Evaluation of two homogeneous methods for measuring high-density lipoprotein cholesterol

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We evaluated the performance of two homogeneous assays for quantifying HDL cholesterol (HDL-C) and compared them with the phosphotungstic acid (PTA)/MgCl2 assay. Both homogeneous HDL-C assays were precise, having a within-run CV of <1.20% and a between-run CV of <4.07%. The HDL-C values (y) measured by the two homogeneous methods correlated well with those by the PTA/MgCl2 method (x): y = 1.00x + 64.98 mg/L, r = 0.987, S_y|x = 27.99 mg/L (n = 152) for the polyethylene glycol-modified enzymes/α-cyclodextrin sulfate (PEGME) assay (Kyowa), and y = 0.84x + 106.51 mg/L, r = 0.984, S_y|x = 26.10 mg/L (n = 152) for the polyanion–polymer/detergent (PPD) assay (Daiichi). The specificity of the PEGME method seemed better than that of the PPD method, as the PPD method was markedly interfered with by supplemental LDL-C. Addition of 20 g/L triglycerides produced a negative error of ~18% in both homogeneous assays. Bilirubin and hemoglobin had little influence on the PEGME method; hemoglobin had little effect on the PPD method. Bilirubin, however, markedly decreased the readings by the PPD method. We found the PEGME assay superior to the PPD assay for routine HDL-C testing, because the PPD assay is relatively inaccurate and not specific.

INDEXING TERMS: methods comparison • triglycerides • atherosclerosis

Monitoring HDL cholesterol (HDL-C) in serum is of clinical importance, since an inverse correlation exists between serum HDL-C concentration and the risk of atherosclerotic diseases [1].4 The National Institutes of Health consensus conference on HDL-C and triglycerides (TG) has concluded that HDL-C is an independent risk factor for coronary heart disease [2, 3]. The National Cholesterol Education Program Adult Treatment Panel II (NCEP ATP II) has revised its guidelines for the diagnosis and treatment of hypercholesterolemia in adults to include HDL-C measurement at the initial screening stage along with total cholesterol [3, 4]. A low HDL-C (<350 mg/L) is thought to increase an individual’s risk of heart disease, and HDL-C ≥600 mg/L has been defined in the NCEP ATP II as a negative risk factor [3, 5]. Concentrations of LDL-C are strongly associated with cardiovascular risk [6, 7], which is calculated in general practice with the Friedewald formula: LDL-C = total cholesterol – HDL-C – TG/5 [8]. This requires that both HDL-C and TG be determined in a laboratory separately. As a result, the demand for HDL-C determination is increasing in clinical practice worldwide.

Several techniques for determining HDL-C in serum have been described, including ultracentrifugation [9], electrophoresis [10], HPLC [11], and precipitation-based methods [12]. Of these, the precipitation-based methods are widely used in the clinical laboratories. Most commonly, HDL-C is measured in the supernatant after use of dextran sulfate or phosphotungstic acid (PTA)/MgCl2 to precipitate the apolipoprotein (apo) B-containing lipoproteins [13, 14]. These methods, however, require relatively large volumes of sera, are time-consuming, not suitable for automated analysis, and are interfered with by high TG concentrations [13]. Recently, two new methods, one homogeneous assay [15] and one magnetic dextran sulfate assay [16], without a centrifugation step, have been developed. The former needs four different reagents, and thus can only be used on a limited number of automated

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4 Nonstandard abbreviations: HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; NCEP ATP, National Cholesterol Education Program Adult Treatment Panel; PTA, phosphotungstic acid; PEGME, polyethylene glycol-modified enzymes/α-cyclodextrin sulfate; PPD, polyanion–polymer/detergent; CHOD-PAP, cholesterol oxidase-peroxidase; 4-aminophenazone; and apo, apolipoprotein.
analyzers; the latter needs a large sample volume (500 μL) and is not fully automated. For these reasons, convenient and reliable methods without any pretreatment are needed to determine HDL-C.

This study evaluated the linearity, detection limit, precision, accuracy, and specificity of two convenient homogenous assays, i.e., the polyethylene glycol-modified enzymes/α-cyclodextrin sulfate (PEGME) assay [17] and the polyanion–polymer/detergent (PPD) assay for HDL-C and compared them with the PTA/MgCl2 method. We also assessed the interference of TG, hemoglobin, and bilirubin.

