Direct Measurement of HDL Cholesterol: Method Eliminating Apolipoprotein E-Rich Particles

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It has been reported that the existing direct method of high density lipoprotein (HDL) cholesterol measures particles enriched with apolipoprotein E (apoE). The aim of our study was to investigate a new analytical protocol to directly measure HDL cholesterol that eliminates apoE-rich particles. The interactions of four lipoproteins (HDL₃, HDL₂, LDL, and VLDL + chylomicron) with surfactants, divalent cations, sugars, and lectins were investigated. By analyzing sera, HDL₃, and HDL₂, we examined the relationships among the measurements obtained by our protocol, a precipitation method using heparin-MnCl₂,

and a commercially available kit for this direct method. A significant difference was found between the direct method and the heparin-MnCl₂ method, but not between our protocol and the heparin-MnCl₂ method. Multiple regression analysis showed that the difference between the direct method and the heparin MnCl₂ method is dependent on sources of apoE-rich HDL. In conclusion, our protocol enables a direct measurement of HDL cholesterol that eliminates apoE-rich particles. *J. Clin. Lab. Anal.* 15:223–229, 2001. © 2001 Wiley-Liss, Inc.

Because of the inverse relationship between high-density lipoprotein (HDL) concentration and coronary heart disease (CHD) risk, attention is being focused on methods for measuring HDL. HDL is usually quantified in terms of its cholesterol content because of its important role in atherogenesis. The second report of the Adult Treatment Panel of the National Cholesterol Education Program, which was released in 1993, affirmed that low HDL cholesterol (<35 mg/dl) is a major risk factor for CHD, whereas high HDL cholesterol (≥60 mg/dl) is a negative risk factor (1).

HDL comprises a complex, heterogeneous population of particles, usually of three species with an anomalous pre- β electrophoretic migration and such major α -migrating components as HDL₂ and HDL₃ (2,3). One of major sources for the free cholesterol appearing in HDL is cell membranes. Cellular cholesterol is initially transferred to the pre- β -migrating HDL, esterified, and then transferred to HDL₃. HDL₃ cholesteryl esters are transferred to HDL₂ through the action of the cholesteryl ester transfer protein (CETP), causing an increase in the lipid content and size of HDL. Larger particles of HDL₂ are enriched with apolipoprotein E (apoE), while HDL₃ is not.

Several techniques to determine HDL-cholesterol in serum have been described, including ultracentrifugation (4), electrophoresis (2,3), gel permeation chromatography (5,6), precipitation-based methods (7), and direct measuring methods

(8,9). Measurements other than the precipitation methods and the direct methods are not widely used in the clinical setting. In the precipitation methods, HDL cholesterol is first separated by precipitating apolipoprotein B (apoB)-containing lipoproteins from serum by using a combination of a polyanion and a divalent cation, such as heparin-MnCl₂, dextran sulfate-MgCl₂ or phosphotungstate-MgCl₂; subsequently, HDL is quantified as cholesterol in the supernate. A protocol of the direct method, for example, measures HDL cholesterol directly in unprocessed serum samples by using polyethylene glycol (PEG)-modified cholesterol esterase and cholesterol oxidase as well as sulfated α -cyclodextrin to produce a catalytic activity that is specific for the cholesterol in HDL particles (9). Since direct measurements require no pretreatments, such as centrifugation, the methods are becoming popular in clinical laboratories.

Although direct methods were only recently developed but not evaluated for their various aspects, it has been reported that one of the methods measures cholesterol in HDL, including HDL particles enriched with apolipoprotein E (apoE)

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(10). The aim of our study was to investigate an analytical protocol that directly measures HDL cholesterol eliminating apoE-rich particles.

