Electrophoretic Separation of High-Density Lipoprotein Cholesterol Evaluated and Compared with the Modified Lipid Research Clinic Procedure

Evan A. Stein,¹,² Susan McNeely,¹ and Paula Steiner²

We evaluated a new agarose-gel-electrophoretic procedure (Corning) (I) for separating and quantitating of high-density lipoprotein cholesterol (HDLc), comparing it with the modified Lipid Research Clinics (LRC) procedure (heparin 183 kilounits/L, MnCl₂ 92 mmol/L) (II). Method I was insensitive to an HDLc concentration of 50 mg/dL, but gave a linear dose-response curve between 130 and 1200 mg/dL. Method II is sensitive to 50 mg/dL and linear from 50–1200 mg/dL. The within-plate CV for the Corning method varied from 26.2% for an HDLc of 168 mg/dL to 6.8% for 580 mg/dL. Within-day between-plate CV for the Corning method ranged from 22.1% at 155 mg/dL to 8.0% at 651 mg/dL, compared to 3.0 and 0.8% for the modified LRC procedure. Between-day CV for method I was 20, 12.6, 4.3, and 3.5% for HDLc concentrations of 175, 435, 542, and 678 mg/dL, respectively; for method II it was 14, 5, 3.5, and 2.6%, respectively. Analysis of HDLc in 100 patients by both procedures showed mean HDLc values to be significantly lower (mean ± SD = 27.8 ± 1.7 mg/dL; p < 0.001) by method I. In 46 patients with HDLc <450 mg/dL, this difference was accentuated (mean ± SD = 40.5 ± 2.6 mg/dL) and clinically significant. Electrophoretic methods offer a promising further alternative method for HDLc separation and quantitation, but the negative bias, present limited sensitivity, and lack of precision at <450 mg/dL indicate that they are not yet optimal for routine clinical use for patients with values <450 mg/dL.

Additional Keyphrases: evaluating heart-disease risk - intermethod comparison - hyperlipoproteinemia

The renewed interest in serum high-density lipoprotein (HDL) has been due, in part, to epidemiological studies (1–3) demonstrating an independent and inverse relationship between its cholesterol content (HDLc) and coronary heart disease. Several other studies have focused on the relationship of HDLc to smoking (4), exercise (5), obesity (6), alcohol intake (7), and other factors (8–10). Because “therapeutic” regimes for consistently increasing HDLc are not yet available, the use of HDLC measurement is at present confined to assessing risk of heart disease and assisting in the diagnosis of other hyperlipoproteinemic states, especially hyperbeta-lipoproteinemia (17).

Assignment of heart-disease risk has for the most part been based on retrospective and prospective data collected by use of the Lipid Research Clinics (LRC) method for isolation and quantitation of HDLC (12). Although there have been several excellent studies (13–16) comparing precipitation techniques for HDLC quantitation in terms of specificity, accuracy, and precision, relatively little has been done to compare results by the more recent electrophoretic methods (17, 18) with those by established methods.

In this report, we evaluate a new commercial agarose gel-electrophoretic separation and enzymatic staining procedure (Corning Medical, Medfield, MA 02052) and compare it to the modified LRC procedure (15) in terms of accuracy, precision, sensitivity, and linearity.

Materials and Methods

Samples

Blood samples were collected at the Cincinnati Lipid Clinic in January and February of 1979 according to established LRC protocol (12). The subjects were apparently healthy volunteers or hyperlipoproteinemic referral patients, 10 to 68 years old. There were 44 female and 56 male subjects. Cholesterol values ranged between 1030 and 5260 mg/dL, triglyceride values between 380 and 9840 mg/dL, and HDLC values between 70 and 1210 mg/dL. Blood was collected into 15-mL Vacutainer Tubes, each containing 22.5 mg of disodium ethylenediaminetetraacetate, mixed thoroughly, and cooled immediately to 4°C. Cells were removed within 3 h by centrifugation at 1500 × g at 4°C for 30 min. Plasma was stored at 4°C, and analyzed within 48 h.

