Heparin–Mn$^{2+}$ Quantitation of High-Density-Lipoprotein Cholesterol: An Ultrafiltration Procedure for Lipemic Samples

G. Russell Warnick and John J. Albers

We describe a modified heparin–Mn$^{2+}$ procedure for high-density-lipoprotein cholesterol quantitation, especially in lipemic samples. High-density-lipoproteins may be estimated as cholesterol remaining in plasma supernates after precipitation of other lipoproteins by heparin and Mn$^{2+}$ treatment. However, in lipemic samples or those from non-fasting individuals, the lower density of the precipitated chylomicrons, very-low-, and low-density-lipoproteins frequently prevents their sedimentation by the usual low-speed centrifugation, and high-density-lipoprotein cholesterol thus is overestimated in the resulting turbid supernates. Sedimentation is improved by a twofold increase in Mn$^{2+}$ concentration to 92 mmol/liter. The procedure reported here produced clear supernates in more than 95% of samples tested. Any remaining turbid supernates can be cleared by a simple, convenient ultrafiltration technique. The filtration removed essentially all of the very-low- and low-density-lipoproteins without removing appreciable amounts of high-density-lipoproteins.

The inverse relationship between HDL$^1$ concentration and cardiovascular disease risk was first identified in 1951 (1). More recently, epidemiological studies have emphasized the importance of HDL as a negative risk factor (2–4). As a result, clinical laboratories have experienced an increased demand for quantitation of this lipoprotein. A method in which heparin and Mn$^{2+}$ are used to precipitate the apoB-containing lipoproteins, primarily VLDL and LDL, with estimation of HDL in terms of the cholesterol remaining in the supernate, was described for serum and subsequently applied to EDTA-treated plasma (5–7). EDTA-treated plasma is preferred to serum for lipid and lipoprotein determinations for the following reasons. For clot formation, blood must stand at room temperature, which may produce changes in lipid/lipoprotein distribution. Chylomicrons may be trapped in the clot and so removed from serum during centrifugation. Serum contains heavy metals that are known to promote autoxidation of the lipids, whereas EDTA in plasma chelates metal ions, preventing autoxidation (7, 8). However, EDTA or the additional proteins such as fibrinogen in plasma may interfere with the heparin–Mn$^{2+}$ precipitation of non-HDL lipoproteins. We observed incomplete precipitation of the apoB-associated lipoproteins from EDTA-treated plasma by the original heparin–Mn$^{2+}$ method with Mn$^{2+}$ at 46 mmol/liter, final concentration, which was remedied by a twofold increase in Mn$^{2+}$ to 92 mmol/liter (9). This increase in Mn$^{2+}$ was incorporated into a method with other modifications for improved accuracy and convenience. Heparin and Mn$^{2+}$ were added to plasma in a combined reagent, decreasing the pipetting manipulations and with volumes better suited for use with manual pipets. Incubation time was reduced to 10 min at room temperature before centrifugation at 1500 × g for 30 min at 4 °C (9).

With lipemic samples or samples from non-fasting subjects, the density of the chylomicron–VLDL–LDL fraction precipitated with heparin–Mn$^{2+}$ is too low for it to sediment. These lipoproteins thus remain suspended in the solution, producing obvious turbidity. Any visible supernatant turbidity immediately after centrifugation indicates incomplete removal of the non-HDL lipoproteins—and hence overestimation of HDL. After overnight storage at 4 °C, a MnO precipitate develops and after three to four days some HDL may precipitate (9). At the higher Mn$^{2+}$ concentration (92 mmol/liter), precipitate density is increased relative to solution density, facilitating sedimentation. In one set of 89 samples, 5% of the supernates were turbid by the modified procedure as compared to 10% after precipitation with 46 mmol of Mn$^{2+}$ per liter (9). In another group of 375 samples, 8.5% were turbid at 46 mmol of Mn$^{2+}$ per liter, as compared to 2.5% by the modified procedure (10).

