used for baseline measurement (control). The lipid analytes examined at various instances included Total Cholesterol (TC), Triglyceride (TG), High density Lipoprotein Cholesterol (HDL-C), Low-density Lipoprotein Cholesterol (LDL-C) and Very Low-density Lipoprotein Cholesterol (VLDL-C). The result showed a statistically significant difference in the concentration of TC, TG, HDL-C and LDL-C for both males and females when frozen at -4^0 C, significant difference in the concentration of TG and VLDL-C for males and TC, TG, LDL-C and VLDL-C for females when frozen -20^0 C. It showed no significant change in the concentration of all the lipid analytes except VLDL-C when frozen at -70^0 C for both males and females as well as no significant difference in the stability of lipid analytes after 7 days storage at -4^0 C, -20^0 C and -70^0 C when compared to immediate freezing-thawing cycles of samples at -4^0 C, -20^0 C and -70^0 C. In conclusion, -70^0 C is the optimum temperature for storage of lipid samples in order to maintain stability of the analytes. Also, maintenance of stability of lipid analytes may not be time-dependent but temperature-dependent. It is therefore recommended that additional studies be conducted on extended freezer storage of lipid samples to really ascertain the effect of extended duration in the maintenance of stability of lipid analytes.

Introduction

Lipid analytes are veritable tools for diagnosis of a number of health disorders mainly cardiovascular diseases. Credibility of biochemical parameters for diagnosis and research purposes depends largely on the stability of representative samples. Most

medical laboratories are constantly faced with challenges which in most cases prevent same-day processing of samples. These challenges may include equipment breakdown, lack of reagents vis-à-vis thousands of samples received daily for processing. Also need for routine batch analysis for research purposes may sometimes necessitate storage or deep-freezing of samples for an extended duration (Kachhawa *et al.,* 2017).

Stability of serum and plasma analytes during long-term storage is of utmost importance in clinical diagnosis and medical science researches. Samples are stored at different temperatures depending on the duration of storage prior to sample analyses. Temperature and storage are therefore two essential parameters that must be considered in order to maintain the composition and integrity of representative samples during the pre-analytical phase (Ono, 1981; Skotol and Nydam, 2005) and guarantee accuracy of analytical results. Studies have shown that 75% of the errors concerning processing of samples occur in the pre-analytical phase (Bonini *et al.,* 2002; Cuhadar *et al.,* 2013). Samples are usually stored in door (4 – 8^0 C) of a refrigerator for short durations or in a deep freezer (- 20^0 C) for longer time periods (Kachhawa *et al.,* 2017). Stability is the capability of keeping the concentrations of analytes minimally affected within an acceptable variation during a period of study (Association of Normatization, 2009).

There is sparse information regarding the stability of biochemical markers such as lipid profile components in serum and plasma following freeze-thawing cycles and storage at different temperatures. Available literatures however reported very controversial findings. In some studies, freezing and storage time increased lipid concentration (Evans *et al.,* 1997; Pini *et al.,* 1990; Tiedink and Katan 1989; Wood *et al.,* 1980); while in other studies, lipid concentrations decreased (Bausserman *et al.,* 1994; Donnelly *et al.,* 1995; Ekbom *et al.,* 1996; Evans *et al.,* 1995; Nanjee and Miller 1990; Simo *et al.,* 2001; Tiedink and Katan 1989); Yet researchers like Kuchmak *et al.,* (1982) and Stokes *et al.,* (1986) found no significant changes in lipid concentration following freezing and storage. In view of the sparse literatures and above controversies therefore, this study was designed to investigate the influence of freezing-thawing and storage on human serum lipid analytes viz; Total Cholesterol (TC), Low Density Lipoproteins (LDL), High Density Lipoproteins (HDL), Very Low Density Lipoproteins (VLDL) and triglycerides.

Materials and Methods

This was a hospital based study conducted at Imo State University health center. The study included samples from One hundred (100) randomly selected outpatient participants between the ages of 20 – 60 years. The aim and objectives of the study were clearly explained to participants in writing and their consent obtained. Ethical consent was also obtained from the University's Ethics Committee. Patients' participation was therefore voluntary, anonymous and confidentiality of the data generated was ensured. Collection of blood samples was done using the antecubital venipuncture. All procedures were conducted in accordance with the guidelines of the Helsinki declaration on human experimentation.

Sample collection and analysis

Blood samples (total of 6ml) of participants were collected in the mornings into 7.5ml plastic vacuette serum clot activation tubes (BD Vacutainer® Serum; BD, Franklin Lakes NJ, USA). Sample tubes were left in upright position for 30mins at room temperature to

allow for complete clot formation, and then were centrifuged at 3500rpm for 10mins. Serum samples were carefully examined for hemolysis and lipemia to prevent possible interference. Serum sample of each subject was divided into five (5) aliquots. Three (3) aliquots were used for analysis of 7 days storage at -4^0 C, -20^0 C, and -70^0 C. One (1) aliquot was used for freeze-thawing analysis at -4^0 C, -20^0 C, and -70^0 C while the remaining aliquot was used for baseline measurement (control).

