Multicenter evaluation of a homogeneous assay for HDL-cholesterol without sample pretreatment

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We evaluated a new homogeneous assay for the measurement of HDL-cholesterol (HDL-C) in six European laboratories. The assay includes two reagents and is applicable to most autoanalyzers, which allows full automation. The total CVs of the new method ranged between 1.3% and 6.7%. Thereby determined HDL-C values were in good agreement with those obtained by precipitation with phosphotungstic acid/MgCl2 or by a combination of ultracentrifugation and precipitation (0.956 < r < 0.994). The assay was linear up to at least 1500 mg/L HDL-C. Hemoglobin did not interfere, whereas icteric samples with bilirubin >100 mg/L showed discrepancies between the homogeneous and the precipitation assay. Lipemia up to total triglyceride concentrations of 8000 mg/L did not interfere with the homogeneous HDL-C assay. The homogeneous HDL-C assay was easy to handle and produced similar results in all laboratories participating in this study. This method will significantly facilitate the screening of individuals at increased risk for cardiovascular disease.

The positive association of coronary heart disease risk with total cholesterol and LDL-cholesterol (LDL-C) concentrations and its negative association with HDL-C concentrations are well established [1–8]. HDL-C concentrations <350 mg/L are considered a cardiovascular risk factor; HDL-C concentrations exceeding 600 mg/L are considered protective [7]. Data from the German PROCAM study clearly show that individuals with increased concentrations of triglycerides and low HDL-C are at a remarkably increased risk for coronary artery disease (CAD) [9]. Together with total cholesterol and triglycerides, the determination of HDL-C allows the calculation of LDL-C according to Friedewald et al. [10].

For these reasons, reliable and easy-to-perform methods are needed to quantify HDL-C. Measurement of HDL-C is still critical from the view of the clinical chemist, because substantial deviations between different laboratories have been observed and because the conventional precipitation procedures are time consuming [11–14]. In addition, they do not meet the HDL-C performance goals of the CDC for 1998, which require for HDL-C a CV of <4% at concentrations >420 mg/L and a SD <17 mg/L at concentrations <420 mg/L [15].

Most frequently, HDL-C is measured in the supernatant after precipitation of apolipoprotein (apo) B-containing lipoproteins by dextran sulfate or phosphotungstic acid (PTA)/MgCl2 [11–14]. All of these methods involve both a precipitation and a centrifugation step, which prevent full automation. In recent years the costs of laboratory procedures have attracted increasing attention. Methods that eliminate manual handling, such as pretreatment with a precipitation reagent in the case of the HDL-C determination, may considerably reduce costs.

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Nonstandard abbreviations: VLDL-C, LDL-C, HDL-C, cholesterol associated with VLDL, LDL, and HDL; CAD, coronary artery disease; apo, apolipoprotein; PTA, phosphotungstic acid; PEG, polyethylene glycol; and NCEP, National Cholesterol Education Program.
Previously, new procedures for the determination of HDL-C were published. They have in common the avoidance of at least the tedious centrifugation step [16–18].

This multicenter study was performed in seven laboratories from three European countries. We evaluated a homogeneous HDL-C assay that includes polyethylene glycol (PEG)-modified enzymes and α-cyclodextrin sulfate to selectively determine HDL-C in serum, as published previously [16]: At neutral pH (7.0) and in the presence of MgCl₂, sulfated α-cyclodextrin and dextran sulfate form water-soluble complexes with LDL, VLDL, and chylomicrons that are not accessible to PEG-modified enzymes. As HDL is not complexed, its cholesterol moiety is readily available for enzymatic quantification by using cholesterol esterase and cholesterol oxidase coupled to PEG.

The aim of this study was to evaluate the new homogeneous HDL-C assay.

**Materials and Methods**

**Samples.** Blood was drawn from 1074 in- and outpatients at the six centers (Bruchsal, Freiburg, Gent, Heidelberg, Münster, Rotterdam). Samples from nonfasting patients were not excluded from the study. Blood was allowed to clot at room temperature and serum was obtained by centrifugation at 1500×g for 15 min. Some of the samples were icteric, hemolytic, or lipemic. Lipid and lipoprotein concentrations in the samples are summarized in Table 1. All analyses with the homogeneous and the precipitation assays were performed within 1 day of blood collection. Measurements of HDL-C with the CDC reference method were performed in aliquots of the samples kept at −20 °C for not longer than 4 weeks.

**Lipid measurements.** Cholesterol and triglycerides were determined enzymatically with the CHOD-PAP and GPO-PAP method, respectively. All reagents and control sera were obtained from Boehringer Mannheim. All measurements were performed on Boehringer Mannheim/Hitachi autoanalyzers types 704, 717, 747, 911, or 917. Control sera Precinorm® L and Precipath® L (both from Boehringer Mannheim) were included in each analytical run.

