Precipitation with Polyethylene Glycol and Density-Gradient Ultracentrifugation Compared for Determining High-Density Lipoprotein Subclasses HDL2 and HDL3

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The purpose of this study was to compare quantification of cholesterol in high-density lipoprotein subfractions HDL2 and HDL3 by precipitation with polyethylene glycol (PEG) with that by density-gradient ultracentrifugation. Fresh serum samples from 32 fasting, obese children were analyzed with precipitation reagent “Quantolip” (Immuno AG), and then fractionated with a Beckman TL 100 ultracentrifuge with a swinging-bucket rotor. After centrifugation we carefully removed the supernatant with a syringe and measured the cholesterol from each fraction enzymatically with CHOD-PAP reagent (Boehringer Mannheim). The low-density lipoprotein (LDL), HDL2, and HDL3-cholesterol values measured by ultracentrifugation did not differ significantly from those obtained by precipitation; the correlation coefficients (r) between the two methods were 0.96 for LDL, 0.75 for HDL2, and 0.96 for HDL3. The relatively simple PEG precipitation method used in this study measures total HDL and its major subclasses HDL2 and HDL3 with accuracy and precision comparable with those of the well-established ultracentrifugation method.

The high-density lipoproteins (HD) of human plasma are a heterogeneous group of particles of differing composition and size within the density range 1.063–1.21 kg/L. On the basis of flotation rate, two major populations of particles have been identified, HDL2 and HDL3 (1), corresponding to densities of 1.063–1.125 and 1.125–1.21 kg/L, respectively. The lower density of HDL2 reflects a greater lipid/protein ratio; other differences include a greater relative content of apoprotein C and a lower content of apoprotein A-II in HDL2 than in HDL3 (2).

It is well documented that the incidence of coronary heart disease in Western countries is negatively correlated with the plasma total HDL-cholesterol (HDL-C) concentration (3). This relationship also may reflect an underlying association between atherosclerosis and the concentration of HDL2 especially (4).

The pathophysiological connection between a high risk for atherosclerotic diseases and low plasma HDL concentration is poorly understood. HDL has a close relationship to triglyceride metabolism, the HDL being created to a great extent during the enzymatic hydrolysis of triglyceride-rich lipoproteins, mainly very-low-density lipoprotein (VLDL), by lipoprotein lipase.

In addition, the hydrolysis of VLDL gives rise mainly to the HDL subfraction HDL2, a lipoprotein subclass considered to be potentially a better risk indicator for atherosclerosis than total HDL. The well-established method for separating and quantifying HDL and its subclasses is ultracentrifugation (5–9), but this laborious and expensive technique is hardly suited for the routine clinical laboratory. Consequently, researchers have developed faster, less-expensive, and automated precipitation methods (10–13).

The purpose of this study was to compare the results of quantifying HDL2- and HDL3-C by precipitation with polyethylene glycol (PEG) with those by density-gradient ultracentrifugation.

Materials and Methods

Blood was sampled from 32 fasting, obese outpatients, ages 10.2–16.1 years. Their triglyceride concentrations ranged from 0.96 to 5.1 mmol/L and total cholesterol from 2.8 to 6.7 mmol/L. We determined LDL-C, HDL2-C, and HDL3-C in aliquots by precipitation with PEG. Samples were stored at –20 °C for no longer than six weeks before lipoprotein fractions were measured by ultracentrifugation with the Beckman TL 100 ultracentrifuge with a swinging-bucket rotor. The method is based on a difference in flotation rate of the HDL subclasses.