Analytes were assayed with reagents manufactured by Roche Diagnostics using kits provided by the manufacturer. Total cholesterol was evaluated by enzymatic colorimetric method. Cholesterol esters were cleaved through the action of cholesterol esterase yielding free cholesterol and fatty acids. Cholesterol oxidase catalyzed the oxidation of cholesterol to form hydrogen peroxide and cholest-4-en-3-one. In the presence of oxidase, the hydrogen peroxide formed affects the oxidative coupling of phenol and 4-aminoantipyrine, forming a quinoneimine red dye. The color intensity is directly proportional to cholesterol concentration, and the absorbance reading is 512 nm. HDL was analyzed by homogeneous colorimetric enzymatic method. In the presence of magnesium ions, dextran sulfate selectively forms water soluble compounds with LDL, VLDL and chylomicrons, which are resistant to polyethylene glycol-modified enzymes. Under the influence of the cholesterol enzyme, the cholesterol esters are decomposed quantitatively into free cholesterol and fatty acids. In the presence of peroxidation, the hydrogen peroxide generated reacts with 4-aminoantipyrine, forming a purple-bluish dye that is directly proportional to cholesterol concentration and is measured photometrically. Triglycerides were measured by colorimetric enzymatic method. This method utilizes the lipoprotein lipase for rapid and complete hydrolysis of triglycerides into glycerol followed by oxidation to dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide reacts with 4-aminophenone and 4-chlorophenol under peroxidase catalytic action to form a red dye. The concentration of triglycerides is proportional to the intensity of the color generated and measured photometrically. LDL was estimated by the Friedewald *et al.,* (1972) formula: LDL = TC – HDL – VLDL (Triglycerides/5).

Statistical analysis

The data generated from the various laboratory analysis were coded in excel sheets and later subjected to statistical analysis using the ANOVA test in the Statistical Packages for Social Sciences (SPSS) version 21 software. Mean ± SEM were calculated and mean values were considered to be statistically significant at 95% level of confidence ($P \leq 0.05$).

Results and Discussion

Lipid profile assessment is an essential parameter which helps in the diagnosis of cardiovascular diseases (Laakso *et al.,* 1993; Shai *et al.,* 2004; Pischon *et al.,* 2005; Walldius and Jungner, 2006) and to a lesser extent diagnosis of diabetes (Jiang *et al.,* 2004; Bruno *et al.,* 2006) and cancer (Santos and Schulze 2012; Muntoni *et al.,* 2009). The temperature at which samples are stored constitutes an important pre-analytical variable that may affect analysis results in the clinical biochemistry laboratory setting. This study was undertaken with the aim of examining the influence of repeated freeze-thawing and storage on the concentration of lipid analytes of human serum. The lipid analytes investigated include total cholesterol (TC), Low density lipoprotein (LDL), High density lipoproteins (HDL), Very low density

lipoproteins (VLDL) and triglycerides. This study revealed a statistically significant difference in the concentration of TC, TG, HDL-C and LDL-C for both males and females when frozen at -4^0 C, significant difference in the concentration of TG and VLDL-C for males and TC, TG, LDL-C and VLDL-C for females when frozen -20^0 C. It showed no significant change in the concentration of all the lipid analytes except VLDL-C when frozen at -70^0 C for both males and females. This finding can be said to be in line with the reports of Paltiel *et al.,* (2008), Comstock *et al.,* 2001), Rudy *et al.,* (2016), Sasaki and Capuzzo (1987)and Metherel *et al.,* (2013). According to Paltiel *et al.,* and Comstock *et al.,* serum cholesterol and triglyceride maintain stability after storage in freezer at -80^0 C. Following investigations of various methods of maintaining stability of samples, Rudy *et al.,* reported that immediate freezing at -80^0 C is ideal to prevent changes in total lipids and fatty acid content while Sasaki and Capuzzo suggested that tissue samples be frozen at -80^0 C upon collection or as soon as possible after sampling or death, Metherel *et al.,* recommended freezing at -80^0 C when compared to -20^0 C (Table 1–4).

Table.1 Effect of freezing to thawing on lipid profile of human male blood sample

Lipid Parameter	Control (fresh)	-4^0 C	-20^0 C	-70^0 C
Total Cholesterol (TC)	165.67 ± 0.51	150.41 ± 0.02*	160.27 ± 0.01	162.22 ± 0.01
Triglyceride (TG)	120.17 ± 0.35	95.26 ± 0.04*	80.24 ± 0.21*	113.25 ± 0.22
HDL-C	59.63 ± 0.54	50.21 ± 0.03*	55.87 ± 0.04	57.78 ± 0.04
LDL-C	101.72 ± 0.40	90.10 ± 0.02*	95.72 ± 0.03	98.62 ± 0.01
VLDL-C	19.83 ± 0.56	15.24 ± 0.08	12.51 ± 0.05*	9.70 ± 0.11*

Values are expressed as mean ± SD, n = 50, * = significantly different from control ($p < 0.05$).

