Loss of High Density Lipoprotein Cholesterol in Serum: Effect of Storage

Panudda Musigavon, MSc*,
Tada Yipintsoi, MB, PhD**

* Department of Pathology, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla
** Department of Medicine, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla

The objectives of the study were to determine the magnitude and pattern of loss of detectable high density lipoprotein cholesterol (HDL-C) as functions of time and temperature of storage. Serum from 29 consented adults were estimated for HDL-C (utilising polyethylene glycol and alpha-cyclodextrin) when freshly obtained and after storage at 4°C, -20°C and -70°C at varied intervals up to a maximum of 120 days. The concentration of fresh HDL-C ranged from 16.9 to 87.2 mg/dl. After 5 days of storing at 4°C, an average of 5% of HDL-C could not be detected. After 120 days of storing at -20°C, 6-8 mg/dl of HDL-C was 'lost', this represented 10-20% of the concentration of the fresh samples. Storage at -70°C resulted in a 'loss' of 3%. The pattern of reduction of detectable HDL-C was such that the initial loss (first few days) was very rapid, reaching a nadir in 20 days. Examining individual serum samples, the loss ranged from minimal up to 30% of the original concentrations for all condition of storage. From further analysis of some portion of the data, there were suggestions that the concentration of triglyceride could be affect the loss of HDL-C with storage. Conclusion: Such rate of loss and heterogeneity of loss of detectable HDL-C, so far unexplained, may have implications in the management of epidemiological surveys involving storage of lipids in tropical countries.

Keywords: *PEG- modified homogeneous enzymatic assay, Storage of HDL-C*

J Med Assoc Thai 2005; 88 (10): 1388-94
Full text. e-Journal: *http://www.medassocthai.org/journal*

At present, health survey includes sampling of blood lipid. Often, whether the study is cross-sectional or longitudinal, the survey involves several geographical sites and by small groups of investigators, necessitating sampling of blood at different time periods. This, coupled with the importance of having a centralized laboratory to maintain quality control, compels investigators to store bloods prior to measurements of lipid. Not every sampling site will have the facility to store blood at a very low temperature, hence it is important to be certain that the lipid constituents remain independent of the conditions of storage.

Previous studies reported various effects of storage temperature on recovery of lipids in blood [this is well reviewed by Shih(1), Nanjee(2) enumerated possible related factors]. Storage at 4°C up to -70°C had minimal to no effect on recovery of total cholesterol (TC) and triglyceride (TG)(2-5), although Shih(1) reported that TC and TG decreased by 2.0 to 2.8% per year when stored at -70°C. For high density lipoprotein cholesterol (HDL-C) most reports confirmed a 'loss' of HDL-C and when specifically studied, the loss occurred in some of its constituents only(2,4-6). The rate of loss depended on both duration and temperature of storage. At storage temperature of 4°C, a 3% loss can be detected by the third day(6-8). The loss can be as high as 25% after 14 days. At storage temperature of minus 20°C, except for Stokes(3), others reported an HDL-C reduction of 10% after 1 month(4), and up to 13% at 1 year(6,9). However if stored at -70°C or under liquid nitrogen(2) no loss of HDL-C occurred(1,2,9), although a very early study by Bachorik(10) suggested a loss of 3% after 3 months at -70°C.

Correspondence to : Musigavon P, Department of Pathology, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand. Fax: 0-7421-2908, E-mail: panudda.m@psu.ac.th

From the literature review, it seemed that different results, some in opposite directions, of HDL-C loss may be related to different techniques of precipitation of other lipid constituents before measuring HDL-C. The reports did not suggest that HDL-C in plasma behaved differently from HDL-C in serum[2,10,11].

At present, the measurement of HDL-C does not need precipitation of other lipid constituents, hence it was thought worthwhile to evaluate the magnitude of loss of HDL-C at various duration and temperature of storage.

Material and Method

Twenty nine volunteers, 10 males 19 females with a mean age of 49.6 years (range 27-77) with previously known levels of serum lipids gave permission to have 15 ml of blood taken for the specific purpose of the present investigation. This allowed the authors to choose various levels of HDL-C to study. The blood was taken non-fasted. The drawn blood was left to clot by standing it for 30 minutes. The serum was separated by centrifugation at 3000 g for 15 minutes and then divided into multiple aliquots to be stored at 4°C,-20°C, and -70°C for intervals of 5 days or up to 120 days. Storage of serum was done immediately after separation. The speed of freezing was not measured.