MATERIALS AND METHODS

Preparation of Chylomicron, VLDL, LDL, HDL₂, and HDL₃

Blood samples were obtained from 48 patients referred to our laboratory for lipoprotein analysis and from 20 healthy volunteers. Chylomicron ($d < 0.950$ kg/l density), very large density lipoprotein (VLDL; $0.950 < d < 1.006$), low density lipoprotein (LDL; $1.019 < d < 1.063$), HDL₂ ($1.063 < d < 1.125$), and HDL₃ ($1.125 < d < 1.210$) were separated by sequentially adjusting the sera to different densities by addition of sodium bromide (NaBr) followed by ultracentrifugation at $401,000 \times g$, 16°C for 8 hr in a Himac centrifuge with a RP80AT rotor (Hitachi Koki, Tokyo, Japan). We thus obtained four solutions of lipoproteins, chylomicron+VLDL, LDL, HDL₂, and HDL₃. Gel-permeation chromatography showed no lipoprotein contamination in each solution.

The apolipoproteins A-I (apoAI), apolipoprotein B (apoB), and apoE concentrations were measured turbidimetrically by using a polyclonal apoAI, apoB, and apoE anti-serum (Daiichi Pure Chemical, Tokyo, Japan), respectively, with a 7450 automatic analyzer (Hitachi, Tokyo, Japan).

Preparation of FPLC Fractions

Blood sample were prepared from 3.4 g/l ethylenediamine-tetraacetic acid dipotassium salt (K₂EDTA)-anticoagulated venous blood of five healthy volunteers and pooled. A 1.39-mL NaBr solution of 1.504 kg/l supplemented with 0.3 mmol/l Na₂EDTA, 1 mmol/l benzamidine hydrochloride, and 10,000 units of aprotinin was added to a 2-mL aliquot of the plasma in each tube and was ultracentrifuged at $401,000 \times g$ at 16°C for 5 hr. Fractions of the obtained lipoprotein solution ($d > 1.210$) were isolated by gel-permeation chromatography on a fast protein liquid chromatography system (FPLC; Pharmacia, Piscataway, NJ) equipped with a Superose-6 column. Samples were loaded and eluted at room temperature in phosphate buffered saline (PBS) supplemented with Na₂EDTA. The sample volume for each run was 500 μL . Eluate was monitored by UV absorbance at 280 nm, and each 0.5-mL fraction was collected by using a fraction collector. Finally we obtained 30 fractions (8 through 23 mL of elution volume).

Analytical Procedure

The interactions of four lipoproteins with 164 surfactants (Triton, Aekatole, etc.), 6 divalent cations (MgCl₂, MnCl₂, etc.), 16 sugars (dextran sulfate, cyclodextrin sulfate, etc.), 4 lectins (wheat germ agglutinin, concanavalin A, etc.), and 8 others (urea, phosphotungstate, etc.) were investigated. We added these substances by varying the combinations, con-

centration, pH, and hydrophile-lipophile balance (HLB) to the aliquots of four lipoproteins, and measured total cholesterol levels enzymatically by using T-CHO(S) assay kit (Denka Seiken, Tokyo, Japan) with a TBA-30FR automated analyzer.

HLB is one of the indicators representing the ratio of the hydrophilicity of a surfactant to its hydrophobicity and is the most effective parameter that alters its specificity. Surfactants with lower HLB values are more oil-soluble, while surfactants with higher HLB values are more water-soluble. In order to vary HLB, two series of each surfactant with different HLBs were purchased and mixed. The HLB value of a mixture of surfactant A (HLB = HLB_A) and surfactant B (HLB = HLB_B) is determined by $(\text{HLB}_A \cdot W_A + \text{HLB}_B \cdot W_B) / (W_A + W_B)$, where W_A and W_B are weight of each surfactant.

Also investigated were four buffers (PIPES: 50 mmol/l, pH 7.0; MES: 50 mmol/l, pH 6.5; HEPES: 75 mmol/l, pH 7.0; and BES: 100 mmol/l, pH 7.0) and enzymes from eight origins.

We first added a surfactant one by one to four lipoproteins by varying the final concentration from 0.05% through 0.3% and then measuring their cholesterol levels. The resulting color, formed in a coupled reaction with peroxidase, was measured spectrophotometrically at dual wavelengths (600 nm for main and 700 nm for subsidiary). Then, we examined the effects of divalent cations, sugars, and so on.

