Use of a Dual-Precipitation Procedure for Measuring High-Density Lipoprotein 3 (HDL₃) in Normolipidemic Serum

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We compared results by a dual-precipitation method (J Lipid Res 1982;23:1206–23) for measuring high-density lipoprotein 3 (HDL₃) cholesterol with those by ultracentrifugation at d 1.125, using 56 fresh and 105 frozen-stored serum samples. For both methods, HDL₂-cholesterol was calculated as the difference between total HDL-cholesterol and HDL₃-cholesterol. In general, for pooled serum samples, agreement was closest with ultracentrifugation when we used a dextran sulfate concentration of 5.0 mg/L to precipitate the HDL₂-rich fraction, although the optimal concentration varied from 3.0 to 6.8 mg/L for different pools. For individual samples, the values for HDL₃ by dual precipitation averaged 12.8% lower than by ultracentrifugation. The coefficients of correlation between the two methods were HDL₃, r = 0.70; and HDL₂, r = 0.92. The dual-precipitation method reflected the expected sex-related differences in HDL₂-cholesterol concentration and inverse relationship with triglyceride concentration.

Additional Keyphrases: ultracentrifugation compared · sample handling

The well-known (1) inverse correlation between concentrations of high-density lipoprotein cholesterol (HDL-cholesterol) in plasma and coronary heart disease is attributable primarily to variations in HDL₃, the less dense of the two major subfractions of HDL, although HDL₂ may also be inversely related to coronary disease (2, 3). Before HDL₂-cholesterol can be measured, HDL must first be isolated from other plasma lipoproteins and HDL₃ separated from HDL₃. The latter separation is most reliably accomplished by ultracentrifugation at a density of 1.25 kg/L, at which density HDL₃ (d 1.063–1.25 d) accumulates as a floating layer and HDL₂ (d 1.125–1.21) sediments. Ultracentrifugation is too cumbersome for most purposes, however, and several methods have appeared within the past few years by which HDL₂ and HDL₃ can be measured by precipitation with polyanions (4–6). In these methods apolipoprotein B (apoB)-containing lipoproteins are first removed by precipitation with a polyanion divalent cation mixture, and total HDL-cholesterol is measured in the supernatant fluid. A separate aliquot of the supernate is treated with a second polyanion precipitant, which precipitates HDL₂ selectively, and HDL₃-cholesterol is then measured in the second supernate. HDL₃-cholesterol is calculated as the difference between total HDL-cholesterol and HDL₂-cholesterol.

In one of these methods, the apoB-containing lipoproteins are precipitated with heparin sulfate and MnCl₂, and HDL₃ is then precipitated with dextran sulfate (4). In another method, the apoB-containing lipoproteins are removed with dextran sulfate and MgCl₂, and a higher concentration of dextran sulfate is used to precipitate HDL₂ (5). Still another method is based on the use of polyethylene glycol (6). None of these methods precipitates HDL₂ specifically, but results from all reportedly agree reasonably well with those by ultracentrifugation methods.

We have compared one of the dual-precipitation methods (4) with ultracentrifugation, and we also examined a modification by which apoB-containing lipoproteins and HDL₂ are precipitated in a single step. There was a relatively high correlation for HDL₂ between the dual-precipitation and ultracentrifugation methods and a negative bias of about 13% for HDL₃.

Materials and Methods

Samples. We used both fresh and frozen-stored serum samples for this study. The fresh specimens consisted of blood from 28 male and 28 female patients of our Lipid Referral Clinic, collected during their regularly scheduled visits after 12 h of fasting. After the blood clotted (room temperature, 45 min), we transferred the serum to storage vials, which we sealed and stored at 4 °C until analysis. The frozen specimens consisted of 105 samples that had been drawn from fasting or nonfasting subjects and stored for several days at −20 °C, then packed on solid CO₂ and shipped to the laboratory, where they were stored at −70 °C for about two weeks until analysis.

Cholesterol, triglyceride, and total HDL-cholesterol analyses. We measured cholesterol and triglycerides enzymatically, using commercially available reagents (cholesterol, CHOL-PAP, cat. no. 704121, Boehringer-Mannheim Diagnostics, Indianapolis, IN; Triglyceride Reagent, cat. no. 6097-05, Abbott Laboratories, Abbott Park, IL). We measured total HDL-cholesterol in the clear supernate after removing apoB-containing lipoproteins by precipitation with heparin sulfate and MnCl₂ in final concentrations of 1.3 g/L and 46 mmol/L, respectively (7).

HDL₃ analysis. We prepared the HDL₃-containing fraction by (a) ultracentrifugation; (b) dual precipitation as described by Gidez et al. (4), in which we added the precipitants sequentially; and (c) a modification of this procedure, in which we added the precipitants simultaneously (8).