**Homogenous HDL-C assay.** The reagents for the homogeneous HDL-C assay were obtained from Boehringer Mannheim. The test was performed according to the manufacturer’s recommendations. In each laboratory, the homogeneous HDL-C assay was calibrated with Precinorm L, a lyophilized control serum in which HDL-C was determined by the manufacturer with the PTA/MgCl₂ precipitation procedure.

**HDL-C determination by precipitation method.** All laboratories used the PTA/MgCl₂ method as the comparison method. The reagents were purchased from Boehringer Mannheim. The test was performed according to the manufacturer’s recommendations.

**HDL-C determination by combined ultracentrifugation and precipitation method.** At laboratory 2 a combined ultracentrifugation and precipitation assay was used as an additional comparison method. Essentially, the protocol of the Lipid Research Clinics Program was followed, with modifications previously described [19, 20]. In brief, VLDL was floated by ultracentrifugation (d = 1.006 kg/L) and LDL was separated from HDL in the infranatant by precipitation with PTA/MgCl₂.

**HDL-C determination according to CDC reference method.** This comparison method, recommended by the National Cholesterol Education Program (NCEP) Lipoprotein Measurement Working Group as the accuracy base, was performed at laboratory 6 in addition to the PTA/MgCl₂ method [15]. The laboratory is a member of the Cholesterol Reference Method Laboratory Network coordinated by the CDC. The CDC reference method includes removal of VLDL by ultracentrifugation, precipitation of LDL from the bottom fraction (d = 1.006 kg/L) with 46 mmol/L heparin–manganese, and analysis of HDL-C in the resulting supernatant by the CDC modified Abell–Kendall method [19, 21].

**Isolation of HDL₂ and HDL₃.** HDL₂ and HDL₃ were isolated by sequential ultracentrifugation with 1.063<d<1.21 kg/L as density limits, respectively [22].

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**Table 1. Summary of total cholesterol, total triglycerides, and HDL-C of the samples included in the intermethod comparisons.**

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Total cholesterol, mg/L</th>
<th>Total triglycerides, mg/L</th>
<th>HDL-C, mg/L</th>
</tr>
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<tr>
<td></td>
<td>Mean</td>
<td>Range</td>
<td>Mean</td>
</tr>
<tr>
<td>1</td>
<td>2460</td>
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<td>6</td>
<td>2261</td>
<td>450–6250</td>
<td>1560</td>
</tr>
</tbody>
</table>

* HDL-C was determined with the PTA/MgCl₂ precipitation method.

**Total cholesterol concentrations were not determined in samples of laboratory 6.
Linearity. Testing of linearity was performed with the Lin-Trol\textsuperscript{®} linearity set for HDL-C from Sigma Diagnostics. The HDL-C Lin-Trol was mixed with HDL-C Lin-Trol diluent at 11 different concentrations ranging from 0% to 100%. In addition, serum from an apo A-I-deficient patient was supplemented with HDL\textsubscript{2} and HDL\textsubscript{3} isolated by ultracentrifugation.

Lowest detectable concentration. The lowest detectable concentration was determined by 21 measurements by using isotonic NaCl solutions as samples. From these results the mean + 3SD was calculated.

Bilirubin measurement. Total bilirubin was measured after its release from albumin with 2,5-dichlorophenyldiazonium salt as the dye. The reagents were purchased from Boehringer Mannheim and Merck.

Interferences. Interference from hemoglobin was analyzed according to Glick et al.\textsuperscript{[23]}. In addition, the influences of hyperbilirubinemia and hypertriglyceridemia were analyzed with the samples of all intermethod comparisons. The bias of HDL-C (homogeneous HDL-C assay minus PTA/MgCl\textsubscript{2} precipitation) were plotted vs bilirubin.

Comparison of serum, lithium heparinate, and EDTA plasma. In five samples, the influence of lithium heparinate and EDTA plasma in comparison with serum was investigated. Each sample was measured five times.

Storage of samples. HDL-C was determined in 50 fresh serum samples by using the homogeneous assay. The samples were stored at −20 and −70 °C for 4 weeks before the samples were reanalyzed.

Statistical methods. Regression analyses were performed with the method of Passing and Bablok\textsuperscript{[24]}. Precision data were calculated according to recommendations of the NCCLS/EP5-T protocol\textsuperscript{[25]}.