Table.2 Effect of freezing-thawing on lipid profile of human female blood sample

Lipid Parameter	Control (fresh)	-4^0 C	-20^0 C	-70^0 C
Total Cholesterol (TC)	180.21 ± 2.01	152.31 ± 0.02*	166.28 ± 0.01*	177.26 ± 0.01
Triglyceride (TG)	115.34 ± 1.50	98.15 ± 0.01*	84.55 ± 0.06*	110.34 ± 0.21
HDL-C	65.62 ± 1.18	54.26 ± 0.03*	57.73 ± 0.04	58.42 ± 0.04
LDL-C	110.15 ± 1.40	91.82 ± 0.02*	99.18 ± 0.03*	104.68 ± 0.01
VLDL-C	21.72 ± 0.52	19.62 ± 0.01	16.83 ± 0.05*	11.27 ± 0.06*

Values are expressed as mean ± SD, n = 50, * = significantly different from control ($p < 0.05$).

Table.3 Effect of 7 day storage at different temperatures on lipid profile of human male blood sample

Lipid Parameter	Control (fresh)	-4^0 C	-20^0 C	-70^0 C
Total Cholesterol (TC)	165.67 ± 0.51	152.11 ± 0.04	162.58 ± 0.04	165.51 ± 0.03
Triglyceride (TG)	120.17 ± 0.35	93.76 ± 0.03	80.26 ± 0.06	115.26 ± 0.02
HDL-C	59.63 ± 0.54	50.29 ± 0.02	48.78 ± 0.03	56.17 ± 0.06
LDL-C	101.72 ± 0.40	89.68 ± 0.05	91.29 ± 0.03	95.62 ± 0.04
VLDL-C	19.83 ± 0.56	14.91 ± 0.04	11.62 ± 0.02	10.74 ± 0.04

Values are expressed as mean ± SD, n = 50.

Table.4 Effect of 7 day storage at different temperatures on lipid profile of Human female blood sample

Lipid Parameter	Control (fresh)	-4^0 C	-20^0 C	-70^0 C
Total Cholesterol (TC)	180.21 ± 2.01	150.24 ± 0.04	160.28 ± 0.04	177.21 ± 0.31
Triglyceride (TG)	115.34 ± 1.50	92.16 ± 0.04	81.68 ± 0.06	108.18 ± 0.52
HDL-C	65.62 ± 1.18	53.18 ± 0.02	51.64 ± 0.03	57.11 ± 0.64
LDL-C	110.15 ± 1.40	86.61 ± 0.05	98.08 ± 0.03	103.22 ± 0.48
VLDL-C	21.72 ± 0.52	18.44 ± 0.05	14.29 ± 0.02	10.45 ± 0.43

Values are expressed as mean ± SD, n = 50.

The present study further revealed no statistically significant difference in the concentration of lipid analytes after 7 days storage at -4^0 C, -20^0 C and -70^0 C when compared to immediate freezing/thawing cycles of samples at -4^0 C, -20^0 C and -70^0 C. This finding is in tandem with the reports of Zivkovic *et al.,* (2009) and Heinz *et al.,* (1995). Storage for 1 week at -4^0 C, -20^0 C and -80^0 C according to Zivkovic *et al.,* had only minor effects on serum lipid composition, with 0 – 4% of metabolites affected in most lipid classes. Heinz *et al.,* reported stability of TC, TG and lipoproteins after 7 days storage in the refrigerator. Also in line with the present finding are the reports of Evans *et al.,* (1995), Franca *et al.,* (2018), Paltiel *et al.,* (2008), Kachhawa *et al.,* (2017), and Cuhadar *et al.,* (2013). According to Evans *et al.,* there is no significant difference in the stability of TC and TG after 10 days storage at -4^0 C and 3 months storage at -20^0 C. Conversely, studies conducted by Tiedink and Katan (1989), Ferrario (1999) and Maduka *et al.,* (2009) revealed changes in the stability of lipid analytes especially the lipoprotein fractions following freezer storage for 11 to 27 weeks, 14 days at -20^0 C and 51 days respectively.

In conclusion, this study revealed a statistically significant difference in the concentration of most lipid analytes when freeze-thawed at -4^0 C and -20^0 C and no significant difference when freeze-thawed at -70^0 C compared to control (fresh samples). It also showed no significant difference in the concentration of lipid analytes after 7 days storage at -4^0 C, -20^0 C and -70^0 C compared to samples immediately freeze-thawed at these temperatures. The study therefore indicated that -70^0 C is the optimum temperature for storage of lipid samples in order to maintain stability of the analytes. Also that maintenance of stability of lipid analytes is may not be time-dependent but temperature-dependent. We therefore recommend storage of lipid samples at -70^0 C (or between -70^0 C to -80^0 C) and the need for additional studies to embark on long-term storage of lipid samples to really ascertain the effect of extended duration in the maintenance of stability of lipid analytes.

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