Measurement of High-Density Lipoprotein Cholesterol in Serum: Comparison of Six Isolation Methods Combined with Enzymic Cholesterol Analysis

P. N. M. Demacker, H. E. Vos-Janssen, A. G. M. Hilmans, A. van't Laar, and A. P. Jansen

We compared six methods for isolating high-density lipoproteins from normal and lipemic serum. Cholesterol was determined by two different enzymic methods. The values for high-density lipoprotein cholesterol obtained with two (centrifugation-speed) modifications of the heparin/Mn²⁺ method and with the phosphotungstate/Mg²⁺, the dextran sulfate/Mg²⁺, and the polyethylene glycol methods were compared with those obtained by ultracentrifugation.

One cholesterol method, in which each determination was blank-corrected, was not interfered with by the various precipitation reagents. The other, in which there was no blank correction, was considerably interfered with by Mn²⁺ and dextran sulfate. Neither method was interfered with by phosphotungstate/Mg²⁺ or by polyethylene glycol.

Use of ethylenediaminetraacetate in either cholesterol reagent was unhelpful (or deleterious).

After correction for all interferences caused by the precipitation reagents and EDTA, values obtained for high-density lipoprotein cholesterol agreed well with those by ultracentrifugation (corrected for apo B-containing lipoproteins in the d > 1.063 kg/L fraction). Rocket immunoelectrophoresis showed no significant co-precipitation of high-density lipoproteins in any method.

Various methods for complete removal of apo B-containing lipoproteins from lipemic serum appeared to vary widely in efficiency, but the optimized phosphotungstate method and the polyethylene glycol method appeared best. With them, only sera with triglyceride concentrations of 7.6 and 18.5 mmol/L, or greater, respectively, could not be fractionated successfully. However, after ultrafiltration, accurate values were also obtained with the other methods.

We conclude that the phosphotungstate method and the polyethylene glycol method are the best methods for high-density lipoprotein cholesterol analysis when enzymic cholesterol analysis is used.

Additional Keyphrases: heart disease • analytical error • intermethod comparison • "rocket" immunoelectrophoresis

Many precipitation methods have been described for determination of HDL-chol.¹ In these methods, HDL-chol is determined in the supernate after precipitating apoprotein B-containing lipoproteins (1–4). The heparin/Mn²⁺ method (5), the most widely used, cannot be used with extremely lipemic sera without first ultrafiltering to clear the turbid supernates (6), and a further disadvantage is that the enzymic cholesterol reagents are interfered with (7–9).

The other precipitation methods—the phosphotungstate/Mg²⁺ (3), the dextran sulfate/Mg²⁺ (3), and the polyethylene glycol 6000 method (4, 10)—do not seem to interfere with enzymic cholesterol analysis. Furthermore, they reported to be effective in the analysis of lipemic sera (2, 10, 11). Soon, one of these methods may be preferred to the heparin/Mn²⁺ method, but to make a justified choice, all current methods should be compared in the same study. Hitherto, in only one study, six different methods were compared (12), but in that study only normal serum was used, and cholesterol was measured with a chemical method.

To get more insight into the suitability of the various methods, we extended these investigations. Using both normal and lipemic serum (TG up to 18.5 mmol/L), we compared two modifications of the heparin/Mn²⁺ method, and the phosphotungstate/Mg²⁺, dextran sulfate/Mg²⁺, polyethylene glycol, and ultracentrifugation methods. The efficiency with which apo B-containing lipoproteins are removed from lipemic sera was assessed. Two different enzymic cholesterol methods were used. From our results, we suggest the best method for determining HDL-chol with enzymic analysis.

Materials and Methods

Sera

Fresh serum samples from overnight-fasting normal persons and from patients with hyperlipoproteinemia were, unless otherwise stated, stored at 4 °C for no longer than four days.

General Fractionation Conditions

In methods 1A, 2, 3, and 4, we precipitated apo B-containing lipoproteins in conical plastic tubes (100 × 15 mm), and, after incubation, centrifuged the tubes at about 2300 × g for 15 min, at room temperature. The supernatant fluid was carefully aspirated with a Pasteur pipette, aspiration being stopped when a slight turbid veil arose from the sediment. After centrifugation and aspiration, the supernate must be clear; that is, it must be as clear as fresh normal serum with a TG concentration between 0.5 and 0.8 mmol/L. When the supernate appeared turbid, it was filtered through a filter of 0.20-µm sw pore size (cat. no 11307; Sartorius, Göttingen, F.R.G.). To minimize clogging of the 0.20-µm filter, the filter holder (Sartorius, cat. no. 16517, diameter 25 mm) also contained an overlying 1.2-µm pre-filter. When the apo B-containing lipoproteins flocculated in a lipemic serum, the isolated supernatant was always filtered, because it was impossible to obtain a clear HDL fraction.