HDL-C was assayed with Hitachi 917 automated analyzer (Roche Diagnostic, Thailand Ltd.) using homogeneous enzymatic colorimetric method[12]. This utilised polyethylene glycol-modified cholesterol esterase and cholesterol oxidase, sulfated alpha-cyclodextrin and magnesium sulphate (Roche Diagnostic, Thailand Ltd.).

The calibrator used was C.f.a.s lipids (catalogue number 1217 2763) from Roche Diagnostic, Thailand Ltd. Three levels of control serum were used to monitor the quality of the HDL-C assay. For the high concentration the authors used the Multiqual level 3 (catalogue number 473700 from Bio-Rad Laboratories Ltd), mean concentration 76.6 mg/dl, CV 2.34%, N = 38. For low concentration of HDL-C, the authors utilised the Precipath HDL-C (catalogue number 1778552 from Roche Diagnostic, Thailand Ltd.), mean concentration 29.0 mg/dl, CV 1.78%, N = 38. The third was the laboratory pooled serum, mean concentration 48.9 mg/dl, CV 1.7%, N = 38. These controls were kept at -70°C.

The fresh serum were analyzed with Hitachi 917 for total cholesterol (using enzymatic CHOD-PAP), the CV for low/high concentrations were 2.23/1.47%; for triglyceride (using enzymatic GPO-PAP), the CV for the low/high concentrations were 1.75/1.32%.

Data Management

The results in terms of concentration of HDL-C (mg/dl) as function of storage (temperature and duration) were examined individually and separated into 3 groups of low, medium and high concentrations of HDL-C [28.4 (n = 10), 56.8 (n = 10) and 83.0 (n = 9) mg/dl respectively]. In looking at "pattern or shape" of changes (i.e. whether the changes occurred at a constant or varying rate), the average percentage loss (with the standard deviation) for groups with low, medium and high concentrations as a function of time were plotted. Absolute changes as well as percentage changes were used to compare HDL-C loss among the groups with different concentrations. Paired t-test was used to denote significance. To appreciate the range of loss, percentage changes in individual sample were plotted as a function of their initial concentrations and linear regression fit was applied. In order to examine the effect of TG on loss of HDL-C with storage, regressions were performed on HDL-C loss (in per cent) against TG and LDL-C (using Friedewald's formula and excluded the one sample whose TG was greater than 400 mg/dl). These were done for each duration and temperature of storage.

Results

The table presents the average concentration and the magnitude of changes for the 3 concentration groups of HDL-C as functions of duration and temperature of storage. The average concentrations of the fresh TC, and TG for each group of HDL-C are given in the legend. As seen, significant loss (when compared to fresh samples) occurred at every storage temperature and at every duration of storage. At 4°C, 5-6% disappeared within 5 days for all the three concentrations. When stored at -20°C the average loss was 6-9% by 5 days and reached 10-20% by 120 days. Also at -20°C, the greatest percentage loss was in the group with the lowest concentration but the absolute loss appeared fix at about 6 to 8 mg/dl for the 3 groups. When the serum was stored at -70°C, only about 3% were not recovered by day 5 and not much more when stored for the full 120 days with an absolute loss of 1.4 to 1.8 mg/dl.

Fig. 1 illustrates the pattern of loss of detectable HDL-C as a function of time. As seen, the basic pattern is shaped like an "L" independent of the average concentration or temperature of storage, i.e., a very rapid fall within 5 days reaching a nadir by day 20. The magnitude of change, as already shown in the table, was most marked among those stored at -20°C.

