A Study of the Use of Polyethylene Glycol in Estimating Cholesterol in High-Density Lipoprotein

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

We studied polyethylene glycol 6000 precipitation of lipoproteins other than high-density lipoproteins, before cholesterol is estimated in the supernate. Other lipoproteins in the supernatant fractions were detected by using rocket immunoelectrophoresis. A polyethylene glycol concentration of 75 g/L in the final mixture appeared to be optimal, and results agreed with those obtained by ultracentrifugation. Differences in serum pH, use of polyethylene glycol from different suppliers, or the presence of ethylenediaminetetraacetate resulted in values that differed significantly (by 40 to 60 μmol/L) from the reference values. Polyethylene glycol did not interfere in four different methods for determination of cholesterol. In combination with an enzymic cholesterol method, the polyethylene glycol method appeared to be very precise, even when lipemic sera (triglycerides up to 5.5 mmol/L) were analyzed that had diminished high-density lipoprotein cholesterol values. We consider this method a method of choice, especially when lipemic sera are tested and enzymic cholesterol analysis is used.

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

HDL-chol\(^1\) in serum seems of increasing interest, owing to the inverse correlation between the HDL-chol concentration and the risk for atherosclerosis (1–3). Therefore an efficient and precise method for its determination is necessary.

HDL in serum is mostly determined by a method involving precipitation. By addition of polyions and divalent cations, the VLDL and LDL are precipitated and HDL is determined as HDL-chol in the supernate. Burstein and Scholnick (4) described several variants of this method, using heparin, phosphotungstate, or dextran sulfate as the polyions and Mn\(^{2+}\) or Mg\(^{2+}\) as the divalent cations. Viikari described another method, based on steric exclusion of the VLDL and LDL with PEG (5).

The heparin/Mn\(^{2+}\) method is the most commonly used. However, in lipemic serum or in plasma containing EDTA, use of this method often results in incomplete removal of VLDL and LDL (6–8). A second disadvantage is interference by Mn\(^{2+}\) ion with the enzymic cholesterol determination (9–11). The Mg\(^{2+}\) ion, used in the dextran sulfate and phosphotungstate methods, does not seem to interfere, nor does PEG (12–16). Therefore, these methods are preferred when enzymic cholesterol analysis is used.

We evaluated the precipitation procedure with PEG for HDL-chol analysis by comparison with five other isolation procedures (17). First, the optimal conditions for this method had to be studied extensively. Opinions differ as to the optimal final PEG concentration in the sample, (5, 16, 18). Our first goal was to settle this disagreement. Secondly, we compared the results with those obtained by ultracentrifugation. Thirdly, we studied some other factors, to get more insight into the usefulness and practicality of the PEG method for HDL-chol analysis.

Materials and Methods

Sera

Serum, freshly obtained from overnight-fasting subjects, was stored at 4 °C for a maximum of four days. In some cases we used serum from nonfasting subjects, which had been stored for one or two days at room temperature.

Procedures

Ultracentrifugation. We isolated the HDL-fractions (d > 1.063 kg/L) by ultracentrifugation as described previously, mixing 3 mL of serum with 3 mL of the d = 1.129 kg/L density solution (9).

Polyethylene glycol precipitation method. When not otherwise indicated, the final PEG concentration in the serum was 75 g/L. The PEG reagent was prepared as follows: 45 g of polyethylene glycol (mean M\(_{\text{r}}\) 6000; Fluka A.G., Buchs, Switzerland) was dissolved in about 60 mL of distilled water and diluted to 100 mL. This reagent was used for no more than two months at room temperature. It was kept in a dark-colored bottle.

We precipitated the non-HDL fraction in plastic 100 \times 15 mm conical tubes. To 1 mL of serum, 200 μL of the 450 g/L PEG solution was added. After thorough vortex-mixing of their contents, the tubes were kept at room temperature for 15 min, then centrifuged at room temperature at about 2300 \(\times\) g for 15 min. The upper part of the supernates was carefully aspirated with a Pasteur pipette. After the cholesterol content of the HDL-fractions was determined, the values were corrected for the dilution caused by the added precipitation reagent.

