Comparison of two homogeneous assays with a precipitation method and an ultracentrifugation method for the measurement of HDL-cholesterol

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We report on the performance of four HDL-cholesterol assays: a homogeneous method based on polyethylene glycol-modified enzymes/α-cyclodextrin sulfate (PEGME; Kyowa); a homogeneous method based on polyanion–polymer/detergent (PPD; Daiichi); the usual precipitation method with phosphotungstic acid/MgCl₂ (PTA); and an ultracentrifugation (UC) procedure. The homogeneous HDL-cholesterol assays (performed with automated analyzers) were precise and correlated well with the PTA and UC assays. The specificity and accuracy of the PEGME method were better than those of the PPD method.

The measurement of HDL-cholesterol (HDL-C) in serum is of clinical importance, given that an inverse correlation between HDL-C concentration in serum and the risk of atherosclerotic disease has been shown in laboratory, clinical, and epidemiological studies. Today, the consensus is that HDL-C is an independent risk factor for coronary heart disease. Therefore, the National Cholesterol Education Program (NCEP) Adult Treatment Panel II has updated its guidelines for the diagnosis and treatment of hypercholesterolemia to include HDL-C quantification in combination with total cholesterol measurement at the initial screening stage. HDL-C concentrations <350 mg/L indicate an increased risk for atherosclerosis, and concentrations >600 mg/L have been defined as protective.

In laboratories, HDL-C has been determined by techniques such as ultracentrifugation (UC), electrophoresis, HPLC, and precipitation-based methods. Of these, the precipitation-based methods have been the most widely used in clinical laboratories. However, they are time-consuming, require large serum volumes, and are not suited to automated analysis; these restrictions have encouraged researchers to focus on homogeneous assays.

Here we report our assessment of the analytical performance of two homogeneous methods designed for use in routine chemical laboratory work: a polyethylene glycol-modified enzymes/α-cyclodextrin sulfate (PEGME) assay from Kyowa and a polyanion–polymer/detergent (PPD) method from Daiichi. We compared the HDL-C concentrations obtained by these procedures with the phosphotungstic acid/MgCl₂ precipitation (PTA) method and a UC method that uses centrifugation to remove triglyceride-rich lipoproteins from plasma.

The PTA method has been chosen because most laboratories use it as a routine method. The UC comparison method has also been included because it is routinely used as the comparison method for determining accuracy.

Materials and Methods

Samples

For the present study we obtained blood samples from 252 patients admitted to the regional Del Río Hortega University Hospital in Valladolid, Castilla y León, Spain. All procedures were in accordance with the Helsinki Declaration of 1975, as revised in 1983. Blood samples were collected in Venoject tubes (Terumo Europe) from patients who had fasted overnight. The samples were centrifuged at 2000g for 15 min. The serum samples thus obtained were processed immediately, and triglycerides, cholesterol, and HDL-C were measured within 1 day.

HDL-C Concentrate Isolation

HDL-C was isolated and concentrated by precipitating the VLDL and LDL particles from a serum pool with a
mixture of 0.44 mmol/L phosphotungstic acid and 20 mmol/L MgCl₂ at a ratio of 1:3.5. After agitation for 30 s (to assure proper homogenization) the mixture was left at room temperature for 10 min and then centrifuged at 4000g for 10 min. The resulting supernatant was dialyzed through a semipermeable membrane with phosphate buffer (pH 7.2) to remove phosphotungstic acid and MgCl₂, and 290 g/L (290 mg/mL) KBr was added to the processed dialyze. The mixture was then centrifuged at 488 000g for 9 h at 16 °C in a TLA100.3 ultracentrifuge rotor (Beckman). The HDL-C concentrate thus obtained was dialyzed with phosphate buffer (pH 7.2) to remove the KBr. Finally, an assay to verify the absence of lipoproteins other than HDL was performed using the Paragon system (Beckman) for electrophoresis and lipoprotein staining.

**HDL-C assays**

*Homogeneous assays.* The PEGME method (Kyowa) involved two reagents in solution form. Reagent 1 contained dextran sulfate, MgCl₂, sodium N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline, and 6-cyclodextrin sulfate. Reagent 2 was reconstituted before use and consisted of a lyophilized enzyme mixture containing PEG-modified cholesterol esterase, PEG-modified cholesterol oxidase, peroxidase, and 4-aminoantipyrine (10).