The existing epidemiological and clinical database relating HDL-cholesterol concentrations to coronary risk is largely based on the Centers for Disease Control and Prevention (CDC)-National Heart, Lung, and Blood Institute's standardized HDL-cholesterol measurements, which are based on the use of the heparin-MnCl₂ method (1). By analyzing the sera, HDL₃, and HDL₂, we examined the relationship among the apolipoprotein content and the measurements obtained by three methods, that is, our protocol, the heparin-MnCl₂ method (Method A), and the commercially available kit for direct method (Determina HDL-C; Kyowa Medics, Tokyo, Japan) (9) (Method B). The heparin-MnCl₂ method was performed in three steps: (1) Ultracentrifugation at $d < 1.006$ kg/l to remove triglyceride-rich lipoproteins; (2) Precipitation of apoB-containing lipoproteins from the ultracentrifugal infranatant with heparin and MnCl₂; (3) Measurement of cholesterol in the supernate. The analyzer was calibrated with materials provided by each manufacturer. For these method-comparison studies, we used a set of 16 sera of the 48 samples.

The protocol was approved by the Regional Ethics Committee on Human Research. Informed consent was obtained from all participants.

Statistical Analysis

Student's *t* and correlation coefficient statistics were applied to the experimental results respectively, and unless otherwise specified, the significance of the obtained values was evaluated at the 0.05 confidence level.

Specificity of each method to apoE-rich HDL was evaluated by the method of least squares (multiple regression analysis) (11).

RESULTS

Reactions of Surfactants, Divalent Cations, and Enzymes

Table 1 presents four surfactants that showed the highest selectivities toward HDL₃ in the four lipoproteins. Emulgen B-66 (polyoxyethylene compound; Kao Co. Ltd., Tokyo, Japan) showed the best result ($P < 0.0001$); that is, 95% (76.0 mg/dl; 1.97 mmol/l) of the cholesterol content in HDL₃ could be measured, followed by 76% (60.8 mg/dl; 1.57 mmol/l) in HDL₂, 25% (20.0 mg/dl; 0.517 mmol/l) in chylomicron + VLDL, and 10% (8.0 mg/dl; 0.207 mmol/l) in LDL.

It was found that only MgCl₂ raised the selectivity of the surfactant toward the HDL, especially HDL₃ ($P < 0.0001$). The minimum required concentration of MgCl₂ was 15 mmol/l (finally 11 mmol/l with sample). We could find no improvements by adding any of the sugars or lectins. By changing buffer, the selectivity of the combinations were examined one by one, and we found that BES seems to be the most appropriate buffer (not significant).

We then obtained 14 different solutions of the surfactant by varying HLB from 12.80 to 14.50, and added each aliquot to the four lipoproteins. Figure 1 shows the relative reactivities of cholesterol in the four kinds of lipoproteins as a function of the HLB, suggesting the best condition was obtained around HLB = 13.5.

Cholesterol esterase is another important factor that affects the selectivity toward HDL₃. We found a big difference among their effects on relative reactivities of cholesterol. The cholesterol esterase from yeast (EC 3.1.1.13; Asahi Chemical, Shizuoka, Japan) showed the best result in terms of the selectivity to HDL₃.

In the final protocol, reagent 1 was composed of 15 mmol/l MgCl₂, 800 U/l cholesterol esterase (from Yeast), 500 U/l cholesterol oxidase (recombinant), 200 KU/l catalase (beef liver), and 0.7 mmol/l N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline, sodium salt in Good's buffer (BES; 100 mmol/l; pH 7.0). Four microliters of a serum sample were

added to 300 µl of reagent 1 and incubated at 37°C for 5 min. To this mixture we added 100 µl of reagent 2, containing 4000 U/l peroxidase (from horseradish), 4 mmol/l 4-aminoantipyrin, and 0.9% Emulgen B-66. The resulting color was measured at dual wavelengths of 600 nm for main and 700 nm for subsidiary. The residual reactivities of cholesterol in lipoproteins other than HDL₂ and HDL₃ were almost completely abolished by adjusting the reading time from 2.5 to 3 min. The automated analyzer was calibrated with pooled sera that had been assayed for HDL-cholesterol by using the Certified Reference Material for Measurement of Total Cholesterol, HDL-Cholesterol, and Triglycerides in Human Serum (WCHL 961H and 962M), which were obtained from the Health Care Technology Foundation, Standard Reference Center (Kawasaki, Japan). This material was prepared in bulk, apportioned into test tubes, and stored at -20°C. On analysis days, the calibration pool was thawed at room temperature, thoroughly mixed, and transferred to a sample cup to serve as a calibrator.