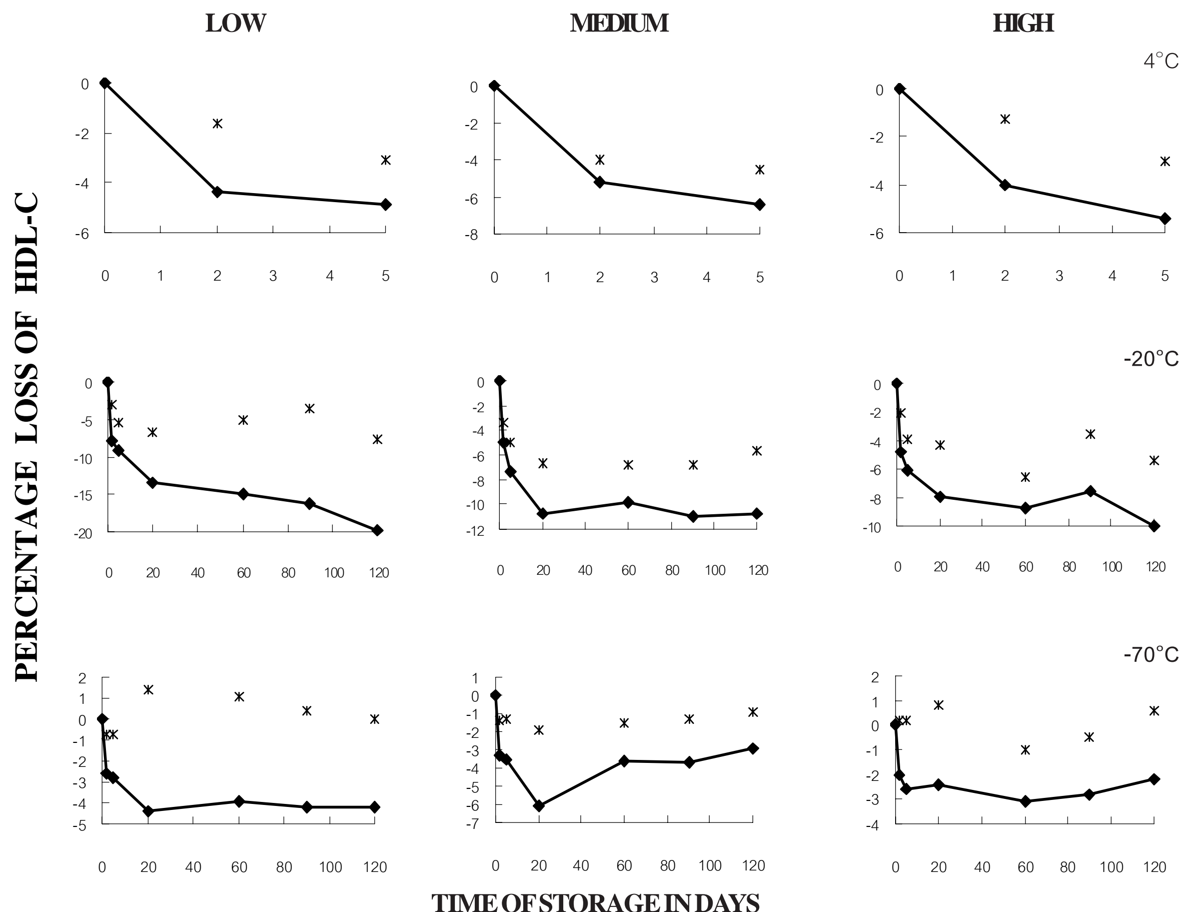

Fig. 1 Pattern of loss of HDL-C (in %) as functions of duration of storage (up to 120 days) for varied concentrations of HDL-C and storage temperatures (4°C, -20°C and -70°C). "LOW, MEDIUM and HIGH" represent HDL-C concentrations for 10, 10 and 9 samples respectively. The connecting symbols are the average percent loss and the asterisks represent an added one standard deviation. Significance of differences from the fresh samples (at time = 0) is expressed in the table

Fig. 2 illustrates the magnitude of percentage loss (only at the end of 5 and 120 days of storage) for individual samples as a function of its initial concentration. The range of loss was very wide. If included data not utilised in the figure, the maximum loss for each set (of storage condition) 4°C, -20°C, -70°C were: -10.4, -32.6 and -12.6%; and the average loss ($\pm$ SD) for the set with these minima were: -5.6 $\pm$ 2.1, -13.6 $\pm$ 9.1 and -3.6 $\pm$ 3.0. Only those samples stored at -20°C showed increasing loss with duration of storage (Fig. 2).

TG concentration affected a loss of HDL-C with storage even when adjusted for calculated LDL-C. These, however, could only be shown for samples stored at -20°C and only from day 20 onwards, while for -70°C, this was seen only from day 60 onwards.

