Simultaneous Determination of Serum Cholesterol in High- and Low-Density Lipoproteins with Use of Heparin, Ca\(^{2+}\), and an Anion-Exchange Resin

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Cholesterol concentrations in serum high-density and low-density lipoproteins are simultaneously determined simply, specifically, and rapidly by use of the precipitation method with heparin, Ca\(^{2+}\), and an anion-exchange resin. The isolation of lipoproteins is reproducible, selective, and complete, as judged by electrophoresis on polyacrylamide gel and by immunoelectrophoresis, with use of samples with very-low-density lipoprotein triglyceride concentrations of less than 3.5 g/liter. The precision of the present method is as good (CV, 2.8–3.1%) as that for the method used by the U.S. Lipid Research Clinics (CV 2.0–3.2%). The present method and the heparin–Mn\(^{2+}\) method of the Clinics gave results that agreed reasonably well (for low-density-lipoprotein cholesterol \(r = 0.935, P < 0.001\); for high-density-lipoprotein cholesterol \(r = 0.897, P < 0.001\)). We also describe the relations between high- or low-density lipoprotein cholesterol and total cholesterol, and between cholesterol concentrations in these two lipoprotein classes.

Measurement of cholesterol in the high-density lipoproteins (HDL) in serum of patients with atherosclerosis is of increased interest. There is evidence of an inverse correlation between HDL-cholesterol and coronary heart disease risk (1–3), and HDL may be important for the transport of cholesterol from tissues to liver (4). On the other hand, the above-normal plasma cholesterol associated with cardiovascular disease is carried primarily with low-density lipoproteins (LDL). If individuals with an increased risk of coronary heart disease are to be more clearly identified, cholesterol concentrations in both the HDL and LDL must be ascertained.

Currently, HDL- and LDL-cholesterol concentrations are measured in plasma lipoproteins fractionated by methods standardized by the U.S. Lipid Research Clinics (LRC): ultracentrifugation and precipitation with heparin–manganese chloride (5). Burstein et al. (6) described an apparently simpler method in which polyamions and divalent cations were used to precipitate LDL and very-low-density lipoproteins (VLDL). This technique has been used for isolation procedures but has not been adapted for routine analyses. However, this method recently has been modified and applied for the routine lipoprotein quantification by turbidimetry (7). We report here a simple, specific, and rapid procedure for simultaneously determining HDL- and LDL-cholesterol by a modified precipitation method.

Materials and Methods

Samples

Venous blood was sampled from normal adults and from hyperlipoproteinemic patients who had fasted overnight, and the serum was promptly separated by low-speed centrifugation.

Reagents

Reagent I: Dissolve 50 mg of sodium heparin (Novo Industri A/S, Copenhagen) and 50 mmol of CaCl\(_2\) in 1 liter of distilled water.

Reagent II: Dissolve 50 mg of sodium heparin and 4 g of sodium chloride in 1 liter of distilled water.

Determination of HDL- and LDL-Cholesterol

HDL-cholesterol: Pipette 0.2 ml of serum into a tube. Add 4.0 ml of Reagent I, shake with a mixer for 1 min, and allow the tube to stand at room temperature for 30 min. Then either centrifuge (10 000 rpm, 20 min) or filter through a 0.22-μm filter (Millipore Corp., Bedford, Mass. 01730). The clear supernate contains only HDL. Cholesterol in the supernatant fluid is determined by the o-phthalaldehyde method (8).

LDL-cholesterol: Pipette 0.1 ml of serum into a tube. Add 4.0 ml of Reagent II, shake with a mixer for 1 min, and allow the tube to stand at room temperature for 15 min. Then add 0.5 g of Amberlite IRA-400 (Organo Co. Ltd., Tokyo) into the tube, shake the mixture, allow it to stand for an additional 10 min, and centrifuge (9000 rpm, 10 min). The supernate contains HDL and LDL. Measure cholesterol in the supernatant fluid (8). LDL-cholesterol concentration is calculated by subtracting HDL-cholesterol from the (HDL + LDL)-cholesterol concentration.

To remove heparin, add 0.5 g of Amberlite IRA-400 into the supernatant fluid obtained with Reagent I, shake, and centrifuge. The supernates are concentrated before electrophoresis. The precipitate obtained with Reagent I and that in the anion-exchange resin are suspended in the same reagent solutions and centrifuged. The washed precipitates are dissolved in 3.0 ml of 0.5 mol/liter sodium chloride. The solutions are dialyzed against distilled water and concentrated to about 0.1 ml for electrophoresis.

