Quantitative Determination of High-Density Lipoprotein Cholesterol by Agarose Gel Electrophoresis

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We have developed a procedure for the determination of high-density lipoprotein cholesterol by agarose gel electrophoresis. Only 2 μL of sample was applied to the gel. After electrophoresis at 90 V for 35 min, an enzymatic reagent was applied. After a 30-min incubation, the high-density lipoprotein cholesterol was quantified by densitometry. Precision for this measurement approaches that reported for the heparin–manganese/Abell–Kendall method (Clin. Chem. 25: 596–609, 1979). We evaluated accuracy by comparing high-density lipoprotein cholesterol concentration measured by electrophoresis to that determined in the Framingham Heart Study procedure (J. Biol. Chem. 195: 357, 1952). The resulting correlation was excellent. By the paired Student's t-test, there was no significant difference between the two methods. The proposed method gives a linear standard curve when the concentration of total cholesterol is between 1.0 and 3.5 g/L. By accurate quantitation of high-density lipoprotein cholesterol, agarose gel electrophoresis can aid in assessment of coronary heart disease risk for a large segment of the population.

Additional Keyphrases: coronary heart disease, Framingham Heart Study, intermethod comparison

Multiple factors have been identified as important in predicting risk of coronary heart disease (1). Recent work has focused on the importance of high-density lipoprotein cholesterol (C-HDL) (2–6) in assessing risk of coronary heart disease. As early as 1951, Barr et al. (7) described an inverse relationship between C-HDL and coronary heart disease. Their finding was largely ignored until 1975, when Miller and Miller (3) published a review of epidemiological studies concerning HDL. Recently, the importance of C-HDL has been reinforced by several extensive epidemiological studies (2, 4, 5). One by Gordon et al. (5) at the Framingham Heart Study revealed that, of all the lipids and lipoproteins present in serum, C-HDL is the most sensitive indicator of coronary heart disease risk for men and women over age 50.

The measurement of C-HDL requires two steps: separation of lipoproteins (LDL and VLDL from HDL) and detection of cholesterol in the separated fraction(s). The classical method of separation is ultracentrifugation (8). However, this method may not be suitable for routine analysis because it is time-consuming and requires expensive equipment. More recently, several precipitation methods have been developed (9–12).

Electrophoresis provides a third method for the separation of HDL.

For many years, the Abell–Kendall method, involving the Liebermann–Burchard method (13) was widely used for detection and quantitation of cholesterol; however, the reagent contains caustic chemicals and the reaction is time dependent. Lipid stains have been used to detect lipoproteins separated by electrophoresis for more than 10 years (14, 15); available stains such as Sudan Black B and Fat Red 7B, however, are general lipid stains and are not specific for cholesterol and cholesterol esters (16–19). Recently, a totally enzymatic reagent has been developed for cholesterol determination (20). This reagent is less time-dependent, less caustic, and more specific for cholesterol than the Liebermann–Burchard reagent or any general lipid stain. The enzymatic assay has been used to detect C-HDL in lipoprotein fractions separated by ultracentrifugation (21) and precipitation methods (10, 22).

A recently reported C-HDL determination by cellulose acetate electrophoresis also involves an enzymatic reagent for cholesterol detection (22). This procedure, however, suffers from poor precision, lack of linearity beyond 1.5 g/L concentrations of cholesterol per fraction, and no reported accuracy against a comparison method. In this paper we describe a method for separation of HDL by agarose gel electrophoresis, and quantitation of C-HDL after an enzymatic cholesterol reagent is applied to the gel. The resulting lipoprotein fractions are quantitated by automatic scanning densitometry. Precision, linearity, and accuracy are improved over previously reported electrophoretic methods (18, 23). The proposed method is rapid and reliable, and commercially available equipment and supplies are used.