Ultracentrifugation. We treated a 5-mL aliquot of serum with heparin and MnCl₂ as described above. After sedimenting the precipitate (30 min, 1500 × g), we adjusted the density of a 5-mL aliquot of the HDL-containing supernate to 1.25 kg/L with NaCl and KBr, then centrifuged the sample at 105 000 × g for 40 h at 10 °C. HDL₃ accumulates as a floating layer; HDL₂ remained in the infranatant layer. We quantitatively measured the cholesterol content of the infranate.

Dual precipitation. We prepared the HDL₃-containing fraction as described by Gidez et al. (4), as follows. First treat the serum with heparin sulfate and MnCl₂ (final concentrations, 1.3 g/L and 92 mmol/L, respectively), centrifuge (1500 × g, 30 min), and treat an aliquot of the...
supernate with dextran sulfate (M, 15 000, lot 4001; Sochibo, Boulogne, France) in a final concentration of 0.5 g/L. Allow the sample to stand at room temperature for 10 min, then remove the precipitate as described above. Treat the clear supernate with NaHCO₃ to give a final concentration of 0.1 mol/L (9), to remove residual MnCl₂, which otherwise would interfere with the cholesterol measurement, and measure its cholesterol content enzymatically as described above.

In a modification of this (8) procedure, we treated the serum with a mixture of heparin sulfate, dextran sulfate, and MnCl₂ (final concentrations, 1.3 g/L, 0.5 g/L, and 92 mmol/L, respectively), and allowed the sample to stand at room temperature for 30 min. The precipitate was removed as described above. We precipitated the excess Mn²⁺ with NaHCO₃, and measured the cholesterol content of the supernate.

The HDL₃-cholesterol concentration was calculated as the difference between total HDL-cholesterol and HDL₂-cholesterol.

We applied the ultracentrifugation method to aliquots of each of the fresh serum samples. Only the two dual-purification procedures were used with individual frozen serum specimens, because we did not have enough of them for ultracentrifugation. In preliminary experiments intended to establish the optimal dextran sulfate concentration, we applied both the ultracentrifugation and dual-purification methods to frozen serum specimens, which we had pooled to provide a sufficient volume of sample for the experiments.

Results

We determined the optimal dextran sulfate concentration by measuring the HDL₃-cholesterol concentration in heparin–MnCl₂ supernates treated with various concentrations of dextran sulfate. We prepared six serum pools by mixing approximately equal volumes of the frozen-stored serum from normolipidemic individuals, treated aliquots of the heparin–MnCl₂ supernate from each pool with dextran sulfate (final concentration, 0.10 to 1.00 g/L), and measured the cholesterol concentration in the dextran sulfate supernate. Aliquots of the heparin–MnCl₂ supernates of each pool were also subjected to preparative ultracentrifugation at d 1.25, and we measured the HDL₃-cholesterol concentration in the ultracentrifugal infranate. Table 1 shows the results.

The cholesterol concentrations in the dextran sulfate supernate decreased rapidly as the dextran sulfate concentration was increased to 0.25 g/L, then decreased more slowly. Table 1 shows the dextran sulfate concentration at which the apparent HDL₃-cholesterol concentration most closely approximated the ultracentrifugal measurement in each pool: at 0.50 g/L in four pools, 0.25 g/L in the fifth, and at 0.75 g/L in the sixth. The actual concentrations at which the two values were the same varied between 0.30 and 0.68 g/L (not shown). On the basis of these results, we used a concentration of 0.50 g/L in the following experiments.

We first compared the cholesterol concentrations of HDL₃ supernates prepared by sequential precipitation (d) and by a variation in which all the reagents were added at the same time, using 105 frozen-stored specimens analyzed over a three-month period. We measured cholesterol in paired HDL₃ fractions at the same time to eliminate run-to-run variation in the cholesterol measurements themselves. The apparent mean (and SD) HDL₃-cholesterol concentrations were 206 (62) and 208 (65) mg/L by the sequential and simultaneous procedures, respectively, and the paired differences were not statistically significant.

Linear-regression analysis revealed the following relationship between the HDL₃-cholesterol values obtained with the two procedures: [\( \text{HDL₃-chol}_{\text{simult.}} = 0.95 \times \text{HDL₃-chol}_{\text{prep.}} + 11.7 \text{ mg/L} \)] (r = 0.91, SEslope = 0.04, SEintercept = 27.7).

The mean (SD) HDL₃-cholesterol concentrations of the samples were 338 (131) and 336 (131) mg/L for the sequential and simultaneous procedures, respectively. For HDL₃-cholesterol, the relationship between the two methods was as follows: [\( \text{HDL₃-chol}_{\text{simult.}} = 0.98 \times \text{HDL₃-chol}_{\text{prep.}} + 4.8 \text{ mg/L} \)] (r = 0.97, SEslope = 0.02, SEintercept = 27.7). These findings indicated the equivalence of the two procedures.