Basic Evaluations

To evaluate the precision of this protocol, we analyzed three serum pools: a pool with low HDL-cholesterol concentration (about 19.2 mg/dl; 0.497 mmol/l), a pool with middle concentration (about 30.4 mg/dl; 0.786 mmol/l), and a pool with high concentration (about 88.5 mg/dl; 2.29 mmol/l) made from patients' sera. Within-run imprecision (CV) was 1.25% and 1.23% at 19.2 and 88.5 mg/dl HDL-cholesterol concentrations, respectively ($n = 30$). The between-assay CV was 0.79 by assaying the same sample (30.4 mg/dl) on 20 different days. These results fulfill the recommendations of the Lipid Standardization Panel of the National Cholesterol Education Program (1, 12–14).

No significant interference was observed when either bilirubin (up to 250 mg/dl; 4275 µmol/l), hemoglobin (up to 4.8 g/dl; 48 g/l), ascorbic acid (up to 500 mg/dl; 28390 µmol/l), fatty acid (up to 5% soybean oil), or EDTA-Na₂ (up to 0.34 g/l) was included during the measurement of HDL cholesterol.

To evaluate matrix effects, we added HDL with cholesterol concentrations of 26.7–51.2 mg/dl (0.690–1.32 mmol/l) to 20 sera and analyzed them with the proposed protocol. The analytical recovery was 104% with a range of 97–109%. Therefore, we can conclude that there is no matrix effect that influences the results.

Method-Comparison Studies

A significant difference was found between Method A and Method B ($P < 0.01$), but not between Method A and our protocol (Fig. 2). The linear regression line of the data obtained by using Method A and Method B yielded a slope = 0.82, intercept = 3.45 ($r = 0.94$, $n = 16$). The linear regression

TABLE 1. Surfactants that are selective toward HDL₃ cholesterol

Surfactant	Concentration (%)	Cholesterol levels (%)			
		Chylo ^a +VLDL	LDL	HDL ₂	HDL ₃
None	—	100	100	100	100
Adekatole NP-720	0.05	34	35	86	87
Emulgen 913	0.1	32	30	79	84
Emulgen 109P	0.1	35	36	80	81
Emulgen B-66	0.3	25	10	76	95

^aChylomicron

Fig. 1. Relative reactivity of the surfactant to cholesterol in the four kinds of lipoproteins as a function of the hydrophile-lipophile balance. VLDL shows VLDL + chylomicron. The reactivity was estimated by measuring relative cholesterol content in each lipoprotein.

line between our protocol and Method A yielded a slope = 0.85, intercept = 6.73 ($r = 0.96$, $n = 16$). We then examined factors that characterized the significant difference between Method A and Method B. We calculated differences (DIF) by subtracting the measurements of Method A from those of Method B and then obtained the following equation according to multivariate regression analysis:

$$\text{DIF} = -1.26 + 0.10X_1 - 13.51X_2 - 0.03X_3 + 2.25X_4$$

where X_1 is the apoAI level and X_2 is the apoE level in each HDL₃ sample. X_3 is the apoAI level, and X_4 is the apoE level in each HDL₂ sample. The multiple correlation coefficient was 0.57 ($P < 0.05$). The partial correlation coefficient between DIF and X_4 (apoE in HDL₂) was 0.43, which was statistically significant. The other partial correlation coefficients were 0.29, -0.38, and -0.07 for X_1 , X_2 , and X_3 , respectively. This result shows that the differences between Method A and Method B are dependent on sources of apoE-rich HDL, and

Fig. 2. Comparisons among measurements by Method A, our protocol, and Method B (* not significant; ** $P < 0.05$).

that Method B is more susceptible to apoE-rich HDL than Method A.

