Homogeneous assay for direct determination of high-density lipoprotein cholesterol evaluated

Matthias Nauck,* Winfried März, Brigitte Haas, and Heinrich Wieland

We evaluated a new homogeneous assay for quantifying high-density lipoprotein cholesterol (HDL-C). The assay included four reagents: polyethylene glycol for “wrapping” chylomicrons, very-low-density lipoproteins (VLDL), and low-density lipoproteins (LDL); antibodies specific for apolipoprotein (apo) B and apo C-III to produce aggregates of chylomicrons, VLDL, and LDL; enzymes for the enzymatic cholesterol determination of the noncomplexed lipoproteins with 4-aminoantipyrine as the color reagent; and guanidine salt to stop the enzymatic reaction and to solubilize the complexes of apo B-containing lipoproteins, which would otherwise interfere with the reading of absorbance. The total CVs of the new method ranged between 2.4% and 8.4%. The HDL-C values (y) were in good agreement with those by a comparison phosphotungstic acid/MgCl₂ method (x): y = 0.987x + 17.2 mg/L (68th percentile of the residuals on the regression line = 21.49; r = 0.970). At triglyceride concentrations of 20 g/L (Intralipid®) the homogeneous HDL-C concentrations increased by 2%. Hemoglobin markedly increased the results, whereas bilirubin reduced them. The homogeneous HDL-C assay was easy to handle and allows full automation. This test should considerably facilitate the screening of individuals at an increased risk of cardiovascular disease.

INDEXING TERMS: enzyme immunoassay • apolipoproteins • screening, risk factors

Epidemiological and clinical studies established total cholesterol as an independent primary risk factor of coronary heart disease [1]. Concentrations of low-density lipoprotein cholesterol (LDL-C) are strongly associated with cardiovascular risk, whereas high concentrations of high-density lipoprotein cholesterol (HDL-C) seem to be protective [2–8].¹ HDL-C concentrations <350 mg/L are thought to be a cardiovascular risk factor, whereas HDL-C concentrations >600 mg/L are considered protective [7]. In addition, the ratio of HDL-C to total cholesterol seems to be a better indicator for the cardiovascular risk than total cholesterol alone [9, 10].

In most clinical studies and in general practice, LDL-C is calculated by using the Friedewald formula: LDL-C = total cholesterol − HDL-C − (triglycerides/5) [11]. This requires that HDL-C and total triglycerides be determined in the laboratory.

For these reasons, reliable and easy-to-perform methods for determining HDL-C are needed. However, the measurement of HDL-C is still problematic. Various expensive and time-consuming methods such as sequential ultracentrifugation and combined ultracentrifugation and precipitation techniques, as well as simpler techniques such as differential precipitation and electrophoresis, have been suggested [12–27].

Most frequently, HDL-C is measured by using dextran sulfate or phosphotungstic acid/MgCl₂ to precipitate the apolipoprotein (apo) B-containing lipoproteins and quantifying the cholesterol in the supernate [20–23]. These methods all depend on a precipitation step and a centrifugation step, which prevents full automation of the measurement. In recent years the costs of laboratory procedures have gained more attention. Methods that eliminate manual handling of samples, e.g., the pretreatment with precipitation reagent in HDL-C determinations, reduce costs. In a recently published homogeneous assay of HDL-C, polyethylene glycol (PEG)-modified enzymes are used with α-cyclodextrin sulfate to provide selectivity of the determination of HDL-C in serum [28]. Here we evaluate another homogeneous assay for HDL-C [29], in which the use of PEG and antibodies against apo B and apo C-III allows the fully automated measurement of HDL-C with a Hitachi 911 automated analyzer.

Materials and Methods

Samples. Blood was withdrawn from 90 patients of the University Hospital of Freiburg. Informed consent was obtained from each patient; all procedures were followed in accordance with the Helsinki Declaration of 1975, as revised in 1983. The blood was allowed to clot at room temperature, and serum was obtained by centrifugation at 1500g for 15 min. The mean

¹ Nonstandard abbreviations: VLDL-C, LDL-C, HDL-C, cholesterol associated with VLDL, LDL, and HDL, respectively; apo, apolipoprotein; and PEG, polyethylene glycol.

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concentrations of cholesterol and triglycerides were 2030 mg/L (range 580-4700 mg/L) and 2400 mg/L (range 300-11070 mg/L), respectively. All analyses were performed in fresh serum, except that the samples used to assess assay precision were stored at −20 °C.