The value for cholesterol content of the HDL fraction was corrected for the dilution caused by the added precipitation reagent.
Specific Procedures

Method 1A and 1B. Heparin/Mn\(^{2+}\) precipitation (13, 14).
One milliliter of serum was mixed with 100 \(\mu\)L of a mixture of equal parts of a 40 g/L sodium heparinate solution (156 USP units/mg; Organon, Oss, The Netherlands) and a 1 mol/L \(\text{MnCl}_2\) solution. After 10 min, the tubes were centrifuged and the HDL fraction was isolated as described above (method 1A). Alternatively, the HDL fraction was isolated after centrifugation at 12 000 \(\times\) g for 15 min (method 1B).

Method 2. Phosphotungstate/Mg\(^{2+}\) (2, 15-17). Optimized phosphotungstate reagent was prepared by dissolving 40 g of phosphotungstic acid (Merck cat. no. 583) in distilled water. The pH of the solution was adjusted to 6.15 with 1 mol/L NaOH (15), and the reagent was diluted to 1 L with water. The magnesium concentration of the 2.5 mol/L \(\text{MgCl}_2\) solution was checked by atomic absorption spectrometry (16, 17).

One milliliter of serum was mixed with 100 \(\mu\)L of the phosphotungstate solution, followed by mixing with 20 \(\mu\)L of the 2.5 mol/L \(\text{MgCl}_2\) solution. After 15 min the HDL fraction was isolated as described.

Method 3. Dextran sulfate/Mg\(^{2+}\) (2, 11). One milliliter of serum was mixed with 100 \(\mu\)L of a mixture of equal parts of 2 mol/L \(\text{MgCl}_2\) and dextran sulfate (sodium salt, 20 g/L, mean \(M_r\) about 500 000; lot no. 1358; Pharmacia, Uppeala, Sweden). The reagent mixture was used within one week. After 5 min the HDL fraction was isolated as described above.

Method 4. Polyethylene glycol (4, 10, 18). The method described by Viikari (4) was modified because we intended to compare the capacity of the different reagents to completely remove the VLDL and LDL in lipemic serum, and so the dilution of the sera had to be kept constant. Therefore, a concentrated PEG solution had to be used. Even the twofold dilution of the serum caused by the addition of an equal volume of a diluted PEG solution to the serum (4, 18) can be the cause for an impaired precipitation.

One milliliter of serum was mixed with 200 \(\mu\)L of PEG solution (45 g/100 mL, mean \(M_r\) 6000; Fluka, Switzerland). The final PEG concentration in the serum was thus 75 g/L. The experiments leading to the choice of this concentration have been described (10). After thorough vortex-mixing, the tubes were maintained at room temperature for 15 min before the HDL fractions were isolated as described above.

Method 5. Ultracentrifugation. The HDL (\(d > 1.063\) kg/L) fractions were isolated by ultracentrifugation as described previously (14). In the analysis of lipemic sera a combined ultracentrifugation/precipitation method had to be used, because these sera had been stored at \(-20^\circ\)C, which may cause aggregation of VLDL and LDL, leading to an increase in the density of these particles and to incomplete isolation of these aggregates in the \(d < 1.063\) fraction. The reference value was obtained by ultracentrifugation at \(d = 1.006\) kg/L, followed by precipitation of the LDL in the isolated \(d > 1.006\) kg/L fraction by means of heparin/Mn\(^{2+}\). The HDL-fraction was isolated as in method 1B (15 min; 12 000 \(\times\) g).

Analytical Methods

The cholesterol concentration in the HDL fractions was determined with the catalase (EC 1.11.1.6) method of Röschlau et al. (19). Unless otherwise stated, the reagent contained 10 mmol of EDTA per liter (14). In the catalase method, each determination is corrected for its own blank. In addition to the catalase method, the “CHOD-PAP” method was used (20). In this method a sample blank determination is unnecessary.

The reagents for both methods and the standards (Preciset) were obtained from Boehringer, Mannheim, F.R.G. (cat. no 124087, 187313, and 125512, respectively). The CHOD-PAP procedure was carried out according to the instructions of the manufacturer, except that 50 \(\mu\)L of sample was used. The modifications in the catalase method have been described (10).

