New immunoseparation-based homogeneous assay for HDL-cholesterol compared with three homogeneous and two heterogeneous methods for HDL-cholesterol

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We evaluated four new commercial methods for HDL-cholesterol determination. The three completely homogeneous assays were an immunoseparation-based (IS) method from Wako, a polyethylene glycol-modified enzyme (PEG) method from Boehringer Mannheim, and a synthetic polymer-based (SP) method from Genzyme. The fourth method was a new heterogeneous method in which lipoproteins are removed using dextran sulfate-coated magnetic beads and Mg\(^{2+}\) (MB, Reference Diagnostics). We compared these methods with the conventional phosphotungstic acid/MgCl\(_2\) precipitation (PTA) procedure. The homogeneous assays had good intraassay imprecision with total CVs <2.3%, whereas the CVs of the MB assay were <5.9%. Adding HDL to serum to achieve HDL-cholesterol (HDL-C) concentrations up to 1000 mg/L revealed nearly complete recoveries in the IS, PEG, and MB assays, whereas the SP assay showed a lower recovery (~70%). The SP HDL-C apparently increased at increasing LDL-cholesterol and VLDL-triglyceride concentrations, whereas the IS, PEG, and MB methods were not influenced by LDL-cholesterol up to 6000 mg/L (MB, 5000 mg/L) and VLDL-triglycerides up to 9000 mg/L. Free fatty acids above ~2 mmol/L produced falsely high HDL-C in the IS and SP assays, the error amounting to as much as 50% in some samples. An intermethod comparison in 291 fresh serum samples yielded correlation coefficients of at least \(r = 0.95\) for all assays, when compared with the PTA procedure. The slopes and intercepts of the regression lines were 1.05 and 57 (IS), 1.12 and 9.9 (PEG), 1.00 and 39 (SP), and 1.0 and 38 mg/L (MB), respectively. The new assays are precise and simplify the determination of HDL-C, but in part they lack specificity or are susceptible to interferences, resulting in discrepancies when compared with the established PTA procedure.

The positive association of coronary heart disease risk with total cholesterol and LDL-cholesterol (LDL-C)\(^{\dagger}\) concentrations and its negative association with HDL-cholesterol (HDL-C) concentrations are well established (1–4). HDL-C concentrations <350 mg/L are considered as a cardiovascular risk factor; HDL-C concentrations exceeding 600 mg/L may be protective (3). According to Friedewald et al. (5), together with total cholesterol and triglycerides, the determination of HDL-C allows the calculation of LDL-C. For these reasons, reliable and easy-to-perform methods are needed to quantify HDL-C.

Most frequently, HDL-C is measured in the supernatant after precipitation of apolipoprotein (apo) B-containing lipoproteins by phosphotungstic acid/MgCl\(_2\) (PTA) or dextran sulfate (6, 7). All of these methods involve both a precipitation and a centrifugation step, which prevent full automation. Previously, new procedures for the determination of HDL-C were published (8–12). They have in common that they avoid at least the tedious centrifugation step.

In 1996, we published the results of an evaluation of a homogeneous HDL-C assay that included polyethylene glycol (PEG) to complex the non-HDL lipoproteins (reagent 1); antibodies against apo B and apo C-III, which aggregate the non-HDL lipoproteins (reagent 2); enzymes for cholesterol determination (reagent 3); and guanidine hydrochloride to stop all reactions (reagent 4) (9). The performance characteristics of this method were good, but

\(\dagger\) Nonstandard abbreviations: LDL-C, LDL-cholesterol; HDL-C, HDL-cholesterol; apo, apolipoprotein; PTA, phosphotungstic acid/MgCl\(_2\) precipitation; PEG, polyethylene glycol; IS, immunoseparation; SP, synthetic polymer; MB, dextran sulfate-coated magnetic bead; FFA, free fatty acid; and NCEP, National Cholesterol Education Program.
the disadvantage of the procedure was that it relied on four different reagents, which limited its application to a small number of automatic analyzers.