Materials and Methods

SAMPLES AND CONTROLS

We collected 152 blood samples from the specimens for HDL-C quantification at National Taiwan University Hospital. All procedures were followed in accordance with the Helsinki declaration of 1975, as revised in 1983. These samples were allowed to clot at room temperature for 1 to 2 h, and then the serum samples were obtained by centrifugation at 1500g for 10 min at room temperature. The hypertriglyceridemic sera were further ultracentrifuged on a Beckman (Fullerton, CA) Airfuge at 122 000g for 10 min to obtain nonlipemic sera. All analyses were performed within 6 h of collection.

To assess within-run and between-run precision, we used three sets of pooled human sera with HDL-C concentrations of 280, 560, and 840 mg/L (PTA/MgCl2 method) as controls, which were stored in aliquots at −20 °C.

HDL-C ASSAYS

PTA/MgCl2 assay. In this assay [12, 18], 200 μL of sample is mixed with 500 μL of solution containing 0.44 mmol/L PTA and 20 mmol/L MgCl2. After a 10-min incubation and precipitation at room temperature, samples were centrifuged at 7000g for 10 min. The supernatant was removed manually for assays, and then HDL-C was determined with an enzymatic end point assay, by using cholesterol oxidase and peroxidase and then a chromogenic reaction with 4-aminophenazone (CHOD-PAP) on a Hitachi (Tokyo, Japan) 7450 analyzer.

HOMOGENEOUS ASSAYS

The PEGME method (Kyowa, Tokyo, Japan) involved two reagents in solution form. Reagent 1 contained 0.5 g/L dextran sulfate, 2 mmol/L MgCl2, 0.96 mmol/L sodium N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline, and 0.5 mmol/L α-cyclodextrin sulfate, which reduces the reactivity of cholesterol, especially in chylomicrons and VLDL [17]. Reagent 2 contained 1 kU/L PEG-modified cholesterol esterase, 5 kU/L PEG-modified cholesterol oxidase, 30 kU/L peroxidase, and 0.5 g/L 4-aminophenazone. These PEG-modified enzymes showed selective catalytic activities towards different lipoprotein fractions, with the reactivity increasing in the following order: LDL < VLDL ≈ chylomicrons < HDL [17]. In the first step, 4 μL of serum was incubated with 300 μL of reagent 1 at 37 °C for 5 min. Then 100 μL of reagent 2 was added and the absorbance was measured bichromatically at 600 nm (main) and 700 nm (subsidiary).

The PPD method (Daichi, Tokyo, Japan) also involved two reagents. Reagent 1, in solution form, contained polyanion and synthetic polymer. Reagent 2 was reconstituted before use and consisted of lyophilized enzyme containing 0.8 kU/L cholesterol esterase, cholesterol oxidase, peroxidase, and 2 mmol/L 4-aminoantipyrine, and solvent containing 1 mmol/L N,N-bis(4-sulfobutyl)-m-toluidine disodium (DSBmT), 60 mmol/L phthalic acid buffer (pH 5.7), and detergent. To assay, 3 μL of serum was combined with 300 μL of reagent 1 and incubated at 37 °C for 5 min, thereby forming LDL–polymer–polyanion and HDL–polymer complexes. Then, 100 μL of reagent 2 was added; the detergent broke down the HDL structure and HDL-C was measured enzymatically. The absorbance was measured at 37 °C at a main wavelength of 546 nm and a subsidiary wavelength of 660 nm.

These two homogeneous assays for HDL-C were performed with a Hitachi 7450 automated analyzer. The two sets of calibrators, as well as their Hitachi 7450 application protocol, were provided by the manufacturers. Each homogeneous method included the calibrating system specified by the original manufacturer.

LDL-C ASSAY

For this analysis, we used the polyvinyl sulfate method (Boehringer Mannheim, Mannheim, Germany) [19]. Briefly, a mixture of 200 μL of serum and 100 μL of reagent containing polyvinyl sulfate was incubated for 15 min at room temperature, and then centrifuged for 15 min at 1500g. After centrifugation, the cholesterol content of the supernatant was determined by the CHOD-PAP method. The LDL-C concentration was calculated from the difference between the total serum cholesterol and the cholesterol in the supernatant.