Plasma Pools

To assess accuracy and precision, we combined selected patients' samples to form four plasma pools containing various total, LDL, HDLC, and triglyceride concentrations (Table 1). These pools were for the most part designed to contain HDLC concentrations either commonly encountered (19) in men and women (labeled "medium" and "normal" pools, respectively) or at concentrations of clinically significant prognostic (1) value ("high" and "low" pools). However, we also took into account other lipid and lipoprotein fractions, to test method
Table 1. Concentrations of Lipid and Lipoprotein Fractions in Plasma Pools Used to Assess Accuracy and Precision

<table>
<thead>
<tr>
<th>Pool</th>
<th>HDLC</th>
<th>Total cholesterol</th>
<th>Calculated LDLc</th>
<th>Triglyceride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>175</td>
<td>3078</td>
<td>2660</td>
<td>1208</td>
</tr>
<tr>
<td>Normal</td>
<td>441</td>
<td>2625</td>
<td>1814</td>
<td>1848</td>
</tr>
<tr>
<td>Medium</td>
<td>537</td>
<td>1836</td>
<td>1033</td>
<td>1329</td>
</tr>
<tr>
<td>High</td>
<td>700</td>
<td>2350</td>
<td>1469</td>
<td>904</td>
</tr>
</tbody>
</table>

Average of eight analyses for each plasma pool.

performance at several clinically significant lipid concentrations. These pools were analyzed (simultaneously with patients' samples) by the two methods described below. The pools were stored at 4 °C; no deterioration in HDLC values was evident during the 14-day study period.

Linearity

We studied linearity and sensitivity by use of 11 specially selected plasma samples (Table 2). The samples were from patients who had similar total cholesterol values, but whose HDLC ranged from 50 to 740 mg/L.

Lipoprotein Separation; HDLC Quantitation

HDLC was separated from VLDL and LDL by the Corning electrophoresis method and the modified LRC procedure (15).

Corning Electrophoresis Method

We applied 2 µL of samples to each of eight wells (1 µL at a time, allowing the sample to diffuse before adding the next microliter) in a Corning Agarose Universal Electrophoresis Film (cat. no. 470100). We then separated the lipoprotein fractions, using a Corning electrophoresis cell and power supply (cat. no. 470130 and 470134) at 90 V for 35 min in 3- (N-morpholino)propanesulfonic acid (cat. no. 470015) buffer (50 mmol/L, pH 7.8). After electrophoresis, excess buffer was drained before inverting the cell cover. After the gel was removed from the cell, it was blotted at the edges and oven dried for 2 min at 60 °C. One milliliter of cholesteryl reagent (4-aminoantipyrine, 6.4 mmol/L final concentration; phenol, 85 mmol/L; peroxidase, EC 1.11.1.7; 200 000 U/L; cholesterol oxidase, EC 1.1.3.6; 2000 U/L; and cholesterol esterase, EC 3.1.1.13, 1250 U/L), reconstituted 15–30 min earlier with the buffer, was then applied to each gel and spread evenly by rolling a 5-mL serological pipette over the surface of the gel. Excess reagent was removed from the gel by the capillary action of the pipette. Any cholesteryl in the separated lipoproteins became visible after incubation for 30 min and was measured at 520 nm with a Corning Model 720 Densitometer (cat. no. 475155). HDLC was evaluated from the scan as a percentage of total cholesterol, which was converted to milligrams per liter by multiplying the value for total cholesterol obtained by LRC method (15).

Modified LRC Method

Specimens were precipitated with heparin and MnCl2 as described in the Manual (12), except that the final MnCl2 concentration was doubled, to 92 mmol/L (21).

Lipid Measurements

All total cholesterol and triglyceride estimations were made with an AutoAnalyzer II (Technicon Instruments Corp., Tarrytown, NY 10591), according to the LRC procedure (12), with use of the serum calibrator supplied by the Center for Disease Control, Atlanta, GA. We similarly quantitated HDLC remaining in the plasma supcrnate after VLDL and LDL precipitation (15). Cholesterol values for the HDLC supernates by the modified LRC method were multiplied by a correction factor of 1.09, to take into account the dilution caused by the extra heparin–Mn2+ reagent (15).

Results

Accuracy and Linearity

We assessed accuracy and linearity by comparing HDLC values obtained by the two methods for a series of samples with various HDLC concentrations, regarding the LRC as the "reference" method. Figure 1 shows that results by the Corning method appear to correlate linearly with the modified LRC method for values between 130 and 740 mg/L. The correlation coefficient was 0.989, with a slope of 0.96 and an intercept on the Corning electrophoresis ($y$) axis of $-33.2$ mg/L.