The aim of this study was to evaluate three homogeneous assays [an immunoseparation-based (IS) assay from Wako, a PEG-based assay from Boehringer Mannheim, and a synthetic polymer-based (SP) assay from Genzyme] and a dextran sulfate-coated magnetic bead-based (MB) procedure from Reference Diagnostics for the determination of HDL-C in parallel with the conventional PTA assay with respect to precision, linearity, interferences, and ease of handling.

Materials and Methods

Samples
Blood was drawn from 291 in- and outpatients of the University Hospital of Freiburg, including nonfasting samples. The blood was allowed to clot at room temperature, and serum was obtained by centrifugation at 1500g for 15 min. All analyses with the homogeneous and the precipitation assays were performed within 1 day of blood collection.

Lipid Measurements
Cholesterol and triglycerides were determined enzymatically with the CHOD-PAP and GPO-PAP methods, respectively. These reagents were purchased from Boehringer Mannheim. All measurements, including the HDL-C assays, were performed on a Boehringer Mannheim/Hitachi automatic analyzer type 911. Control sera Precinorm® L and Precipath® L (both from Boehringer Mannheim) were included in each analytical run. In addition, the nonesterified fatty acids were determined with the NEFA C assay, purchased from Wako, on a Wako 30R automatic analyzer in a subset of 130 samples. All analyses with the homogeneous and the precipitation assays were performed within 1 min. In severe hypertriglyceridemic samples, this separation can take up to 30 min. The cholesterol content of the HDL particles that remain in the supernate can easily be measured by the CHOD-PAP method. We applied a modified version of this assay, in which the accuracy at low cholesterol concentrations has been claimed to be improved by using a higher sample volume. The dilution of the sample was accounted for by multiplying the results by 1.2 (10).

HDL-C Determination by a PTA-Based Method
We used the PTA procedure as the comparison method. The reagents were purchased from Boehringer Mannheim. Two hundred microliters of serum were mixed with 500 µL of serum was incubated with 100 µL of reagent containing 250 mmol/L Mg²⁺ and MBs. After the cups were mixed for a short period on a vortex-type mixer and incubated for 10 min, they were transferred to a magnet-containing tube, which was then directly placed in the Hitachi analyzer. The dextran sulfate binds all apo B-containing particles, which are pulled down in the magnetic field within 1 min. In severe hypertriglyceridemic samples, this separation can take up to 30 min. The cholesterol content of the HDL particles that remain in the supernate can easily be measured by the CHOD-PAP method. We applied a modified version of this assay, in which the accuracy at low cholesterol concentrations has been claimed to be improved by using a higher sample volume. The dilution of the sample was accounted for by multiplying the results by 1.2 (10).

HDL-C Determination by a Combined Ultracentrifugation and Precipitation Method
In 130 samples, a combined ultracentrifugation-precipitation assay was used as the comparison method. Essentially, the protocol of the Lipid Research Clinics Program was followed, with modifications previously described (13, 14). In brief, VLDL were floated by ultracentrifugation (density = 1.006 kg/L), and the LDL was separated from the HDL in the infranatant by PTA.
Isolation of VLDL, LDL, and HDL, and preparation of lipoprotein-deficient serum

VLDL, LDL, and HDL were isolated by sequential ultracentrifugation using density <1.006 kg/L, 1.006 < density < 1.063 kg/L, and 1.063 < density < 1.21 kg/L as density limits (15). Lipoprotein-deficient serum was obtained by 2 × repeated ultracentrifugation at 150 000 g for 48 h after adjusting the serum to density = 1.25 kg/L by adding solid KBr.

Apo A-I

Apo A-I was measured turbidimetrically on the Wako-30R analyzer with reagents purchased from Greiner Biochemica. The assay was calibrated against the IFCC apo A-I standard.

Linearity

Testing of linearity was performed by adding HDL isolated by ultracentrifugation to serum. To a constant volume of serum, increasing amounts of HDL were added. To modify the matrix as minimally as possible, the samples were brought to equal volumes with lipoprotein-deficient serum.