We next compared the simultaneous precipitation procedure with preparative ultracentrifugation in 56 fresh serum samples analyzed over a three-month period (Table 2). The HDL₃-cholesterol values obtained with the precipitation method (y) averaged 33 mg/L lower than the ultracentrifugal (x) values (P < 0.001). The linear-regression equation relating the two procedures is as follows: y = 0.56x + 85 mg/L (r = 0.70, SEslope = 0.078, SEintercept = 34.7).

Thus, at an HDL₃-cholesterol concentration of 150 mg/L, the predicted value would be 169 mg/L, and at 350 mg/L, the precipitation value would be 281 mg/L. There was a higher correlation between the precipitation (y) and ultracentrifugal (x) methods for the HDL₃-cholesterol measurements: y = 1.029x + 30 mg/L (r = 0.92, SEslope = 0.058,

### Table 1. Titration of Heparin–MnCl₂ Supernates with Dextran Sulfate

<table>
<thead>
<tr>
<th>Dextran sulfate concn, g/L</th>
<th>HDL₃-chol, mg/L, in serum pool</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (total HDL-cholesterol)</td>
<td>500</td>
<td>450</td>
<td>560</td>
<td>440</td>
<td>520</td>
<td>580</td>
<td></td>
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<tr>
<td>0.10</td>
<td>380</td>
<td>380</td>
<td>450</td>
<td>410</td>
<td>410</td>
<td>450</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>260</td>
<td>310</td>
<td>240</td>
<td>290</td>
<td>340</td>
<td>360</td>
<td></td>
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<tr>
<td>0.50</td>
<td>200</td>
<td>250</td>
<td>220</td>
<td>220</td>
<td>260</td>
<td>270</td>
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<tr>
<td>0.75</td>
<td>180</td>
<td>220</td>
<td>200</td>
<td>190</td>
<td>250</td>
<td>240</td>
<td></td>
</tr>
<tr>
<td>1.00</td>
<td>180</td>
<td>210</td>
<td>200</td>
<td>210</td>
<td>220</td>
<td>220</td>
<td></td>
</tr>
<tr>
<td>HDL₃-chol (ultracent.)</td>
<td>240</td>
<td>230</td>
<td>220</td>
<td>240</td>
<td>300</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>Total chol</td>
<td>1930</td>
<td>1810</td>
<td>1620</td>
<td>1510</td>
<td>1910</td>
<td>1840</td>
<td></td>
</tr>
<tr>
<td>Triglyceride</td>
<td>1330</td>
<td>1510</td>
<td>880</td>
<td>870</td>
<td>860</td>
<td>780</td>
<td></td>
</tr>
</tbody>
</table>

*Values in italics most closely approximate HDL₃-cholesterol concentrations determined by ultracentrifugation.*


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**Notes:**

- Table 1: Titration of Heparin–MnCl₂ Supernates with Dextran Sulfate
- 1391
Table 2. HDL-Cholesterol Measured in Fresh Serum by Ultracentrifugation (UC) and Simultaneous Heparin–MnCl₂ and Dextran Sulfate Precipitation (Pptn)\(^a\)

<table>
<thead>
<tr>
<th>Sex</th>
<th>n</th>
<th>Total HDL-chol</th>
<th>HDL₂-chol</th>
<th>HDL₃-chol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>UC</td>
<td>Pptn</td>
<td>UC</td>
</tr>
<tr>
<td>All</td>
<td>56</td>
<td>446</td>
<td>173</td>
<td>208</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(121)</td>
<td>(100)</td>
<td>(111)</td>
</tr>
<tr>
<td>Males</td>
<td>28</td>
<td>399</td>
<td>137</td>
<td>159</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(88)</td>
<td>(62)</td>
<td>(72)</td>
</tr>
<tr>
<td>Females</td>
<td>28</td>
<td>498</td>
<td>206</td>
<td>252</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(133)</td>
<td>(109)</td>
<td>(131)</td>
</tr>
</tbody>
</table>

\(^a\) The mean (SD) cholesterol and triglyceride concentrations in the samples were: all—2359 (522) and 1455 (802); males—2230 (490) and 1280 (550); females—2400 (710) and 1640 (960) mg/L, respectively.

\(^b\) Paired Student’s t-Test.

SE\(_{\text{intercept}} = 43.7\). At an HDL₂-cholesterol value of 150 mg/L, the precipitation method would give a value of 184 mg/L, and at 350 mg/L, the predicted value would be 390 mg/L.