Results

Analytical performance. Two commercial control sera with low and medium HDL-C concentrations and human serum pools were used to assess the precision of the new homogeneous HDL-C assay in five laboratories (Table 2). The within-day variance was determined as the average of the variances obtained at each day. The between-day variance was calculated from the variance of the means obtained each day, which was then adjusted for the within-day variance component. The total precision CVs ranged from 1.3% to 6.7%. Intraassay CVs ranged from 0.6% to 6.4%, interassay CVs from 1.1% to 3.1%.

In each run, two control samples were measured as duplicates. The results of the Precinorm L and Precipath L in laboratories 1 to 5 ranged from 94.3% to 105.5% (mean: 99%) and from 98.5% to 114.8% (mean: 103%) of the manufacturer’s stated values (530 mg/L and 330 mg/L, respectively, as determined with the PTA/MgCl\textsubscript{2} precipitation assay). The total CVs of the homogeneous HDL-C determined calculated from these samples were between 1.1% and 4.6%.

Linearity. Results of the homogeneous HDL-C assay with the Lin-Trol dilution experiments revealed linearity up to

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Hitachi analyzer</th>
<th>Sample</th>
<th>Mean, mg/L</th>
<th>SD, mg/L</th>
<th>CV, %</th>
<th>SD, mg/L</th>
<th>CV, %</th>
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<td>273</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>PNL</td>
<td>504</td>
<td>(8.7)</td>
<td>(2.5)</td>
<td>(8.7)</td>
<td>(2.5)</td>
<td>(12.3)</td>
<td>(3.6)</td>
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<tr>
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<tr>
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<td>7.3</td>
<td>1.4</td>
<td>6.8</td>
<td>1.4</td>
<td>10.0</td>
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<tr>
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<td>2.5</td>
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<tr>
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Two control sera and one human serum pool were analyzed in each laboratory. Pools were divided into 300-μL aliquots and stored at 4 °C until tested. One aliquot of each pool was assayed in duplicate at each of 21 days. Calculations for within-run and between-day SD were based on 21 samples; for within-day SD on a pair of 21 samples. SD\textsubscript{total} = SD\textsubscript{within} + SD\textsubscript{between}.

HPS, human pool serum; PNL, Precinorm L; PPL, Precipath L.

In laboratory 4 the data in parentheses are obtained if one outlier (190 mg/L) is disregarded. Laboratory 6 used lots of the control sera different from those of the other laboratories. In laboratory 6 the control sera were analyzed at 20 different runs with identical lots for the whole series.
at least 1500 mg/L ($y = 0.971x + 11.7$ mg/L, $r = 0.9998$). Similar results were obtained in experiments in which HDL$_2$ and HDL$_3$, isolated by sequential ultracentrifugation, were added at increasing amounts to serum from a patient with apo A-I deficiency (HDL$_2$: $y = 0.991x + 39.6$ mg/L, $r = 0.9987$; HDL$_3$: $y = 1.037x - 3.5$ mg/L, $r = 0.9996$).

Lowest detectable concentration. The lowest HDL-C concentration that could be measured reliably with the homogeneous assay was ~30 mg/L.

Intermethod comparison. The homogeneous HDL-C assay was compared with a conventional PTA/MgCl$_2$-based precipitation method in all laboratories. A total of 1074 fresh unfrozen sera were analyzed in parallel. Mean total cholesterol and total triglycerides ranged from 1967 to 2639 and from 1702 to 2944 mg/L, respectively. Maximum cholesterol and triglyceride concentrations were 8080 and 24260 mg/L, respectively. HDL-C concentrations ranged between 12 and 1560 mg/L, determined with the PTA/MgCl$_2$ method. The correlation coefficients were between 0.956 and 0.994 (Table 3). When we estimated the parameters of the regression lines according to Passing and Bablok, slopes and intercepts ranged from 0.99 and 1.08 and −23.0 and 24.1 mg/L, respectively [24].

In addition to the PTA/MgCl$_2$ precipitation of native sera, two different methods for HDL-C quantification involving ultracentrifugation at $d = 1.006$ kg/L were investigated. Table 3 and Fig. 1A show that the combined ultracentrifugation and PTA/MgCl$_2$ precipitation method and the new homogeneous HDL-C assay were strongly correlated. However, in some of the icteric samples the homogeneous assay revealed HDL-C values lower than the comparison method (see below).

The new homogeneous HDL-C assay was in good agreement with the CDC reference method as well (Table 3, Fig. 1B). None of the samples analyzed with the CDC reference method was hypertriglyceridemic or icteric.

Interferences. Hemoglobin at concentrations up to 10 g/L did not interfere with the new homogeneous HDL-C assay (data not shown). We examined the results obtained in icteric samples of the intermethod comparison study. This revealed that the homogeneous assay produced low HDL-C in samples with bilirubin concentrations >100 mg/L, compared with the PTA/MgCl$_2$ precipitation (Fig. 2).