Interferences

The influences of LDL and VLDL on HDL-C measurements were investigated in experiments in which they were added to samples. In addition, free fatty acids (FFAs) and bilirubin were measured in the samples of the intermethod comparisons. Interference from hemoglobin was analyzed according to Glick et al. (16).

Statistical methods

Precision data were calculated according to recommendations of the NCCLS/EP5-T protocol (17). Regression analyses were performed using the method of Passing and Bablok (18). Total error was calculated as the sum of the systematic error plus random error (19, 20). Systematic error is calculated from the linear regression equation $y = b_0 x + a$, where $b$ is the slope of the regression line and $a$ is the $y$-axis intercept. At an HDL-C concentration of $x_c$, systematic error is the absolute value of $y - y_c$. Random error is $1.96 \times SD$ from the run-to-run precision study.

Results

Precision

Two commercial control sera with low and medium HDL-C concentrations and one human serum pool were used to assess the precision of the HDL-C assays (Table 1). The within-day imprecision values were determined as the average of the imprecision obtained each day. The between-day imprecision values were calculated from the imprecision of the means obtained each day, which were then adjusted for the within-day imprecision component. The total CVs ranged from 1.1% to 2.3% for the three homogeneous assays, from 3.9% to 5.9% for the MB assay, and from 3.1% to 3.7% for the PTA assay, respectively.

Linearity

The new HDL-C assays were linear up to at least 1000 mg/L, as revealed by increasing amounts of HDL prepared by sequential ultracentrifugation to sera (Fig. 1). For the SP assay, a slope of ~0.7 was obtained when the

<table>
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<th>Assay</th>
<th>Sample</th>
<th>Mean, mg/L</th>
<th>Within-daya</th>
<th>Between-day</th>
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<td>CV, %</td>
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a Two control sera and one human pool serum were analyzed with each HDL-C assay. Pools were divided into 300-μL aliquots and stored at 4 °C until tested. One aliquot of each pool was assayed in duplicate on each of 21 days.

b Calculations for within-day and between-day SD were based on 21 samples; for within-day SD, calculations were based on a pair of 21 samples.

c $SD_{total} = SD_{w} + SD_{b}$, where $SD_{w} = \text{within-day standard deviation}$, and $SD_{b} = \text{between-day standard deviation}$.

d HSP, human serum pool; PNL, Precinorm L; PPL, Precipath L.
measured HDL-C was plotted against HDL-C added, suggesting that the recovery of this assay was \(<100\%\).

**SPECIFICITY**

HDL-C concentrations are plotted against added LDL-C and VLDL-triglycerides, respectively, in Fig. 2.

Cross-reactivity with LDL varied between assays. The IS assay was not influenced by LDL-C up to 6000 mg/L. The PEG assay showed a minimal increase over the whole concentration range, but deviations >5% occurred only in samples with LDL-C above 6000 mg/L. LDL-C increased the HDL-C values of the SP assay at any concentration. The MB assay was markedly influenced at LDL-C concentrations above 5000 mg/L.

Cross-reactivity with VLDL also varied between assays. Triglycerides did not substantially interfere with the IS and PEG assays up to 9000 mg/L VLDL-triglycerides. The HDL-C values of the SP assay increased over the whole concentration range, whereas VLDL-triglycerides interfered with the MB assay at concentrations of \(~5000\) mg/L. Both the IS and the MB assays showed an unexpected negative bias with increasing VLDL-triglycerides.

**INTERMETHOD COMPARISONS**

As the comparison method, we used the PTA procedure either in whole serum (n = 161), or after removal of VLDL by ultracentrifugation (n = 130).

A total of 291 fresh sera were analyzed with each of the new methods and the comparison method. Mean total cholesterol and total triglycerides were 1690 and 1680 mg/L, respectively. Maximum cholesterol and triglyceride concentrations were 3670 and 6830 mg/L, respectively. HDL-C concentrations ranged between 35 and 906 mg/L, determined with the PTA method.