LIPID MEASUREMENTS

Concentrations of cholesterol and TG were determined enzymatically with the CHOD-PAP (Amresco, Solon, Ohio) and glycerophosphate oxidase–peroxidase-4-aminophenazone (GPO-PAP) methods (Boehringer Mannheim), respectively, on a Hitachi 7450 analyzer. The interassay CV for determinations of total cholesterol and total TG varied between 1.02% and 2.71% and between 2.01% and 3.35%, respectively. Control sera were purchased from Baxter Diagnostics (Deerfield, IL) and Bio-Rad (Anaheim, CA).
Lipoproteins were isolated by sequential ultracentrifugation on a Beckman L8-M ultracentrifuge at 125 000 g for 20 h. The lipoproteins were identified at the following densities: 1.006 kg/L for VLDL, between 1.030 and 1.050 kg/L for LDL, and between 1.063 and 1.21 kg/L for HDL.

We added various amounts of HDL fraction isolated by sequential ultracentrifugation to lipoprotein-deficient serum obtained by ultracentrifugation to examine the linearity capacity of both homogeneous assays. Interferences from TG, hemoglobin, and bilirubin were also analyzed. The serum pool, containing total cholesterol 1880 mg/L, HDL-C 600 mg/L, LDL-C 1040 mg/L, VLDL-C 130 mg/L, and TG 1180 mg/L, was supplemented with Intralipid (Kabi Pharmacia, Stockholm, Sweden), hemoglobin, or bilirubin at various concentrations and was also used for the specificity study. Reagents for evaluating interference of hemoglobin and bilirubin were purchased from International Reagent Corp. (Kobe, Japan). The specificity of both homogeneous assays was evaluated by adding various amounts of VLDL and LDL, isolated by sequential ultracentrifugation, to a serum pool. The serum pool contains total cholesterol 1128 mg/L, HDL-C 360 mg/L, LDL-C 680 mg/L, VLDL-C 80 mg/L, and TG 708 mg/L after diluting with 9 g/L saline.

**Statistical Methods**

The mean, SD, and CV were calculated with Microsoft Excel Version 5.0 (Microsoft, Redmond, WA). Parametric paired t-test and linear regression analyses were calculated with Statistica (Statsoft, Tulsa, OK). The paired t-test was significant at $P < 0.05$.

We calculated the total errors by adding the systematic errors and the random errors [21, 22]. At an HDL-C concentration of $x_c$, systematic error was equal to $y_c - x_c$, where $y_c = bx_c + a$ (linear regression equation). Random error was 1.96 SD from the between-run precision study [22, 23]. $S_{yp}$ represented the standard deviation of the regression line.
Results

With the three different concentrations of pooled human sera, the precision of the two homogeneous HDL-C assays was satisfactory. Within-run CV ranged from 0.79% to 1.20%, and between-run CV ranged from 0.86% to 4.07% (Table 1). Therefore, both assays fulfilled the performance criteria for HDL-C imprecision of <5% established by both the CDC and the NCEP [23].

The linearity studies (Fig. 1) showed the PEGME method to be linear up to 1500 mg/L HDL-C, with a detection limit (mean of blanks plus 3 SD of blank readings) of 4.5 mg/L HDL-C. The PPD method was linear up to 1200 mg/L HDL-C, and the detection limit was 4.9 mg/L HDL-C. Moreover, recovery studies were performed after adding known amounts of HDL-C to six serum samples with HDL-C concentrations of 280–770 mg/L. Percentages of recovery for both homogeneous methods were 90.7% to 112.5% (Table 2).

With the 152 fresh samples, the accuracy of the two homogeneous HDL-C methods was established by comparison with the conventional PTA/MgCl₂ precipitation method. The mean total cholesterol and total TG concentrations were 2232 mg/L (range 1420–4100 mg/L) and 2522 mg/L (range 300–21 480 mg/L), respectively, for these samples. Mean HDL-C concentrations were 447 mg/L by the PTA/MgCl₂ method, 512 mg/L by the PEGME method, and 483 mg/L by the PPD method, with respective ranges of 105–1260, 145–1255, and 162–1096 mg/L. The paired t-test showed significant difference (P <0.05) between these two homogeneous methods and the

Fig. 2. Scattergrams comparing three methods.
A and D, PEGME method and PTA/MgCl₂ precipitation method. A, TG <4000 mg/L; D, TG >4000 mg/L. B and E, PPD method and PTA/MgCl₂ precipitation method. B, TG <4000 mg/L; E, TG >4000 mg/L. C and F, PPD method and PEGME method. C, TG <4000 mg/L; F, TG >4000 mg/L.
precipitation method, indicating the existence of a systematic error.