Three procedures have been described for circumventing turbid supernates in lipemic samples. Turbid supernates may be cleared by centrifugation at a higher speed; 12 000 × g for 10 min (Method B) usually causes the unsedimented lipoproteins to layer over the clear subnatant solution (9). Chylomicrons and VLDL can be removed by 20-h ultracentrifugation at density 1.066 kg/liter before heparin–Mn$^{2+}$ treatment (Method C) (7). A disadvantage of both these procedures is that an ultracentrifuge is required, and additionally for Method C, the time and manipulations required to obtain the d > 1.066 fraction. A third approach (Method D) is to dilute the lipemic sample with isotonic saline solution before adding heparin and Mn$^{2+}$ reagents, thus decreasing the solution density and facilitating sedimentation (5). The dilution, however, also proportionately reduces the already-low HDL cholesterol value and may magnify technical error.

We have investigated an alternative method (Method A), using ultrafiltration to remove the supernatant turbidity associated with lipemic samples (11). Various ultrafilters and
prefilters were tested, to determine the most suitable combination for specific removal of the apoB-associated lipoproteins, primarily VLDL and LDL, without retention of HDL. Lipoprotein cholesterol and triglyceride concentrations in filtrates were compared to those in supernates obtained by centrifugation. Apo-A-I, the major protein of HDL, was measured by radial immunodiffusion assay. Removal of the apoB-associated lipoproteins was checked by an radial immunodiffusion assay in which antibody to apoB was used. The ultrafiltration approach to HDL quantitation in lipemic samples was compared to the other three methods—B, C, and D—in terms of cholesterol, triglyceride, and apoA-I cholesterol in filtrate or supernate.

**Materials and Methods**

**Samples**

Plasma samples were obtained from normal and hyperlipidemic subjects according to the Lipid Research Clinic Protocol (7). Blood was collected from the antecubital vein into 15-ml Vacutainer Tubes containing 22.5 mg of dry disodium EDTA (3218-XP282-3875; Becton-Dickinson, East Rutherford, N.J. 07073). Immediately after thorough mixing, samples were cooled to 4°C and within 2 h cells were removed by centrifugation.

**Heparin—Mn²⁺ Precipitation**

The apoB-associated lipoproteins were precipitated from plasma by heparin—Mn²⁺ treatment as described previously (9). The following method is designated the "modified procedure" (Mn²⁺ concentration, 92 mmol/liter). Prepare a 1.06 mol/liter solution of MnCl₂·4 H₂O. Add 0.6 ml of sodium heparin (Lipo-Hepin, 40 × 10⁶ USP units/liter, about 280 g/liter; Riker Laboratories, Northridge, Calif. 91324) to 10.0 ml of 1.06 mol/liter MnCl₂ solution. This combined heparin—Mn²⁺ solution, stored at 4°C, is stable for at least a month. Heparin from other sources may also be suitable, but each lot should be tested to initial concentration. To accommodate heparin preparations of different initial concentration, adjust the concentration of the original MnCl₂ solution to obtain a Mn²⁺ concentration of 1.0 mol/liter and a heparin concentration of about 16 g/liter in the combined solution. Add 0.2 ml of the combined heparin—Mn²⁺ solution to 2.0 ml of plasma, with thorough mixing. Allow the samples to stand for 10 min at room temperature, then centrifuge at 1500 × g for 30 min at 4°C. Aspirate the clear supernate for analysis. Filter any turbid supernates through a 0.22-μm filter protected by two depth prefilters as follows: Place a 0.22-μm filter, 25 mm in diameter, on the lower support of a 25-mm Swinnex filter holder. Place the silicone gasket on the filter and fit an AP 15 and an AP 20 glass depth prefiter, each 22 mm in diameter, within the gasket. Tighten the upper unit of the Swinnex holder over the filter assembly (Millipore Corp., Bedford, Mass. 01730). Force the turbid supernate through the filter assembly with moderate positive pressure from a syringe and collect the clear filtrate for analysis.

This filter combination was selected after the following experiments.

First, clear supernates were filtered to determine if HDL was removed by ultrafiltration. Normo-lipidemic plasma samples (7.0 ml) each were treated with the combined heparin—Mn²⁺ solution (0.7 ml) and the apoB-associated lipoproteins were removed by centrifugation at 1500 × g for 30 min. Two-milliliter portions of the supernatant solutions were filtered through 0.22-, 0.45-, and 0.8-μm filters without prefilters. Cholesterol, apoA-I, and triglyceride concentrations in the filtrates were compared to those in the remaining unfiltered supernatant solutions.