The correlation coefficients were between 0.949 and 0.987 (Table 2). When we estimated the parameters of the regression lines according to Passing and Bablok, slopes relating the homogeneous HDL-C IS and PEG assays to the PTA assay were significantly higher than 1.00 (P<0.05), whereas the SP and MB methods produced slopes close to 1.00 (18). All intercepts were significantly (P<0.05) different from 0.0 mg/L, which produced signif-
icantly ($P<0.05$) higher HDL-C values for all new methods, compared with the conventional precipitation procedure. The bias plots of the IS and PEG assays revealed small but systematic deviations, which increased slightly as HDL-C concentrations increased (Fig. 3). At HDL-C concentrations <350 mg/L, some of the IS results showed a deviation of approximately ~200 mg/L, whereas the PEG assay produced only one outlier. The bias plot of the

<table>
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<th>Assay</th>
<th>Correlation coefficient</th>
<th>Regression line</th>
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<th>New procedure for HDL-C, y</th>
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<td>0.980</td>
<td>1.05$^c$</td>
<td>56.8$^d$</td>
<td>336</td>
</tr>
<tr>
<td>PEG</td>
<td>0.987</td>
<td>1.12$^c$</td>
<td>9.9$^d$</td>
<td>336</td>
</tr>
<tr>
<td>SP</td>
<td>0.949</td>
<td>1.00</td>
<td>39.0$^d$</td>
<td>336</td>
</tr>
<tr>
<td>MB</td>
<td>0.987</td>
<td>1.00</td>
<td>37.8$^d$</td>
<td>336</td>
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</table>

$^a$ Regression analyses were done according to Passing and Bablok (18).
$^b$ Concentrations are given in mg/L.
$^c$ Slopes are significantly different from 1.00.
$^d$ Intercepts are significantly different from 0.00.
$^e$ The means were significantly higher, compared with the PTA method.

Fig. 3. Bias plots of HDL-C (new assay minus PTA assay) in the IS (A), PEG (B), SP (C), and MB (D) assays.
SP assay showed substantial scattering over the entire range. The results of the MB assay were negatively biased throughout the entire concentration range, with only a small scattering.

The correlation coefficients between the apo A-I measurements and the HDL-C concentrations of the IS, PEG, SP, and MB assays were 0.92, 0.93, 0.89, and 0.93, respectively.

TOTAL ERROR
The total errors were calculated at the decision points of 350 mg/L and 600 mg/L, respectively. As shown in Table 3, the systematic deviations contributed much more to the total errors than did the random errors. The highest total errors were found for the IS and PEG assays.

FREE FATTY ACIDS
Conditions with increased FFAs occur mainly in inpatients receiving intravenous heparin therapy. At concentrations >2 mmol/L, there is substantial binding of FFAs to lipoproteins (21). This increases the electrophoretic mobilities of the lipoproteins and may change their interaction with precipitating reagents (22–24). The results obtained in those serum samples of the intermethod comparisons that had FFA concentrations >2.0 mmol/L (n = 22) indicated that the IS and SP assay were susceptible to interference from FFAs, whereas the assays from PEG and MB were not (Fig. 4). Experiments with samples enriched in FFA, according to the method of Spector and Hoak (25), completely confirmed these findings (data not shown).

INTERFERENCES
Hemoglobin at concentrations up to 2 g/L did not interfere substantially with the new homogeneous HDL-C assays using IS and PEG (16). The SP assays yielded lower values, whereas in the MB and the PTA method HDL-C increased markedly when hemoglobin was added (Fig. 5).

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). Thus 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 but are tedious in respect to the centrifugation step involved (26, 27). Recently, several new HDL-C assays have been devised which have–with exceptions–satisfactory performance characteristics (8–12).