Discussion

Using the present regimen of detecting HDL-C from human serum and despite the relatively small number of samples, 3 aspects of the present study will be stressed. In one, HDL-C loss reached a nadir soon after storage, the pattern is possibly independent of concentration of HDL-C and of the temperature of storage. The second is that the loss at 120 days was most marked when stored at –20°C and was minimal at –70°C. On day 5, the samples stored at –20°C appeared to loose as much if not more than samples stored at 4°C. Third, the percentage loss is not uniform in every sample. This could be near zero per cent and as high as 30%. These three features had not been directly addressed in previous studies, except in a report comparing 2 methods for determining HDL-C [13]. The L-shaped

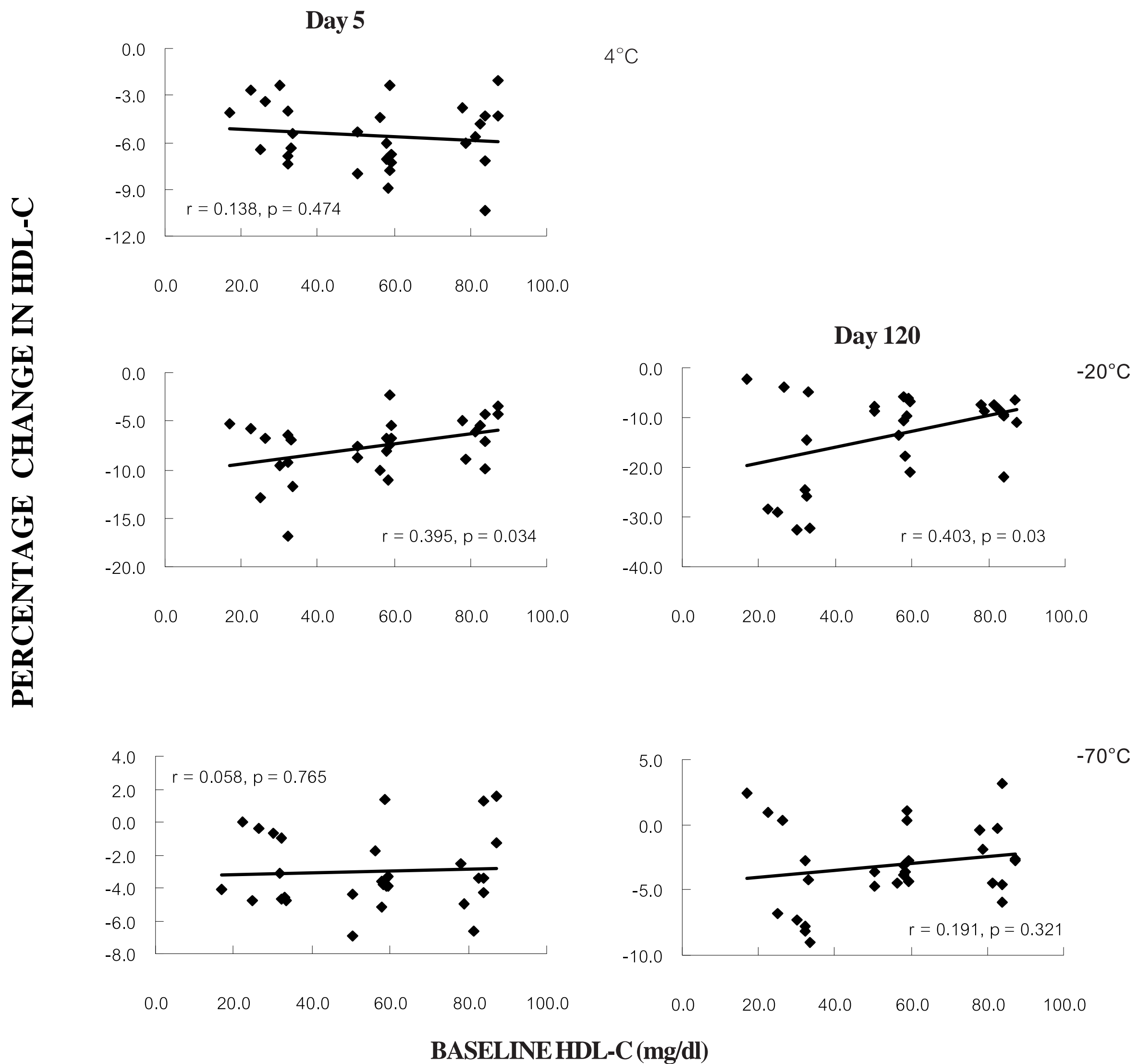

Fig. 2 Percentage loss of individual HDL-C sample as function of its initial concentration in mg/dl, shown only for the 5th day and the 120th day of storage and for the 3 temperatures studied. "r" and "p" describe the linear fit and its statistical significance. Note that significance is seen only for the -20°C storage

pattern, if proven, strongly suggests that the "loss" which is probably just the inability to detect total HDL-C occurred very early and that after a certain period, this phenomena ceased or was markedly decreased.

The varied magnitude of loss of HDL-C suggests that there may be constituents in the serum that protected or vice versa that encouraged non-detectability of HDL-C. Previous studies(2,4-6) reported a loss in total HDL-C and HDL_3-C fractions and suggested that the apparent loss was due to redistribution among the different lipoprotein fractions especially those related to triglyceride. This may explain the relationship between magnitude of loss of HDL-C to the baseline level of triglyceride (but not LDL-C) as reported by Bachorik 1982(10). Cohn et al(14) recently reported