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Very long-term stability of lipid biomarkers in human serum

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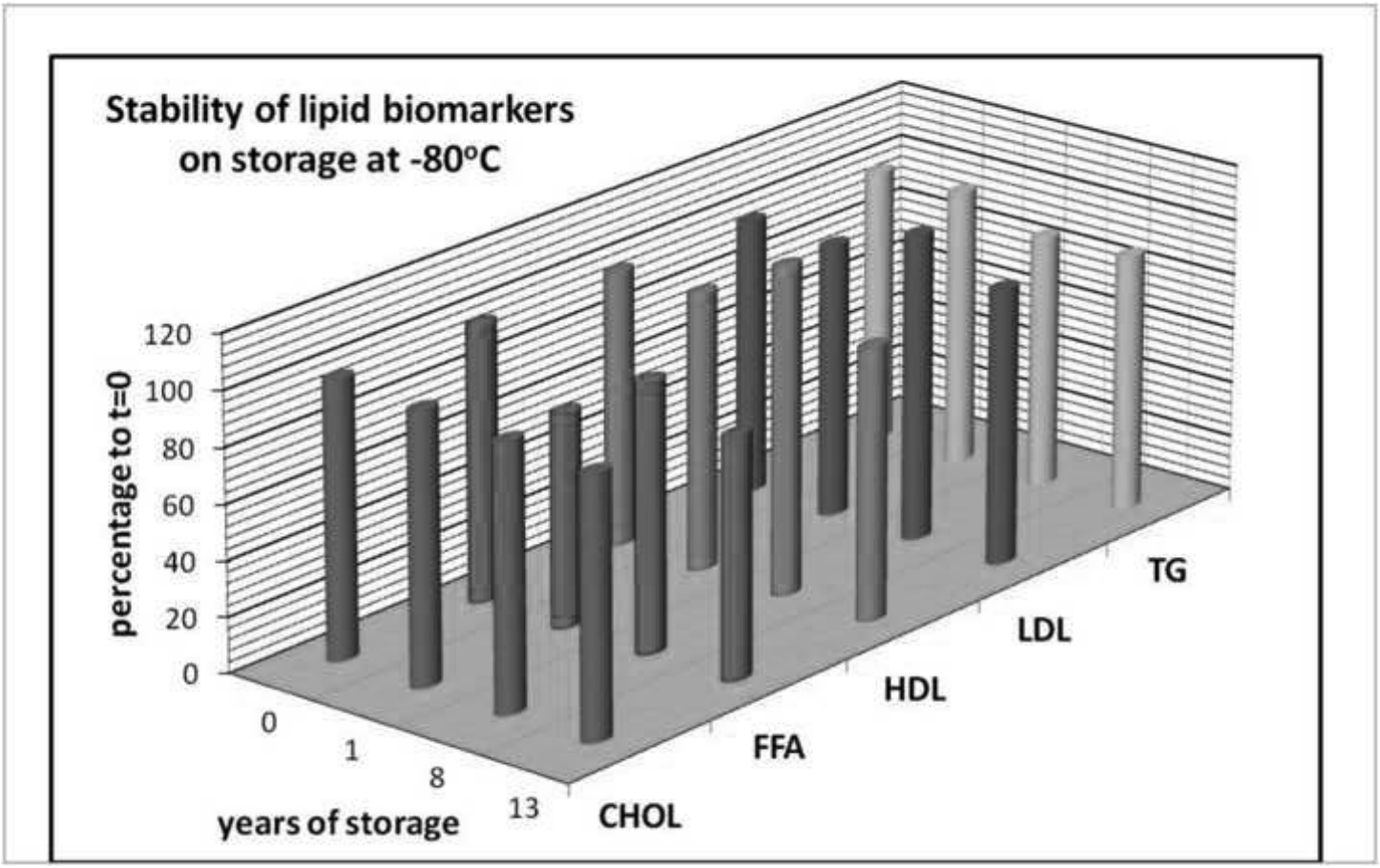

Full title: Very long-term stability of lipid biomarkers in human serum

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Abstract
The assessment of the stability of biomarkers is very important for epidemiological studies. In this study, the stability of five lipid parameters (total cholesterol, triglycerides, HDL- and LDL-cholesterol and free fatty acids) has been tested in 16 human serum samples after storage at -80 °C up at time points 0, 1, 8 and 13 y.
The majority of the lipid biomarkers were stable during storage conditions, except for cholesterol. The correlations between the samples were very good at time points.
Therefore, long-term storage of human serum samples allows lipid biomarker determination, provided that the samples are stored at -80 °C.

Key words: storage stability; human serum; lipid biomarkers.