The between-day precision during this study, given as the coefficients of variation for three control sera with concentrations of approximately 3.2, 5.4, and 7.3 mmol of cholesterol per liter, was better than 2.3, 2.0, and 1.7\%, respectively, in both methods (\(n = 18\)). Both methods satisfy the criteria for precision established by the Center for Disease Control (CDC), Atlanta, GA. With the catalase method, the values obtained in the normal and pathological range of human serum were approximately 3\% lower than the CDC’s target values. For the CHOD-PAP method, these values were 10\% lower. After extrapolation of these results to a concentration of 1.20 mmol/L, the normal value for HDL-cholesterol values by the CHOD-PAP method agreed well with the CDC values. The catalase method gave values that were approximately 0.2 mmol/L higher.

Triglycerides were determined by semiautomated colorimetry (21). “Sinking” pre-B lipoproteins and pre-B(10) and pre-B(11) lipoproteins in the \(d > 1.063\) kg/L fractions were detected by the previously described agarose gel electrophoresis method (22). Rocket immunoelectrophoresis, performed as previously described (10), was used to examine whether the HDL fractions were contaminated with apo B-containing lipoproteins and whether apo B-lipoproteins had been co-precipitated.

Statistical Analysis

Student’s \(t\)-test for paired observations was used to test the differences for significance. The correlations between results obtained with different methods or under different conditions were calculated by using Pearson’s correlation test.

Results

Validation of the Ultrafiltration Method

We studied effectiveness of the ultrafiltration method for removing the precipitated apo B-containing lipoproteins as follows. The HDL-cholesterol concentration of 35 sera was determined with method 1B. After testing a part of the clear supernatant fractions for cholesterol, we resuspended the precipitated lipoproteins in the remaining supernate by vortex-mixing. Subsequently, the sample was ultrafiltered and the filtrate was again tested for cholesterol. A possible systematic difference in the cholesterol determination was excluded by determination of all the samples in the same series. The mean values before and after filtration were 1.29 and 1.27 mmol/L, respectively. The results did not differ significantly (\(p > 0.4\)).

We conclude that our filtration method is reliable for separating VLDL and LDL from turbid HDL fractions.

Interference of the Precipitation Reagents with the Enzymic Catalase and CHOD-PAP Method

Manganese interferes with reagents used for enzymic cholesterol analysis. Addition of EDTA to the reagent prevents this, but the pseudocholesterol concentration of the precipitation-blank solution (distilled water instead of serum diluted with the precipitation reagents) is usually not zero (7, 14). The precipitation reagents used in methods 2, 3, and 4 do not seem to interfere with enzymic cholesterol reagents (2, 3, 10). We checked these data for both cholesterol methods. The HDL fractions were isolated from 10 fresh sera by the six methods (range in the HDL-cholesterol concentration was 0.73-3.28 mmol/L). Cholesterol was determined with the catalase and the CHOD-PAP methods. The pseudocholesterol concentrations of the precipitation-blank solutions were determined with and without EDTA in the cholesterol reagents (10 mmol of EDTA per liter). Also, the accuracy of the various HDL-cholesterol methods...
was studied after we cleared up the problems of interferences in the cholesterol methods and the role of EDTA.

In the catalase method, the pseudocholesterol concentration obtained for different precipitation-blank solutions was negligible. Apparently, there is no interference with this cholesterol method—or interference is effectively corrected for. The latter possibility seems the more probable. When the heparin/Mn\textsuperscript{2+} precipitation-blank solution was analyzed with the catalase method, the absorbances of the determination blank (cholesterol reagent without oxidase) and of the real determination (cholesterol reagent with oxidase) were significantly—and equally—higher than the absorbances of the other precipitation-blank solutions. This was independent of the use of EDTA.

