Original Article

The effect of time, temperature and P-chloro-mercuriphenylsulfonic acid during serum storage on HDL$_t$-C and HDL$_3$-C concentration

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Abstract

Background: Accurate measurement of clinical laboratory parameters plays an essential role in the correct interpretation of clinical biochemistry abnormalities. The purpose of this study was evaluation the time and temperature effect on HDL$_t$-C, HDL$_2$-C and HDL$_3$-C stability during storage. **Materials and Methods:** 50 adult healthy subjects were participated. For the isolation of HDL$_t$-C, we used precipitation method and HDL$_t$-C data was analyzed by the Abell-Kendal cholesterol reference method. The remaining serum were dispensed into 12 sample tubes and divided into two groups. One of each group was stored upright at room temperature (approximately 25 ºC) while another at 4 ºC and the half of each group tubes were received p-chloro-mercuriphenylsulfonic acid (PCMPS). The stored serum aliquots from all temperature and time points were analyzed on 1, 2, 3 days post collection. **Results:** HDL$_t$-C concentration at the temperature of room in 24 hours is not changed significantly but over the time decreased (7.2% in 3 days). In addition of PCMPS inhibitor, the concentration is increased by 17.3% in 3 days. But in 4ºC, with or without PCMPS, there is no a significant change in the HDL$_t$-C concentration. HDL$_3$-C was found to be the most stable lipoprotein studied because of non-significant effect of storage time and temperature on it. **Conclusion:** The results suggest 4ºC as the ideal storage condition for the preservation of human serum samples for HDL$_t$-C assay. Also it is suggested that HDL concentration estimation should be performed in the first 24 hours of samples collection. PCMPS addition didn't affect HDL subtypes concentration in 4ºC.

Keywords: Cardiovascular disease, HDL, Storage Time, Storage Temperature, LCAT enzyme.

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Introduction

Cardiovascular diseases (CVDs) are one of the important cause of death in the world (1). CVDs are responsible for about 17.7 million deaths in 2015, representing 31% of all global deaths. Of these deaths, an estimated 7.4 million were due to coronary heart disease and 6.7 million were due to stroke. Also, the number of people, who die from CVDs, mainly from heart disease and stroke, will increase to reach 23.3 million by 2030 (2). About 50% of annual deaths are related to this group of diseases in Iran (3).

Among the variables involved in determining of the CVDs, Dyslipidemias characterized by disorders in the levels of circulating lipids, are important due to correlation with excessive caloric ingestion, with high rate of fat and total cholesterol (TC) and fraction cholesterol of low-density lipoprotein (LDL-C) (4). Diminished fraction of high density lipoprotein cholesterol (HDL-C), hypertension, smoking, diabetes and obesity are associated to advanced atherosclerosis lesions and a greater risk of clinical manifestations of

atherosclerotic disease (5). So nowadays, lipid profile is one of the most prescribed laboratory tests worldwide.

According to the particle size, HDL-C can be divided into 10 subtypes (HDL_1-HDL_{10}) using lipoprint HDL analyzer, 1-3 types were large particle types (HDL-L), 4-7 types were intermediate particle types (HDL-M), and 8-10 types were small particle types (HDL-S). HDL-L may be protected factor for atherosclerosis, whose decrease was closely related with the increase of risk of chronic heart disease (6). Other studies suggest high density lipoprotein sub fractions difference in relationship with atherosclerosis (7).

Despite advances in clinical chemistry testing, poor blood sample quality continues to impact laboratory operations and the quality of results (8).

Sol F. Green demonstrates that pre-analytical errors are a significant financial burden to total hospital costs. Pre-analytical errors not only increase redraw costs within the laboratory, but also impact on the time and resources required for follow-up or delays in patient care (9). How do we prevent or reduce these errors and conserve valuable resources?

Studies showed collection, storage and transport of samples as pre-analytical factors in quality of laboratory tests (10).