Second, heparin—Mn²⁺ treated plasma samples were filtered without centrifugal removal of the apoB-associated lipoproteins to determine the best filter for their specific removal. Heparin—Mn²⁺ solution (0.7 ml) was added to 7.0-ml plasma samples and thoroughly mixed. Two-milliliter portions were filtered through 0.22-, 0.45-, and 0.8-μm filters without prefilters. The apoB-associated lipoproteins were removed from the remaining unfiltered heparin—Mn²⁺ treated plasma by centrifugation. HDL recovery was determined by comparing cholesterol and apoA-I concentrations in filtrates to those in the corresponding supernates. LDL/VLDL removal was determined by measuring the apoB-associated cholesterol in filtrates.

Third, similar experiments were done except that two glass depth prefilters, an AP 15 and an AP 20, were added to prevent plugging of the ultrafilters.

In the fourth series of experiments, the ultrafiltration procedure (Method A) was compared to methods B, C, and D for HDL cholesterol quantitation in hypertriglyceridemic samples. Samples were treated with the combined heparin—Mn²⁺ solution as explained previously. Method A: An aliquot of each turbid supernate was filtered with the 0.22-μm filter with an AP 15 and an AP 20 prefiter assembled as above. Method B: A second aliquot was subjected to centrifugation at 12 000 × g for 10 min in a SW 41 rotor (Beckman Instruments, Palo Alto, Calif. 94302). The clear subnatant solution was obtained by aspirating with a 23-gauge needle through the side of the tube (9). Method C: Heparin—Mn²⁺ (0.2 ml) precipitation was simultaneously performed on 2.0 ml of the d >1.006 fraction obtained previously from each sample. Five milliliters of plasma was overlayed with 1.5 ml of 0.15 mol/liter NaCl in a cellulose nitrate tube for the 40.3 rotor (Beckman Instruments) and centrifuged at 37 000 rpm for 20 h at 10°C. The d >1.006 fraction was recovered by a tube slicing technique and readjusted to the original plasma volume (7). Method D: A 1.0-ml portion of the lipemic plasma was diluted with 1.0 ml of 0.15 mol/liter NaCl solution before 0.2 ml of the combined heparin—Mn²⁺ solution was added. In all methods samples were incubated for 10 min at room temperature before filtration or centrifugation, and in Methods C and D precipitates were sedimented by centrifugation at 1500 × g for 30 min.

In the original heparin—Mn²⁺ precipitation procedure, Mn²⁺ was at 46 mmol/liter final concentration, half that used in these experiments (7). We performed a parallel series of experiments to validate the ultrafiltration approach for removal of precipitates at the lower Mn²⁺ concentration. In this method, designated "46 mmol/liter Mn²⁺," heparin (final concentration, 1.3 g/liter) and Mn²⁺ solutions were added to plasma sequentially, with mixing, followed by incubation at 4°C for 30 min before centrifugation or filtration, which were done as described above for the modified procedure. Methods A—D were compared in hypertriglyceridemic samples after precipitation with 46 mmol of Mn²⁺ per liter.

**Lipid Analysis**

Cholesterol and triglycerides were quantitated by Lipid Research Clinic continuous-flow (AutoAnalyzer II) procedures (7). Supernates and filtrates were mixed thoroughly to re-suspend any precipitates before extraction into isopropanol in the presence of zeolite mixture. Cholesterol was determined by a Liebermann—Burchard reagent method and triglyceride by a fluorometric 2,4-pentanediol procedure. Standard solutions and quality control samples were provided by the Lipid
Table 1. Recovery of Cholesterol, Triglycerides, and ApoA-I from 10 Clear Heparin–Mn$^{2+}$ (92 mmol/liter) Supernates after Ultrafiltration

<table>
<thead>
<tr>
<th>Unfiltered supernates</th>
<th>Mean concentrations, mg/liter</th>
<th>ApoA-I</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cholesterol</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>0.22-μm filtrate</td>
<td>517 (23)a,b</td>
<td>162 (48)a,c</td>
</tr>
<tr>
<td>0.45-μm filtrate</td>
<td>527 (16)b</td>
<td>177 (59)b</td>
</tr>
<tr>
<td>0.8-μm filtrate</td>
<td>526 (17)b</td>
<td>118 (11)</td>
</tr>
</tbody>
</table>

* Number in parentheses is the paired standard deviation of the difference between concentrations in the filtrate and the corresponding supernatant (Δdifference$^2/2n^2$)/

b Paired Student's t-test P < 0.005.