**Lipid measurements.** Cholesterol and triglycerides were determined enzymatically with the CHOD-PAP and GPO-PAP methods, respectively, with reagents purchased from Boehringer Mannheim, Mannheim, Germany. The interassay CV for determinations of total cholesterol and total triglycerides varied between 0.97% and 1.05% and between 1.52% and 2.07%, respectively. All measurements were performed with a Hitachi 911 analyzer. The control sera (Precinorm L and Precipath L) were also purchased from Boehringer Mannheim.

**HDL-C determination by precipitation.** VLDL and LDL were precipitated with phosphotungstic acid/MgCl₂ (Boehringer Mannheim) after a 5-min incubation of 200 μL of serum with 500 μL of 0.44 mmol/L phosphotungstic acid containing 20 mmol/L MgCl₂. HDL-C remained in the supernate after centrifugation at room temperature at 7000g and was determined enzymatically with the CHOD-PAP method.

**Homogeneous HDL-C assay.** The homogeneous assay of HDL-C was performed on the Hitachi 911 analyzer at 37 °C. The assay requires four different reagents, all from International Reagents Corp., Kobe, Japan. In the first step a 4-μL sample is incubated with 60 μL of PEG (4000 Da) for 1.5 min to complex the chylomicrons, VLDL, and LDL. In the second step, 100 μL of antibodies specific for apo B and apo C-III is added to produce aggregates of the chylomicrons, VLDL, and LDL. After an additional incubation of 3.5 min, 130 μL of enzymatic cholesterol reagent is added, consisting of cholesterol esterase (EC 3.1.1.13), cholesterol oxidase (EC 1.1.3.6), peroxidase (EC 1.11.1.7), and 4-aminoantipyrine dye. The enzymes react with the cholesterol of the noncomplexed lipoproteins for 5 min. Then 200 μL of guanidine hydrochloride is added and incubated with the reaction mixture for 5 min to stop the enzymatic reactions and to solubilize the aggregates formed in steps one and two, which would otherwise interfere with the subsequent reading of the absorbance of the product formed by the cholesterol oxidase/peroxidase reaction. After the 5-min interval, the absorbance is measured bichromatically at 600 (signal) and 700 nm (noise).

**Apo A-I.** Apo A-I was measured turbidimetrically on the Hitachi 911 with Tina-quant® reagents purchased from Boehringer Mannheim. This assay was calibrated against the IFCC apo A-I standard.

**Isolation of VLDL, LDL, and HDL.** Lipoproteins were isolated by sequential ultracentrifugation at the following densities: <1.006 kg/L for VLDL, between 1.019 and 1.063 kg/L for LDL, and between 1.063 and 1.21 kg/L for HDL [12].

### Table 1. Assay precision of homogeneous test for HDL-C.

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Mean</th>
<th>SDw, mg/L</th>
<th>CV, %</th>
<th>SDb, mg/L</th>
<th>CV, %</th>
<th>SDt, mg/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precinorm L</td>
<td>414.3</td>
<td>9.6</td>
<td>2.3</td>
<td>10.9</td>
<td>2.6</td>
<td>18.0</td>
<td>4.3</td>
</tr>
<tr>
<td>Precipath L</td>
<td>224.5</td>
<td>10.1</td>
<td>4.5</td>
<td>6.9</td>
<td>3.1</td>
<td>18.8</td>
<td>8.4</td>
</tr>
<tr>
<td>Human serum</td>
<td>604.5</td>
<td>9.8</td>
<td>1.6</td>
<td>5.3</td>
<td>0.9</td>
<td>14.6</td>
<td>2.4</td>
</tr>
</tbody>
</table>

*Pools were divided into 300-μL aliquots and stored at −20 °C until tested. One aliquot from each was assayed in duplicate at each of 21 days.

**Interferences.** Interferences from triglycerides, hemoglobin, and bilirubin were analyzed according to Glick et al. [30]. A serum pool was supplemented with Intralipid®*, hemoglobin, or bilirubin at various concentrations.

**Statistical methods.** Statistical regression analyses were performed by the method of Passing and Bablok [31]. Precision data were calculated according to recommendations of the NCCLS/ EP5-T [32].

### Results

**Analytical performance.** Two commercial control sera with low and medium HDL-C concentrations and one human plasma pool with a high HDL-C concentration were used to assess the precision of the new homogeneous procedure for HDL-C (Table 1). The within-day variance was determined as the average of the variances obtained at each day. The between-days 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 2.4% to 8.4%. Intrassay CVs ranged from 1.6% to 4.5%. Interassay CVs were between 0.9% and 3.1%.

Using the homogeneous HDL-C assay, we obtained recoveries of HDL-C in both control sera, i.e., Precinorm L and Precipath L, of 89.5% and 89.4% of the target value declared by the manufacturer for the phosphotungstic acid/MgCl₂ method.