"Rocket" immunoelectrophoresis. The presence of apo B containing lipoproteins (VLDL and LDL) in the HDL-fractions obtained by ultracentrifugation or by precipitation was quantitatively determined by rocket immunoelectrophoresis. The \(\alpha\)-lipoproteins present in the HDL-fractions were also determined by this technique.

Antisera against human \(\beta\)-(apo B) or \(\alpha_1\)-(HDL) lipoproteins and the \(\beta\)-lipoprotein control serum (700 mg of apo B per liter; 3.31 mmol of cholesterol per liter) were obtained from Behringwerke A.G., Marburg, F.R.G. The method was performed according to Laurell (19), with agarose from BDH (no. 33006; BDH Ltd, Poole, U.K.). The agarose concentration in the barbitral buffer (50 mmol/L, pH 8.6) was 7 g/L. The concentration of the antisera in the gel was 8 mL/L. For the electrophoresis we used the LKB Multiphor 2117, cooled with running tap water. The field strength was 20 V/cm and an electrophoresis run lasted 4 h. The control sera and the HDL fractions were diluted (10–40 fold) with the barbitral buffer. The immunoprecipitins were stained with a 5 g/L solution of Coomassie Brilliant Blue R-250 (Serva GmbH, Heidelberg,
F.R.G.). Later in our study, the immunoprecipitins were made visible with manganese chloride/sodium phosphotungstate according to Hueck et al. (20).

To minimize methodological imprecision of the rocket anti-α-method, all the fractions obtained of one serum were tested on the same plate.

Analytical Methods

Cholesterol concentration was determined with the catalase (EC 1.1.1.16) method of Röschlau et al. (21). Reagent and standards (Preciset) were obtained from Boehringer (no. 124087 and 125512; Boehringer, Mannheim, F.R.G.). The procedure was carried out according to the instructions supplied by the manufacturers, except that 50 μL instead of 20 μL of sample was used. After the sample was mixed with 5 mL of reagent, about half of the mixture was decanted into another tube. To one portion cholesterol oxidase (EC 1.1.3.6) was added and the other portion was used as the blank. It is not necessary for the 2.5 mL of the sample–reagent mixture to be exact, because the cholesterol oxidase is present in excess. It is important that during the measurement of absorbances any slight drift in the photometer be immediately noticed, by regularly blanking the apparatus, because a drift of only 3 milli-absorbance units causes a bias of 0.04 mmol/L in the HDL-chol values. We found the between-day precision of the method, expressed as the coefficient of variation for three control sera with concentrations of 3.2, 5.4, and 7.3 mmol/L, to be 2.3, 1.4, and 1.2%, respectively (n = 18).

To study possible interference of PEG with various cholesterol methods, we examined the above-mentioned catalase method together with the enzymic "CHOD-PAP" method (22) with reagent obtained from Boehringer (no. 187313), the chemical method of Huang et al. (23), and the Abell-Kendall method (24).

Triglycerides were determined by a semi-automated colorimetric method (25).

The presence of "sinking" pre-β-lipoproteins and of β- and pre-β-lipoproteins in the HDL fractions was detected by the previously described agarose-gel electrophoresis method (26).

Results

The Choice of the Optimal PEG Concentration

To 10 fresh sera ranging in HDL-chol concentration from 0.90 to 1.94 mmol/L, we added increasing volumes (0–1000 μL) of concentrated PEG solutions (450–600 g/L) and determined the supernatant cholesterol concentration (Figure 1). Below a final PEG concentration of 65 g/L, the cholesterol concentration in the supernate decreased strongly, at 65 g/L the decrease was less abrupt, and from 65 to 92 g/L there was a slight and gradual decrease. From 92 to 140 g/L the decrease became greater. In all cases, values obtained with a given final PEG concentration differed significantly from those obtained with other concentrations in the series tested.

We analyzed the fractions obtained in this manner by use of the anti-α-rocket immunoelectrophoresis method. Samples obtained by use of a final PEG concentration of 0–50 g/L were tested after appropriate dilution with buffer; the samples obtained with PEG >50 g/L were tested undiluted.