Table 1. Recovery of Cholesterol, Triglycerides, and ApoA-I from 10 Clear Heparin–Mn$^{2+}$ (92 mmol/liter) Supernates after Ultrafiltration

Standardization Laboratory of the Center for Disease Control, Atlanta, Ga. 30333. CV’s of 0.6, 0.9, and 1.4% were obtained for CDC pools (Q6, Q3, and MQ-2) with cholesterol concentrations of 2760, 1540, and 690 mg/liter, respectively, analyzed four times per day for 12 months. The corresponding triglyceride CV’s for Q6 (2480 mg/liter) and Q3 (690 mg/liter) were 1.1 and 3.0%, respectively. Mean values for these pools were within 20 mg/liter of Lipid Standardization Laboratory reference target values. Analysis conditions were designed to maximize the measurement precision in the supernates and filtrates that have low HDL concentrations. To eliminate between-run and between-tray variation, comparison samples from each subject were analyzed consecutively in one tray. Systematic bias effects of within-tray baseline drift and scale expansion were minimized by randomizing the order of the comparison samples for each subject. Sample interaction (carryover) was minimized by analyzing samples in duplicate, sequentially, and reporting only the second value. Instrument response did not deviate significantly from linearity, even at very low concentrations. Serial dilutions to 1:16 of a 650 mg/liter heparin–Mn$^{2+}$ supernate were all within 10 mg/liter of the expected values. Lipid results from the continuous-flow analyzer were corrected for the dilution caused by the added heparin–Mn$^{2+}$ reagent and the NaCl solution.

Apolipoprotein Quantitation

ApoA-I was quantitated in supernate and filtrate solutions by a radial immunodiffusion method (12). Samples were diluted with an equal volume of tetramethyleurea (Burdick and Jackson Laboratories, Muskegon, Mich. 49442), further diluted five-fold with tris(hydroxymethyl)aminomethane buffer (10 mmol/liter, pH 8.0) containing 8 mol of urea per liter and incubated at 23 °C for 30 min. After incubation samples were applied to 1.8-mm wells in gels of 1.0% agarose (Sigma Chemical Co., St. Louis, Mo. 63178), containing, per liter, 20 mmol of tris(hydroxymethyl)aminomethane buffer, pH 8.0, 0.15 mol of NaCl, 500 mg of EDTA, 500 mg of NaN$_3$, 5.0 g of bovine serum albumin (Sigma), and rabbit antibody specific for human apoA-I. The gels were incubated for 72 h at 37 °C in a humidity chamber. Precipitation ring diameters were measured with a calibrated viewer (Transidyne General Corp., Ann Arbor, Mich. 48106). ApoA-I standards were purified from human HDL and standardized against albumin as described previously (12). Concentrations of six standards ranging from 500 to 2500 mg/liter were linearly related to precipitation ring diameter squared.

LDL-VLDL in heparin–Mn$^{2+}$ supernatant and filtrate solutions were estimated as apoB-associated cholesterol by a similar radial immunodiffusion procedure (13). Samples were applied to 1.8-mm wells in 1% agarose gels containing the same mixture just described, except that the rabbit antibody was specific for human apoB. The gels were incubated at 37 °C for 72 h in a humidity chamber before precipitation ring diameters were measured. A standard was prepared by pooling plasma d > 1.066 fractions (with NaN$_3$, 500 mg/liter, as preservative) and quantitating LDL cholesterol as total cholesterol minus HDL cholesterol. Concentrations of five dilutions of this standard ranging from 10 to 200 mg of apoB-associated cholesterol per liter, prepared freshly for each assay, were linearly related to precipitation ring diameter squared. The antibody concentration in the gels was adjusted to provide sensitivity to about 5 mg of LDL cholesterol per liter.