Specificity. In Fig. 3 the bias of the homogeneous HDL-C assay minus HDL-C determined by the combined ultracentrifugation and PTA/MgCl$_2$ precipitation method are plotted against triglycerides, VLDL-C, and LDL-C, respectively. Neither triglycerides, VLDL-C, nor LDL-C systematically influenced the differences of the two HDL-C measurements up to concentrations of 9000, 2600, and 3000 mg/L, respectively.

Comparison of serum, lithium heparinate, and EDTA plasma. There were no significant differences between HDL-C measured in either serum or heparin plasma: In four samples, deviations were ≤8.7 mg/L (data not shown). In one sample the deviation was 20.4 mg/L. The average bias between serum and heparin plasma was −6.2 mg/L, the relative deviation being <1.9% in each case. EDTA plasma yielded lower results compared with serum: The overall bias was −46.6 mg/L; the percentage deviations ranged from 4.7% to 11.5%.

Storage. In 46 of 49 samples, excellent agreement was obtained when the homogeneous assay was used to measure HDL-C in fresh native sera and in sera stored at

| Table 3. Summary of intermethod comparisons. |
|---|---|---|---|---|---|---|---|
| Laboratory | Comparison method | Hitachi analyzer | n | Slope | Intercept | $r$ | Comparison HDL-C $\bar{x}$ | Homogeneous HDL-C $\bar{y}$ |
| 1 | PTA | 747 | 277 | 1.03 | 9.6 | 0.970 | 560 | 581 |
| 2 | PTA | 717 | 121 | 1.07$^a$ | −0.3 | 0.975 | 535 | 572 |
| 3 | PTA | 911 | 147 | 1.08$^a$ | −23.0$^b$ | 0.965 | 450 | 459 |
| 4 | UC/PTA | 911 | 147 | 1.07$^a$ | −15.9$^b$ | 0.967 | 446 | 458 |
| 5 | UC/PTA | 704 | 108 | 1.02$^a$ | 2.8 | 0.956 | 392 | 392 |
| 6 | UC/HEP | 911 | 210 | 1.02$^a$ | 24.1$^b$ | 0.971 | 439 | 463 |
| 7 | UC/HEP | 911 | 112 | 1.01$^a$ | −1.1 | 0.983 | 528 | 529 |
| 8 | UC/HEP | 911 | 99 | 0.99 | −12.1$^a$ | 0.994 | 429 | 437 |

UC/HEP, ultracentrifugation/heparin MnCl$_2$.
Concentrations are given in mg/L.

$^a$ Slopes are significantly different from 1.00; $^b$ Intercepts are significantly different from 0.00.

$^a$ The assay for HDL-C with the UC/HEP CDC reference method was performed in samples stored at −20 °C for not longer than 4 weeks.

Regression analyses were done according to Passing and Bablok [24].
20 and 70 °C for 2 and 4 weeks, respectively. The slopes ranged between 0.97 and 0.99 and the intercepts were negligible. However, three samples showed deviations of 260, 200, and 380 mg/L, respectively, compared with the results in fresh native sera. The total cholesterol and the total triglyceride content of these samples was 2500 mg/L.

Discussion

As shown in many epidemiological and clinical studies, a low concentration of HDL-C is an important risk factor for CAD [1, 4, 5].

So far, HDL-C has widely been determined by methods in which apo B-containing lipoproteins are precipitated with polyanions and bivalent cations. These methods are precise and agree well with ultracentrifugation methods [26, 27]. However, they involve removal of the complexes of lipoproteins and polyanions by centrifugation, which is tedious and prevents full automation. Recently, some of us reported the evaluation of a homogeneous HDL-C assay that involves PEG and antibodies specific for apo B and apo C-III to exclude cholesterol of non-HDL particles from the enzymatic detection [17]. The performance characteristics of that assay were satisfactory. However, because four different reagents were used, the assay was confined to the availability of special autoanalyzers. We now present the results of a multicenter evaluation of another homogeneous assay that overcomes this limitation because it involves two reagents only [16].