The PPD method (Daiichi) also involved two reagents, both in solution. Reagent 1 contained a polyanion and a synthetic polymer. Reagent 2 consisted of a mixture of cholesterol esterase, cholesterol oxidase, peroxidase, 4-aminoantipyrine, and solvent containing disodium N,N-bis(4-sulfobutyl)-m-toluidine, phthalic acid buffer (pH 5.7), detergent, and other constituents, as listed in the manufacturer’s instructions (11).

These two homogeneous assays for HDL-C were performed with a Hitachi 917 automated analyzer (Boehringer Mannheim) according to the manufacturer’s specifications.

**PTA assay.** In the PTA assay, the samples were mixed with a precipitating solution containing phosphotungstic acid and MgCl₂ (Boehringer Mannheim). After incubation and precipitation at room temperature, samples were centrifuged, and the supernatant was collected for assays. HDL-C was determined with an enzymatic endpoint assay using cholesterol oxidase, peroxidase, and a chromogenic reaction with 4-aminophenazone (CHOD-PAP) on a Hitachi 917 analyzer. The reagents were purchased from Boehringer Mannheim, and the assay was carried out according to the application protocol provided by the manufacturer.

**UC method.** In the UC method, 1.5 mL of NaCl solution (density, 1.006 kg/L) was added to 1.5-mL serum samples and then centrifuged at 488 000g for 3 h at 16 °C in 13 × 51 mm polycarbonate tubes in a TLA100.3 rotor. A 1.5-mL aliquot of the supernatant (the fraction containing the VLDL and chylomicrons) was isolated. The HDL-C in the infranatant (the fraction containing the LDL and HDL) was determined by the PTA method described above.

**Cholesterol and triglyceride measurements.** Cholesterol and triglycerides were determined enzymatically with a Hitachi 917 automated analyzer, using the CHOD-PAP and glycerophosphate oxidase-peroxidase-4-aminophenazone (GPO-PAP) methods, respectively, according to manufacturers’ recommendations and with reagents purchased from Boehringer Mannheim.

**Performance evaluation**

*Precision.* Four commercial control sera with low and medium HDL-C concentrations from Boehringer Mannheim (Precipath® HDL/LDL-C and Precinorm® L) and ITC Diagnostics (cat. no. 767660, levels 1 and 2) were used to compare the precision of the two homogeneous HDL-C assays.

The interassay imprecision (CV) was calculated from 20 different analyses of aliquots of the four controls performed on 20 different days. The intraassay CV was determined as the average of the variances obtained with 20 assays in the same analytical run in 1 day.

*Total error.* Total error is the sum of systematic error plus random error (18). The systematic error of each homogeneous assay was calculated by linear regression analysis comparison with the UC “reference” method (22). Random error was 1.96 × the total CV, which includes both intraassay and interassay CVs (18).

*Linearity.* Linearity was tested by adding various amounts of isotonic 9 g/L NaCl to the previously described HDL-C serum pool concentrate. In addition, another dilution series was obtained with the Lin-Trol® linearity set for HDL-C from Sigma Diagnostics; the HDL-C Lin-Trol was mixed with HDL-C Lin-Trol diluent at concentrations from 0% to 100%.

*Interferences.* Interference from hypertriglyceridemia was analyzed according to Glick et al. (19). Sera from hypertriglyceridemic subjects were centrifuged in solutions with the same densities as the sera, which allowed triglyceride-rich lipoproteins to rise to the top of the sample. After isolation, these lipoproteins were added to pooled serum at various concentrations (20) to obtain triglyceride concentrations of 2500–15 000 mg/L.

*Sample storage.* HDL-C was determined both in two pools of fresh serum samples (at low and medium concentrations) and two plasma pools (heparin- and EDTA-treated samples). The pools were divided into aliquots; of these, some were stored for 40 days at 4 °C; others were stored at −20 °C for the same period. The samples were then...
reanalyzed. The HDL-C concentration in each pool was measured 10 times during the study with the three field methods (the homogeneous PEGME and PPD assays and the PTA precipitation assay).