Results and Discussion

Portions of 10 clear (free of apoB-associated cholesterol) heparin–Mn$^{2+}$ supernates, obtained from normolipidemic plasma by the modified procedure, were filtered through 0.22-, 0.45-, and 0.8-μm filters without prefilters (Table 1). Cholesterol in the supernate was decreased slightly but significantly from a mean of 545 mg/liter to 517, 527, and 526 mg/liter on use of the 0.22-, 0.45-, and 0.9-μm filters, respectively. A corresponding decrease was observed for ApoA-I, from a mean of 1318 mg/liter to 1264, 1275, and 1269 mg/liter, respectively. This slight decrease in HDL, approximately 3–9%, is probably a result of adsorption onto the ultrafiltration membranes. The slight decrease is statistically significant because of the excellent precision in the cholesterol analyses. The triglyceride concentrations were increased significantly with the 0.22- and 0.45-μm filters, from a mean of 115 mg/liter to 162 and 177 mg/liter, respectively. This increase in apparent triglyceride, was attributable to a non-triglyceride fluorescent contaminant leaching from some lots of filters into the filtrate, and was also observed in de-ionized water after 0.22-μm filtration. Because HDL is generally quantitated in terms of its cholesterol moiety, the artificial increase in triglyceride is only a concern in studies of HDL composition. Presumably, only fluorometric triglyceride methods would be affected.

Similar results were obtained for filtrates of three supernates obtained after precipitation at 48 mmol of Mn$^{2+}$ per liter. Filtrate cholesterol concentrations averaged 1% and apoA-I concentrations averaged 4% less than the original values for the supernates.

Portions of 16 plasma samples after heparin–Mn$^{2+}$ addition (92 mmol of Mn$^{2+}$ per liter), but without centrifugation, were filtered through 0.22-, 0.45-, and 0.8-μm filters without depth prefilters. Filtration rates through the 25-mm diameter filters with moderate pressure from a 5-ml syringe were low, <1 ml/h. Filtrate cholesterol and apoA-I concentrations, as compared to those in clear supernates of the same samples obtained by centrifugation at 1500 X g for 30 min averaged, respectively: 0.22-μm filter, 68 and 71%; 0.45-μm, 82 and 85%; and 0.8-μm, 124 and 93%. The corresponding mean filtrate apoB-associated cholesterol values were 22, 12, and 50 mg/liter for the 0.22-, 0.45-, and 0.8-μm filters, respectively. The apoB-asso-
Fig. 1. Removal of lipoproteins precipitated with heparin-Mn$^{2+}$ (92 mmol/liter) from normolipidemic plasma by centrifugation at 1500 × g for 30 min (x) compared to ultrafiltration with 0.22- or 0.45-μm filters with two depth prefilters (y).

The solid line represents y = x. The relationship for 0.22 μm is y = 0.96x + 10.0, r² = 0.996, and for 0.45 μm is y = 1.02x + 20.3, r² = 0.975. Note the very slight negative bias with the 0.22-μm filters and the relatively high positive bias in some samples with the 0.45-μm filters.

Associated lipoproteins precipitated by heparin-Mn$^{2+}$ treatment plugged the unprotected filters, decreasing the filtration rate and causing retention of HDL. In addition, under the prolonged pressure required to force samples through the filters, some of the apoB-associated lipoproteins leaked through the filter. This was particularly evident with the 0.8-μm filter, as indicated by the high values for cholesterol and apoB-associated cholesterol.

In contrast, addition of two depth prefilters to the same pore size filters produced rapid filtration of the heparin-Mn$^{2+}$-treated samples with very little HDL retention (Table 2). Filtration through 0.22-μm filters protected by AP 15 and AP 20 depth prefilters resulted in a mean cholesterol concentration in the filtrate of 521 mg/liter and a mean apoA-I concentration of 1310 mg/liter, 98% or greater of those values for the corresponding supernatant fractions: for cholesterol, 533, and for ApoA-I, 1340 mg/liter. The small paired standard deviation of the bias between filtrates and supernates (12 mg/liter) indicates a consistent and statistically significant difference (P < 0.005), even though the mean bias was only 11 mg/liter. The negligible apoB-associated cholesterol concentrations in supernates and in the 0.22-μm filtrates indicated essentially complete removal of LDL-VLDL by either method. By contrast, the mean cholesterol concentration of 565 mg/liter in the 0.45-μm filtrates, 106% of the mean supernatant concentration, and the mean apoB-associated cholesterol concentration, 48 mg/liter, suggests permeability of the 0.45-μm filter to the precipitated VLDL-LDL.

These conclusions are supported by the data in Figure 1 illustrating the relation between cholesterol concentrations for 0.22-μm and 0.45-μm (with depth prefilters) filtrates with those in the corresponding control supernates (x). The relationship for the 0.22-μm filter, y = 0.96x + 10.0 mg/liter, with a correlation coefficient of 0.998, demonstrates the very slight negative bias, but a high correlation as compared to the usual centrifugation procedure. The relationship for the 0.45-μm filter combination; y = 1.02x + 20.3 mg/liter, with a correlation coefficient of 0.975, indicates significantly poorer agreement (P < 0.005) with the filter of larger pore size. In about 20% of the samples, the cholesterol concentration in the 0.45-μm filtrates was markedly higher than that in the corresponding supernates. The apoB-associated cholesterol concentrations in these filtrates indicated LDL-VLDL contamination.