Sensitivity

At an HDLC concentration of 50 mg/L, no HDLC fraction was discernible by the Corning electrophoretic method, as confirmed by the inability of the Corning method to differ-

![Fig. 1. Accuracy and linearity studies—Corning electrophoresis and modified LRC procedure (mean ± SD)](https://example.com/fig1.png)

Table 2. Lipid and Lipoprotein Concentrations in 11 Selected Patients' Plasma Samples Used for Sensitivity and Linearity Studies

<table>
<thead>
<tr>
<th>HDLC (mean ± SD)</th>
<th>Total cholesterol (mg/L)</th>
<th>Triglyceride (mg/L)</th>
<th>LDLc (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>53 ± 15</td>
<td>2970</td>
<td>2190</td>
<td>2480</td>
</tr>
<tr>
<td>130 ± 0</td>
<td>2120</td>
<td>440</td>
<td>1900</td>
</tr>
<tr>
<td>170 ± 0</td>
<td>2690</td>
<td>970</td>
<td>2330</td>
</tr>
<tr>
<td>250 ± 5.7</td>
<td>2490</td>
<td>2920</td>
<td>1680</td>
</tr>
<tr>
<td>280 ± 0</td>
<td>2830</td>
<td>910</td>
<td>2370</td>
</tr>
<tr>
<td>320 ± 0</td>
<td>2490</td>
<td>3400</td>
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<tr>
<td>400 ± 5.7</td>
<td>2620</td>
<td>1820</td>
<td>1860</td>
</tr>
<tr>
<td>510 ± 5.7</td>
<td>2770</td>
<td>950</td>
<td>2070</td>
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<tr>
<td>570 ± 5.7</td>
<td>2780</td>
<td>1510</td>
<td>1910</td>
</tr>
<tr>
<td>660 ± 0</td>
<td>2500</td>
<td>1080</td>
<td>1630</td>
</tr>
<tr>
<td>740 ± 5.7</td>
<td>2720</td>
<td>810</td>
<td>1820</td>
</tr>
</tbody>
</table>

Average of triplicate assays of each by the modified LRC procedure.
entiate between the three plasma samples with HDLC values of 250, 280, and 320 mg/L (Figure 1).

**Precision**

To assess within-plate precision of the Corning method, we ran each pool on all eight tracks per plate. The within-plate coefficient of variation (CV) was 20.4 and 26.2% for the low-HDLC pool, 23.7 and 10.2% for the medium-HDLC pool, and 9.3 and 6.8% for the high-HDLC pool.

The within-day (between-plate) CV for the Corning method (Table 3) was 22.1% for the low pool (mean HDLC found, 155 mg/L), 7.8% for the normal pool (mean HDLC found, 372.5 mg/L), 4.1% for the medium pool (mean HDLC found, 524.3 mg/L) and 8.0% for the high pool (mean HDLC found, 651.4 mg/L). In assessing between-plate precision, we used the same sample track on every plate, to minimize within-plate variation. The within-day between-tray CV for eight analyses of each of the same pools by the modified LRC procedure was 3.0, 0.8, 2.2, and 0.5%, respectively (Table 3). Between-day precision for the two methods was assessed by analyzing the same pools on nine consecutive working days. The between-day CV for the Corning electrophoretic method was 20.0, 12.6, 4.3, and 3.5% for the low, normal, medium, and high HDLC pools, respectively (Table 3); the CV for the modified LRC precipitation procedure for the same days and pools was 14.0, 5.0, 3.5, and 2.6% respectively.

**Comparison of HDLC by Corning Electrophoresis and the Modified LRC Method**

HDLC concentrations in plasma from 100 subjects were quantitated by the Corning electrophoretic (455.4 ± 203.8 mg/L, mean ± SD) and modified LRC procedures (483.2 ± 188.0 mg/L). The mean values for total plasma cholesterol and triglyceride in these 100 subjects were 2416 ± 687 and 1687 ± 1290 mg/L, with a cholesterol range of 1030 to 5260 mg/L and triglyceride range of 380 to 9840 mg/L. HDLC values by the modified LRC procedure were slightly but consistently higher (mean difference ± SD = 27.8 ± 1.7 mg/L). This difference was significant by paired t-test (t = 6.7, p < 0.001) and by two-way analysis of variance (f = 44.28; df 1,99; p < 0.001). There was a close correlation between HDLC as measured by the two methods (r = 0.981) with a slope of 1.086 and an intercept on the Corning HDLC (y) axis of -70.3 mg/L (Figure 2).

In a subset of the population, with HDLC values of 450 mg/L or less (Figure 2), the difference between the modified LRC (343.3 ± 85.6 mg/L) and Corning methods (302.5 ± 93.4 mg/L) was greater (mean difference ± SD = 40.5 ± 2.6 mg/L). This difference was again significant by the paired t-test (t = 7.8; p < 0.001). The correlation between the two methods at <450 mg/L was still good (r = 0.928), with a slope of 1.01 and an intercept on the Corning HDLC axis of -45.3 mg/L.