Materials and Methods

Reagents

Materials purchased for development of the cholesterol reagent were: cholesterol esterase (EC 3.1.1.13, from pancreas), cholesterol oxidase (EC 1.1.3.6, from Brevibacterium sp.), peroxidase (EC 1.11.1.7, from horseradish), and sodium cholate, all from Microbics Division, Beckman Instruments, Inc., Carlsbad, CA 90028; 4-aminoantipyrine from Aldrich Chemical Co., Milwaukee, WI 53233; and phenol from Mallinckrodt, Inc., St. Louis, MO 63147.

The cholesterol reagent composition was, per liter, 4-aminoantipyrine, 7.2 mmol; phenol, 96 mmol; peroxidase, 225 000 U; cholesterol oxidase, 2300 U; cholesterol esterase, 2000 U; cholate, 26 mmol; and sodium phosphate (mono- and dibasic), 377 mmol, such that pH (if reconstituted with water) = 7.5 (30 °C).

Total cholesterol was determined with the "Spin Chem Reagent Test for Cholesterol" from SmithKline Instruments, Inc., Sunnyvale, CA 94086.
Samples

For most studies, we collected serum from fasting subjects and stored it at 3–5 °C. Samples were analyzed within one to three days after collection.

Electrophoretic Method

Lipoproteins were separated with the use of commercially available equipment and supplies (Corning Medical, Palo Alto, CA 94306). Two 1-μL portions of sample were applied to each of eight wells in an agarose gel (10 g/L barbital buffer, pH 8.6 (universal gel). 3-(N-Morpholino)propanesulfonate buffer (50 mmol/L, pH 7.8) was used in the electrode wells.

We separated the lipoproteins by electrophoresis at 90 V for 35 min. One vial of the lyophilized cholesterol reagent was reconstituted for each gel with 1.0 mL of 2-(N-morpholino)-ethanesulfonate buffer (50 mmol/L, pH 6.2).

After electrophoresis, we blotted the gels at the edges and placed them in a 60 °C oven (with air flow) for 2 min. This removed moisture on the gel, minimizing band diffusion and maximizing reagent uptake by the gel in the following step. We used a 5-mL serological pipette, placed lengthwise along the cathode edge of the gel, about 25 mm from the edge, to dispense 1 mL of reagent evenly across the gel, on the anode side of the pipette. The reagent was evenly applied to the gel surface by pushing the 5-mL pipette toward the anode edge of the gel, stopping about 25 mm from the edge, pulling the pipette back to its original position, and finally pushing the pipette off the anode edge of the gel.

After incubation in a dark, humid chamber at 37 °C for 30 min, the back of the gel was wiped and the resulting patterns were scanned within 30 min on a densitometer (Corning Model 720) with the 500-nm filter and C-HDL output cards. (This densitometer automatically sets the gain and zero.) Percent C-HDL was read directly off the densitometer digital display. Peak and baseline voltages were measured with a digital meter (Model 3800; Dana Laboratories, Inc., Irvine, CA 92715) by measuring the voltage between ground and the TP3 and TP1 connections, respectively, on the “Peak Detector” printed-circuit board at the rear of the Model 720 densitometer.

Comparison Method

We evaluated the accuracy of our method by comparing the Framingham Heart Study method (13, 24). Serum samples from the Framingham laboratory were analyzed by agarose gel electrophoresis within 1 to 2 days after analysis by the Framingham Heart Study group. Sera were refrigerated at all times. Comparison values were supplied by the Framingham laboratory after electrophoretic values were recorded.

Results

Because the electrophoresis was similar to an existing procedure for separation of lipoproteins in an agarose gel (25), most of the work in this study involved optimization of the subsequent steps.

Dehydration. Preliminary observations showed that occasionally some of the quinoneimine dye (formed in the final step of the cholesterol reaction sequence) was extracted from the gel into excess reagent remaining on the gel surface. A short drying step just before reagent application facilitated reagent absorption, thus eliminating the excess reagent. To determine optimum dehydration time and to examine oven temperature effects, we partly dried the gels for 2, 4, and 6 min at 55, 65, and 75 °C. Precision of the C-HDL measurement was the point of comparison.

