High-Density Lipoprotein Subfractions Measured in Stored Serum
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We compared the effects of freezing serum on the determination of high-density lipoprotein (HDL) subfractions by two dual-precipitation methods, heparin and manganese chloride/dextran sulfate (HM/DS) (Gidez et al., J Lipid Res 1982;23:1206–23) and DS/DS (Warnick et al., Clin Chem 1982;28:1574), and by ultracentrifugation. Storing serum for 1 month at −70°C resulted in reduced HDL3-cholesterol by ultracentrifugation and reduced total and HDL3-cholesterol by the DS/DS method. There was no change in either total HDL-cholesterol or HDL3-cholesterol with the HM/DS method. Additional studies involving only HM/DS indicated that total HDL-cholesterol in serum stored at 4°C begins to decline after 3 days (−3.1 ± 3.5%, P < 0.1). HDL was stable at −20°C for 2 weeks but both total and HDL3-cholesterol decreased significantly after 1 month. Storage of serum at −70°C resulted in no changes for 1 year; however, at 18 months, HDL3-cholesterol was reduced 13% (P = 0.002). We conclude that HDL subfractions can be determined accurately in stored serum as well as in plasma after storage at −70°C for up to 1 year.

Indexing Terms: cholesterol/sample handling

High-density lipoprotein (HDL)-cholesterol is now well established as a risk factor for coronary artery disease (CAD) (1, 2). Two major subpopulations of HDL, differing in density and composition, have been identified. HDL2, the less-dense subfraction, accounts for most of the variability in total HDL-cholesterol concentration (3). Some studies suggest that HDL2-cholesterol is a stronger marker for CAD than is total HDL (4, 5), whereas another (6) has demonstrated an association between HDL2-cholesterol and risk and found little advantage to measuring HDL subfractions. Nonetheless, the potential significance of HDL subfractions in assessing risk for CAD has generated considerable interest in large-scale studies of HDL subfraction distribution, for which accurate yet practical methods of measurement must be identified.

Isolation of HDL subfractions by ultracentrifugation of fresh plasma or serum remains the standard with which other methods are compared, but this technique is impractical for large epidemiological studies because it is expensive and time consuming. Electrophoretic techniques (7, 8) require only small volumes but are likewise labor-intensive. Double-precipitation methods (9–12) are popular because they are rapid, require no specialized techniques or equipment, use small volumes, and allow simultaneous analyses of large numbers of samples.

Another important consideration in selecting a method for large-scale studies is accuracy in stored as well as fresh samples. Nanjee and Miller (13), reviewing reports from several laboratories including their own on the effects of storage on HDL subfraction determination, concluded that storage of either plasma or sera at 4°C or −20°C was inadequate. However, total HDL and HDL3 remained stable in plasma stored as long as 1 year at −70°C (13). Because storage of plasma often leads to precipitation of fibrin, which requires clarification of samples by centrifugation before analysis, we prefer to store serum. We are aware of only two reports describing the effects of storage on analysis of HDL subfractions in serum. One found a significant decrease in both HDL-cholesterol and HDL3-cholesterol stored 90 days at −20°C (14), and the other reported an increase in HDL3-cholesterol after 4 days at −20°C (15). The effect of storage at −70°C on HDL subfractions in serum has not been described. Therefore we report here the effects of storage on serum HDL-cholesterol and HDL3-cholesterol determined by three methods.

Materials and Methods
Serum Samples
Serum was obtained from healthy volunteers, 26 men and 22 women, after a 12-h fast. Informed consent was obtained in accordance with guidelines of the Miriam Hospital Clinical Research Review Board. Mean lipid values for these subjects were cholesterol 5.43 ± 1.37 mmol/L; triglyceride 1.43 ± 0.81 mmol/L; HDL-cholesterol 1.29 ± 0.41 mmol/L; and low-density lipoprotein cholesterol 3.72 ± 1.16 mmol/L. Subsets of samples from this group were used for all studies except for the comparison of storage at −20 and −70°C, for which we used pooled sera from subjects with total cholesterol <6.46 mmol/L and triglycerides <2.82 mmol/L. The mean triglyceride concentration for each study is stated in the appropriate Results section.