redistribution of lipoproteins between plasma and HDL-C, but this resulted in higher HDL apoC-III and apoE after freezing. This process was shown to be related to high TG. The present study was not designed to explore this relationship. However, the regressions of loss of HDL-C on TG (higher loss with higher concentration of TG) did support this possibility although seen only in certain samples particularly at -20°C.

Table 1. Effect of storage conditions on varied concentration of HDL-C

	4°C			-20°C			-70°C		
	L	M	H	L	M	H	L	M	H
D_0	28.4 (5.6)	56.8 (3.5)	83.0 (3.2)	28.4 (5.6)	56.8 (3.5)	83.0 (3.2)	28.4 (5.6)	56.8 (3.5)	83.0 (3.2)
D_2	27.0 (5.1)	53.8 (3.6)	79.7 (4.0)	26.1 (5.1)	54.0 (3.8)	79.0 (4.0)	27.6 (5.4)	54.9 (4.0)	81.3 (3.9)
D_{2-0}	- 1.3 (0.8)*	- 2.9 (0.7)*	- 3.3 (2.2)*	- 2.3 (1.5)*	- 2.8 (0.9)*	- 4.0 (2.2)*	- 0.7 (0.6)*	- 1.9 (1.0)*	- 1.7 (1.9)*
D_5	26.9 (5.1)	53.2 (3.5)	78.5 (3.9)	25.7 (4.8)	52.6 (3.9)	78.0 (4.1)	27.6 (5.3)	54.8 (4.1)	80.9 (4.8)
D_{5-0}	- 1.4 (0.7)*	- 3.6 (1.1)*	- 4.5 (2.0)*	- 2.7 (1.3)*	- 4.2 (1.4)*	- 5.0 (1.8)*	- 0.8 (0.6)*	- 2.0 (1.2)*	- 2.1 (2.3)*
% D_{5-0}	95.1 (1.8)	93.6 (1.9)	94.6 (2.4)	90.8 (3.7)	92.6 (2.4)	93.9 (2.2)	97.2 (2.1)	96.5 (2.2)	97.4 (2.8)
D_{20}				24.4 (4.3)	50.7 (4.6)	76.5 (4.8)	26.9 (4.5)	53.4 (4.9)	81.0 (5.2)
D_{20-0}				- 4.0 (2.3)*	- 6.0 (2.2)*	- 6.5 (3.0)*	- 1.4 (1.6)*	- 3.4 (2.3)*	- 2.0 (2.6)
D_{60}				23.9 (4.5)	51.2 (3.8)	75.8 (3.4)	27.1 (4.8)	54.8 (4.2)	80.4 (4.2)
D_{60-0}				- 4.5 (3.1)*	- 5.6 (1.8)*	- 7.2 (1.8)*	- 1.3 (1.6)*	- 2.0 (1.1)*	- 2.6 (1.7)*
D_{90}				23.6 (5.1)	50.5 (3.7)	76.8 (4.2)	27.0 (4.9)	54.7 (3.9)	80.7 (4.5)
D_{90-0}				- 4.8 (3.7)*	- 6.2 (2.5)*	- 6.2 (3.3)*	- 1.3 (1.7)*	- 2.1 (1.4)*	- 2.3 (1.9)*
D_{120}				22.6 (5.0)	50.6 (3.8)	74.6 (4.4)	27.0 (4.7)	55.2 (4.0)	81.2 (3.5)
D_{120-0}				- 5.8 (3.7)*	- 6.2 (3.1)*	- 8.4 (3.9)*	- 1.4 (1.3)*	- 1.6 (1.1)*	- 1.8 (2.3)*
% D_{120-0}				80.2 (12.2)	89.2 (5.1)	90.0 (4.6)	95.8 (4.2)	97.1 (2.0)	97.8 (2.8)