Although many investigations have been undertaken on the stability of proteins and lipids, but the results are widely divergent. According to the MONICA Manual, isolation of HDL and TC should preferably be done on fresh serum aliquots on the day of blood collection (11) while in the UK National Health Surveys, the samples were dispatched immediately after blood taking (12).

LCAT (Lecithin Cholesterol Acyltransferase) is an enzyme which converts FC (Free Cholesterol) to EC (Esterified Cholesterol) and HDLt-C to HDL_3-C. This enzyme plays a key role in the HDL metabolism (13).

Hence, the present study intends to evaluate the effect of storage time and temperature and p-chloro-mercuriphenylsulfonic acid (PCMPS) as LCAT enzyme inhibitor on the measured density of HDL_3-C and HDLt-C in the samples of human serum under various storage conditions.

Methods

Sample collection. 50 adult healthy persons (All men and with age between 45-55) were participated. All subjects had been fasting for 12 hrs. Blood samples (50 ml) were collected from antecubital vein using sterile needles (18 gauge) directly into clean dry sterile glass tubes without anticoagulant. Serum (20 ml) was harvested after 30 minutes following clot formation and by centrifugation for 10 minutes at 2000 rpm.

Isolation of HDL-C. HDLt-C measurements are performed after first removing of the other lipoproteins (VLDL-C and LDL-C) from the sampleS by heparin-Mn++ precipitation (14). The HDLt-C in the supernatant was analyzed by the Abell-Kendal cholesterol reference method (15). "HDL-C" was precipitated by addition of Dextran Sulfate solution. After thorough mixing, the sample was left at room temperature for 20 min, and then was centrifuged for 30 min at 4°C at 2700 rpm. An aliquot of the supernatant was used for cholesterol analysis (HDL_3-C). The difference between total HDL cholesterol and "HDL_3" cholesterol represented HDL_2-C (16).

Measurement. The remaining serum were dispensed into 12 sample tubes, closed tightly and divided into two groups. One of each group was stored upright at room temperature (approximately 25 ºC) while another was stored at 4 ºC and the half of each group tubes were received PCMPS (0.01 mg/dl). The stored serum aliquots from all temperature and time points were analyzed on 1, 2, 3 days' post collection.

Ethics. Informed consent was obtained from all human adult participants. The protocols of the current study were approved by ethics committee of Tehran University of Medical Sciences (No.1396.3408).

Statistical analysis. To test the significant differences in concentration between storage temperatures and to assess the significant trends over time at each temperature, the data was analyzed statistically using non-parametric Friedman test for repeated-measures to examine the influence of storage time. Finding the significant differences ($P < 0.05$), Wilcoxon signed-rank test was used for those groups. Post-hoc analysis was conducted with a Bonferroni

correction applied, resulting in a significance level set at P < 0.017 (0.05/3 = 0.0166). To determine the clinically significant variations the percentage relative bias from the baseline sample was calculated by the formula: [(Cn-C1)/C1)] × 100. (C1: the median result of the T_0 sample, Cn: the median result of the stored sample). Percentage relative bias for paired groups (T_0-T_1, T_0-T_2, T_0-T_3, etc.) was compared with the current analytical quality specifications for desirable bias taken from the Westgard QC (10), which was updated at 2014 by Ricos et al (17).

Results

The results of the present investigation at T_0 are presented in Table 1 along with the Intra-assay coefficient of variations determined at three levels for each analyte.

The statistical analysis of serum +/- PCMPS under time and temperature effects is presented in Table 2 and 3.

Time-course plots illustrate various stability situations are presented in Figure 1 and 2.

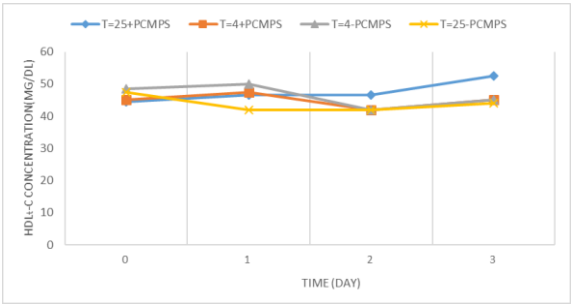

Figure 1. Time and temperature effects on the concentration of HDL_1-C (mg/dl).

