Five Methods for Determining Low-Density Lipoprotein Cholesterol Compared

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We evaluated three precipitation methods for determination of low-density lipoprotein cholesterol in serum and an indirect method involving the Friedewald formula (Clin Chem 18: 499–502, 1972) by comparison with results by ultracentrifugation. The results of all methods for 83 sera, including 59 hyperlipidemic type IIa, IIb, and IV sera agreed very well, at least for concentrations of serum triglycerides below 8 mmol/L. The accuracy of the Friedewald formula was confirmed in 285 other sera, including 66 sera with triglycerides content between 4.52 and 8.0 mmol/L. For type III sera, the precipitation methods produced similar values to those obtained with the Friedewald formula, all being much higher than the ultracentrifugation values. Density-gradient ultracentrifugation showed that the very-low-density lipoprotein remnants in type III sera almost completely coprecipitated with the low-density lipoproteins. The precipitation methods are not only accurate but also very precise (CV <5%); they can therefore be used in clinical laboratories to measure atherosogenic low-density lipoproteins plus the remnants of very-low-density lipoproteins. However, when serum triglycerides and high-density lipoprotein cholesterol also are determined, the Friedewald formula is a reliable alternative.

Additional Keyphrases: Friedewald formula · lipoprotein precipitation · type III hyperlipoproteinemia · atherogenesis · triglycerides · very-low-density lipoprotein remnants · hyperlipidemia · ultracentrifugation compared

The contribution of serum cholesterol to the risk for atherosclerosis is determined by its distribution among the various lipoprotein fractions. A relatively large amount of cholesterol in the low-density lipoprotein (LDL) fraction is atherogenic, whereas HDL-cholesterol appears to be protective (1). For distinguishing survivors of coronary heart disease from healthy persons, the LDL-cholesterol/HDL-cholesterol ratio is reportedly threefold more sensitive than the measurement of total serum cholesterol (2). Thus, for selection of high-risk patients, simple but reliable methods for determining lipoprotein-associated cholesterol, especially LDL-cholesterol, should be available.

Ultracentrifugation for LDL-cholesterol is time-consuming and expensive and therefore not suitable for large-scale use. Recently, three different procedures for specific precipitation and determination of LDL-cholesterol have become available (3–6). At least one of these methods is considered suitable for analyzing serum from patients with hyperlipoproteinemia (HLP) type III (3) as well as from patients with type IV or V HLP (4). Therefore, we compared these methods with a combined ultracentrifugation/precipitation method (7, 8) for various sera from normo- and hyperlipemic subjects. In addition, we considered the usefulness of the indirect method of Friedewald et al. (9), which is based on the determination of serum cholesterol, triglycerides, and HDL-cholesterol.

Materials and Methods

Sera

Fresh serum samples from overnight-fasted normal persons and from patients with various types of HLP were stored at 4 °C for no longer than four days, unless stated otherwise. Patients were classified into the different phenotypes according to the criteria of Fredrickson et al. (10) with cutoff limits for serum cholesterol, triglycerides, and LDL-cholesterol of 7.3, 2.0, and 5.2 mmol/L, respectively (11). HLP was classified as type III when high concentrations of serum lipids were combined with a VLDL-cholesterol/serum TG ratio >0.69 and the absence of apoprotein E-3 and E-4 in the isoelectric focusing pattern of the VLDL apoproteins (12). Because it was our intention to propose a clear criterion at which the precipitation methods and the Friedewald formula are equally accurate or inaccurate, we arbitrarily classified sera as type IV or V HLP when LDL-cholesterol was not increased and serum TG were less or more than 8 mmol/L, respectively. We judged this adaptation to be necessary because of the disagreement of the current electrophoresis and refrigeration tests for detecting chylomicrons in serum of different phenotypes (12). In our opinion, the interpretation of these tests is rather subjective, especially in the analysis of type IV and V sera.

Procedures

Comparison method. The method we used for reference in this study was a combined ultracentrifugation/precipitation procedure. VLDL-cholesterol was determined directly in the VLDL-fraction isolated by ultracentrifugation (7) and HDL-cholesterol was determined after precipitation of the VLDL and LDL in whole serum by polystyrene glycol 6000 (8). LDL-cholesterol was calculated by subtraction.

Precipitation procedure A (3, 4). The reagents were obtained as a test-kit (LDL-cholesterol, cat. no. 14992; Merck A.G., D-6100, Darmstadt 1, F.R.G.). The procedure was performed according to the manufacturer’s directions: add 100 μL of serum to 1000 μL of sodium citrate (64 mmol/L, pH 5.12) containing 50 000 U of heparin per litre. After vortex-mixing, incubate the tubes for 10 min at room temperature, centrifuge for 15 min at 2800 × g at room temperature to remove LDL, and, using a Pasteur pipette, aspirate the supernate, which contains the VLDL and HDL. Measure the cholesterol in the supernate within 1 h.