The negative bias of the HDL₃-cholesterol measurements was about 20 mg/L greater in specimens from females than from males (Table 2). The reason for this is unknown. The HDL₂-cholesterol values calculated from the dual-precipitation method were somewhat higher than the ultracentrifugal values because of the negative bias of the HDL₃ values. The dual-precipitation method did reflect the higher HDL₃-cholesterol concentrations in the females, and the correlations (\(r\)) between HDL subfractions and total HDL₃-cholesterol and between HDL₂ and serum triglyceride concentration with the two methods (ultracentrifugation and dual precipitation) were respectively as follows: HDL₃ vs total HDL, 0.87, 0.92; HDL₂ vs total HDL, 0.57, 0.40; HDL₃ vs triglyceride, −0.33, −0.29. Thus, the dual-precipitation values reflected the correlations obtained by ultracentrifugation.

Discussion

We used the dual-precipitation method of Gidez et al. (4) to estimate HDL₃- and HDL₃-cholesterol concentrations in fresh or frozen-stored serum. The dextran sulfate concentration at which the HDL₃-cholesterol measurements with the dual-precipitation and ultracentrifugal methods were the same varied by over twofold. The two methods most closely agreed for most serum pools when we used 0.5 g/L dextran sulfate. Higher concentrations increased the negative bias of the HDL₃ measurements. These findings differ somewhat from those of others (4, 10). Gidez et al. (4) found a concentration of 1.3 g/L to be optimal, whereas Demacker et al. (10), who also found that the optimal concentration varied, ultimately selected a concentration of 0.87 g/L. The reasons for these differences are unknown. Gidez et al. (4), however, developed the method for use in EDTA-plasma; Demacker et al. (10) used serum. Both workers prepared the initial heparin–MnCl₂ supernates by using MnCl₂ at 92 mmol/L. The higher dextran sulfate concentration needed in plasma may have resulted from the presence of the EDTA, which complexes some of the Mn\(^{2+}\), lowering its effective concentration in the heparin–MnCl₂ supernate. This, in turn, may have increased the amount of dextran sulfate needed. Various results have been obtained even when serum was used, however. Demacker et al. (10) found that HDL₃-cholesterol was overestimated at dextran sulfate concentrations <0.78 g/L, whereas our study indicated that HDL₃-cholesterol was underestimated at concentrations >0.5 g/L. Simpson et al. (11) reported a negative bias in serum HDL₂-cholesterol measurements at a dextran sulfate concentration of 1.1 g/L, whereas Whitaker et al. (8) found close agreement with ultracentrifugation when using a concentration of 1.3 g/L. The conditions of precipitation were essentially the same in all of the studies, and the findings may have been influenced by lot-to-lot variations in dextran sulfate or by the lipoprotein concentration of the samples. The lot numbers were not specified in most of the studies (4, 8, 11).

The original method described by Gidez et al. (4) required two centrifugations, one to remove the apoB-containing lipoproteins and another to remove the HDL₂-rich fraction from the heparin–MnCl₂ supernate. Whitaker et al. (8) suggested a modification in which the dextran sulfate was added immediately to the serum–heparin–MnCl₂ mixture. They found that the two variations gave equivalent HDL₃-cholesterol values in 15 specimens. Our more extensive evaluation in 105 specimens gave similar results.

Finally, we compared the dual-precipitation and ultracentrifugal methods in 56 individual serum samples. The dual-precipitation method gave a negative bias of 33 mg/L for HDL₂-cholesterol, but the HDL₃ and HDL₃ values correlated reasonably well with ultracentrifugal values. The correlation for HDL₂-cholesterol (0.92) was similar to those reported by most other workers—0.88 (2); 0.94 (10); 0.91 (8); 0.80 (11); that for HDL₃-cholesterol (0.70) was lower than reported by Demacker et al. (10) (0.97), intermediate between those found by the two other groups (0.61 (4), 0.84 (8)), and considerably higher than that reported (0.36) by Simpson et al. (11). The method reflected the sex-related difference in HDL₂-cholesterol values and showed correlations with triglyceride and total HDL-cholesterol concentrations in serum similar to those based on the ultracentrifugal measurements.

It is known that the dual-precipitation method does not specifically precipitate HDL₂ (8, 10). The method does provide an estimate of HDL₃ and HDL₃-cholesterol concentrations, and reflects the expected variations in HDL₂ concentration with sex, race, and triglyceride concentration (4, 8, 12). It can be simplified by adding heparin, MnCl₂, and dextran sulfate to the specimen at the same time. The method seems best suited for use in large-scale studies, but the optimal dextran sulfate concentration must be determined for each lot of reagent and should be deter-
mined in the same kind of specimens to which the method will be applied.

This work was supported in part by NIH Contract #NO1-HV-78102. We thank Carol McGeeney for preparing the manuscript.

References