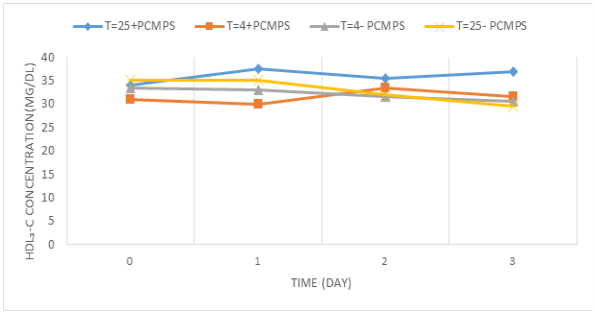

Figure 2. Time and temperature effects on the concentration of HDL_3-C (mg/dl).

Table1. Intra-assay coefficient of variations determined at three levels and T_0 (median and interquartile range) (mmol/L).

Analyte	CV1 (%)	Level1	CV2 (%)	Level2	CV3 (%)	Level3	Median (IQR)
HDL_1-C	3.3	1.89	4.6	1.62	5.3	0.84	1.29 (0.61-2.03)
HDL_3-C	4.6	0.68	6.2	1.26	6.8	1.57	0.9 (0.54-1.74)

Table2. The time and temperature effects on the concentration (mmol/L) of HDL_1-C in samples during their storage with and without PCMPS.

Temperature (ºC) / Time(day)	With PCMPS		Without PCMPS		Desired bias
	25	4	25	4	
0	1.15	1.16	1.25	1.22	5.6%
1	1.20 (4.3%) P=0.437	1.22 (5.2%) P=0.142	1.29 (3.2%) P=0.972	1.08 (-11.4%) P=0.505	
2	1.20 (4.3%) P=0.428	1.08 (- 6.9%) P=0.191	1.08 (-13.6%) P=0.385	1.08 (-11.4%) P=0.023	
3	1.35 (17.3%) P=0.862	1.16 (0.0%) P=0.278	1.16 (-7.2%) P=0.936	1.13 (-7.3%) P=0.750	

Table3. The time and temperature effects on the concentration (mg/dl) of HDL_3-C in samples during their storage with and without PCMPS.

Temperature (ºC) / Time(day)	With PCMPS		Without PCMPS		Desired bias
	25	4	25	4	
0	0.87	0.80	0.86	0.90	
1	0.96 (10.3%) P=0.806	0.77 (-3.7%) P=0.138	0.85 (-1.1%) P=0.372	0.90 (0.0%) P=0.495	
2	0.91 (5.7%) P=0.083	0.86 (7.5%) P=0.145	0.81 (-5.8%) P=0.224	0.82 (-8.8%) P=0.233	4.0%
3	0.95 (9.1%) P=0.593	0.81 (1.2%) P=0.249	0.78 (-10.4%) P=0.279	0.76 (-15.5%) P=0.589	

HDL_t-C and HDL_3-C concentrations decreased markedly beginning from the first day to second day of storage in the serum (by 10.4% for HDL_t-C and 4.7% for HDL_3-C just in one day). In addition of LCAT inhibitor (PCMPS), the concentration began to increase (10.3% for HDL_3-C for first day and 17.3% for HDL_t-C over 3 days), But in 4ºC, with or without PCMPS, there isn't significant change in the HDL_t-C and HDL_3-C concentration.