Precipitation procedure B (5). This precipitation reagent was also obtained as a test-kit (LDL-cholesterol, cat. no.
imetry (2100/2020) accuracy

Results

Evaluation of the accuracy of the Friedewald formula. The accuracy of the Friedewald formula (LDL-cholesterol = total cholesterol - HDL-cholesterol - 0.45 × serum TG) is of course influenced by the accuracy of the methods used for the determination of cholesterol, TG, and HDL-cholesterol. Furthermore, the relation between the concentration of serum TG and VLDL-cholesterol is dependent on age, sex, ethnic, and sociodemographic variations of the subjects studied (18).

We determined the accuracy of the Friedewald formula with 285 serum samples from normal individuals and from patients with various forms of HLP who were visiting our outpatient clinic. Patients with type I, III, or V HLP were not included, so that this group we studied had serum TG between 0.4 and 8.0 mmol/L. These subjects were divided into two groups, with serum TG concentrations greater or less than 4.52 mmol/L (400 mg/100 mL). The ratios for VLDL-cholesterol to serum TG in these groups were 0.41 ± 0.10 (mean and SD; n = 219) and 0.47 ± 0.12 (n = 66), respectively. The average ratio in the whole group was 0.42 ± 0.11, which is in good agreement with Friedewald's value of 0.45. In the following section we used the 0.42 value to calculate LDL-cholesterol concentrations; these results (4.51 ± 1.75 mmol/L) agreed excellently with those obtained by ultracentrifugation/precision (4.49 ± 1.66 mmol/L). The linear regression equation for this was LDL (calculated) = 1.03 (± 0.03) LDL (reference) - 0.12 (± 0.13); SEE was 0.38, r was 0.98, and the mean difference between LDL obtained with both methods was -0.01 ± 0.39 mmol/L (p = 0.90, n = 285).

Analytical recoveries of cholesterol in the ultracentrifugation and precipitation method. The sum of cholesterol in the VLDL and the LDL + HDL fractions was 103.7 ± 2.6% (mean ± SD, n = 28) of the value in total serum. The sum of cholesterol recovered in the supernates containing the VLDL + HDL and in the precipitated LDL dissolved with 0.1 mol/L NaOH was 97.9 ± 2.0%, 99.0 ± 4.6%, and 101.6 ± 5.1%, in precipitation methods A, B, and C, respectively (n = 28). These results indicate that the ultracentrifugation/precipitation method is accurate and that none of the precipitation reagents interferes with the cholesterol determination.

Comparison of the five LDL methods in normal and hyperlipidemic sera. We applied the various methods for determining LDL-cholesterol in normal sera (n = 32) and in sera of patients with type II A (n = 21), type II B (n = 9), type III (n = 9), type IV (n = 21), and type V (n = 5). The results obtained for the sera of patients with type III and V HLP were analyzed separately. In the nine sera of patients with type III HLP, the LDL-cholesterol concentrations obtained by the four methods under study were significantly greater than and did not correlate significantly with those obtained by ultracentrifugation/precipitation (Table 1).

Surprisingly, the LDL-cholesterol values obtained by the precipitation method A, B, and C did not differ significantly from the values calculated by the Friedewald formula: respective correlation coefficients were 0.92, 0.98, and 0.94 (S_{xy} = 0.57, 0.37, and 0.51, respectively).

### Table 1. LDL-Cholesterol Values in Sera of Nine Patients with Type III Hyperlipoproteinemia as Determined by the Five Different Methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean (SD)</th>
<th>S_{xy}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Friedewald formula</td>
<td>5.89 (1.60)</td>
<td>0.57</td>
</tr>
<tr>
<td>Method A</td>
<td>5.69 (1.37)</td>
<td>0.37</td>
</tr>
<tr>
<td>Method B</td>
<td>6.08 (1.66)</td>
<td>0.37</td>
</tr>
<tr>
<td>Method C</td>
<td>5.73 (1.39)</td>
<td>0.51</td>
</tr>
</tbody>
</table>

*Significantly different (p < 0.001) from results by the other four methods.
LDL-chol values obtained by the four methods under study in type V sera occasionally disagreed by more than ±1 mmol/L. Use of these methods in type V sera is therefore not recommended.

In the 83 sera with TG concentrations less than 8.0 mmol/L (mean and SD: 2.47 and 1.68 mmol/L), the correlation between the results obtained by the four methods under study and our reference method was excellent (Table 2). The accuracy of all three precipitation methods was very satisfactory. Surprisingly, the values found with the Friedewald formula correlated best and gave the lowest $S_{xy}$ (0.35 vs 0.52–0.56).