Comparison of serum, lithium heparinate, and EDTA plasma. Lithium heparinate and EDTA plasma samples were compared with serum for HDL-C in 26 patients. The samples were obtained from a single stick in no particular order. EDTA plasma was obtained with blood samples (3 mL) collected in tubes containing 0.06 mL of 0.19 mol/L K3EDTA. Each sample was measured once.

Intermethod comparison. We compared the two homogeneous HDL-C assays with the conventional PTA method, analyzing 199 samples in parallel (group I). In addition, for patients with sufficient blood sample volume (88 samples), the homogeneous assays were compared with the UC method (group II). In both groups I and II we used subjects with triglyceride concentrations <4000 mg/L (<400 mg/dL). In addition, we compared the two direct HDL-C assays with the UC method, using 27 sera from subjects with triglycerides >4000 mg/L (>400 mg/dL; group III).

STATISTICAL ANALYSIS
Descriptive statistics (means, SDs, and CVs) were calculated with Microsoft Excel, Ver. 5.0® (Microsoft). Regression analysis was calculated according the Passing and Bablok method by the Astute Statistics Add-in program for Microsoft Excel (1993–1995 DDU software, University of Leeds, UK).

Results

Analytical Performance

Precision. The assay precision results for the PEGME, PPD, and PTA methods are shown in Table 1. The total CVs were 2.9–4.0%. The intraassay CVs were 1.6–3.0%. The interassay CVs were 2.4–3.1% for the homogeneous methods and 3.3–5.5% for the PTA method.

<table>
<thead>
<tr>
<th>Assay and control</th>
<th>Intraassay</th>
<th>Interassay</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean, mg/L</td>
<td>SD, mg/L</td>
<td>CV, %</td>
</tr>
<tr>
<td>PEGME</td>
<td></td>
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<td></td>
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<tr>
<td>Precinorm L</td>
<td>444.00</td>
<td>7.54</td>
<td>1.7</td>
</tr>
<tr>
<td>Precipath</td>
<td>341.50</td>
<td>8.12</td>
<td>2.4</td>
</tr>
<tr>
<td>PPD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level 2</td>
<td>614.50</td>
<td>10.00</td>
<td>1.6</td>
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<tr>
<td>Level 1</td>
<td>316.50</td>
<td>9.33</td>
<td>3.0</td>
</tr>
<tr>
<td>PTA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precinorm L</td>
<td>423.50</td>
<td>13.87</td>
<td>3.3</td>
</tr>
<tr>
<td>Precipath</td>
<td>273.00</td>
<td>14.90</td>
<td>5.5</td>
</tr>
</tbody>
</table>

Fig. 1. Interference in the homogeneous assays for HDL-C.

Table 1. Assay precision of homogeneous and precipitation methods for HDL-C.

Assay bias (overestimated triglyceride-rich lipoproteins result minus true result, as a percentage) as a function of triglyceride concentration.

Total error. The linear regression equations for systematic error calculations were: \( y = 0.962x + 23.45 \) for the PEGME method; and \( y = 0.956x + 38.82 \) for the PPD method. Using the PEGME method, we obtained total error values for HDL-C in low (331 mg/L) and high (428 mg/L) concentrations of 10.90% and 8.03%, respectively. With the PPD method, the total error values at low (304 mg/L) and high (598 mg/L) HDL-C concentrations were 16.19% and 7.75%, respectively.

Linearity. To define the linearity of the homogeneous HDL-C methods, we plotted the measured HDL-C values resulting from each method as a function of the expected HDL-C concentrations for a dilution series obtained with 9 mg/L NaCl. Statistical analysis revealed that the slopes of the linear regression equations (\( y = 1.004x - 0.56 \) for the PEGME method and \( y = 1.019x - 4.6 \) for the PPD method) did not differ significantly from the lines of identity of the scattergrams up to at least 1500 mg/L for the PEGME method and up to at least 1200 mg/L for the PPD method. These results suggest an almost complete analytical recovery for both methods.
Data from both the PEGME and PPD homogeneous HDL-C assays from the Lin-Trol dilution experiments indicated that the PPD assay was not as good as desired (recovery, 31.5%), whereas the PEGME method was very superior in this aspect (91%). The linearity for the PEGME method followed the linear regression equation: \( y = 1.009x - 10.7 \).