To compare the HDL-C values obtained by the two homogeneous methods and the PTA/MgCl\(_2\) method, samples were separated into two groups according to their TG concentration: group I, TG <4000 mg/L, n = 135; group II, TG ≥4000 mg/L, n = 17. The correlation coefficients between the PEGME method and the PTA/MgCl\(_2\) method in both groups were excellent, but the intercepts were relatively large: y = 0.99x + 71.53, r = 0.987, slope = 0.99 (95% confidence interval 0.965–1.018), S\(_{\text{y|x}}\) = 28.35 mg/L in group I and y = 0.98x + 56.57, r = 0.953, slope = 0.98 (95% confidence interval 0.768–1.092), S\(_{\text{y|x}}\) = 20.81 mg/L in group II (Fig. 2A, D).

In group I, readings obtained by the PPD method and the PTA/MgCl\(_2\) method showed significant deviation from the line of identity: y = 0.84x + 105.33, r = 0.986, slope = 0.84 (95% confidence interval 0.816–0.864), S\(_{\text{y|x}}\) = 24.49 mg/L (Fig. 2B). In group II, the PEGME method seemed to correlate better with the precipitation method than did the PPD method (r = 0.95 vs 0.86) (Fig. 2D, E).

We also compared the HDL-C values of hypertriglyceridemic samples (group II, TG range 4690–21 480 mg/L, mean 13 085 mg/L) using each method before and after ultracentrifugation with a Beckman Airfuge. The data showed that the PEGME assay was less interfered with by lipemia than the PPD assay: y = 1.05x + 25.23, r = 0.975, slope = 1.05 (95% confidence interval 0.928–1.172), S\(_{\text{y|x}}\) = 16.30 mg/L and y = 0.85x + 58.62, r = 0.945, slope = 0.85 (95% confidence interval 0.698–1.002), S\(_{\text{y|x}}\) = 21.98 mg/L (Fig. 3).

Table 3 shows the calculated total errors of both homogeneous HDL-C assays. This calculation was based on (a) the interassay variation presented in Table 1, as the random errors, and (b) the estimates of the regression lines of the 152 samples (groups I plus II) measured in parallel by the homogeneous assays and the PTA/MgCl\(_2\) procedure, as the systematic errors. At 280 mg/L both homogeneous assays failed to meet the forthcoming 1998 NCEP total error goal of <13% [24] because of large systematic errors. At 560 and 840 mg/L, these criteria were just met, while the total error estimated was highly borderline (13.29%) for the PEGME assay at 560 mg/L (Table 3).

With the PEGME method, there were negligible changes in HDL-C readings even after addition of 410 mg/L VLDL-C to yield a final concentration of 490 mg/L (+3%) or after addition of 2300 mg/L LDL-C to yield a final concentration of 2980 mg/L (+10%) (Fig. 4). With the PPD method, HDL-C readings increased from 346 to 388 mg/L (+12%) after adding 410 mg/L VLDL-C and increased from 344 to 453 mg/L (+32%) after adding 2300 mg/L LDL-C, indicating a poorer specificity of the PPD assay for HDL-C (Fig. 4).

We plotted the differences between the results of the PPD and PEGME methods on the one hand and that of the PTA/MgCl\(_2\) method on the other hand against the ratio of LDL-C to HDL-C (Fig. 5). This analysis confirmed that the PPD but not the PEGME method was susceptible to interference by LDL-C. The higher the LDL-C/HDL-C ratio of sera, the larger the bias between the PPD method and the PTA/MgCl\(_2\) (P <0.0001, Fig. 5B). The same trend did not show statistical significance with the PEGME method (P = 0.21, Fig. 5A).

Finally, analysis of the interference from TG, hemoglobin, and bilirubin showed that addition of 20 g/L TG to the pooled serum, baseline HDL-C concentration of 366 mg/L after diluting with 9 g/L saline, produced a negative error in the results of both homogeneous assays (18%). Hemoglobin up to 12 000 mg/L had little influence on both homogeneous methods (<5%). Bilirubin up to 400 mg/L showed no effect on the PEGME method, whereas a decrease of HDL-C up to 42% was seen in the PPD method (Fig. 6).