The untreated serum, tested in various dilutions, was used for the calibration curve. At final PEG concentrations of 0, 25, 50, and 65 g/L, the mean percentage of apo B-containing lipoproteins present in the supernates, compared to the untreated serum, was 100, 104, 21.9, and 1.6%, respectively (Figure 1). The amount of apo B-containing lipoproteins in the 50 and 65 g/L fractions corresponded to 0.86 and 0.06 mmol of cholesterol per liter, respectively. Furthermore, no apo B-containing lipoproteins could be detected in fractions obtained at final PEG concentrations of 70 g/L or greater. We conclude that a final PEG concentration of 70 g/L or greater is advisable if one is to obtain HDL fractions free from apo B-containing lipoproteins. The decrease in the cholesterol concentration in the supernate at increased PEG concentrations from 70 to 140 g/L therefore can not be the result of removal of trace amounts of these lipoproteins.

We tested for possible co-precipitation of α-lipoproteins at the final PEG concentrations in the range 0–120 g/L, by use of the rocket anti-α-immunoelectrophoresis procedure. From 0–80 g of PEG per liter in the sample, no decrease in the α-lipoprotein concentration in the supernatant fluid could be noticed (Figure 1). At 100 g/L there was a tendency for co-precipitation of α-lipoproteins. The mean value for α-lipoprotein was 3.3% lower than the value for untreated serum, but this was not significant (0.05 < p < 0.1). At 120 g of PEG per liter, the α-lipoprotein concentration was significantly lower, by 25.2% (p < 0.06). Using these PEG concentrations for HDL-chol analysis in normal serum would therefore reduce the HDL-chol value by 0.10 and 0.37 mmol/L, respectively. We conclude that the optimal PEG concentration for the determination of HDL-chol is between 70 and 80 g/L. Therefore we chose 75 g/L for routine use.

Comparison of the PEG-HDL-chol Method with the Ultracentrifugation Method

Generally the ultracentrifugation method is used as a reference method for HDL-chol determination. However, this method gave higher values than the heparin/Mn2+ precipiti-
tation method when the samples contained sinking pre-\( \beta \) lipoproteins (9). In the absence of sinking pre-\( \beta \) lipoproteins, both methods gave values that differed insignificantly. Therefore, the ultracentrifugation method was again used as the reference method. We analyzed 32 fresh sera by both methods. None of the HDL fractions obtained by ultracentrifugation demonstrated a clear sinking pre-\( \beta \) band on agarose gel. However, all HDL fractions showed a slight coloring in the \( \beta \) and pre-\( \beta \) region, indicating the presence of minor amounts of these lipoproteins. Albers et al. (15), using an immunological test, came to the same conclusion. They arbitrarily corrected the values obtained by ultracentrifugation for the apo B-associated cholesterol in the \( d > 1.063 \) kg/L fraction (0.15 mmol/L) and also by 5% for losses in the isolation procedure. In our procedure, the lipoproteins in the \( d < 1.063 \) kg/L fraction were isolated by aspiration; the volumes were determined after weighing the fractions and correction for the density. Furthermore, cholesterol was determined with an enzymic method. We found that the analytical recovery of cholesterol in the top and bottom fractions was 100.6 \( \pm \) 2.5%. Calibrated on the \( \beta \)-lipoprotein control serum, the amount of apo B-associated cholesterol in 25 other (\( d > 1.063 \) kg/L) fractions as determined by the rocket method averaged 0.10 \( \pm \) 0.04 mmol of cholesterol per liter (range, 0.06-0.34 mmol/L). We therefore corrected all HDL (\( d > 1.063 \)) values by subtracting 0.10 mmol/L.

There was a good agreement between the results obtained by the two methods (Figure 2). The mean values for HDL-cholesterol obtained with the ultracentrifugation method (\( x \)) and the PEG method (\( y \)) were 1.26 \( \pm \) 0.44 mmol/L and 1.29 \( \pm \) 0.43 mmol/L, respectively (\( p > 0.1 \), Student's t-test for paired samples).