**Interferences.** The assay bias data (overestimated triglyceride-rich lipoprotein result minus the true result, as a percentage) plotted as a function of triglyceride concentration are shown in Fig. 1. Triglyceride concentrations between 2500 and 15,000 mg/L led to a 32% increase in the HDL-C result in the PEGME method and a 15% increase in the PPD method.

**Storage.** The stability of HDL-C as determined by the three methods in serial measurements of four pools stored for 40 days at 4 °C (●) and −20 °C (△) for pooled serum with a low HDL-C concentration (a); pooled serum with a medium HDL-C concentration (b); pooled EDTA-treated samples (c); and pooled heparin-treated samples (d).

### Table 2. Comparison of serum, lithium heparinate plasma, and EDTA plasma.

<table>
<thead>
<tr>
<th>Method</th>
<th>Means Serum</th>
<th>Intercept Serum</th>
<th>Slope Serum</th>
<th>Means EDTA</th>
<th>Intercept EDTA</th>
<th>Slope EDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEGME*</td>
<td>585/596</td>
<td>8.75</td>
<td>0.975</td>
<td>541/596</td>
<td>−10.77</td>
<td>0.923(^b)</td>
</tr>
<tr>
<td>PPD*</td>
<td>590/585</td>
<td>10.00</td>
<td>1.000</td>
<td>556/585</td>
<td>11.00</td>
<td>0.933(^b)</td>
</tr>
<tr>
<td>PTA*</td>
<td>559/565</td>
<td>17.85</td>
<td>0.961</td>
<td>537/565</td>
<td>−9.83</td>
<td>0.965</td>
</tr>
</tbody>
</table>

\(^a\) n = 26; means and intercepts in mg/L.

\(^b\) Slopes are significantly different from 1.0.
show the percentage of change in the HDL-C concentrations of the four pools over time. Excellent agreement was obtained with the PEGME method (the largest decrease (<9%) was shown at 31 days in the low-concentration serum pool at 4 °C). The PTA method also showed low deviations compared with the results either in fresh native sera or in heparin- and EDTA-treated samples (the greatest overall decrease was 12% for the low-concentration serum pool stored at 4 °C). However, aliquots of the low-concentration serum pool stored at 4 °C showed decreases of 18% at 21 days when assayed with the PPD method.

Comparison of serum, lithium heparinate plasma, and EDTA plasma. The PEGME, PPD, and PTA methods showed no significant differences between HDL-C measured in either serum or heparin plasma. For determinations by the PEGME and PPD methods, EDTA plasma yielded lower HDL-C results compared with serum (Table 2).

INTERMETHOD COMPARISON

The means and SDs for cholesterol, triglycerides, and HDL-C in the samples included in the comparison of the homogeneous assays with the PTA method (group I) and the UC method (groups II and III) are shown in Table 3. The data in Table 4 show that all the homogeneous HDL-C assays and the PTA and UC methods were strongly correlated. When we estimated the parameters of the regression lines according to Passing and Bablok, the slopes and intercepts ranged from 0.974 to 1.038 mg/L and −10.87 to 40.00 mg/L, respectively.

**Discussion**

The performance of the PEGME and PPD methods, as shown by the interassay CVs, indicates that both methods are more precise than the PTA method. The NCEP performance goals for 1998 require that the CVs of HDL-C determinations be <4% at concentrations >420 mg/L and that SDs be <17 mg/L at concentrations <420 mg/L (21), criteria that were met by the homogeneous HDL-C assays being evaluated. These results confirm previous results reported in the literature (12, 17, 22–24).

In the present study, the results of total error with the PEGME method are in agreement with established NCEP criteria (21) and were better than those reported by Harris et al. (22) and Huang et al. (12). The PPD method also met the 1998 NCEP performance goal (<13%) for samples with 598 mg/L HDL-C; however, at low concentrations (304 mg/L) it failed because of large systematic errors. Analogous results were also reported by Harris et al. (11) and Huang et al. (12) for this method.