Discussion

Sample storage is one of the most effective factors in concentration of composite, especially lipid and lipoproteins which are metabolized fast and this fact influence laboratory results catastrophically. Therefore, we should pay attention to environmental factors and elements in blood that affect their metabolism. One of these factors in the blood is LCAT which is secreted into plasma by the liver and associates mostly with HDL-C, but also with LDL-C. It catalyzes the conversion of FC on the surface of lipoprotein particles to EC (Esterified Cholesterol). LCAT is also believed to be a key enzyme in the reverse cholesterol transport (RCT) pathway, because esterification of cholesterol on HDL-C increases the concentration gradient for the movement of FC from cells onto HDL-C (18). HDL-C metabolism implies a continuous transfer of phospholipids from VLDL-C and IDL-C particles. A part of EC is transferred to chylomicrons and VLDL-C by cholesteryl ester transfer protein (CETP) activity. The combined action of LCAT and CEPT result HDL_3-C changes into HDL_2-C (13). Briefly, this enzyme has been seen in blood samples influences the concentration of FC, EC, HDL_t-C and HDL_3-C so in this study, we used the inhibitor of LCAT(PCMPS) to evaluate how it influence HDL_t-C and HDL_3-C stability for probable use of this enzyme in sample storage.

HDL_t-C concentration at the temperature of room in 24 hours is not changed significantly but over the time decreased (7.2% in 3 days). Our observations were consistent with those of authors of similar studies (19,20,21).

We revealed in 4ºC, with or without PCMPS, there is no significant change in the HDL_t-C concentration. Heins M et al. showed HDL-C tubes at 4 ºC were stayed stable up to 36-h (22). Maduka Ignatius et al. reported non-significant changes up to 48-h in serum and significant change from day 2-10 at 4ºC (23). Heins et al. reported increases in HDL concentrations in plain serum, but found as stable at 9 ºC (22). According above results, there are discrepancies between the results of other studies and the present study, it might be explained by differences in analytic methods, by different storage temperatures and assay intervals.

Although studies about PCMPS in Vitro kinetics are limited, but there are literatures about enzyme deactivation in decreased or increased temperatures (24). Hence, we can explain stable concentration of HDL$_t$-C with deactivation of PCMPS as an inhibitor of destructive enzyme of HDL under the effect of temperature.

HDL$_3$-C was found to be the most stable studied lipoprotein because of non-significant effect of storage time and temperature on it. To compare with other studies Gao et al. emphasized that HDL$_2$-C are remodeled faster and thus are less stable than HDL$_3$-C. They evaluated the reason and found that "the smaller the particle, the higher the rupture temperature and the slower the protein unfolding" (25). Therefore, smaller spherical HDL-C are more stable than their larger competitors. This fact reported here is consistent with study by Pownall and colleagues of HDL disruption by streptococcal serum opacity factor that causes HDL –C fusion and dissociation of a fraction of apoA-I. They showed that smaller HDL-C undergo slower disruption and fusion than their larger counterparts (26).

Thus, increased propensity of HDL$_2$-C to fuse and rupture, together with increased affinity for HDL receptor (27) may contribute to preferential lipid uptake from these large particles over their smaller protein-rich counterparts (HDL$_3$-C) and cause reduced stability.

There are two main limitations that need to be acknowledged regarding the present study. Because of time consuming nature of precipitation methods for HDL isolation and material limitation, we repeated measurements 3 times which supposed to be not enough for statistical evaluation. Therefore, we suggest studies with more repeated data for the better statistical significance. It was difficult to select appropriate assay intervals, but we used previous studies in this way. We suggest studies with shorter assay intervals, because of the fast change of lipoproteins.

Conclusion

The results suggest 4ºC as the ideal storage condition for the preservation of human serum samples for HDL$_t$-C and HDL$_3$-C assay. Also it is suggested that HDL$_t$-C concentration estimation should be performed in the first day of samples collection. PCMPS addition didn't affect HDL$_t$-C and HDL$_3$-C concentration in 4ºC.

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Conflict of Interest

None.

Abbreviations

HDL-C: High density lipoprotein cholesterol

HDL-S: High density lipoprotein small particle cholesterol

HDL-M: High density lipoprotein intermediate particle cholesterol

HDL-L: High density lipoprotein large particle cholesterol

LCAT: Lecithin cholesterol acyltransferase

PCMPS: P-chloro-mercuriphenylsulfonic acid

TC: Total cholesterol

CVD: Cardiovascular diseases

CETP: Cholesteryl Ester Transfer Protein

VLDL: Very low density lipoprotein

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