Concentrations in mg/dl are expressed as mean (SD). L, M, H = low, medium and high concentrations of HDL-C. D_n and D_{n-o} represent duration of storage in 'n' days and difference in concentration from day '0'. % D_{n-o} = the average of the % remaining after the specified duration of storage. Average concentrations [in mg/dl (SD)] of fresh total cholesterol (TC) and triglyceride (TG) for each of the HDL-C groups were: Low: TC 196.6 (93.7), TG 205.8 (125.7); Medium: TC 207.7 (39.8), TG 130.5 (46.9); High: TC 219.3 (56.3), TG 65.9 (22.1)

* = $p < 0.05$ by paired t-test

sera. Clin Chem 1986; 32: 995-9.

There are 2 deficiencies in the present study. In one, no evaluation was made on loss of TG or TC with storage. It was assumed not to be present from previous reports(2-5). The other was that only the Roche PEG-modified homogenous enzymatic assay was utilised. No attempt at comparison with other methods was made(13).

In conclusion the potential and appreciable rapid loss of detectable HDL-C in some (not all) serum at ordinary temperature and especially at -20°C suggested that one should alter the present method of managing blood/serum samples for HDL-C measurement. The sample should not be left too long before separating out the serum after blood collection and not delayed before transporting it to an acceptable storage site for storage or for estimation. This may be important in surveys in tropical countries. Since each sample can behave differently (range of loss can be minimal to as much as 20%), one cannot use a 'correction factor'. Finally, more in depth study should be done to try and determine what fractions of the HDL-C are lost from detection and what components in the serum exaggerate this alteration.

Acknowledgements

The study was funded by the Faculty of Medicine, Prince of Songkla University and the Thai Research Fund administered by the National Health Foundation. Ms S Panphon assisted in the preparation of the manuscript and illustrations. Ms A Lim helped in certain aspects of the statistic.