In the CHOD-PAP method without EDTA, the pseudocholesterol concentrations of the heparin/Mn\textsuperscript{2+} precipitation-blank solution and of the dextran sulfate/Mg\textsuperscript{2+} precipitation blank were 0.14 and 0.30 mmol/L, respectively. Those of the phosphotungstate/Mg\textsuperscript{2+} and of the PEG precipitation-blank solutions were negligible. Thus, heparin/Mn\textsuperscript{2+} and dextran sulfate/Mg\textsuperscript{2+} interfere with the CHOD-PAP reagent in the absence of EDTA. With EDTA, the pseudocholesterol concentrations of the phosphotungstate/Mg\textsuperscript{2+} blank and of the PEG precipitation blank were zero, while those of the heparin/Mn\textsuperscript{2+} blank and of the dextran sulfate/Mg\textsuperscript{2+} precipitation-blank solutions were 0.03 and 0.31 mmol/L, respectively. Apparently, EDTA decreases the pseudocholesterol concentration of the heparin/Mn\textsuperscript{2+} precipitation blank, but not of the dextran sulfate/Mg\textsuperscript{2+} precipitation-blank solution. In the next sections, all HDL-cholesterol values obtained with the various isolation methods were corrected for the pseudocholesterol concentration in the corresponding precipitation blanks.

As for the use of EDTA, the mean HDL-cholesterol values obtained after the different isolation methods and analyzed with the catalase method did not change significantly after addition of EDTA to the cholesterol reagent, except for the dextran sulfate fractions. There, the addition of EDTA resulted in a decrease of the HDL-cholesterol values with 0.07 mmol/L (p<0.02, Table 1).

In the CHOD-PAP method, addition of EDTA significantly increased the HDL-cholesterol values obtained with the heparin/Mn\textsuperscript{2+} methods (1A and 1B) by 0.12 ±0.03 mmol/L (p<0.001). It should be mentioned that these values were corrected for the pseudocholesterol concentration of the precipitation blanks, which amounted to 0.14 and 0.03 mmol/L, respectively, with and without EDTA. When not corrected for these pseudocholesterol values, the HDL-cholesterol values with and without EDTA were in better agreement (Table 1). As in the catalase method, the mean uncorrected HDL-cholesterol value obtained for the dextran sulfate fractions with the CHOD-PAP method was significantly decreased by the addition of EDTA, by 0.03 mmol/L (p<0.05, Table 1).

As to the accuracy of the HDL-cholesterol values, we compared the values obtained with the various precipitation methods with those obtained after ultracentrifugation. Cholesterol was determined with both reagents, with and without EDTA (Table 1). The ultracentrifugation values were corrected for contamination with apo B-containing lipoproteins as described further (mean 0.09 ± 0.05 mmol/L determined with rocket immunoelectrophoresis). For good insight into the results, it should again be mentioned that the catalase method produces higher values than the CHOD-PAP method (see Materials and Methods). Indeed, the HDL-cholesterol values obtained with isolation methods 2–5 were lower when analyzed with the CHOD-PAP method as compared with the corresponding catalase values. In contrast, the CHOD-PAP values obtained with the heparin/Mn\textsuperscript{2+} methods (1A and 1B) were higher, especially with EDTA present. Generally, the mean values obtained with the various isolation methods agree with the reference values when analyzed with the same cholesterol reagent. Any disagreement was mainly caused by the interference of the precipitation reagents with the cholesterol reagent and by the use of EDTA. For example, when corrected for the pseudocholesterol concentration of the precipitation-blank solution, the mean value obtained for the dextran sulfate fractions analyzed with the CHOD-PAP method is considerably too low. When not corrected, the mean value agrees better with the reference value. Addition of EDTA to both cholesterol reagents in analyzing the dextran sulfate fractions also resulted in significantly lower results. Owing to interference with the CHOD-PAP reagent, values obtained for the heparin/Mn\textsuperscript{2+} samples were too high, whether or not EDTA was used.
Table 2. Determination of HDL-Cholesterol in 32 Normal and Slightly Lipemic Sera