Here we present the results of a comparison study that included three principally different homogeneous HDL-C assays: the first (IS) uses antibodies for the separation of non-HDL lipoproteins coupled to a conventional enzymatic cholesterol assay; the second (PEG) uses MgCl2, sulfated a-cyclodextrin, dextran sulfate, and PEG-coupled enzymes; the third (SP) uses synthetic polymers, polyanions, and detergents coupled with a conventional enzymatic cholesterol assay for the quantification of HDL-C. The fourth method (MB) is based on the absorption of apo B-containing lipoproteins to dextran sulfate-coated magnetic beads. All of these assays were compared with the conventional PTA procedure.

General advantages of the homogeneous methods are that they obviate pretreatment of the samples and that only small sample volumes are needed. These features allow the fully automated determination of HDL-C even in the emergency laboratory, where HDL-C may be useful in the diagnosis of serious infections, such as malaria, or to monitor patients after liver transplantation (28, 29).

The National Cholesterol Education Program (NCEP) performance goals for 1998 demand that the CV of HDL-C determinations be <4% at concentrations >420 mg/L (30), a criterion that was met by all investigated HDL-C assays. The NCEP precision goal for HDL-C concentrations <420 mg/L is a SD <17 mg/L (30). The homogeneous assays and the PTA procedure fulfilled these criteria. The MB assay produced a SD >17 mg/L in one of three pools examined. Thus, the NCEP precision goals will obviously be met by all homogeneous assays considered here at both high and low HDL-C concentrations, whereas the MB assay may need further improvement. With regard to the precision, the differences between the three homogeneous HDL-C assays are small and appear not clinically relevant. These data agree with previous publications (9–12).

The slopes of the experiments with added HDL indicated nearly complete recoveries with the IS and PEG assay. Our data confirm that the specificity of the PEG method is good (10, 12, 30, 31). Furthermore, they indicate that the antibody concentrations of the IS assay are

<p>| Table 3. Total error for the new HDL-C assays. |
|-------------------------------|------------------|------------------|------------------|</p>
<table>
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<tr>
<th><strong>Assay</strong></th>
<th><strong>Systematic error, %</strong></th>
<th><strong>Random error, %</strong></th>
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<td></td>
<td>600 mg/L HDL-C</td>
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</table>
sufficient to complex all non-HDL particles. The specificity of the IS assay is thus at least as good as that of the homogeneous HDL-C from International Reagents Corporation, Kobe, which also contains antibodies to form stable aggregates of the non-HDL lipoproteins. The latter method, however, has the disadvantage of including four different reagents.

On the other hand, only ~70% of the added HDL was recovered with the SP method. The addition of LDL and VLDL produced falsely high HDL-C concentrations, indicating limited specificity of this method, as described previously.

The MB assay was specific, but the interferences from LDL-C and VLDL-triglycerides started at lower concentrations than in the IS and PEG assays.

Recently, Demacker et al. (27) showed that the PTA procedure agrees well with the CDC reference method. Therefore, and because the PTA procedure is very common in laboratories, it is well suited as a comparison method.

We obtained excellent correlation coefficients when we compared the homogeneous HDL-C assays with the PTA procedure. The slope of the IS assay was >1.00, which caused the positive slope of the bias plot (Fig. 3). This was due to a
In a previous premarking evaluation of the PEG assay, a positive bias of 3% was observed (31). The optimistic data of this previous multicenter study were not reproduced in the current study. The PEG assay showed a slope significantly \( P<0.05 \) exceeding unity. This deviation was due to a falsely high declaration of the calibrator, which may have occurred on large-scale production. Finally, a very recently performed evaluation of the PEG method performed in our laboratory showed that the positive bias had disappeared, indicating that this assay is now working well \( y = 0.98x + 11.9 \text{ mg/L}; r = 0.997; n = 100 \).

The unsatisfactory results of the SP assay have been reported by other investigators as well (11, 12). The low specificity of this assay is hampering the use of this method in the clinical chemical laboratory, in spite of the good imprecision and a slope of 1.0 in the intermethod comparison. The scattering of the bias plots, caused by the nonspecificity of this method, is not acceptable.