**Precision of the precipitation methods and stability of the sera stored at −20 °C or 4 °C.** We divided into 1-mL aliquots fresh serum from a patient with type III HLP (cholesterol 10.3 mmol/L, TG 4.9 mmol/L), from a patient with type IV HLP (cholesterol 6.5 mmol/L, TG 3.6 mmol/L), and from four normolipidemic persons. Portions were analyzed when fresh and after storage at 4 °C or at −20 °C for 1, 6, 10, 13, and 21 days.

The apparent LDL-chol values in HLP type III serum stored at −20 °C, as measured with reagents A and C, increased by about 14%, but when these samples were stored at 4 °C, the values gradually decreased by as much as 50%. With reagent B we obtained widely variable results because of incomplete sedimentation of the precipitated lipoproteins.

For the other five sera, the LDL-chol values obtained with reagents B and C at days 1, 6, 10, 13, and 21 at either temperature did not differ significantly from the values obtained in the fresh samples. CVs less than 5.1% were obtained with both precipitation procedures (Table 3). Procedure A tended (0.05 < $p$ < 0.1) to give 4–5% lower values after storage at −20 °C for 10, 13, or 21 days. The LDL-chol values for the samples stored at 4 °C and measured with reagent A decreased as much as 18%, which explains the higher CV obtained under these conditions (Table 3).

**Analysis of LDL precipitates by density-gradient ultracentrifugation.** The presumed LDL precipitates obtained with reagent A from a serum with normal lipid values and a type III serum were analyzed by density-gradient ultracentrifugation as described above. In the normal serum 83% of the lipoprotein-associated cholesterol was found in the 0 < $S_{r}$ < 15 fraction and had the flotation characteristics of LDL; the remainder was isolated in the fractions with $S_{r}$ > 100 (2%) and 15 < $S_{r}$ < 100 (15%). In the type III serum these percentages were 30, 23, and 47%, respectively. Thus, in the type III serum not less than 70% of the precipitated lipoprotein-associated cholesterol was non-LDL.

**Discussion.**

The three recently introduced precipitation methods have been claimed to specifically precipitate LDL without co-precipitating the VLDL and HDL. Our results confirm this. LDL-chol values obtained by the precipitation methods and by ultracentrifugation correlated very well. However, till now the precipitation behavior of VLDL remnants accumulated in serum of patients with type III HLP, but also present in fasting sera of normolipemics and hyperlipemic non-type III subjects, remained in question. The VLDL remnants have physicochemical characteristics intermediate between VLDL and LDL, being primarily 1.006 < $d$ < 1.019. In type III HLP, large quantities of these remnants are present in the VLDL fraction. Earlier (19) we studied with the same ultracentrifugation method 15 patients with type III HLP, whose serum lipids and LDL-chol values were similar to those of the patients in the present study. We found that the mean concentration of LDL$_{1}$-cholesterol (d 1.006–1.019) in non-type III patients was 0.38 to 0.81 mmol of cholesterol per litre, and was related to the amounts of intermediate β-pre-β lipoproteins (VLDL remnants) on agarose gel electrophoresis (19). The mean LDL$_{1}$-cholesterol concentration in type III patients was 1.47 mmol/L. Thus LDL-chol corrected for remnant-associated cholesterol in the present study should be approximately 3.74 − 1.47 = 2.27 mmol/L, considerably less than the LDL-chol concentration found by precipitation (5.80 mmol/L). This indicates that VLDL remnants are precipitated even when their density is <1.006 kg/L. This is confirmed by density-gradient ultracentrifugation: in a normal serum and a type III serum, 17 and 70% of the precipitated lipoprotein-associated cholesterol had flotation properties characteristic for VLDL remnants. Binding of heparin or other polyanions to the LDL particles does not explain these results, because we found that binding of heparin does not influence the density distribution of LDL.

Taken together, the precipitation methods evaluated here measure the same apparent LDL-chol values as ultracentrifugation but also precipitate the VLDL remnants. All precipitation methods were equally effective, at least in non-type III sera with TG concentrations less than 8 mmol/L, and all were equally inaccurate in the analysis of type III sera.