FPLC Patterns

We obtained 30 lipoprotein fractions from a pool of healthy subjects with FPLC. For each fraction, HDL cholesterol was measured by using our protocol and Method B (Fig. 3A). Also apoE concentration was measured for each

fraction (Fig. 3B). Each peak in the FPLC pattern had already been identified by using ultracentrifuged samples and by measuring apolipoproteins (4,6). The immunochemical characterization of the FPLC fractions gave the following results: ApoE was associated with larger particles of HDL (eluted early in the HDL peak; fractions 24–28); cholesterol obtained by our protocol for the fractions enriched with apoE showed slightly lower levels than those by Method B. This

Fig. 3. FPLC patterns. HDL cholesterol measured by using our protocol (closed circle) and Method B (open circle) (A), and apoE (closed circle; 10 times of concentration) levels (B) are plotted on a FPLC pattern (represented as total cholesterol measurements).

difference was statistically evaluated and found to be significant ($n = 3$, $P < 0.05$).

DISCUSSION

Although it has been pointed out that there are large discrepancies among data obtained by different methods of HDL-cholesterol measurement (15-18), little consideration has been given to the detailed relationship between the distribution of HDL particle size and selectivity by these methods. The close similarity in physicochemical characteristics of HDL subclasses makes the development of a specific method for their separation difficult.

A lipoprotein is a particle consisting of a core of hydrophilic lipids surrounded by a shell of polar lipids and apolipoproteins. The most abundant are phospholipids. The phospholipids have a polar head group and two hydrophilic fatty acid tails. Several apolipoproteins, such as A-I, A-II, B, and E, have been isolated and characterized. ApoAI is a major protein component of HDL₃ and HDL₂. This apolipoprotein, a 243-amino-acid protein with a mass of 28,000 D, has an amphipathic nature. ApoAII, a smaller protein with 77 amino acids (17,400 D), is another protein component of HDL₃. And apo E, a 299-amino-acid glycoprotein (34,000 D), is highly lipophilic. These apolipoproteins solubilize highly hydrophobic lipids and play an important role in characterizing the particle size of lipoproteins and receptor mediated interactions. In this study, we found that HDL₃ was selectively recognized by a non-ionic surfactant. Although the mechanism is not clear, this surfactant may solubilize the polar lipids via a specific interaction with a combination of HDL apolipoproteins except for apoE. In views of the fact that surfactant is indispensable for direct measurements of lipoproteins, the reactivity of the surfactants toward the combination of the apolipoproteins must be clarified in the future.

The reason behind our choice of reagent is based on a trial and error procedure, and the justification has been done by two experimental results; the results presented in Figure 1 and in the method-comparison studies. Also the comparison of our protocol and Method A using patient samples yielded a good result, which supports our point that the protocol can adequately measure patient samples as good as the heparin-MnCl₂ method. All existing precipitation method or direct methods for HDL cholesterol were devised by trial and error to quantify the same mass of cholesterol as estimated by a "gold standard" procedure. The "gold standard" is ultracentrifugation. However, since it is difficult to estimate apoE-rich HDL contents in fractions obtained by ultracentrifugation, we did not compare our protocol to this gold standard.

What is the rationale in eliminating apoE-rich HDL from the routine assay? Wilson summarized published data from eight studies about the relation between HDL subclasses and CHD (19). Although these data showed that HDL₃ and HDL₂

were associated with CHD, their conclusions were conflicting. Lane et al. reported that diabetic patients had decreased levels of HDL₃ and not of HDL_{2a}, compared with control subjects (20). Since larger particles of HDL₂ are enriched with apoE, while HDL₃ is not, this study supports that apoE-rich HDL particles are less significant.

In conclusion, the data presented here offer evidence that the proposed protocol provides a new direct method for determining cholesterol content in HDL with less inclusion of apoE-rich HDL particles than the existing direct method does.

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