Influence of pH on the Precipitation of Apo B-
containing Lipoproteins by PEG-6000

According to Polson et al. (27), not only the final PEG-6000 concentration but also the final pH of the solution determines the selective precipitation of serum proteins. The pH of an aqueous PEG solution used by Vikari was 4.7-4.8. After addition to plasma, the pH was 7.9 owing to the buffering capacity of the plasma. Our PEG solutions in the same concentration range had a pH of 5.0-5.2. We also studied the change in pH of the serum after addition of PEG, using both fresh and aged sera. The latter serum samples had been stored for two days at room temperature. The pH of the sera was 7.90 \( \pm \) 0.05 \( (n = 12) \) and 8.65 \( \pm \) 0.24 \( (n = 24) \), respectively. After addition of 0.2 mL of a 450 g/L PEG solution to 1 mL of serum and thoroughly mixing, the pH was consistently 0.2 higher than before: the mean pH of the fresh sera was now 8.18 \( \pm \) 0.05 and of the aged sera 8.75 \( \pm \) 0.18. Thus, after PEG is added to serum samples to a final concentration of 75 g/L, the pH will not be the same in all samples. The ultimate pH after PEG addition appears to depend on the pH before addition—and, in turn, on the age of the serum.

The relation between serum pH and supernatant cholesterol concentration, as determined with the PEG method, was studied as follows. We mixed 0-50 \( \mu \)L of 0.5 mol/L \( H_2SO_4 \) or 1 mol/L NaOH with 2 mL of serum. After measuring the pH, we determined the cholesterol concentration of the supernatant with the PEG method. The results (mean of five sera) are shown in Figure 3. Evidently the supernatant cholesterol concentration is slightly increased as a result of increasing the serum pH from 6.4-9.6. An increase in the serum pH of 7.9 to 8.65 causes a significant increase in the HDL-cholesterol concentration of 0.04 mmol/L (\( p < 0.02 \)).

Comparison of Several PEG-6000 Preparations

It has been suggested that variations in PEG-6000 preparations obtained from different sources may be responsible for a bias in the HDL-cholesterol concentration (18, 28). We therefore determined HDL-cholesterol in 20 sera using the PEG-6000 preparations, final concentration 75 g/L, of Fluka (no. 81260), BDH (no. 29577), Merck (no. 807491), Baker (no. 1616), and Sigma (no. P-2139). The results obtained with a given preparation differed significantly from those obtained with the Fluka preparation (Table 1), which was arbitrarily chosen as the basis for comparison. The highest and lowest mean values (with the preparations of Sigma and Merck) differed by 0.06 mmol/L.

To study whether these differences were caused by differences in the pH, we measured the pH of the PEG solutions after 10-fold dilution (Table 1) and found large differences. However, no significant correlation was found between mean HDL-cholesterol and pH of the various PEG preparations (\( p > 0.1 \)).

Precision of the PEG-HDL-cholesterol Method

Five fresh sera with TG concentrations in the range 2.0 to 5.5 mmol/L and with rather low HDL-cholesterol concentrations were divided into 1.5-mL aliquots and stored for 14 days at \(-20^\circ C\) or at \( 4^\circ C \). During 15 days portions were analyzed 10 times. (Table 2). Even at these low-HDL-cholesterol-concentrations the precision of the method is satisfactory. It is independent of the TG concentration of these sera. The coefficient of variation for the HDL-cholesterol values in the sera held at \( 4^\circ C \) is higher than those in the sera held at \(-20^\circ C \). Furthermore, the HDL-cholesterol values for samples stored at \( 4^\circ C \) are slightly but insignificantly lower (\( p > 0.05 \)). We conclude that serum stored for 14 days at \( 4^\circ C \) or at \(-20^\circ C\) still gives reliable HDL-cholesterol values. Even though the differences in the CVs and the HDL-cholesterol values obtained for the sera stored under the different conditions are negligible, it seems advisable to store samples at \(-20^\circ C\).