The linearity results for both the PEGME and PPD homogeneous HDL-C assays were as good as those reported in the literature (12, 17). We observed that the PEGME method yields more ideal results than the PPD assay in Lin-Trol dilution experiments. The poor analytical recovery of the PPD method with the latter materials

<table>
<thead>
<tr>
<th>Group</th>
<th>Intermethod</th>
<th>Intercept</th>
<th>Confidence interval</th>
<th>Slope</th>
<th>Confidence interval</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (n = 199)</td>
<td>PEGME/PTA</td>
<td>1.13</td>
<td>−21.20–20.00</td>
<td>1.037</td>
<td>1.000–1.080</td>
<td>0.964</td>
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<tr>
<td></td>
<td>PPD/PTA</td>
<td>20.00</td>
<td>20.00–20.00</td>
<td>1.000</td>
<td>1.000–1.000</td>
<td>0.988</td>
</tr>
<tr>
<td></td>
<td>PPD/PEGME</td>
<td>20.00</td>
<td>0.00–39.15</td>
<td>0.974</td>
<td>0.936–1.000</td>
<td>0.976</td>
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<tr>
<td>II (n = 88)</td>
<td>PEGME/UC</td>
<td>−10.87</td>
<td>−56.11–20.16</td>
<td>1.025</td>
<td>0.969–1.111</td>
<td>0.941</td>
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<tr>
<td></td>
<td>PPD/UC</td>
<td>10.00</td>
<td>−17.25–45.00</td>
<td>1.000</td>
<td>0.937–1.050</td>
<td>0.969</td>
</tr>
<tr>
<td>III (n = 27)</td>
<td>PEGME/UC</td>
<td>40.00</td>
<td>−20.00–106.0</td>
<td>1.000</td>
<td>0.800–1.200</td>
<td>0.939</td>
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<tr>
<td></td>
<td>PPD/UC</td>
<td>20.00</td>
<td>−70.00–102.2</td>
<td>1.000</td>
<td>0.777–1.307</td>
<td>0.922</td>
</tr>
</tbody>
</table>
could be attributable to our use of a different reagent system than the one recommended by the manufacturer (who specifies that only cholesterol-precipitating reagents containing phosphotungstate may be used with Lin-Trol). Nevertheless, this lack of linearity could be nothing but the result of matrix effects.

Analysis of triglyceride interference showed a positive bias in both direct assays being compared. The positive error for our results is similar to that seen by Harris et al. (11). Notwithstanding the presence of a significant positive bias, both homogeneous assays met the NCEP total error goal (bias <5%), suggesting acceptable calibration of the methods and adequate specificity for HDL-C, at least in hypertriglyceridemic specimens with concentrations <10 000 mg/L.

In storage studies, our data showed that the PEGME method can be appropriately applied in samples stored for 40 days either at 4 °C or −20 °C. Nevertheless, with the PPD method, the serum samples stored at 4 °C should be either analyzed before 21 days or frozen; the heparin and EDTA-treated samples stored at 4 °C should be analyzed before the 16th day; and the heparin- and EDTA-treated samples stored at −20 °C should be analyzed before the 40th day. All these conclusions should be interpreted cautiously because of the inherent random error.

Highly significant differences in HDL-C were obtained when EDTA plasma results were compared with serum for the two homogeneous methods. These differences in HDL-C results are in agreement with analogous average bias results recently reported by Nauck et al. (17). Thus, with EDTA-treated samples there would be disadvantages in analyzing HDL-C.

The statistical study on comparison methods showed that the PEGME method yields the same results as both the PTA and UC methods (i.e., the slopes did not differ significantly from 1, and the intercepts not were significantly different from 0). These findings are better than those of earlier work that report results handicapped by slope or intercept values significantly different from 1 and 0, respectively (12, 17, 22, 24). We are convinced that the PEGME method allows a reliable determination of HDL-C. Concerning the PPD assay, we found that this method gives results similar to those obtained with the UC method. Nevertheless, readings obtained by the PPD were 20 mg/L higher than those obtained by the PTA method because of a constant systematic error.

In conclusion, the two HDL-C homogeneous assays studied represent a significant improvement in our methodology to quantify HDL-C: both use small sample volumes, are easy to handle, are suitable for totally on-line automation, and produce results in 10 min. As shown in this and earlier studies, both homogeneous assays correlated highly and compared very well with the UC method used as a reference procedure. Therefore, we found that the PEGME and PPD methods can play an important role in routine HDL-C testing.

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References