Preparation of Lipoprotein Fractionation by Ultracentrifugation

Measure 1-mL aliquots of serum into 2-mL (11 × 34 mm) polyallomer centrifugation tubes. Layer 1 mL of 1.006 kg/L KBr solution over the surface. Centrifuge the tubes and their contents in the Beckman TL S55 swinging-bucket rotor for 18 h (10 000 × g, 10 °C). This is Spin I, during which the fraction defined as VLDL is floated to the top of the tube.
Using a syringe and a 0.50 × 23 mm needle, remove the upper 1 mL of sample from the centrifuge tubes. Transfer the bottom fractions into clean tubes, layer 1 mL of 1.063 kg/L KBr solution over the surface, and re-centrifuge (24 h, 10 000 × g, 10 °C). This is Spin II, and the top 1 mL contains the LDL fraction. Repeat these procedures for each HDL subfraction, HDL2 and HDL3, centrifuging for 48 h at 10 000 × g and 10 °C (Spins III and IV), using KBr solutions of 1.125 and 1.21 kg/L, respectively.

To adjust the density for the serum samples, use solid KBr, according to the following calculation (14):

\[
x = \frac{V_i (d_f - d_i)}{1 - V d_f}
\]

in which \(x\) is the grams of solid KBr to be added, \(V_i\) is the initial volume of the plasma, \(d_f\) is the final density after adjustment, \(d_i\) is the initial density, and \(V\) is the partial specific volume of KBr.

For example, to increase the density of 1 mL of serum from 1.006 to 1.063 kg/L with solid KBr; add

\[
\frac{1.0(1.063 - 1.006)}{1 - 0.298(1.063)} = 0.0834 \text{ mg of KBr}
\]

After centrifugation, measure the cholesterol content of each fraction (we used the enzymatic CHOD-PAP reagent from Boehringer Mannheim, Mannheim, F.R.G.).

Precipitation of HDL Subfractions

To precipitate HDL subfractions, we used a commercially available kit ("Quantolip"; Immuno Diagnostics, Vienna, Austria). The test kit consists of two precipitation solutions, A and B, with the following compositions: solution A—PEG 20 000, 95 g/L in 0.1 mol/L sodium phosphate buffer, pH 6.5; solution B—PEG 20 000, 150 g/L in 0.1 mol/L sodium phosphate buffer, pH 7.5. The addition of PEG solutions of different concentrations and pH values selectively precipitates the different lipoprotein fractions.

We mixed 100-μL aliquots of sample with 200 μL of solution A or solution B; after 10 min at room temperature, we centrifuged the reactants at 400 × g for 15 min. The cholesterol contents of 100 μL of the supernates were determined by mixing with 1 mL of the CHOD-PAP reagent.

LDL-C was measured according to the Lipid Research Clinica procedure (15), wherein samples are reprecipitated after the triglyceride-rich lipoprotein fraction <1.006 kg/L is removed.

For statistical analysis of the results we used Student's t-test and standard linear regression.

Results

To evaluate the potential usefulness of the stepwise precipitation procedure for measuring cholesterol in HDL subfractions, we compared the results with the measured cholesterol concentrations of HDL2 and HDL3 isolated by density-gradient ultracentrifugation. Table 1 summarizes the lipid values for 32 plasma specimens in this comparative study. The concentrations determined after ultracentrifugation were not significantly different from those measured after precipitation.

To determine the accuracy of the procedure, we compared the concentrations of HDL2-C and HDL3-C determined in 32 human sera by both methods. The HDL2 and HDL3 results mean (SD) were in acceptable agreement—0.48(0.18) vs 0.51(0.20) and 0.69(0.28) vs 0.77(0.27) mmol/L, respectively—and the biases were <0.1 mmol/L for both subclasses (16). Precision studies for HDL2 and HDL3 determinations in pooled human sera gave within-run and run-to-run CVs <5% (Table 2) except for HDL2-C (run-to-run CV = 8.3%), correlating well with previous reports (17-19).