Blood was allowed to clot for 20 min at room temperature and cells were removed. Serum was aliquoted into freezer-safe cryoules and immediately frozen for storage studies. Fresh samples were analyzed the same day.

HDL Determination
Ultracentrifugation. Total HDL-cholesterol was determined after precipitation of lower-density lipoproteins by using the Lipid Research Clinic (LRC) combined heparin/Mn2+ reagent containing 46 mmol/L Mn2+ (16).
Cholesterol was determined by an enzymatic method on an Impact 400 (Gilford, Oberlin, OH) computer-driven analyzer (17). Tris buffer was used instead of phosphate, to prevent interference by the heparin/Mn⁴⁺ reagent. For isolating subfractions, we treated 5.5 mL of serum with the LRC reagent, incubated this at room temperature for 10 min and centrifuged at 1500g to remove the precipitate. Part of the supernate (5 mL), adjusted to a density of 1.125 kg/L with KBr, was underlayered with 0.5 mL of KBr solution at 1.21 kg/L, and overlayered with KBr solution at 1.125 kg/L. Samples were centrifuged at 143 000g (min. avg.) in a Beckman (Beckman Instruments, Fullerton, CA) 40.3 rotor for 48 h. Supernatant and infranatant fractions were analyzed for cholesterol and corrected for dilution. Recoveries of HDL-cholesterol averaged 89.6% ± 5.6%.

Double precipitation. Two precipitation methods were compared. The first (DS/DS) used dextran sulfate (DS) and MgCl₂ as described by Warnick et al. (10, 11). Apolipoprotein B-containing lipoproteins were precipitated by 1.0 mL of serum by the addition of 0.1 mL of 10 g/L DS (Dextralip 50, M, 50 000; Sochibo, Boulogne, France) in 0.5 mol/L MgCl₂. After a 10-min incubation at room temperature, the precipitate was removed by centrifugation at 1500g for 30 min. HDL₂ was precipitated from 0.5 mL of the supernate by the addition of 0.05 mL of 10 g/L DS in 1.5 mol/L MgCl₂.

The second method, heparin and manganese chloride (HM/DS), was that of Gidez et al. (9). Serum (1.0 mL) was treated with 0.1 mL of combined HM reagent to give final concentrations of 1.26 g/L heparin and 91 mmol/L MnCl₂. Mixtures were incubated for 10 min at room temperature and the precipitates were pelleted by centrifugation at 1500g for 30 min. DS (M, 15 000; Sochibo), 1.43 g/L in 0.15 mol/L NaCl, was added to 0.5 mL of the supernate to give a final concentration of 1.3 g/L. The precipitate was removed by centrifugation as above after a 20-min incubation at room temperature. For both methods cholesterol determined in the supernates was corrected for dilution by reagent.

Quality Control

Serum pools prepared from combined sera with low (3.33 mmol/L), medium (5.87 mmol/L), and high (8.48 mmol/L) concentrations of total cholesterol were run twice in each assay. The same set point, based on total cholesterol, was used to calibrate the instrument for the duration of each study. Low (0.80 mol/L) and high (1.45 mol/L) HDL pools were also run as controls for the precipitation steps. Separate aliquots were stored at −70°C and the same pools were used for all studies for as long as 12 months. Assay CVs were: 2.8%, high total HDL; 5.6%, high HDL₃; 2.3%, low total HDL; and 4.3%, low HDL₂ by the HM/DS method.

Statistics

Paired t-tests were used for comparisons of methods. Repeated-measures analyses of variance (ANOVAs) were used for storage studies. The Bonferroni correction was applied to adjust for the number of comparisons.