In all participating laboratories, the precision of this assay was better than the precision of the conventional precipitation methods [11–13]. The NCEP performance goals for 1998 demand that the CV of HDL-C determinations be <4% at concentrations >420 mg/L [15], a criterion that was met by the new homogeneous HDL-C assay. The NCEP precision goal for HDL-C concentrations <420 mg/L is a SD <17 mg/L [15]. In the human serum pools used for the determination of the imprecision the lowest HDL-C concentrations were 273 and 253 mg/L, respectively. The SDs for these HDL-C concentrations were 8.1 and 4.6 mg/L, respectively, and thus far below the
permitted value of 17 mg/L. Thus, the NCEP precision goals will obviously be met by the homogeneous assay at both high and low HDL-C concentrations. Calculations of the total error were not performed because a preliminary calibration was used in this study. The definite calibration of the homogeneous assay, which will differ from that used here by <3%, will further diminish the differences between the homogeneous HDL-C assay and conventional methods to measure HDL-C (Cobbaert et al., submitted).

Comparison of the homogeneous HDL-C assay with a conventional PTA/MgCl₂ procedure in 1074 samples produced ample correlation coefficients. The slopes of the regression lines ranged between 0.99 and 1.08 with small positive or negative intercepts, respectively. Therefore, in none of the participating centers did the mean HDL-C values of the homogeneous assay differ significantly from those of the PTA/MgCl₂ procedure. The parameters of the regression lines were highly consistent throughout the different laboratories. This is in contrast to previous multicenter evaluations of methods for HDL-C determination and shows that the new homogeneous assay for HDL-C is robust [11–14].

The homogeneous assay for HDL-C was compared with reference methods, including ultracentrifugation, in two laboratories. In laboratory 6 only normolipidemic samples were analyzed; this resulted in a correlation coefficient of 0.992 and a difference of the mean of 2 mg/L. Slope and intercept were not significantly different from 1 and 0, respectively. Similar results were obtained between a combined ultracentrifugation and precipitation method in laboratory 2 if icteric samples were disregarded.

The accuracy of the homogenous HDL-C assay was not impaired by free hemoglobin up to 10 mg/L [23]. In some samples bilirubin at concentrations of >100 mg/L brought about marked differences of HDL-C in the homogeneous assay, compared with the PTA/MgCl₂ procedure. Supplementation experiments with ditaurobilirubin and unconjugated bilirubin revealed, however, that the PTA/MgCl₂ method was more susceptible to these interferences than the new homogeneous assay (data not shown). The influence on HDL-C determined with both the homogeneous assay and the PTA/MgCl₂ procedure was more pronounced with unconjugated bilirubin compared with ditaurobilirubin, respectively. Analyses of the data of laboratory 2 indicate that total triglycerides and VLDL-C did not affect the homogeneous HDL-C assay up to a triglyceride concentration of at least 8000 mg/L.

The homogeneous assay allows the use of lithium heparinated plasma instead of serum. HDL-C measurements in EDTA plasma yielded lower results than expected from the dilution introduced by the EDTA solution, which reduces the results by ~3% compared with serum. This may be due to the fact that the binding of α-cyclodextrin to apo B-containing lipoproteins depends on Mg²⁺ and that EDTA, through its ion capturing
capacity, reduces the availability of Mg$^{2+}$ [16]. Another explanation could be the osmotic effect of EDTA, which causes a shift of water from cells to plasma, with a dilution of the plasma by $\sim$3%.

Freezing of samples should be avoided. Our data show that in most samples freezing did not influence the results, but three outliers of 49 samples suggest that freezing may affect the results in particular situations. There was no obvious reason for the discrepancies in the three samples, as lipids and lipoproteins as well as the appearance of the samples themselves were absolutely normal. The addition of sucrose to serum might help to overcome this problem [28].

Because the assay is convenient to use in routine laboratories, costs have to be considered. The reagents for the homogeneous HDL-C assay are approximately fivefold more expensive than conventional precipitation reagents for HDL-C. However, total assay costs depend on many other aspects, such as the number of HDL-C determinations per day, materials needed for the pretreatment of samples (conventional assay), type of dilutor (manual or automated), and the time of the technical assistant to perform the analysis. In addition, the possibility of errors such as misidentification or loss of the sample (during centrifugation) is much higher with the conventional HDL-C assay. A further advantage of the new homogeneous HDL-C assay is the small sample volume needed. We are convinced that in most laboratories the higher reagent costs will be compensated by economizing the laboratory work and that the homogeneous HDL-C assay will improve the accuracy of the HDL-C determination.

In summary, the new homogeneous assay produces precise and accurate determinations of HDL-C. Even hypertriglyceridemic samples up to at least 8000 mg/L and samples with bilirubin <100 mg/L showed unbiased results. Thus this homogeneous assay represents a significant improvement of our methodology to quantify HDL-C and may facilitate the identification of individuals at increased risk of atherosclerosis.

We thank all technical assistants of the participating laboratories who were involved in this study. We also thank Boehringer Mannheim, Germany, for providing test kits free of charge.

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