Discussion

The possible role of HDL as a risk indicator of atherosclerosis, and especially the suggested roles of the subfractions HDL2 and HDL3, make it necessary to develop a technique for measuring these fractions suitable for large-scale use. Several methods for separating HDL2 and HDL3 have been presented (8, 20–24). However, these methods are often complicated, time-consuming, or inaccurate. Patash et al. (8) used rate-zonal ultracentrifugation to separate lipoproteins; however, that technique can fractionate only one sample at a time, and as much as 20 mL of serum must be applied if all density classes are to be separated. The usefulness of their method is limited to particular problems. Gambert et al. (20) concluded that the two main HDL subfractions separated by gel electrophoresis are not identical to those separated by ultracentrifugation.

The precipitation methods described so far (17, 20) suffer from several drawbacks: the method described by Gidez and Miller (20), involving heparin-Mn²⁺, lacks specificity; takes two steps, which increases the chance of error and presents difficulties for full automation; gives values that are too high in comparison with the reference method (ultracentrifugation); and cannot be applied to hyperlipemic samples.

In this report the density-gradient ultracentrifugation technique, which takes only 1 mL of serum sample, allowed us to compare the cholesterol concentration of

| Table 1. Comparison of LDL-C, HDL2-C, and HDL3-C Values Obtained by PEG Precipitation and by Density-Gradient Ultracentrifugation (UC) for 32 Sera |
|---|---|---|---|
| | Mean (SD), mmol/L | U C | P E G | r | S E |
| LDL-C | 3.0 (0.56) | 2.90 (0.72) | 0.96 | 0.05 |
| HDL2-C | 0.48 (0.18) | 0.51 (0.20) | 0.75 | 0.11 |
| HDL3-C | 0.69 (0.28) | 0.77 (0.27) | 0.84 | 0.10 |

SE, standard error of the estimate.

* Each significantly correlated at P <0.001.
Table 2. Precision of Cholesterol Measurement in Pooled Sera by Density Ultracentrifugation

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Mean (SD), mmol/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Within-run</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL-C</td>
<td>4</td>
<td>3.54 (0.11)</td>
<td>3.0</td>
</tr>
<tr>
<td>HDL2-C</td>
<td>3</td>
<td>0.66 (0.03)</td>
<td>4.5</td>
</tr>
<tr>
<td>HDL3-C</td>
<td>3</td>
<td>1.0 (0.03)</td>
<td>3.0</td>
</tr>
<tr>
<td><strong>Run-to-run</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL-C</td>
<td>8</td>
<td>3.45 (0.12)</td>
<td>3.6</td>
</tr>
<tr>
<td>HDL2-C</td>
<td>6</td>
<td>0.61 (0.04)</td>
<td>8.3</td>
</tr>
<tr>
<td>HDL3-C</td>
<td>6</td>
<td>1.0 (0.03)</td>
<td>4.3</td>
</tr>
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both HDL2 and HDL3 subfractions, directly isolated from serum, with the measured HDL fraction and the derived HDL2 subfraction obtained with the precipitation technique, even though the methods are based on different principles, i.e., charge/particle size in the precipitation method and density/particle size in the density gradient ultracentrifugation. For flotation of lipoprotein fractions, calculations show that 24 h of centrifugation is sufficient for quantitative recovery of all LDL (1.019–1.063 kg/L). In addition, within 48 h all HDL, HDL2 as well as HDL3, can migrate to the top of the 1.125–1.21 kg/L gradient; furthermore, the separation of the HDL subfractions is critically determined by the shape of the rotor (9, 11).

In summary, the simple PEG precipitation method used in this study may allow precise and direct assessment of the cholesterol content of all the fractions possibly involved in the development of atherosclerosis: LDL, which is accepted to be one of the principal atherogenic factors in serum, and VLDL, which, according to some investigators, also has a positive correlation with atherosclerosis. Furthermore, this method provides a simple technique for the determination of HDL and HDL subclasses, which are of increasing clinical importance because of their strong negative correlation with coronary heart diseases. Finally, a systematic use of this method is likely to provide a better understanding of serum lipoprotein distribution in normolipoproteinemic and dyslipoproteinemic subjects as a function of time, drug therapy, and dietary manipulations.

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References