<table>
<thead>
<tr>
<th>Ptn method \ HDL-Chol concn, mmol/L</th>
<th>Linear regression and correl. coeff.</th>
<th>r</th>
<th>Student's t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catalase method</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1A</td>
<td>$y = 0.94x + 0.10$</td>
<td>0.98</td>
<td>$-0.02 (0.08)$</td>
</tr>
<tr>
<td>1B</td>
<td>$y = 0.90x + 0.13$</td>
<td>0.90</td>
<td>0.01 (0.07)</td>
</tr>
<tr>
<td>2</td>
<td>$y = 0.88x + 0.14$</td>
<td>0.98</td>
<td>0.02 (0.10)</td>
</tr>
<tr>
<td>3</td>
<td>$y = 0.89x + 0.08$</td>
<td>0.99</td>
<td>0.06 (0.07)</td>
</tr>
<tr>
<td>4</td>
<td>$y = 0.96x + 0.08$</td>
<td>0.99</td>
<td>$-0.02 (0.07)$</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>CHOD-PAP method</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1A</td>
<td>$y = 1.14x$</td>
<td>0.99</td>
<td>$-0.14 (0.08)$</td>
</tr>
<tr>
<td>1B</td>
<td>$y = 1.07x + 0.05$</td>
<td>0.99</td>
<td>$-0.13 (0.06)$</td>
</tr>
<tr>
<td>2</td>
<td>$y = 0.95x + 0.01$</td>
<td>0.98</td>
<td>0.05 (0.08)</td>
</tr>
<tr>
<td>3</td>
<td>$y = 0.94x + 0.04$</td>
<td>0.99</td>
<td>0.03 (0.07)</td>
</tr>
<tr>
<td>4</td>
<td>$y = 1.03x - 0.03$</td>
<td>0.99</td>
<td>0.00 (0.07)</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0.99</td>
<td></td>
</tr>
</tbody>
</table>

* Results obtained with the precipitation methods (y) were compared with those by ultracentrifugation (x). The latter values were corrected for the amount of apo B-containing lipoproteins (0.09 ± 0.05 mmol/L). Cholesterol concentrations were determined with the catalase method (with EDTA) and also with the CHOD-PAP method (without EDTA). For the dextran sulfate fractions determined with the CHOD-PAP method, values are given with and without correction for the pseudocholesterol concentration of the precipitation-blank solution.

Accuracy of the Six HDL-Methods for Normal to Slightly Turbid Serum

We prepared 32 samples with a large range in HDL-chol values by mixing high- and low-value samples, and isolated the HDL-fractions according to the six methods within four days. When a certain HDL-fraction was or seemed turbid, incomplete removal of apo B-containing lipoproteins was expected and the sample was cleared by ultrafiltration. Immediately after the fractions were isolated, cholesterol was determined by both cholesterol methods (Table 2). Because at that time we did not know the results described in the preceding section, we added EDTA to the catalase reagent in all cases. The values were corrected for the pseudocholesterol concentration of the precipitation blanks. The catalase method again produced higher values than did the CHOD-PAP method (see Materials and Methods). As mentioned, one cannot correct for the pseudocholesterol concentration of the precipitation-blank solution when dextran sulfate fractions are analyzed with the CHOD-PAP reagent. Therefore, in the statistical calculation given in Table 2 no correction is made for the dextran sulfate precipitation-blank values. For a given cholesterol method, there is generally good agreement between the results and the reference-method values. The correlation coefficients are high and the regression equations are acceptable. However, as demonstrated, the systematic difference of 0.06 ±0.07 mmol/L obtained with the catalase reagent in analyzing the dextran sulfate fractions is due to the use of EDTA in the cholesterol reagent. Furthermore, with use of the CHOD-PAP reagent without EDTA, the systematic differences of $-0.14 ±0.08$ and $-0.13 ±0.06$ mmol/L obtained for the heparin/Mn²⁺ fractions (1A and 1B) can be ascribed to interference of manganese with this cholesterol reagent. Compared with the reference-method values, the mean HDL-chol values for the precipitation methods obtained with the catalase method had an inaccuracy of maximally 0.06 mmol/L. However, this inaccuracy was obtained in analyzing the dextran sulfate fractions with cholesterol reagent containing EDTA. When this is taken into account, the various precipitation methods are negligibly inaccurate.

In the CHOD-PAP method, the various precipitation methods, when corrected for the pseudocholesterol concentrations, had an inaccuracy of $-0.45$ to $+0.14$ mmol/L. After correction for all interferences in this cholesterol method, as mentioned in the previous section, the inaccuracy diminished to no more than 0.06 mmol/L.

Immunoechemical Examination of the Fractions

The presence of apo B-containing lipoproteins and the absence of α-lipoproteins in the HDL fractions were determined quantitatively in 10 fresh sera by rocket immunoelectrophoresis (10). To diminish the methodological imprecision in the α-lipoprotein determination, the six HDL fractions obtained for each serum were not only tested in the same dilution, but also simultaneously on the same plate. None of the HDL fractions, except that isolated by ultracentrifugation, contained an appreciable amount of apo B-containing lipoproteins. The apo B-associated cholesterol present in the ultracentrifugation fractions averaged 0.09 ±0.05 mmol/L (range 0.06-0.34 mmol/L).