**Discussion**

We used the modified LRC method for HDLC isolation and quantitation as a "reference" method in this study because it is more convenient than ultracentrifugation and because extensive previous and recent studies by Warnick et al. (13, 15) have shown that HDLC values obtained with plasma samples do not differ significantly from those with the ultracentrifugation method (d > 1.063 corrected). In addition, with a Mn²⁺ concentration of 92 mmol/L, all of the apo-B-associated lipoproteins are precipitated (13, 15, 21).

The Corning method correlated highly (r = 0.981) with the modified LRC procedure over a wide range of plasma total cholesterol, LDLC, HDLC, and triglyceride concentrations; however, plasma HDLC values by the Corning method were significantly (p < 0.001) lower (by 5–6%) than those by the modified LRC procedure. In those subjects with HDLC values <450 mg/L, again there was a good correlation between the two methods (r = 0.928); however, the difference between the two methods was even more substantial, with the Corning method giving results 11–12% lower than those obtained by the modified LRC procedure.

![Fig. 2. Comparison of patient plasma HDLC values by Corning electrophoresis and the modified LRC procedure](image-url)
The cooperative lipoprotein phenotyping study (23), involving 6859 individuals, demonstrated that an HDLC of 450 mg/L in men and 550 mg/L in women was associated with average risk for heart disease, and higher and lower HDLC concentrations with lower and higher CHD risk. In males, HDLC values of 400, 350, 300, and 250 mg/L were associated with 1.22, 1.49, 1.82, and 2.0 times the average risk, respectively. These large increases in heart-disease risk for relatively small changes in HDLC must be stressed, as well as the fact that these clinical and epidemiological data were derived with the LRC method and a Mn2+ concentration of 46 mmol/L (25). In the study by Warnick et al. (13), HDLC by the heparin-Mn2+ (46 mmol/L) method gave results slightly higher than those obtained with a Mn2+ concentration of 92 mmol/L. Thus, the differences between the Corning method and the modified LRC procedure, especially at <450 mg/L, are sufficient to have significant clinical implications. The problem in clinical interpretation is additionally complicated by the lack of sensitivity of the Corning method. The linearity studies (Figure 1) demonstrate the difficulty of differentiating between HDLC of 250, 280, 320, and 380 mg/L with the electrophoretic procedure, an important range of values in terms of assigning heart-disease risk. Possibly the sensitivity for HDLC can be increased by additional applications of plasma to the sample well. However, in doing so, the cholesterol concentrations in the other lipoprotein fractions (LDL and VLDL) may be increased sufficiently to cause exhaustion of the substrate in the cholesterol-oxidase staining reagent and other analytical problems.

However, the Corning method does appear to have better precision at similar HDLC concentrations than does a previously described electrophoretic method on cellulose acetate (17). Schifman (24) has suggested for the American College of Clinical Pathologists' Check Sample that an alternative method be used if day-to-day precision for HDLC is less than 8% at 400 mg/L. In our hands the Corning method did not clearly differentiate the VLDL from the LDL fraction, or allow direct quantitation of LDL.

It has been proposed (25, 26) that heart-disease risk can be sufficiently assessed by the total cholesterol/HDLC ratio or the percent HDLC [(HDLC/total cholesterol) × 100], or both. The almost normal indexes found in a patient with well-documented familial hypercholesterolemia (Table 4) demonstrates the potentially serious diagnostic misclassification inherent in the use of such ratio calculations and shows the value of obtaining specific lipoprotein cholesterol concentrations.

In view of the accuracy, sensitivity, and precision needed for HDLC measurement so that results will be clinically relevant, we conclude that the electrophoretic methods, while promising, are not yet optimal for routine clinical use, and the method of choice for isolation and quantitation would seem to still be the modified LRC precipitation procedure (13, 15, 21).

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References

<table>
<thead>
<tr>
<th>Total cholesterol (TC)</th>
<th>Patient Interpretation</th>
<th>Age &amp; sex percentile (19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3080 mg/L</td>
<td>Markedly high risk (27)</td>
<td>&gt; 99</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>Normal</td>
<td>70</td>
</tr>
<tr>
<td>HDLC</td>
<td>Normal</td>
<td>50</td>
</tr>
<tr>
<td>LDLC (calculated)</td>
<td>Markedly high risk (27)</td>
<td>&gt; 99</td>
</tr>
<tr>
<td>TC/HDLC</td>
<td>Average risk (25, 26)</td>
<td></td>
</tr>
<tr>
<td>(HDLC/TC) X 100</td>
<td>Average risk (25, 26)</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Atherosclerotic Risk by Quantitation of HDLC and Calculation of LDLC vs Total Cholesterol/HDLC Ratio or HDLC in 10-year-old Male