List of Abbreviations:

CHOL	total cholesterol
TG	triglycerides
HDL-C	HDL-cholesterol
LDL-C	LDL-cholesterol
FFA	free fatty acids

1. Introduction

In both clinical and epidemiological studies, the stability of serum or plasma parameters during short- and long-term storage of human samples should be assessed, being of great importance. Especially, in long-term studies, such as prospective multi-centre studies, the stability of the measurement of certain biomarkers in stored samples should be checked before science-based conclusions can be drawn regarding relationship between health outcomes and biomarkers. These stability studies are part of the process of good clinical practice starting from blood withdrawal, processing to serum or plasma, and storage of the final samples. The time between blood withdrawal and processing, the time delay until storage, the final storage temperature and period until analysis of the samples, all contribute to the process of gathering a reliable set of data for statistical analysis [1,2,3].
In the present study, the storage stability of a number of lipid biomarkers, including total cholesterol (CHOL), triglycerides (TG), HDL- and LDL-cholesterol (HDL-C, resp. LDL-C) and free fatty acids (FFA) have been determined during storage at -80 °C up to 13 years.

2. Materials and Methods

For the stability study of TG, FFA, CHOL, HDL-C and LDL-C, human serum samples of 16 healthy human volunteers (blood donors, 8 males and 8 females with an age range of 20 to 60 y) were used. Samples were obtained from the Central Blood Laboratory of the Red Cross

(Amsterdam, the Netherlands) with written permission of the volunteers. The appropriate institutional approval of the review board was obtained and the principles outlined in the Declaration of Helsinki for human experimental investigations have been followed. At the day of blood withdrawal 2 x 8 mL blood was collected in serum tubes (Vacuette serum clot activation tubes, Greiner, Alphen aan den Rijn, the Netherlands). The serum samples were prepared by centrifugation (for 10 min at 3200 rpm) within two hours, divided in aliquots and stored at -70 ^{o}C.

Determination of lipid parameter concentrations

At the time points, the biomarkers have been determined in human serum. The measurements at day 0 and after 1 year were performed with an auto-analyzer (Hitachi 912, Roche Diagnostics, Almere, the Netherlands) using the kits supplied by Roche Diagnostics. The measurements of FFA were performed with the NEFA kit (Non-Esterified Fatty Acids) from Wako Chemicals (Neuss, Germany). The measurements were performed as a single measurement, except on day 0 (duplicate measurements). Each day, a number of quality control samples were included to exclude the potential effect of long-term assay drift as much as possible. The inter assay coefficients of variation were determined by daily measurements in duplicate using quality control samples (Precinorm L) obtained from Roche Diagnostics for CHOL, TG and HDL-C and LDL-C; AHSL-1 serum obtained from Randox (Crumlin, United Kingdom) for FFA. The inter assay CVs were 5.6% for CHOL at the level of 4.39 mmol/L, 7.5% for TG at the level of 1.40 mmol/L, 1.8% for HDL-C at the level of 1.20 mmol/L, 6.5% for LDL-C at the level of 2.43 mmol/L, 8.5% for FFA at the level of 1.28 mmol/L.

At time points 8 and 13 years, the measurements were performed on a clinical auto-analyzer LX-20 Pro from Beckman-Coulter (Woerden, The Netherlands). A comparative study showed no statistically significant shift in the levels of all biomarkers after a change in equipment. The assays were also obtained from Beckman-Coulter, except the FFA assay (NEFA kit from Wako Chemicals, Neuss, Germany). The QC data were obtained with Synchron reference sera (from Beckman-Coulter, Woerden, the Netherlands) at 3 levels in duplicate. CVs (%) were 0.93, 0.87, 2.05, 0.44 and 1.12 for CHOL, FFA, HDL-C, LDL-C and TG, respectively.

At time point 13 years, the measurements were performed with the same auto-analyzer and kits as for the 8 y time point. The QC data were obtained with Synchron reference sera at 3 with N=6. CVs (%) were 0.86, 2.36, 1.63, 1.03 and 3.33 for CHOL, FFA, HDL-C, LDL-C and TG, respectively.

For long-term stability, samples were stored for 1, 8 and 13 years at -70 ^{o}C for one year and at -80 ^{o}C for the remaining period. Samples stored at -70 ^{o}C were kept in a freezer equipped with temperature recorder and sound alarm. From 1 year onwards, the samples were stored at -80 ^{o}C in a freezer equipped with an automatic temperature registration system with an alarm function.

At all time points the statistical difference (95 % confidence interval) of the values from the initial value at t=0 were determined with paired two-samples t-test.