Interference Studies

Interference of PEG-6000 in some methods used to determine cholesterol. Possible interference of PEG-6000 in four methods for determination of cholesterol was examined: the
enzymatic catalase method, the CHOD-PAP method, and the chemical (Huang et al. and Abell–Kendall) methods. Either 0.2 mL of distilled water or 0.2 mL of 450 g/L PEG solution was added to 1 mL of Preciset standard (4000 mg/L) and cholesterol was determined. The differences between the cholesterol concentrations found and expected in these diluted standard solutions were less than 1% in all four methods. Thus PEG does not interfere in these methods, at least in the determination of free cholesterol.

**Interference of anticoagulants in the PEG-HDL-chol method.** Possible interference of the anticoagulants heparin and EDTA in the PEG method was studied. We divided each of 45 serum samples into three 1-mL portions. To one portion EDTA (1 mg/mL) was added; to another portion 20 μL of heparin solution (3 g of lithium heparinate per deciliter) was added. HDL-chol in all three portions was determined with the PEG method (Table 3). Based on the resulting regression equation and the correlation coefficient we conclude that there is reasonably close agreement between the results obtained for the treated and untreated samples. However, the addition of EDTA resulted in values that were significantly lower (p < 0.001) as compared to the untreated serum samples. The heparin-treated samples did not differ significantly from the untreated samples (p > 0.6).

**Discussion**

This study has revealed some important conditions for an effective performance of the PEG–HDL-chol method. Concerning the optimal PEG concentration in the sample, conflicting results have been reported. Initially a concentration of 120 g/L was used (5), a concentration based on ultracentrifugal and electrophoretic analyses. Others (16) considered 100 g/L as optimal, based on double rocket immunoelectrophoresis. These results were not compared with another HDL-chol method, although the reference ranges of the HDL-chol values agreed well with those reported by others. Warnick et al. (18) also used 120 g/L. The HDL-chol values they obtained were about 12 and 20% lower than values by the heparin/Mn³⁺ method and the ultracentrifuge method, respectively. They suggested that a concentration between 60 and 80 g/L might give more specific results, which we confirm in our study. Using rocket immunoelectrophoresis, we detected no apo B-containing lipoproteins in the supernatant fluid when precipitation had been performed with a PEG concentration >70 g/L. At 80 g/L or greater there was a tendency for co-precipitation of α-lipoproteins. Therefore, we chose a final concentration of 75 g/L. As a result of the co-precipitation of α-lipoproteins, the HDL-chol values obtained at PEG concentrations of 100 and 120 g/L were 0.1 and 0.37

**Table 1. Comparison of PEG-6000 from Different Suppliers**

<table>
<thead>
<tr>
<th>Supplier</th>
<th>pH</th>
<th>Linear regression and corr. coeff.</th>
<th>Concentration</th>
<th>Student's t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDH</td>
<td>5.80</td>
<td>y = 1.03x + 0.06</td>
<td>Mean (SD), mmol/L</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Merck</td>
<td>5.13</td>
<td>y = 1.01x + 0.01</td>
<td>(0.15)</td>
<td>r = 0.99</td>
</tr>
<tr>
<td>Baker</td>
<td>6.70</td>
<td>y = 1.02x - 0.05</td>
<td>(0.15)</td>
<td>r = 0.99</td>
</tr>
<tr>
<td>Sigma</td>
<td>7.05</td>
<td>y = 1.01x - 0.05</td>
<td>(0.15)</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Fluka</td>
<td>6.00</td>
<td>—</td>
<td>1.00</td>
<td>(0.14)</td>
</tr>
</tbody>
</table>

* After adding PEG solutions (final conc, 75 g/L), we determined HDL-chol concentration in 20 sera. Results obtained with each preparation (y) were compared to those obtained with the Fluka preparation (x). There was no significant correlation between the mean HDL-chol values and the pH of the 10-fold diluted PEG reagents.

* Difference between x and y.