Lipoprotein electrophoresis and immunochemical analysis. Lipoprotein electrophoresis is performed on polyacrylamide gel by using the QUICK-DISC QDL reagent kit (Ames Co., Elkhart, Ind. 46514). The samples are prestained with Sudan Black B and electrophoresed at room temperature for 40–60 min with continuous current of 3 mA per column.

Immunochemical analysis are performed by immunoelec-
trophoresis in 10 g/liter agar, using antibodies against human whole serum, HDL, and LDL.

Ultracentrifugation and HDL- and LDL-cholesterol determination. We followed the protocol used in the LRC Program (5). VLDL are separated from LDL and HDL by ultracentrifugation of 3 ml of serum (d 1.006; 24 h; 105 000 x g; Beckman no. 40-3 rotor). Subsequently, LDL in the supernatant fraction are precipitated from HDL with heparin (6 g/liter) and MnCl₂ (0.2 mol/liter). In all fractions (VLDL, LDL + HDL, HDL) cholesterol is determined (8). LDL-cholesterol concentrations are calculated by subtraction of HDL-cholesterol from the corresponding (LDL + HDL)-cholesterol.

VLDL, LDL, and HDL are isolated for experiments with single lipoprotein fraction by fractionation with the ultracentrifuge at d 1.006 (20 h; 105 000 x g) for VLDL, after the separation of VLDL at d 1.063 (24 h at 105 000 x g) for LDL, and for HDL at d 1.21 (40 h at 105 000 x g).

Results
Isolation of HDL and LDL

Disc electrophoresis of a concentrated solution of the isolated HDL with Reagent I (supernate) on polyacrylamide gel revealed only one component with the mobility corresponding to α-lipoprotein (Figure 1). Electrophoresis of the concentrated HDL plus LDL with Reagent II (supernate) revealed two bands, in the α and β regions. Almost no VLDL and chylomicrons could be detected in the supernatant fluids. After heparin was removed with anion-exchange resin, the mobilities of lipoproteins on polyacrylamide gel were slightly different than before. The purities of the isolated HDL or HDL plus LDL were confirmed after immunoelectrophoresis of these preparations. On the other hand, electrophoresis of the concentrated solution dissolved from the precipitates on polyacrylamide gel revealed bands with mobility corresponding to LDL and VLDL, and to VLDL, respectively (Figure 1).

To exclude the possibility that precipitation of LDL and VLDL might be insufficient in sera with very high LDL or VLDL, or both, we investigated sera from patients with hyperlipoproteinemia. Even at high LDL and/or VLDL concentrations (<5.0 g of total cholesterol per liter and/or 3.5 g triglyceride per liter of serum), the isolation of HDL and LDL by the present method was generally satisfactory within the limits of electrophoretic detectability.

Linearity and Recovery

The curves prepared with various volumes of serum sample in the same volume (4.0 ml) of reagents were linear up to 0.3 and 0.5 ml of serum in the cases of HDL- and (HDL + LDL)-cholesterol, respectively (Figure 2). When we use more than 0.3 ml of serum for 4.0 ml of Reagent I, the incomplete precipitation of LDL leads to overestimation of HDL-cholesterol concentration.

Other curves prepared with various volumes of the purified HDL with Reagent I and the purified LDL with Reagent II, in the presence of albumin at concentration of 50 g/liter, were linear to at least 2.5 and 5.0 g of LDL- and LDL-cholesterol, respectively, per liter. In these experiments, the analytical recoveries of the purified HDL and LDL were approximately 80% in both cases with Reagents I and II.

Reproducibility

The precision of the present method was assessed by performing 10 replicate assays of pooled normal and hyperlipoproteinemic sera. The means for normal serum were 0.33 and 1.36 g/liter, with coefficients of variation (CV) of 2.8 and 3.1%, for cholesterol concentrations with Reagent I (HDL) and Reagent II (HDL + LDL), respectively. We also found within-day precisions (CV) for hyperlipoproteinemic serum of 3.8 and 2.7% and mean cholesterol values of 0.62 and 2.38 g/liter with Reagents I and II, respectively.
Comparison Study

To further test the validity of our method, we compared results to those obtained by the LRC method, better to observe the relations between HDL- or LDL-cholesterol and the total concentration of cholesterol in serum, and the relation between HDL- and LDL-cholesterol concentrations.