3. Results

The mean concentrations of CHOL, FFA, HDL-C, LDL-C and TG at the various time points are shown in Figure 1. The starting mean concentration was set to 100%. The mean CHOL concentrations showed lower values during the time course of this study, decreasing from 100% to 98.0, 95.8 and 93.8% at time points 1, 8 and 13 years, respectively. Only the mean value at 13 years is statistically significantly lower compared with the mean value at t=0 (P= 0.00051). The mean FFA concentrations changed from 100% to 77.1, 96.8 and 86.4% at time points 1, 8 and 13 years, respectively. The mean values at 1 and 13 years were statistically significantly lower compared with the mean value at t=0 (P= 6.3*E-11 and P= 9.6*E-5, respectively). The mean values of HDL-C changed from 100% to 100.6, 118.4, and 98.1% at time points 1, 8 and 13 years, respectively. The mean value after 8 years was statistically

significantly higher compared to the mean value at t=0 (P= 4.9*E-10). The mean value of LDL-C changed from 100% to 99.3, 111.3 and 99.9% at time points 1, 8 and 13 years, respectively. The mean value after 8 years was statistically significantly higher compared with the mean value at t=0 (P= 2.5*E-8). The mean value of TG changed from 100% to 100.6, 91.8 and 93.2% at time points 1, 8 and 13 years, respectively. The mean values at 8 and 13 years were statistically significantly lower compared to the mean value at t=0 (P= 3.2*E-6 and P= 0.0014, respectively).

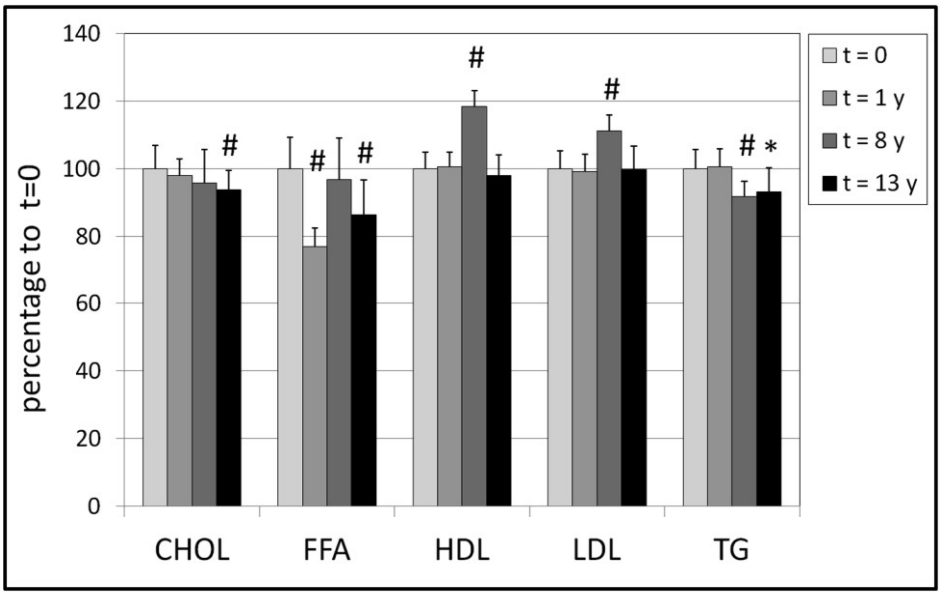

Fig. 1. Stability of CHOL, FFA, HDL-C, LDL-C and TG after storage for 1, 8 and 13 years at -80 °C. The concentrations have been expressed as percentage of the mean value of their concentrations at t=0. Statistically significant differences have been indicated in the figure. Statistics: * P<0.01; # P<0.001.

In addition, we checked whether there was a good correlation among the data on the various lipid biomarkers obtained after 1, 8 and 13 y. In Table 1, the characteristics of the correlation lines between the data of 1, 8 and 13 y and those of t=0 (date before storage) are shown for CHOL, FFA, HDL-C, LDL-C and TG, respectively.

Table 1: Summary of the slopes and correlation coefficients (R^2) of the correlation lines between t=0 and t=1, 8 and 13 years of storage at -80 °C.

	1 y	8 y	13 y	1 y	8 y	13 y
biomarker	slope	slope	slope	R^2	R^2	R^2
CHOL	0.754	0.288	0.711	0.674	0.084	0.850
HDL	0.858	1.230	0.685	0.924	0.949	0.862
LDL	0.869	1.015	0.850	0.742	0.824	0.601
FFA	0.907	1.040	1.115	0.941	0.811	0.868
TG	0.974	0.953	0.960	0.897	0.918	0.827

4. Discussion

The present paper is a follow-up of a study started in 2003, which presented the first results on storage stability after 1 year [4].