A similar comparison in five samples of 0.22- and 0.45-μm filtration (with prefilters) to centrifugation after heparin-Mn$^{2+}$ treatment (46 mmol of Mn$^{2+}$ per liter) produced similar results. The mean cholesterol and apoA-I concentrations, respectively, were 650 and 1584 mg/liter for the supernates obtained by centrifugation, as compared to 634 and 1573 mg/liter in the 0.22-μm filtrates and 650 and 1578 mg/liter in the 0.45-μm filtrates. Only the 0.45-μm filtrates had significant apoB-associated cholesterol, a mean of 22 mg/liter.

The addition of two depth prefilters protected the ultrafilters from plugging and minimized HDL retention. Flow rates were acceptable, approximately 1 ml/min. The 0.22-μm filter effectively removed the precipitated apoB-associated lipoproteins, and the cholesterol and apoA-I values in 0.22-μm filtrates correlated highly with those for the corresponding supernates. The very slight negative bias in the 0.22-μm filtrates, <2% in these precise paired analyses, would not be an important source of variation in routine HDL cholesterol quantitation.

These experiments demonstrate the effectiveness of appropriate ultrafiltration for specifically removing heparin-Mn$^{2+}$ precipitated LDL-VLDL from normolipidemic samples. The real utility of the procedure is in removing the precipitated VLDL-LDL that does not sediment during the initial low speed centrifugation and is manifested as supernatant turbidity.

Ultrafiltration with the 0.22-μm filter and two prefilters (Method A) was compared to methods B, C, and D in lipemic samples. Summary results for supernatant or filtrate cholesterol, triglycerides, and apoB-associated cholesterol after heparin-Mn$^{2+}$ treatment by either the modified (92 mmol/liter) or the 46 mmol/liter procedure are presented in Table 2.

### Table 2. Filtration Compared to Centrifugation for Lipoprotein Removal from Heparin-Mn$^{2+}$-Treated Plasma

<table>
<thead>
<tr>
<th>Mean concentrations, mg/liter</th>
<th>Supernate after centrifugation</th>
<th>Filtrate, 0.22-μm</th>
<th>Filtrate, 0.45-μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>ApoA-I</td>
<td>ApoB-associated cholesterol</td>
<td></td>
</tr>
<tr>
<td>533</td>
<td>1340</td>
<td>&lt;5</td>
<td></td>
</tr>
<tr>
<td>521 (12)$^{b,c}$</td>
<td>1310 (56)$^{b}$</td>
<td>&lt;5 (4)$^{b}$</td>
<td></td>
</tr>
<tr>
<td>565 (35)$^{c}$</td>
<td>1300 (53)$^{d}$</td>
<td>48 (48)$^{c}$</td>
<td></td>
</tr>
</tbody>
</table>

$^{a}$ Alloquots of 21 normolipidemic plasma samples after heparin-Mn$^{2+}$ (92 mmol/liter) treatment subjected to centrifugation (1500 × g, 30 min) or to ultrafiltration with 0.22- or 0.45-μm filters with two depth prefilters.

$^{b}$ Numbers in parentheses is the paired standard deviation of the difference between concentrations in the filtrate and the corresponding supernate (2 × difference$^{2}/2n$)$^{1/2}$.

$^{c}$ Paired Student's t-test P < 0.005.

$^{d}$ P < 0.05.
Table 3. Comparison for Four Methods for HDL Estimation in Hypertriglycerideremic Samples

<table>
<thead>
<tr>
<th>Method</th>
<th>Cholesterol</th>
<th>Triglycerides</th>
<th>ApoB-associated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD, mg/liter</td>
<td>Mean ± SD, mg/liter</td>
<td>Mean ± SD, mg/liter</td>
</tr>
<tr>
<td>Modified procedure (92 mmol/liter Mn2+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Method A</td>
<td>292 ± 89</td>
<td>265 ± 115</td>
<td>3 ± 5</td>
</tr>
<tr>
<td>Method B</td>
<td>307 ± 91</td>
<td>241 ± 118</td>
<td>6 ± 10</td>
</tr>
<tr>
<td>Method C</td>
<td>291 ± 77</td>
<td>167 ± 80</td>
<td>3 ± 5</td>
</tr>
<tr>
<td>Method D</td>
<td>297 ± 92</td>
<td>228 ± 121</td>
<td>0'</td>
</tr>
</tbody>
</table>