The amount of α-lipoproteins in the HDL fractions obtained by precipitation was compared with that obtained by ultracentrifugation. Owing to the good recoveries with the
ultracentrifugation procedure (10, 14), we assumed that ultracentrifugation does not result in loss of α-lipoproteins. The percentage of α-lipoproteins recovered in the HDL fractions prepared by the different precipitation methods amounted to 98.4% or more (98.4-103.5%). Results obtained with each of the precipitation methods did not differ significantly from the results obtained by ultracentrifugation (p >0.2-0.7).

Application of the Six HDL-Methods to Turbid and Milky Sera

From 22 lipemic serum samples, the HDL-fractions were isolated by the six methods. The TG values of the sera averaged 92 ±4.1 mmol/L (range from 2.5 to 18.5 mmol/L). The sera had been stored for about a month at −20 °C. After thawing, the fractions were isolated within four days and their cholesterol concentration was determined immediately after. Ultrafiltration was used to clear turbid or apparently turbid supernates. Table 3 gives the percentage of sera that were clear after centrifugation, and also indicates at which TG concentration the fractionation was unsuccessful and the supernate appeared turbid. There were large differences in the percentages of HDL fractions that could be obtained by the different precipitation methods (Table 3).

After we determined the cholesterol concentration in the fractions by the catalase method with EDTA present, values obtained with a given precipitation method and the reference method generally agreed well (Table 4). Only the results obtained with the heparin/Mn²⁺ method (1B) were significantly too low by 0.05 ±0.07 mmol/L (p <0.005). The good agreement between the results was valid for all fractions isolated clear and also for the fractions that appeared turbid after isolation. Thus, the selection of the samples on the basis of turbidity was in our hands a good criterion for possible contamination with apo B-containing lipoproteins. The percentages given in Table 3 can therefore be considered to be the percentages of sera from which the apo B-containing lipoproteins could be removed successfully.

With the heparin/Mn²⁺ precipitation reagent (method 1A), only slightly lipemic serum could be fractionated successfully. The removal of apo B-containing lipoproteins could be improved when the HDL fractions were isolated at 12 000 × g (method 1B). However, this was only effective for sera with TG concentration of <4.1 mmol/L. The phosphotungstate method removed apo B-containing lipoproteins with reasonable efficiency and was effective in sera with TG concentrations <7.6 mmol/L. The percentage of clear fractions obtained with the dextran sulfate method was small. The PEG method was the most efficient for removing apo B-containing lipoproteins from lipemic sera. Only sera with a TG concentration of 18.5 mmol/L or greater showed a (slightly) turbid supernate.

Discussion

Precipitation methods are widely used for determining HDL-cholesterol. However, they do not always give similar results when compared with the ultracentrifugation method. Some reasons for disagreement may be: incomplete precipitation and removal of apo B-containing lipoproteins; coprecipitation of α-lipoproteins; presence of sinking pre-β lipoproteins (14) or of apo B-containing lipoproteins in the d >1.063 kg/L fraction, and interference of the precipitation reagents or of the KBr used in the ultracentrifugation procedure with the cholesterol measurement. Because of the excellent precision of the enzymic cholesterol determination in serum (23) and the absence of interference with KBr (14), we also use this procedure for HDL-cholesterol analysis. Precipitation with heparin/Mn²⁺ may then result in inaccurate HDL-cholesterol values, caused by interference of the manganese with the cholesterol reagent (7, 8, 24). Other precipitation reagents do not seem to interfere.

| Table 4. Results for Determination of HDL-Cholesterol in 22 Lipemic Sera with the Catalase Reagent, with Use of EDTA*

<table>
<thead>
<tr>
<th>HDL method</th>
<th>Linear regression and correl. coeff.</th>
<th>HDL-cholesterol, mmol/L</th>
<th>ρ (Student's t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin/Mn²⁺, 2300 × g</td>
<td>$y = 0.96x + 0.05$</td>
<td>0.81 (0.17)</td>
<td>-0.02 (0.05)</td>
</tr>
<tr>
<td>Heparin/Mn²⁺, 12 000 × g</td>
<td>$y = 0.89x + 0.04$</td>
<td>0.74 (0.16)</td>
<td>0.05 (0.07)</td>
</tr>
<tr>
<td>Phosphotungstate/Mg²⁺</td>
<td>$y = 0.98x + 0.03$</td>
<td>0.81 (0.16)</td>
<td>-0.01 (0.05)</td>
</tr>
<tr>
<td>Dextran sulfate/Mg²⁺</td>
<td>$y = 1.02x - 0.01$</td>
<td>0.80 (0.18)</td>
<td>-0.01 (0.06)</td>
</tr>
<tr>
<td>Polyethylene glycol</td>
<td>$y = 1.04x - 0.02$</td>
<td>0.80 (0.18)</td>
<td>-0.01 (0.03)</td>
</tr>
<tr>
<td>Ptn. in the d &gt;1.006 kg/L fraction</td>
<td>—</td>
<td>0.79 (0.17)</td>
<td>—</td>
</tr>
</tbody>
</table>