Standard linear regression was used to compare frozen vs fresh samples and the change in HDL vs triglyceride.

Results and Discussion

Initially we compared the effect of freezing serum on HDL-cholesterol determined by all three methods (Fig. 1). We found differences in total HDL-cholesterol measured in fresh serum by the LRC precipitation method (16) and by the two methods used for subfraction determination. HDL-cholesterol was reduced 8.2% ± 3.5% (P <0.001, n = 19) by HM/DS and 14.9% ± 5.1% (P <0.001) by DS/DS. The difference in the LRC and HM/DS precipitation methods was not unexpected because the LRC precipitation method uses a lower concentration of Mn⁴⁺ (46 vs 91 mmol/L). The percentage change in measured values did not correlate with either HDL concentration (range 0.75–2.33 mmol/L, mean 1.34 ± 0.41 mmol/L) or triglyceride (range 0.56–3.52 mmol/L, mean 1.65 ± 0.46). Freezing (−70°C for 1 month) did not affect total HDL-cholesterol determined by the LRC method (mean change 1.1% ± 5.2%) or by HM/DS (mean change 0.5% ± 3.5%) but did reduce the total HDL-cholesterol determined by DS/DS by 8.3% ± 2.5% (n = 10, P <0.001). HDL₃-cholesterol determined by ultracentrifugation (mean change 7.5% ± 10%, P <0.05) and by DS/DS (mean change 7.9% ± 3.4%, P <0.001) was significantly reduced after freezing. HDL₃-cholesterol determined by HM/DS was unaffected (mean change 0.3% ± 5.5%) by freezing. Both increased (18) and decreased (19) HDL₃-cholesterol have been reported after freezing at −20°C for 76 days or at −70°C for 14 days, respectively, when DS was used as precipitant in both steps. Patsch et al. (19) also reported decreased total HDL-cholesterol analyzed by DS after freezing at −70°C. Because the HM/DS method yielded results for total HDL-cholesterol more closely approximating that obtained by the LRC precipitation method (r = 0.99, P <0.0001) and because results were not altered by freez-
ing \( (r = 0.99, P < 0.001) \), further studies were conducted
with the HM/DS method only.

Numerous studies involve refrigeration of samples for 1 to 5 days before analysis. Therefore we compared results for serum stored at 4 and \(-70^\circ\text{C}\) for as long as 1 month. As above, total HDL-cholesterol and HDL₃-cholesterol were unaffected by storage at \(-70^\circ\text{C}\) for 1 month (Fig. 2). However, both total HDL-cholesterol \( (P < 0.001, \) repeated-measures ANOVA) and HDL₃-cholesterol \( (P = 0.01) \) were decreased by storage at \( 4^\circ\text{C} \). The percent change in total HDL-cholesterol was statistically different from 0 by day 3 \( \text{(mean } -3.1\% \pm 3.5\%, P < 0.01) \). The reduction in HDL-cholesterol was related to neither the initial HDL concentration nor the triglyceride concentration \( \text{(mean } 0.88 \pm 0.44 \text{ mmol/L) at day 3 or at day 28.} \) The percentage change in HDL₃ was not significant during the first week of storage.

Comparison of samples stored at \(-20\) and \(-70^\circ\text{C}\) for as long as 6 months indicated significant decreases in both total HDL-cholesterol \( (P < 0.001) \) and HDL₃-cholesterol \( (P = 0.002) \) at \(-20^\circ\text{C}\) and no change at \(-70^\circ\text{C}\) (Table 1). Total HDL-cholesterol remained stable for as long as 18 months when stored at \(-70^\circ\text{C}\) (Fig. 3). HDL₃-cholesterol was stable at 12 months \( \text{(mean decrease} 1.1\% \pm 10\% \), but decreased by 13.4\% \pm 13.6\% \( (P = 0.003) \) by 18 months. The magnitude of the decrease was not related to the baseline value of either total HDL-cholesterol \( (r = -0.21) \) or HDL₃-cholesterol \( (r = -0.13) \) but was related to the serum triglyceride concentration \( (r = -0.64, P = 0.014, \text{mean triglyceride } 1.60 \pm 0.49 \text{ mmol/L).} \) Thus, greater decreases in HDL₃-cholesterol were observed in samples with increased triglycerides. Similarly, no change was observed in mean values of control pools stored 1 year at \(-70^\circ\text{C}\) \( \text{(total HDL-cholesterol} 1.31 \pm 0.03 \text{ vs } 1.33 \pm 0.04 \text{ mmol/L at 1 year; HDL₃-cholesterol} 0.74 \pm 0.04 \text{ vs } 0.73 \pm 0.06 \text{ mmol/L; } n = 25; \) however, the standard deviations for both measures appeared to increase.