Temperature variations had no significant effect on precision, and precision with the 2-min drying time (at 65 °C, within-gel CV = 3.3%) was better than with the 4-min (at 65 °C, CV = 4.8%) or the 6-min time.

Incubation. After electrophoresis of a sample with total cholesterol of 2.1 g/L (18% of which was C-HDL), the gels were incubated at 37 °C for 20, 30, and 50 min. We found that the maximum signal (measured as voltage of the highest peak) was obtained after 30 min; moreover, the percentage of C-HDL did not change between 30 and 50 min.

During the course of the incubation studies we incubated several gels for only 10 and 20 min at room temperature. Scanning tracks from these gels showed that although the colored reaction product in the HDL band was almost completely developed, the low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL) bands showed less color development. That is, the rate of the color development reaction of the HDL fraction was greater than the rate for the LDL or VLDL fractions. This was also observed in an earlier report on a cellulose acetate method (23).

After incubation, the colored quinoneimine formed in the lipoprotein bands begins to fade. By 1 h after incubation, color intensity (measured densitometrically) of the C-LDL band had diminished by about 30%. To determine whether the rate of color loss was equivalent in all fractions and to determine the safe time limit for scanning (such that precision and accuracy would not be adversely affected), we assayed five samples in quadruplicate. The average change in percent C-HDL in 1 h was only +0.2%, suggesting that the rate of color loss is equivalent in both fractions. The average overall precision changed from a CV of 5.5% initially to 6.8% 1 h later. On the basis of these findings, we decided 30 min was a safe time limit for scanning.

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<th>Table 1. Precision Data</th>
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<td>Fraction</td>
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* n = 9, samples run on three gels per day for three days.
* n = 72, samples run on eight tracks per gel, three gels per day for three days.
Scanning. We noticed that scanning cholesterol patterns on the Model 720 densitometer with the current Model 720 output card resulted in spiking at the sample well and baseline that did not return to zero (Figure 1, left). To eliminate these problems, we developed a modified output card. The card has a shortened scanning area, so that scanning is initiated just anodal to the well. This modification resulted in the elimination of spikes, lower baselines, and improved precision (Figure 1, right).

Analytical Parameters

**Precision.** To determine precision of this method, we assayed a pool of apparently normal serum (total cholesterol 2.66 g/L, C-HDL 0.57 g/L) on eight tracks per gel, three gels per day, for three days. Using the resulting values we calculated within-gel precision for each gel and computed the representative coefficient of variation (CV%) by analysis of variance. The between-gel precision was calculated by comparing the mean values for three plates on a given day, followed by analysis of variance for the three groups. Overall precision was calculated by including all 72 values in one group. Results are shown in Table 1.

**Accuracy.** We compared C-HDL values obtained by the electrophoresis method with those obtained by the Framingham Heart Study method (13, 24) in a double-blind study. The study included samples from 40 men and 58 women between the ages of 44 and 87 (average, 67 years old). Total cholesterol ranged from 1.56 to 3.75 g/L, with C-HDL between 0.25 and 0.92 g/L. C-HDL ranged from 10 to 45% of total cholesterol.

**Correlation analysis of the data is shown in Figure 2. These results indicate excellent agreement ($r = 0.95, S_{yx} = 52 \text{ mg/L}$) between the present electrophoretic method and the method used in the Framingham Heart Study.**

**Linearity.** Linearity is usually limited by reagent depletion in samples containing high concentrations of C-LDL. Defining upper-end linearity is important because incomplete C-LDL development will result in false increases of percent C-HDL. Furthermore, we have found that for samples with low total cholesterol the accuracy of the method is limited by precision. To determine the linear range of the method, we serially diluted with saline a serum sample with 4.06 g of total cholesterol and 1.22 g of C-HDL per liter. Each dilution was electrophoresed eight times (on one gel) and assayed for total cholesterol.