* The results obtained with the direct precipitation methods (y) and the combined ultracentrifugation/precipitation method (x) are compared. When needed, fractions ultrafiltered to eliminate apo B-containing lipoproteins.
The possible interference of the various precipitation reagents was checked by using two different cholesterol reagents. Apparently, the catalase method was not interfered with by the various precipitation reagents. Although the heparin/Mn\textsuperscript{2+} precipitation blank solution resulted in higher absorbances, the net effect was zero, because in this cholesterol method each determination is corrected for its own blank. Thus, none of the precipitation reagents really interfered with the catalase reagent. Not only is use of EDTA not necessary, its use results in lower values in the analysis of dextran sulfate fractions.

The CHOD-PAP method was less suitable for analysis of HDL fractions obtained with the heparin/Mn\textsuperscript{2+} and the dextran sulfate/Mg\textsuperscript{2+} method and the dextran sulfate/Mg\textsuperscript{2+} method. These precipitation reagents interfere with the CHOD-PAP method. The pseudocholesterol concentrations of the precipitation-blank solutions were 0.14 and 0.30 mmol/L, respectively. Use of EDTA in the CHOD-PAP reagent diminished the pseudocholesterol value of the heparin/Mn\textsuperscript{2+} precipitation-blank solution to 0.03 mmol/L. The value obtained for the dextran sulfate/Mg\textsuperscript{2+} precipitation-blank solution was not influenced by EDTA. Values obtained with the CHOD-PAP reagent for the heparin/Mn\textsuperscript{2+} fractions and for the dextran sulfate/Mg\textsuperscript{2+} fractions were inaccurate, at least when the values were corrected for the pseudocholesterol concentration of the precipitation blanks. The results for the dextran sulfate fractions agreed better with the reference values when not corrected for the pseudocholesterol concentration. This can be explained as follows. Dextran sulfate interferes with the CHOD-PAP reagent. In the precipitation-blank solution the dextran sulfate concentration is higher than in the HDL fractions obtained with the dextran sulfate method. In the HDL fractions most if not all of the dextran sulfate has been removed by complexing with apo B-containing lipoproteins. The pseudocholesterol concentration caused by the remaining dextran sulfate in the HDL fractions is considerably lower than that of the precipitation-blank solution and it is uncertain whether or not a correction for the pseudocholesterol concentration is necessary. Furthermore, it may be possible that this correction varies with the amount of VLDL and LDL removed from the serum. According to this line of reasoning, the CHOD-PAP method, then, is not suitable for obtaining accurate HDL-chol values for dextran sulfate fractions unless each cholesterol determination is corrected for its own blank, as in the catalase method.

The interference of the heparin/Mn\textsuperscript{2+} solution cannot be explained in the same way. Although for the dextran sulfate fractions more nearly accurate values were obtained when not corrected for the pseudocholesterol value of the precipitation blank, it was demonstrated that for the heparin/Mn\textsuperscript{2+} fractions more nearly accurate values were obtained when corrected for the pseudocholesterol concentration of the heparin/Mn\textsuperscript{2+} precipitation blank. In addition, this value for the heparin/Mn\textsuperscript{2+} precipitation-blank solution could be diminished by the use of EDTA in the cholesterol reagent. We speculate that the side reaction of the manganese with the cholesterol reagent is optimal at a manganese concentration corresponding to that in the HDL fractions and is less at higher manganese concentrations such as are present in the precipitation-blank solution. It is also possible that serum components enhance the side reaction of the manganese with the cholesterol reagent. In any case, this interference can only accurately be corrected by making a cholesterol blank determination for each cholesterol determination by omitting the cholesterol oxidase, as in the catalase method. EDTA diminished the pseudocholesterol concentration of the heparin/Mn\textsuperscript{2+} precipitation-blank solution, but resulted in a larger inaccuracy of the HDL chol values obtained with the CHOD-PAP method. Thus, EDTA is not needed in the CHOD-PAP method in the analysis of phosphotungstate- and PEG fractions, while it results in inaccurate values when heparin/Mn\textsuperscript{2+} fractions and dextran sulfate fractions are analyzed. As mentioned, it also is not needed in the catalase method.