Our results contrast with those of Gidez et al. \( (9) \), who found no change in plasma HDL-cholesterol or HDL₃-cholesterol stored at \(-20^\circ\text{C}\) for 1 month. Studies with serum stored at \(-20^\circ\text{C}\) have found that HDL₃-cholesterol decreased when HM/DS was used \( (14) \) and increased after precipitation with polyethylene glycol and DS \( (15) \). Similarly, plasma HDL₃-cholesterol increased

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**Table 1.** Effect of storage at \(-20\) and \(-70^\circ\text{C}\) on serum HDL subfraction determination by dual precipitation with HM/DS.

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<thead>
<tr>
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<th>Total HDL-cholesterol</th>
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<th>HDL₃-cholesterol</th>
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<tbody>
<tr>
<td></td>
<td>(-20^\circ\text{C})</td>
<td>(-70^\circ\text{C})</td>
<td>(-20^\circ\text{C})</td>
</tr>
<tr>
<td>mmol/L</td>
<td>%</td>
<td>mmol/L</td>
<td>%</td>
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<tr>
<td>Baseline</td>
<td>1.24 ± 0.44(^a)</td>
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<td>1.24 ± 0.44</td>
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<tr>
<td>2 weeks</td>
<td>1.22 ± 0.45</td>
<td>98 ± 3</td>
<td>1.24 ± 0.43</td>
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<tr>
<td>1 month</td>
<td>1.19 ± 0.42</td>
<td>96 ± 2(^a)</td>
<td>1.24 ± 0.43</td>
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<tr>
<td>6 months</td>
<td>1.09 ± 0.38</td>
<td>88 ± 2(^a)</td>
<td>1.24 ± 0.45</td>
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<tr>
<td>Overall change</td>
<td>(^c) P &lt; 0.001</td>
<td>NS</td>
<td>(^c) NS</td>
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\(^a\) Mean ± SD.

\(^b\) Significantly different from baseline, \( P < 0.02. \)

\(^c\) By repeated-measures ANOVA; \( n = 12. \)

NS, not significant.
after 1 week at −20°C with DS/DS (18). Two studies involving heparin/Mn2+ have reported decreases in total HDL-cholesterol stored in plasma at −70°C (19, 20). In contrast, Curb et al. found an increase in HDL-cholesterol in serum stored for 4 weeks at −60°C (21). Only two studies have reported the effects of storage at −70°C on HDL3-cholesterol, and both analyzed plasma. One found decreases in both total HDL-cholesterol and HDL3-cholesterol with DS/DS after 2 weeks of storage at −70°C (19). The other involved HM/DS and found no change after 1 year (13). Our results with serum are consistent with both of these studies. Thus, HDL3-cholesterol decreased after storage at −70°C when the DS/DS method was used but no changes were found with HM/DS for up to 1 year.

In conclusion, serum appears to be stable for 1 year at −70°C for analyses of both total HDL-cholesterol and HDL subfraction cholesterol when the HM/DS method is used. Moreover, it appears to be advantageous to freeze samples immediately rather than holding them at 4°C if they cannot be analyzed within 24 h after they are drawn.

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References