**Table 2. Precision of the PEG-HDL-chol Method**

<table>
<thead>
<tr>
<th>TG concn, mmol/L</th>
<th>Mean HDL-chol, mmol/L (and CV, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Storage at 4 °C</td>
</tr>
<tr>
<td>2.0</td>
<td>0.98 (2.2)</td>
</tr>
<tr>
<td>2.5</td>
<td>0.77 (3.3)</td>
</tr>
<tr>
<td>3.3</td>
<td>0.67 (3.4)</td>
</tr>
<tr>
<td>3.8</td>
<td>0.89 (1.8)</td>
</tr>
<tr>
<td>5.5</td>
<td>0.75 (2.7)</td>
</tr>
</tbody>
</table>

* The HDL-chol concentration was determined in portions of sera with above-normal TG concentration and rather low HDL-chol concentration. Portions were stored at 4 °C and -20 °C. Each was assayed 10 times during 15 days.

**Table 3. Influence of Anticoagulants in the PEG–HDL-chol Method**

<table>
<thead>
<tr>
<th>Anticoagulants</th>
<th>Mean concn (and SD)</th>
<th>Linear regression and corr. coeff.</th>
<th>Student's t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA samples</td>
<td>y = 1.02x - 0.08</td>
<td>1.13 (0.25)</td>
<td>0.05 (0.04)</td>
</tr>
<tr>
<td>Heparin samples</td>
<td>y = 0.91x + 0.1</td>
<td>1.18 (0.23)</td>
<td>0.00 (0.04)</td>
</tr>
<tr>
<td>Untreated</td>
<td>—</td>
<td>1.18 (0.24)</td>
<td>—</td>
</tr>
</tbody>
</table>

* On 45 serum samples containing EDTA (1 mg/mL) or lithium heparinate (0.5 g/L) the PEG–HDL-chol assay was performed (y) and results were compared with those for untreated serum (x).
mmol/L too low. Using 75 g/L, we could demonstrate good agreement with corrected values obtained by the ultracentrifugation technique.

Keeping the final concentration constant (75 g/L), we examined whether differences in the various PEG preparations could be responsible for the discrepancy in reported optimal concentrations. We used the PEG-6000 preparation of Fluka, as did Viikari (5). PEG from BDH, Merck, Baker, and Sigma gave significantly different HDL-chol values as compared with values obtained with the Fluka preparation, the maximal difference (0.06 mmol/L) being obtained with the preparations of Sigma and Merck. It seems unlikely that the discrepancy in reported optimal PEG concentration is ascribable to differences in the various preparations.

On storage of serum at room temperature its pH increases. We show that the HDL-chol value obtained with the PEG method increases when the serum pH increases. Therefore, it may be expected that stored serum will give a higher HDL-chol value when determined with the PEG method. The maximal increase amounted to 0.04 mmol/L. Although the various PEG preparations had, after dilution, a large variation in pH, we could demonstrate that this did not influence the HDL-chol value. There was no significant correlation between the mean HDL-chol values obtained with the various PEG solutions and the pH of these solutions (p > 0.10). Besides, the differences in the pH would largely be neutralized by the buffering capacity of the serum.

With the PEG–HDL-chol method, results for EDTA-containing sera are significantly lower than for untreated samples, about 0.05 mmol/L. The cause is not clear. Possibly there is, in contrast to the untreated samples, a slight co-precipitation of HDL with a PEG concentration of 75 g/L. Decreasing the concentration to about 70 g/L might solve this problem, but other causes have not been ruled out.

In our hands, results by the PEG–HDL-chol method agreed well with the corrected ultracentrifuge values. Furthermore, the precision of the method was good, even for sera with low HDL-chol concentrations and was independent of the TG concentration of the serum. Sera with a TG concentration up to 5.5 mmol/L always gave clear supernates, indicating the absence of VLDL and LDL. Therefore, this method seems effective for analysis of lipemic sera. In addition, polyethylene glycol 6000 did not interfere with two enzymic cholesterol methods or with the Huang et al. or Abell–Kendall methods, at least in the determination of free cholesterol.

Although several factors may slightly influence the accuracy of the PEG method, we consider it a method of choice for HDL-chol determination. In searching for a more reliable method, which can also be used in lipemic sera and in combination with enzymic cholesterol analysis, the PEG method should be considered together with methods involving dextran sulfate or phosphotungstate.

References