The MB assay showed the best agreement with the comparison method, including an ideal slope of 1.0. It is worth noting that all results of the MB assay were slightly higher than the PTA procedure, caused by a small positive intercept of the regression line.

The NCEP goals for 1998 demand a total error of <13% (30). In the current study, the IS and PEG homogeneous assays have not met these criteria because of high systematic error caused by an incorrect calibration (slopes >1 plus a positive y-intercept in the intermethod comparison). The SP assay had the lowest total error because of a very small systematic error. Obviously, the limited specificity of the SP assay for HDL is balanced by a suitable calibrator, which accounts for the fact that LDL-C and VLDL-C are in part determined as HDL-C. The MB assay meets the NCEP criteria, the total error amounting to 13.2% at 350 mg/L.

A common denominator of the present study and of many earlier reports is that homogeneous assays tend to overestimate HDL-C in the range of \( \sim 10\% \). This raises the suspicion that homogeneous assays may yield an HDL fraction that differs in some respect from HDL found in the supernate of precipitation reactions. Such a systematic difference between homogeneous and conventional methods would not be entirely surprising. HDL represents a polydisperse system of particles markedly differing by physicochemical characteristic, apolipoprotein composition, and pathobiochemical importance. On the basis of their apo E content, two populations of HDL particles can be distinguished, those without apo E and those with apo E. The latter accounts for \( \sim 10\% \) of the total HDL-C. In the PTA assay, the apo E-containing HDL particles are precipitated and therefore not included in the HDL-C (32). In the homogeneous PEG assay, in contrast, these particles contribute to HDL-C (33), a finding that may at least in part account for the bias found between the homogeneous and the precipitation methods. It is currently not known how different HDL subfractions behave in the other homogeneous methods.

What are the consequences of these differences in practice? The large epidemiological studies, which established the role of low HDL-C concentrations as a risk factor of coronary artery disease, were performed with precipitation methods adjusted to the CDC reference method. These studies will probably not be repeated in the future because they are expensive and the results would be biased through the widespread use of lipid-lowering therapies, even in primary prevention. It will probably not be feasible to change the cutoff point for HDL-C from 350 (without apo E-containing HDL-C) to 380 mg/L (including apo E-containing HDL-C). More pragmatic, however, will be adjusting the new methods to the CDC reference method (31, 34), thereby deliberately disregarding the fact that the homogeneous assays yield an HDL fraction that may physicochemically be distinct from HDL produced by precipitation reactions.

The reagents for the homogeneous HDL-C assay are approximately threefold more expensive than conventional precipitation reagents. With the exception of the SP assay, the homogeneous assays evaluated here are convenient to use in routine laboratories. The MB assay, which also avoids centrifugation, is substantially cheaper than the homogeneous assays and therefore represents an alternative for laboratories with a workload of <50 samples per day. However, in most laboratories, the higher reagent costs for the new HDL-C assays will probably be offset by economizing the laboratory work.

In summary, the homogeneous IS and PEG assays produce precise and accurate determinations of HDL-C. Even hypertriglyceridemic samples up to at least 9000 mg/L revealed unbiased results. The MB assay showed the best agreement with the PTA procedure, except in of hypertriglyceridemic and hemolytic samples. This assay is cheaper than the homogeneous assays but is not fully automated. All new HDL-C assays, with the exception of the SP assay, represent a major improvement in our methodology to quantify HDL-C and may facilitate the identification of individuals at increased risk of atherosclerosis. Although it was the only homogeneous assay to meet the NCEP performance goals, the SP assay proved nonspecific and is therefore not recommended for use in the clinical laboratory.

We are grateful for the excellent technical assistance of Gabi Herr, Brigitte Kreisel, and Sibylle Rall. We also thank Wako, Boehringer Mannheim, Greiner, and Reference Diagnostics for providing HDL-C assay kits free of charge.
References