Figure 3 shows the mean and one standard deviation limits for C-HDL and C-LDL vs total cholesterol. Even at the maximum concentration tested, there was no deviation from linearity. The within-gel CV for C-HDL was <5% when total cholesterol was as little as 1.86 g/L (C-HDL = 0.59 g/L). At total cholesterol = 1.48 g/L (C-HDL = 0.45 g/L), the CV for C-HDL was 6.1%; at total cholesterol = 0.91 g/L (C-HDL = 0.28 g/L), the CV for C-HDL was 7.2%.

Sample Studies

We also examined possible plasma-serum differences in C-HDL determination. Of particular concern was heparinized plasma because, in the presence of sufficient quantities of certain divalent cations, heparin precipitates VLDL and LDL (9).

From each of 18 subjects we collected blood into three tubes: plain (serum), containing heparin (plasma), and containing ethylenediaminetetraacetic acid (EDTA) (plasma). All tubes were treated similarly, and total cholesterol and percent C-HDL were determined on all samples.

The resulting mean ($n = 18$) values for percent C-HDL were: serum = 28.7%, heparin-treated plasma = 29.1%, and EDTA-treated plasma = 28.3%. The paired Student's $t$-test indicated that the differences between the three groups were insignificant at $p = 0.05$. Therefore, either serum or EDTA-or heparin-treated plasma may be used with the proposed method without significantly affecting the percent C-HDL.

We did note, however, that samples collected in heparin-containing tubes exhibit poor VLDL resolution. Therefore, when LDL/VLDL information is sought, heparin should not be used.

In a related study, we examined effects of storage conditions. Eight serum and eight plasma (EDTA-treated) samples were drawn, divided into aliquots, and stored at 4 and $-20^\circ C$. All samples were tested on the day drawn, and one day, three days, one week, two weeks, and two months after. Because we had found no difference between values for serum and EDTA-treated plasma (see above), results for the two categories were combined in analysis of the data.

The resulting mean ($n = 16$) values for percent C-HDL were: fresh = 27.0%, two weeks at 5 °C = 26.9%, two months at 5 °C = 23.9%, and two months at $-50^\circ C = 26.8%$. The paired Student's $t$-test revealed that the differences between the fresh and the stored samples were insignificant ($p = 0.05$) except for the samples stored for two months at 5 °C. Therefore, percent C-HDL was stable for at least two weeks, but not two months, at 5 °C. Some of the two-month-old frozen serum and plasma samples exhibited LDL trailing into the sample well, which, although not affecting quantitation significantly, is a good reason to limit the recommended storage time to one month.

Comparison of scans revealed that, for refrigerated samples, VLDL resolution decreased with time, such that after one week, resolution had decreased noticeably. Frozen samples also exhibited decreased VLDL resolution, possibly a result of either breakdown or modification of the VLDL particle. Therefore, when LDL/VLDL information is sought, samples should be stored refrigerated and analyzed as soon as possible after collection.

Eight samples from patients with high amounts of triglycerides (six with 4.2–6.4 g/L, one with 2.8 g/L, and one with 21.2 g/L) were examined to determine whether there was any interference in C-HDL measurement by the lipemic nature of these samples. Lipid stain electrophoresis of these samples revealed that only one contained detectable chylo microns. No trailing of electrophoretic patterns was observed, even in the sample with >20 g/L triglycerides. The mean C-HDL by
agarose electrophoresis was 0.26 g/L, compared with 0.27 g/L by the Framingham method. The paired Student's t-value was 0.87. Therefore, lipemic samples do not require any special handling (e.g., dilution) when evaluated by the proposed method. Samples with type V hyperlipoproteinemia can readily be detected by their striking creamy appearance. If allowed to stand undisturbed overnight in a refrigerator, they will have a creamy layer floating on top (chylomicrons) and marked turbidity in the subnate layer (VLDL).