Surprisingly, the LDL-chol values calculated by the Friedewald formula were also accurate, except for sera from

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**Table 2. Results for LDL-Chol in 83 Sera (TG < 8.0 mmol/L) by the Four Methods (y) Compared with Those by the Ultracentrifugation Method (x)**

<table>
<thead>
<tr>
<th>Method</th>
<th>Linear regression equation</th>
<th>$r$</th>
<th>$S_{xy}$</th>
<th>Mean</th>
<th>$\bar{x} - \bar{y}$</th>
<th>$\rho$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Friedewald</td>
<td>$y = 1.020(0.02)x - 0.10(0.13)$</td>
<td>0.96</td>
<td>0.38</td>
<td>5.00 (1.83)</td>
<td>0.01 (0.36)</td>
<td>0.87</td>
</tr>
<tr>
<td>Method A</td>
<td>$y = 0.97(0.03)x + 0.25(0.17)$</td>
<td>0.96</td>
<td>0.52</td>
<td>5.10 (1.70)</td>
<td>-0.03 (0.52)</td>
<td>0.13</td>
</tr>
<tr>
<td>Method B</td>
<td>$y = 1.03(0.04)x - 0.14(0.19)$</td>
<td>0.96</td>
<td>0.56</td>
<td>5.04 (1.90)</td>
<td>-0.03 (0.56)</td>
<td>0.62</td>
</tr>
<tr>
<td>Method C</td>
<td>$y = 0.98(0.03)x + 0.08(0.16)$</td>
<td>0.96</td>
<td>0.54</td>
<td>5.00 (1.81)</td>
<td>0.00 (0.53)</td>
<td>0.95</td>
</tr>
</tbody>
</table>

*Sera of patients with type III HLP excluded. *Mean LDL-chol by ultracentrifugation was 5.00 (SD 1.76) mmol/L. *No. in parentheses indicate SD.

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**Table 3. Precision (CV, %) of the LDL-Precipitation Methods and of Total Cholesterol Measurement in Sera at Different Storage Conditions**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Mean</th>
<th>Range</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>−20 °C</td>
<td>3.7</td>
<td>1.9–6.6</td>
<td>7.0</td>
<td>3.8–10.8</td>
</tr>
<tr>
<td>−2 °C</td>
<td>3.2</td>
<td>1.7–5.0</td>
<td>2.8</td>
<td>1.5–4.8</td>
</tr>
</tbody>
</table>

Five sera divided into 1-mL aliquots were analyzed freshly or after storage at −20 °C or 4 °C for 1, 6, 10, 13, and 21 days by the three precipitation methods. For comparison, the CV for the total cholesterol measurement is also given. See also text.
patients with type III and V HLP. As mentioned earlier, the mean value we obtained for the VLDL-chol/serum TG ratio in 286 sera was close to that found by Friedewald et al. (9). In contrast to their original report, we also found the Friedewald formula to be reliable in sera with TG concentrations between 4.5 and 8 mmol/L. This disagreement strengthens the assumption by Friedewald et al. (9) that the larger percentage of errors seen in this group of type IV patients might be due in part to laboratory errors in the ultracentrifugation calculation of LDL-chol rather than greater inaccuracy of the estimation procedure. Our findings enhance considerably the number of sera in which ultracentrifugation can be omitted for the determination of LDL-chol, provided that the rare type III HLP has been excluded.

Both the precipitation methods and the Friedewald formula produced similar values for type III sera, but these were considerably higher than the values obtained by ultracentrifugation/precipitation. This is reasonable because the Friedewald formula is based on the assumption that the chol/TG ratio of the VLDL fraction is normal; contamination of this fraction with VLDL remnants invalidates the formulas. The good agreement between the apparent LDL-chol values produced by the precipitation methods or by the Friedewald calculation again confirms that in type III sera most, if not all, of the VLDL remnants also precipitate. However, the co-precipitation of VLDL remnants is in disagreement with the data of Wieland and Seidel (3), who reported preliminary findings that type III VLDL is not precipitated.

The precipitation methods appeared to be precise (except for HLP type III serum), and the stability of serum stored at −20 °C was good. Using the same storage conditions as for HDL-chol should permit reliable results for LDL-chol by precipitation. We recommend storing the sera at −20 °C (maximum one month), preferably, or at 4 °C (one week). Precipitation method A is the most sensitive to changes in the storage conditions, perhaps because during storage the pH of serum increases (8), which may influence the precipitation of LDL by reagent A (3).

From our results, we conclude that the introduction of methods for specific precipitation of LDL seems superfluous, because we obtained the same or even better results by using the Friedewald formula. However, this is only a good alternative for those laboratories that have accurate and precise methods for the determination of both serum TG and HDL-chol. Determination of HDL-chol in sera with abovenormal TG concentrations may be an essential source of error (20).

If LDL-chol is precipitated to differentiate between LDL-chol and HDL-chol, we recommend using the Friedewald formula. The interference of VLDL remnants in the precipitation method for LDL is, in our opinion, rather an advantage than a disadvantage because both classes of lipoproteins are atherogenic in contrast to ordinary VLDL (21). Thus, by using the precipitation methods or the Friedewald formula, one can more nearly accurately quantify the concentrations of atherogenic lipoproteins present, even in type III HLP. In this respect these methods are superior to ultracentrifugation.

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References