In the analysis of sera, the inaccuracy of the HDL-chol values obtained with the various precipitation methods is mainly ascribable to interference of these precipitation reagents with the cholesterol reagents. Also, the use of EDTA sometimes results in inaccurate HDL-chol values. After correction for this interference, the inaccuracy of the various mean HDL-chol values was negligible. Evidently the various precipitation methods are rather specific. Their specificity was examined by rocket immunoelectrophoresis. The ultracentrifugation method, used by us as the reference method, was not specific. The d > 1.063 kg/L fractions were not free of apo B-containing lipoproteins, thus increasing HDL-chol by 0.09 ± 0.05 mmol/L (range 0.06–0.34 mmol/L). In contrast, the fractions obtained by precipitation contained only negligible amounts of these lipoproteins. Possible co-precipitation of α-lipoproteins was also investigated. From other studies (14) the reliability of the ultracentrifugation method was known. Contrary to Warnick et al. (12), we did not measure the presence of α-lipoproteins in the sediments VLDL and LDL, but we estimated the loss of α-lipoproteins in the HDL-fractions obtained by precipitation by comparison with the concentration in the ultracentrifugal fractions. Our procedure is probably less sensitive and is possibly more influenced by the imprecision of the rocket method. We therefore improved the precision by analysis of all HDL fractions obtained by the different methods from one serum on the same plate. The advantage of our procedure is that the HDL fractions obtained by ultracentrifugation and precipitation can be handled in the same way. Washing of the VLDL + LDL precipitates, which may be a source of error, is not necessary. Within the experimental error of the method we could not perceive a significant loss of HDL in the different isolation procedures. These findings disagree with those of Warnick et al. (12), who found a slight co-precipitation of apo A1 in all precipitation methods.

There were large differences in the percentages of clear HDL fractions obtained by the different precipitation methods. We could demonstrate that when supernates were clear—having the same clarity as fresh serum with a TG value between 0.5 and 0.8 mmol/L—the HDL-chol values were accurate, indicating complete removal of apo B-containing lipoproteins. The same was found when turbid fractions were filtered. In analyzing lipemic serum samples, the PEG method resulted in the highest percentage of clear fractions (96%), followed by the phosophotungstate method (64%). The methods were effective for sera with TG concentrations <18.5 and <7.6 mmol/L, respectively. The efficiency of the heparin/Mn\textsuperscript{2+} method (1A) for removing apo B-containing lipoproteins from lipemic serum was low. This is in agreement with the literature (6, 25–27). HDL-fractions obtained from sera with a TG concentration of 2.8 mmol/L or greater were turbid. More intense centrifugation (method 1B) was less effective than precipitation in the d > 1.006 kg/L fraction (method 5). Because this is a laborious modification, ultracentrifugation is indicated. The ultracentrifugation procedure we introduced (14) appeared to be very effective in removing apo B-containing lipoproteins. This procedure, slightly modified, was also successfully used by others (6).

The dextran sulfate method removes apo B-containing lipoproteins poorly. This is in contradiction with the findings of Finley et al. (11), who could successfully fractionate serum with a very high TG concentration.

In this study it became clear that the optimized phosphotungstate method and the PEG method are the best.
methods for HDL-chol analysis when enzymic cholesterol analysis is used. Warnick et al. (12) found that the phosphotungstate method resulted in values that were about 5% too low, but we could not confirm this. Besides, this method appeared to be very sensitive to variations in the reagent concentrations or in the temperature (12). In the polyethylene glycol method the source of the PEG preparation and the freshness and the pH of the sera slightly influence the accuracy (10). Other precipitation methods also seem to be influenced by the pH of the mixture or the freshness of the serum (12, 28, 29). With the PEG method, slightly lower values were found for samples containing EDTA, for unknown reasons (10).

In summary, HDL-chol values obtained with the various precipitation methods and the ultracentrifugation method agreed well for both normal and lipemic sera, if turbid supernates were ultrafiltered. Any differences were largely ascribable to interferences with the enzymic cholesterol reagents. When enzymic cholesterol analysis is used, the phosphotungstate method or the PEG method should be preferred, and the latter method covers an especially wide range of TG concentrations.

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