During the course of this study, we noticed abnormal samples containing a variety of LDL/VLDL relationships. Three quite different examples are shown in Figure 4. (A sample from a normal, fasting subject is shown in Figure 1.)

Discussion

Recent reports in the literature have described electrophoretic methods for the measurement of C-HDL. The polyacrylamide gel procedure (18) requires staining the sample with Sudan Black B before electrophoresis. In a cellulose acetate method (23) an enzymatic cholesterol reagent is overlaid on the cellulose acetate after electrophoresis, to detect the cholesterol-containing lipoproteins. Unfortunately, neither electrophoretic method provides the accuracy and precision required for C-HDL quantitation. The agarose gel procedure described here is quite accurate and approaches the C-HDL precision described by the current comparison methods. Like the cellulose acetate and polyacrylamide methods, the agarose procedure determines C-HDL as a percentage of total cholesterol. Percent C-HDL can then be multiplied by total cholesterol (g/L) to obtain C-HDL in g/L.

Overall precision for C-HDL determination was found to be 6.5% (CV). This represents a marked improvement over both cellulose acetate (23) and polyacrylamide gel (18) electrophoretic methods, for which reported CV's were 17.0% and 11.5%, respectively. The precision of the agarose electrophoretic method is more like that of the heparin–manganese precipitation method, with reported CV's of 4.3% (26) and 4.5% (22).

For the cellulose acetate method (23), accuracy was reported by comparison of C-HDL values obtained by electrophoresis for 10 age- and sex-specific populations with values published in 1967. The number of subjects for each group ranged between 11 and 36. Significant differences (p = 0.05) were found in two of the 10 groups. For the polyacrylamide gel method, accuracy was evaluated by comparing the C-HDL values of 123 samples with the values determined on the same samples by ultracentrifugation. The reported correlation coefficient was 0.945 and the standard error of the estimate was 107 mg/L (18). The agarose electrophoretic method we describe produced data with a correlation coefficient of 0.947, standard error of the estimate of 52 mg/L, and a paired Student's t-value of 0.66 for C-HDL determination, a marked improvement in accuracy over both of the previously reported electrophoretic C-HDL methods.

Linearity of the standard curve for the cellulose acetate method extends upward to 1.5 g cholesterol per liter per fraction; because the mean C-LDL concentration for most adult populations is between 1.3 and 1.6 g/L (27), quite often samples must be diluted before analysis. The standard curve for the agarose method exhibits linearity up to at least 2.8 g/L cholesterol per fraction, a value that includes more than 99% of most adult populations (27). Samples exceeding 2.8 g/L cholesterol per fraction can be accurately analyzed by simply assaying only 1 μL of sample (instead of the usual 2 μL). Samples with less than 1.2 g of total cholesterol or less than 0.3 g of cholesterol per liter in any lipoprotein fraction can be accurately analyzed by using 3 μL of sample (if C-LDL is less than 2.0 g/L) or by repeated determinations and averaging of results.

In addition to the accuracy, precision, and linearity improvements of the present agarose method over previous electrophoretic techniques, no gel preparation steps are necessary. The cellulose acetate method requires overnight soaking of the gel; for the polyacrylamide gel method, 24-h storage of gels before quantitation is highly recommended.
Currently, there are several precipitation methods for C-HDL determination (26). Electrophoretic C-HDL methods require less sample volume than these methods, provide permanent, visible records of results, and produce semi-quantitative information on LDL and VLDL. This LDL/VLDL information may be useful in detection of abnormalities in the metabolism of lipoproteins (28). Finally, a recent report comparing various precipitation methods suggested that they may not be equivalent with respect to precipitation of LDL and VLDL and C-HDL quantitation (26).

Because of its improved precision, accuracy, and linear range over previously reported electrophoretic methods (18, 23), the agarose method is suitable for routine use in the clinical laboratory for determination of C-HDL and risk assessment of coronary heart disease.

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References