46 mmol/liter Mn2+ h

- Method A: 375 ± 127, 246 ± 49, 17 ± 18
- Method B: 381 ± 130, 191 ± 75, 18 ± 27
- Method C: 374 ± 127, 140 ± 45, 0'
- Method D: 388 ± 119, 180 ± 112, 0'

* Twenty-four plasma samples with triglyceride distribution of 5376 ± 4586 mg/liter (mean ± standard deviation).
* Turbid supernates filtered with 0.22-μm filter with two depth filters.
* Turbid supernates subjected to centrifugation (12000 X g, 10 min).
* Heparin-Mn2+ precipitation of d > 1.006 fraction obtained by ultracentrifugation.
* Plasma diluted with an equal volume of 0.15 mol/liter NaCl before addition of heparin-Mn2+ solution.
* Paired Student's t-test (vs. Method A) P < 0.005.
* p < 0.05.
* Twelve plasma samples with triglyceride distribution of 3077 ± 2311 mg/liter.

3. Mean cholesterol results were in good agreement (within 20 mg/liter) for Methods A–D at either Mn2+ concentration; only Method B cholesterol was significantly different (P < 0.005) from that by Method A. Somewhat poorer agreement was observed in mean-triglyceride values, ranging from 167 to 265 mg/liter after precipitation by the modified heparin–Mn2+ procedure and from 140 to 246 by the procedure in which 46 mmol of Mn2+ per liter is used. As observed previously, filtrate triglyceride concentrations (Method A) were significantly greater as compared to the other methods. Removal of LDL-VLDL, monitored by the apoB-associated cholesterol, was nearly complete by all methods at the higher Mn2+ concentration. After precipitation with 46 mmol of Mn2+ per liter, Method A filtrates contained an average of 17 mg of apoB-associated cholesterol per liter and Method B subnatant solutions averaged 18 mg/liter, consistent with previous reports of incomplete LDL-VLDL precipitation at this Mn2+ concentration (9).

Compared to ultrafiltration, the other methods have important disadvantages. Method B, recentrifugation of turbid supernates at 12000 X g for 10 min, requires access to a high-speed centrifuge. Careful technique is required to obtain an uncontaminated sample of the subnatant solution; the somewhat higher cholesterol and apoB concentrations by this method may be a result of slight contamination from the overlayered apoB-associated lipoproteins.

The 20-h ultracentrifugation to obtain the d > 1.006 fraction, free of VLDL and chylomicrons for Method C is inconvenient and many laboratories do not have the necessary equipment. Also the method may underestimate HDL cholesterol as compared to the other methods in some samples. Two samples of the 36 we tested by Method C had cholesterol concentrations that were 60 and 75% of the mean of the other three methods.

About 40% of the total apoA-I of these two samples was in the d < 1.006 fraction, as compared to the usual proportion of <1%. Association of HDL with VLDL has been reported (14). In some samples, part of the apoA-I-containing lipoproteins may remain associated with VLDL and float at density 1.006 during ultracentrifugation, but not precipitate on heparin–Mn2+ treatment of plasma.

Supernatant cholesterol by Method D, dilution of the lipemic sample before heparin–Mn2+ is added, agreed with results by the other procedures in this comparison. However, analytical conditions here were designed to minimize imprecision; the cholesterol response was linear throughout the HDL cholesterol range, and sample interaction (carryover) effects were eliminated by the technique described. In routine analyses, nonlinearity, sample interaction, and other sources of variation can diminish the accuracy and precision in measuring the low cholesterol concentrations by this method. Measurement errors are then enhanced in correcting for the isotonic saline dilution.

We conclude that the ultracentrifugation approach allows accurate estimation of HDL cholesterol in lipemic samples, particularly when used in conjunction with the modified heparin–Mn2+ procedure (9, 10). Ultrafiltration is convenient, inexpensive, and readily accessible to clinical laboratories. Results are in good agreement with those by other current methods for lipemic samples.

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References