References

1. Shih WJ, Bachorik PS, Haga JA, Myers GL, Stein EA. Estimating the long-term effects of storage at -70°C on cholesterol, triglyceride, and HDL-cholesterol measurements in stored sera. Clin Chem 2000; 46: 351-64.
2. Nanjee MN, Miller NE. Evaluation of long-term frozen storage of plasma for measurement of high-density lipoprotein and its subfractions by precipitation. Clin Chem 1990; 36: 783-8.
3. Stokes YM, Salmond CE, Carpenter LM, Welby TJ. Stability of total cholesterol, high-density lipoprotein cholesterol, and triglycerides in frozen sera. Clin Chem 1986; 32: 995-9.
4. Evans K, Mitcheson J, Laker MF. Effects of storage at 4°C and -20°C on lipid, lipoprotein, and apolipoprotein concentrations. Clin Chem 1995; 41: 392-6.
5. Evans K, Mitcheson J, Laker MF. Effects of storage at -70°C on lipid, lipoprotein, and apolipoprotein concentrations. Clin Chim Acta 1997; 258: 219-29.
6. Bausserman LL, Saritelli AL, Milosavljevic D. High-density lipoprotein subfractions measured in stored serum. Clin Chem 1994; 40: 1713-16.
7. Khan SR, Elkeles RS. Effects of storage on the precipitation procedure for the measurement of HDL subclasses in serum. Clin Chim Acta 1984; 144: 59-63.
8. Kronenberg F, Lobentanz EM, Konig P, Utermann G, Dieplinger H. Effect of sample storage on the measurement of lipoprotein[a], apolipoproteins B and A-IV, total and high density lipoprotein cholesterol and triglycerides. J Lipid Res 1994; 35: 1318-28.
9. Demacker PN, Jansen AP. Stability of high-density lipoprotein cholesterol in frozen sera. Clin Chem 1983; 29: 567-8.
10. Bachorik PS, Walker RE, Kwiterovich PO Jr. Determination of high-density lipoprotein cholesterol in human plasma stored at -70°C. J Lipid Res 1982; 23: 1236-42.
11. Bachorik PS, Walker R, Brownell KD, Stunkard AJ, Kwiterovich PO. Determination of high-density lipoprotein cholesterol in stored human plasma. J Lipid Res 1980; 21: 608-16.
12. Sugiuchi H, Uji Y, Okabe H, Irie T, Uekama K, Kayahara N, et al. Direct measurement of high-density lipoprotein cholesterol in serum with polyethylene glycol - modified enzymes and sulfated α-cyclodextrin. Clin Chem 1995; 41: 717-23.
13. Arranz-Pena M, Tasende-Mata J, Martin-Gil FJ. Comparison of two homogeneous assays with a precipitation method and an ultracentrifugation method for the measurement of HDL-cholesterol. Clin Chem 1998; 44: 2499-505.
14. Cohn JS, Rodriguez C, Jacques H, Tremblay M, Davignon J. Storage of human plasma samples leads to alterations in the lipoprotein distribution of ApoC-III and ApoE. J Lipid Res 2004; 45: 1572-9.

การสูญเสีย เอช ดี แอล โคเลสเตอรอล ในซีรัม : ผลของการเก็บรักษา

ปนัดดา มุสิกวัณณ์, ธาดา ยิบอินซอย

ศึกษาความเปลี่ยนแปลงของระดับ เอช ดี แอล โคเลสเตอรอล เมื่อเก็บซีรัมที่อุณหภูมิและช่วงเวลาแตกต่างกันใช้ ซีรัมจากอาสาสมัคร 29 คน ที่มีค่า เอช ดี แอล โคเลสเตอรอล ระหว่าง 16.9-87.2 มก./ดล. วิเคราะห์ระดับ เอช ดี แอล โคเลสเตอรอล ทันทีหลังปั่นแยกซีรัมและหลังเก็บที่อุณหภูมิ 4°C, -20°C และ -70°C ตามช่วงเวลาต่าง ๆ จนครบ 120 วัน วิเคราะห์โดยวิธี PEG-modified enzyme.

ผลการศึกษาพบว่าระดับ เอช ดี แอล โคเลสเตอรอล ลดลงเฉลี่ยร้อยละ 5 หลังจากเก็บซีรัมที่ 4°C เป็นเวลา 5 วัน หลังจากเก็บซีรัมที่ -20°C เป็นเวลา 120 วัน ระดับ เอช ดี แอล โคเลสเตอรอล ลดลง ร้อยละ 10-20 (6-8 มก./ดล.) แต่ระดับนี้ลดลงเพียงร้อยละ 3 เมื่อเก็บรักษาไว้ที่ -70°C อัตราลดนี้ไม่สม่ำเสมอ ลดลงอย่างรวดเร็วในวันแรก ๆ และหลังจากเก็บรักษา 20 วัน ระดับจะเกือบคงที่ ถ้าพิจารณาอัตราลดแต่ละรายจะพบว่าบางรายแทบไม่ลด แต่บางรายลดลงร้อยละ 30 จากการวิเคราะห์บางส่วนของข้อมูลมีความเป็นไปได้ว่าความเข้มข้นของไตรกลีเซอไรด์ อาจทำให้ระดับ เอช ดี แอล โคเลสเตอรอล ลดลง โดยสรุป ถึงแม้ยังไม่สามารถอธิบายว่าทำไมแต่ละซีรัม ลดในปริมาณไม่เท่ากัน แต่การสูญ "หาย" ของ เอช ดี แอล โคเลสเตอรอลนี้มีผลในการศึกษาที่พึ่งการตรวจสอบไขมันชนิดนี้ โดยเฉพาะในภูมิประเทศเขตร้อน