**Linearity.** HDL isolated by sequential ultracentrifugation was added at increasing amounts to a serum pool. The baseline HDL-C concentration of the serum pool was 250.5 mg/L after diluting it with an equal volume of 9 g/L NaCl. Fig. 1 shows that the homogeneous HDL-C determination is linear up to at least 1500 mg/L. Moreover, as estimated from the slope of the regression line in Fig. 1, the recovery of the added HDL-C was 98.6%. The lowest HDL-C concentrations that could reliably be measured were ~30 mg/L.

**Specificity.** The apparent HDL-C concentration increased from 247 to 277 mg/L (+12%) when a serum pool initially containing LDL-C at 1500 mg/L was supplemented with LDL-C to yield a final concentration of 4000 mg/L (Fig. 2A). However, addition of 5000 mg/L LDL-C (final concentration 6500 mg/L) increased the HDL-C by ~50%. Supplementation of the pool with 553 mg/L VLDL-C (final concentration 835 mg/L, which corresponds to 3800 mg/L total triglycerides) did not substan-
tially increase the measured HDL-C (+6%; see Fig. 2B). Increasing VLDL-C to a final concentration of 2500 mg/L (corresponding to total triglycerides >11 g/L) caused an overestimation of HDL-C by 46%.

Intermethod comparison. We compared the homogeneous assay of HDL-C with a conventional phosphotungstic acid/MgCl$_2$-based precipitation method, analyzing 90 samples in parallel. Mean total cholesterol and total triglycerides were 2031 and 2402 mg/L, respectively; maximum cholesterol and triglycerides were 4703 and 11 070 mg/L, respectively. Mean HDL-C concentrations by the homogeneous method and the phosphotungstic acid/MgCl$_2$ precipitation method were respectively 414 and 403 mg/L, with respective ranges of 71–1115 mg/L and 51–1121 mg/L. The correlation between the two methods was excellent: $r = 0.970$ (Fig. 3). For a nonparametric equivalent of the standard deviation of the residuals, we calculated the 68th percentile of the residuals on the regression line according to Passing and Bablok [31]; it was 21.49. When we estimated the parameters of the regression line according to Passing and Bablok, the slope and intercept were 0.987 (95% confidence interval 0.956–1.019) and 17.2 mg/L (4.13–26.4 mg/L), respectively [31]. Further statistical analyses according to Passing and Bablok revealed that the slope did not significantly differ from 1, whereas the intercept was significantly different from 0. These data suggest that the new homogeneous assay for HDL-C allows reliable determination of HDLs by their cholesterol content. In addition, apo A-I correlated well with both the homogeneous HDL-C assay and the comparison precipitation method ($r = 0.890$ and 0.916, respectively; data not shown graphically).

As shown in Fig. 3, results of the homogeneous assay of HDL-C disagreed with the results of the precipitation method for two samples. Total triglycerides in these samples were 3092 mg/L (HDL-C 1121 mg/L by phosphotungstic acid/MgCl$_2$...
assay) and 1387 mg/L (HDL-C 271 mg/L by phosphotungstic acid/MgCl₂ assay). Taking all samples together, the ratio of apo A-I to HDL-C (homogeneous assay) was on average 2.1 but was 3.6 in the outlier with the higher HDL-C concentration, which suggests that the result of the homogeneous assay was falsely low. On the other hand, the apo A-I to HDL-C ratio in the other outlier was 2.3, which is normal. In contrast, the ratio of apo A-I to HDL-C (phosphotungstic acid/MgCl₂ assay) in this second outlier was 0.28, suggesting a falsely high result of precipitation assay.

Interferences. Fig. 4A shows that triglycerides at concentrations of up to 20 g/L increased the HDL-C result by only 2%. In contrast, hemoglobin increased the signal for HDL-C continuously, starting at 1000 mg/L, whereas bilirubin at concentrations of 60 mg/L or more lowered the results (Fig. 4B, 4C).

Discussion
As shown in many epidemiological and clinical studies, a low concentration of HDL-C is an important risk factor for coronary artery disease [1, 4, 5]. Here we evaluated a new method that allows a direct and fully automated determination of HDL-C in serum.

In most clinical laboratories HDL-C is determined by methods in which VLDL and LDL are precipitated with dextran sulfate, sodium phosphotungstic acid/MgCl₂, or PEG. These precipitation methods are precise and give results in excellent agreement with those of ultracentrifugation methods [26, 33]. However, the complexes generated by the addition of polyions have to be removed by a centrifugation step, which is tedious and prevents full automation. A reliable assay for HDL-C that can be run fully automated would have two advantages: screening for low HDL-C values would be significantly facilitated, and LDL-C could be calculated from the Friedewald formula without the need for a previous precipitating step [11]. One should keep in mind, however, that the caveats precluding the use of the Friedewald formula in postprandial or excessively hypertriglyceridemic samples also apply to the new homogeneous assay for HDL-C.