In the case of CHOL, there was a small, but steady decrease in the concentration levels. After 13 years, statistically significant decrease in concentration was observed (drop to 94%). The other lipids, FFA, HDL-C, LDL-C, and TG, did not show statistically significant, consistent trend

at all time points. The reason might be a change of auto-analyzer, although the QC parameters were correctly adjusted. Thus, we can conclude that all lipid biomarkers are stable upon long-term storage up to 13 years.

In the case of CHOL, the correlations between time t=0 and the other time points (1, 8 and 13 years) were rather low, probably because of the small concentration range. Especially the data at year 8 had rather bad correlation (R^2 = 0.08). Probably, the change of auto-analyzer has caused this discrepancy. However, the correlation coefficients (R^2) for the other lipid biomarkers (FFA, HDL-C, LDL-C and TG) between time t=0 and the other time points were generally between 0.81 and 0.97, except of LDL-C, where the correlations (R^2) were between 0.60 and 0.82.

Our results are in general agreement with the previously published studies. Apart of several short-term studies [5,6,7], only limited number of long-term studies on the storage stability of lipid parameters at various temperatures was published. The FFA composition of plasma lipid constituents was reported to be preserved for 4 years at -80 °C [8]. Another study showed an increase of the initial content of FFA after storage for 300 days at -15 °C [9]. Ito et al. [10] reported that in a single pooled serum sample, concentration of CHOL and TG decreased (to 90%) after 6 years of storage at -80 °C. In another study [11], all lipid parameters showed no decrease after storage for 6 years at -80 °C and -180 °C. Some parameters showed lower stability after storage at -20 °C and -40 °C. But experimental details of this study were not given. Kronenberg et al. [12] demonstrated stability of lipid parameters in 8 EDTA-plasma samples. Small increase was observed in the case of total and HDL-cholesterol at -20 °C and -80 °C, which was attributed to the freezing process.

A potential limitation of the present study is the fact that the serum samples originated from healthy volunteers. Serum samples from diseased participants were not included.

In conclusion, we have demonstrated that most of the lipid biomarkers are stable during long-term storage for 13 years at -80 ºC. The correlations among the samples at different time points remained very good. Therefore, long-term storage conditions of serum samples for lipid biomarker measurements are recommended to be -80 °C.

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Declaration of Competing Interests

The authors declare no conflicts of interest.

References

1. Lippi G, Chance JJ, Church S, Dazzi P, Fontana R, Giavarina D, et al. Preanalytical quality improvement: from dream to reality. Clin Chem Lab Med 2011;49:1113-26.
2. Zhang DJ, Elswick RK, Miller WG, Bailey JL. Effect of serum-clot contact time on clinical chemistry laboratory results. Clin Chem 1998;44:1325-33.
3. Drammeh BS, Schleicher RL, Pfeiffer CM, Jain RB, Zhang M, Nguyen PH. Effects of delayed sample processing and freezing on serum concentrations of selected nutritional indicators. Clin Chem 2008;54:1883-91.
4. Jansen EHJM, Beekhof PK, Schenk E. Long term stability of parameters of lipid metabolism in frozen human serum: triglycerides, free fatty acids, total-, HDL- and LDL-cholesterol, apolipoprotein-A1 and B. J Mol Biomark Diagn 2014;5:4.
5. Trichopoulou A, Kalaidzidou C, Kalandidi A. The relationship between plasma non-esterified fatty acids concentration and conditions of storage. Clin Chim Acta 1976;69:355-6.

6. Jackson C, Best N, Elliott P. UK Biobank Pilot Study: stability of haematological and clinical chemistry analytes. Int J Epidemiol 2008;37Suppl 1:i16-22.
7. Tanner M, Kent N, Smith B, Fletcher S, Lewer M. Stability of common biochemical analytes in serum gel tubes subjected to various storage temperatures and times pre-centrifugation. Ann Clin Biochem 2008;45:375-9.
8. Hodson L, Skeaff CM, Wallace AJ, Arribas GL. Stability of plasma and erythrocyte fatty acid composition during cold storage. Clin Chim Acta 2002;321:63-7.
9. Hron WT, Menahan LA. A sensitive method for the determination of free fatty acids in plasma. J Lipid Res 1981;22:377-81.
10. Ito Y, Nakachi K, Imai K, Hashimoto S, Watanabe Y, Inaba Y et al. Stability of frozen serum levels of Insulin-like growth factor-I, Insulin-like growth factor-II, Insulin-like growth factor binding protein-3, Transforming growth factor ß, soluble Fas, and superoxide dismutase activity for the JACC Study. J Epidemiol 2005;15:S67-73.
11. Elliott P, Peakman TC; UK Biobank. The UK Biobank sample handling and storage protocol for the collection, processing and archiving of human blood and urine. Int J Epidemiol 2008;37:234-44.
12. Kronenberg F, Lobentanz EM, König 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.