**Discussion**

Conventional HDL-C procedures in the clinical laboratory included a chemical precipitation step, which requires both centrifugation and recovery of the supernatant. Of these procedures, the PTA/MgCl\(_2\) method is one of the
In this study, we evaluated the performance of two newly developed homogeneous HDL-C assays that can be readily adapted to automated analyses as on-line procedures to meet the increasing demand for rapid and accurate determination of HDL-C in daily laboratory practice, and compared the results with those obtained by the PTA/MgCl₂ method.

Both homogeneous assays were precise but had a large systematic error compared with the PTA/MgCl₂ method. Warnick et al. found that the HDL-C concentration measured by the PTA/MgCl₂ precipitation method was consistently 5% to 10% lower than that by ultracentrifugation method, the reference method [14]. This discrepancy may be caused by the incomplete capability to measure all the HDL-C fraction by the PTA/MgCl₂ method [25], since the precipitation method would fail to measure the cholesterol molecules in the high-M₄ apo E-rich HDL [26]. We also found that both homogeneous assays were calibrated falsely high (Table 4). This could also partly explain the reasons for the overestimation of HDL-C by the two new assays.

The PPD method showed a systematic deviation over the whole range, with a positive y-intercept and a slope being significantly <1, resulting in falsely high results for HDL-C at concentrations ~<600 mg/L and falsely low concentrations at ~>600 mg/L (Fig. 2B). With this method, there would be disadvantages in calculating the LDL-C with the Friedewald formula [8]. The underestimation of the HDL-C concentrations >600 mg/L by the PPD method would result in a reciprocal error in the LDL-C estimate and produce a considerable shift in the ratio of LDL-C to HDL-C.

Table 3. Percentage error (difference from the PTA/MgCl₂ method) of the two homogeneous assays.

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<thead>
<tr>
<th>HDL-C, mg/L</th>
<th>Systematic error, %</th>
<th>Random error, %</th>
<th>Total error, %</th>
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<tr>
<td></td>
<td>PEGME</td>
<td>PPD</td>
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<td>560</td>
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<tr>
<td>840</td>
<td>7.74</td>
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Fig. 4. Specificity of the two homogeneous methods after adding VLDL and LDL to pooled human serum.

A, added VLDL up to 410 mg/L; B, added LDL up to 2300 mg/L. □, PEGME method; ●, PPD method.

Fig. 5. Comparison of the bias (HDL-C readings by the homogeneous methods minus the readings by PTA/MgCl₂ method) against the serum LDL-C/HDL-C ratio of sera with TG <4000 mg/L (n = 135).

A, PEGME method; B, PPD method.
We found the PPD assays not as specific for HDL-C as desired (Fig. 4). This lack of specificity may be a result of incomplete wrapping of non-HDL with the reagents, so that the enzymes used in the cholesterol assays may also measure the cholesterol in the chylomicrons, VLDL, and LDL, resulting in falsely high HDL-C concentrations. Only with the PPD method did we find a statistically significant relation ($P < 0.0001$) between serum LDL-C/HDL-C ratios and the difference between the results of the homogeneous assays and the PTA/MgCl2 method (Fig. 5). This relation implies that in samples with a high LDL-C/HDL-C ratio, falsely high HDL-C concentrations will be obtained with the PPD method. For these samples, a lower ratio of total cholesterol/HDL-C will ensue and give a falsely low risk of coronary heart disease with the PPD assay.

Hemoglobin seems to have little influence on either homogeneous assay. However, 20 g/L TG decreased the HDL-C readings by $\sim 18\%$ in both assays. Its volume effect may have contributed to the negative interference. In contrast to the PEGME method, the PPD method was markedly interfered with by bilirubin (Fig. 6). This could be caused by the reaction between bilirubin and H$_2$O$_2$, and the subsequent reduction of the amount of peroxide available for the formation of the colored complex.

Both the PEGME and PPD assays have the advantages of requiring small sample volumes, convenience, and rapidity. Both also need no pretreatment and are ready for full automation. However, the systematic deviation between the two homogeneous assays and the PTA/MgCl2 assay were substantial. One of the reasons seemed to be in the calibration systems of both assays, which needed to be improved. We found the PPD assay not as specific and accurate as desired, and the PEGME method superior in many aspects. Thus, before they can be generally applied in clinical laboratories, restandardization of the calibration systems are mandatory and the specificity of the PPD assay has to be improved.

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### References