The precision of the homogeneous HDL-C assay is close to the precision of the conventional precipitation methods [20–22]. The linearity of the new homogeneous HDL-C assay also is excellent.

Comparison of the homogeneous HDL-C assay with a conventional phosphotungstic acid/MgCl₂ procedure showed ample correlation and a slope of 0.987; the intercept, although significantly different from 0, was negligible. A crucial point of the homogeneous HDL-C assay is the "wrapping" of chylomicrons, VLDL, and LDL with PEG and the subsequent generation of aggregates by addition of anti-apo B and anti-apo C-III. If the wrapping is incomplete, the enzymes of the cholesterol assay will also measure the cholesterol of the chylomicrons, VLDL, and LDL, resulting in falsely high HDL-C concentrations. As expected, the addition of large amounts of LDL (cholesterol up to 6500 mg/L) or VLDL (triglycerides up to 3800 mg/L) showed that the capacity of the reagent to complex the non-HDL lipoproteins was limited, ultimately leading to falsely high HDL-C values.

If LDL-C is calculated according to the Friedewald formula, an overestimation of HDL-C 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. Fortunately, this limitation is not relevant for the vast majority of samples, as shown in the intermethod comparison study: The homogeneous HDL-C assay did not produce high values, not even in sera with triglycerides up to 11 070 mg/L. In addition, the homogeneous assay did not produce higher HDL-C values than expected in the 5 (of 90) samples in which LDL-C exceeded 2300 mg/L (maximum LDL-C concentration 3175 mg/L). Given indications in the supplementation studies that LDL-C concentrations >3000 mg/L interfere with the homogeneous assay, results obtained in samples with total cholesterol concentrations of 3500 mg/L or more should be interpreted with caution. This constraint should not affect >1% of the samples encountered in general Western populations.

The new assay includes three different steps: wrapping the apo B- and apo C-III-containing lipoproteins; the enzymatic cholesterol reaction; and precipitation of the lipoprotein complexes and antibodies. The wrapping of apo B and apo C-III is accomplished by the sequential addition of PEG and the antibodies for apo B and apo C-III. To reduce the number of reagents in this assay, one could premix these two reagents; however, attempts to combine PEG and the antibody solution produced highly inconsistent results and were therefore abandoned early in the development of the homogeneous assay (Shiro Komori; personal communication).

No experience is currently available on the use of cholesterol reagents other than the CHOD-PAP method. However, because all complexed lipoproteins and antibodies are removed with guanidine hydrochloride before the absorbance is read, other chromophores and other wavelength combinations are likely to function as well.

For two samples the two methods for HDL-C yielded largely discrepant values, one presumably a falsely high result by the phosphotungstic acid/MgCl$_2$ method, the other presumably a low value by the homogeneous assay. The fact that 1 of 90 measurements was falsely low in the homogeneous assay may not relate to the assay itself but might have a technical reason, e.g., an obstructed pipettor. However, because we did not reanalyze the sample, we cannot exclude the possibility that in some instances samples may not produce the expected results with the homogeneous assay.

The accuracy of the homogeneous HDL-C assay was not impaired by adding Intralipid up to total triglycerides of 24 g/L [30]. However, when we increased the total triglycerides to 3800 mg/L or more by adding purified VLDL, we observed marked increases in the results of the homogeneous assay. An explanation for these findings is that lipemia per se does not interfere with the assay, whereas the apo B- and apo C-III-containing VLDL may completely absorb the antibodies present in the reaction mixture. The presence of free hemoglobin resulted in higher values, whereas bilirubin reduced the signal, leading to lower readings. These interferences are a general problem of enzymatic cholesterol reactions and hence also apply to precipitation methods [34].

The reagents for the homogeneous HDL-C assay are ~20-fold more expensive than conventional precipitation reagents for HDL-C. However, assuming 30 HDL-C tests per day, a technician would spend ~30 min to pipette samples and precipitation reagents before starting the automated cholesterol measurements. The tradeoff in technician time might approximately compensate for the higher costs of the reagents needed in the homogeneous assay.

A disadvantage of the new method is the need of four different reagents, which considerably reduces the number of automated analyzers suitable for adaptation. However, new instruments and software may emerge that will allow implementation of the homogeneous assay on additional analyzers. In summary, the new homogeneous assay for HDL-C determines HDL-C precisely and accurately and shows an excellent correlation with the phosphotungstic acid/MgCl$_2$ method. It thus represents a substantial improvement in our methodology to quantify HDL-C and may facilitate identification of individuals at an increased risk of atherosclerosis.

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References


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