We analyzed 56 samples obtained from normal individuals and from patients with hyperlipoproteinemia (cholesterol range, 0.9–3.2 g/liter) for HDL- and LDL-cholesterol by the LRC method and the present method (Figure 3). The correlation for LDL-cholesterol by the two methods was excellent ($r = 0.935, P < 0.001$). That for HDL-cholesterol, however, was lower in both procedures ($r = 0.837, P < 0.001$), due, at least in part, to the low proportion of cholesterol in HDL and to the narrow range in HDL-cholesterol concentration.

Figure 4 shows the linear regressions and correlations between HDL- or LDL-cholesterol and total serum cholesterol in 82 samples in which cholesterol varied widely. The relationship between HDL-cholesterol and total cholesterol was not significant ($r = -0.156$). On the other hand, there was a good correlation ($r = 0.742, P < 0.001$) between LDL-cholesterol and total cholesterol concentration. There was no significant relationship between HDL- and LDL-cholesterol concentrations ($r = -0.166$).

Values of HDL- and LDL-Cholesterol

Application of the present method to the evaluation of serum HDL- and LDL-cholesterol concentrations in a group of “normal” subjects on our hospital staff is shown in Table 1.

Discussion

Although several methods have been proposed earlier for estimating HDL- or LDL-cholesterol, based on the property of complex formation with polyanionic (6, 9) or polycationic (10) macromolecules, these methods do not simultaneously quantitate HDL- and LDL-cholesterol. In the past, HDL-cholesterol has been measured primarily as part of an indirect procedure for quantitation of LDL-cholesterol (5, 11). On the other hand, Wilson and Spiger (12, 13) succeeded in the simultaneous estimation of HDL- and LDL-cholesterol concentrations. However, the main advantages of the present method, as compared with their method, are that it is rapid, relatively simple to perform, reproducible, and requires only minute amounts of serum.

The enzymic method or the Liebermann–Burchard reaction is widely used for cholesterol determination in the routine analyses. In the present study, however, the cholesterol de-
termination was disturbed by Ca\(^{2+}\)-detergent interaction in the case of the enzymic method and by the dilution in the case of isopropanol extraction preliminary to the Liebermann-Burchard reaction used with the AutoAnalyzer. It will be necessary to modify these analytical methods for the present procedure.

It is well known that heparin can form soluble and insoluble complexes with serum LDL and VLDL in the absence and presence of divalent cations, respectively. It has been shown (7, 14) that it is possible to isolate LDL, VLDL, and chylomicrons by changing the ionic strength with increasing molarity of sodium chloride at constant concentrations of heparin and Ca\(^{2+}\). Thus by appropriate reagent selection the lipoprotein classes can be selectively precipitated. Because sodium heparin and anion-exchange resin (Cl\(^{-}\) form) increase the ionic strength of the reagent, we use the reagent with lower concentration of sodium chloride than that in the turbidimetric method for isolation of lipoproteins (7, 14). Although Burstein et al. (6) dialyzed against BaCl\(_2\) solution to remove heparin, the anion-exchange resin we used in the present study is rapid and convenient.

Incomplete precipitation of LDL and VLDL with polyanion and divalent cation, observed with grossly hypertriglyceridemic samples, leads to overestimation of HDL-cholesterol concentration (15, 16). The isolation of HDL and LDL with the present method is satisfactory, within the limits of electrophoretic detectability, for samples with triglyceride concentrations of less than 3.5 g/liter. In the cases of hypertriglyceridemic samples, however, the filtration after reaction with Reagent I was done to obtain clear supernates, because floating precipitates form. We have not been able to test the present method with samples having VLDL-triglyceride concentrations greater than 3.5 g/liter. On the other hand, the total cholesterol concentration in serum (<5.0 g/liter) has no effect on the present method.

Srinivasan et al. (17) and Warnick and Albers (16) found that about 0.25 of isolated HDL was precipitated by heparin–Mn\(^{2+}\) treatment. Warnick and Albers (16) also reported that the addition of the other plasma proteins in the d >1.21 kg/liter fraction, as well as albumin, virtually eliminated HDL precipitation by heparin–Mn\(^{2+}\) treatment. In the present study, however, analytical recoveries of isolated HDL and LDL were about 80% in all HDL and LDL concentrations examined, even in the presence of albumin. The reason for this incomplete recovery is at present unknown.

It is well recognized that serum cholesterol in Japanese people is lower than that in the Western people. The present result indicates that the low concentration of total cholesterol is largely attributable to a lower LDL-cholesterol concentra-

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<th>Table 1. Plasma HDL- and LDL-Cholesterol Concentrations in Normal Japanese Adults</th>
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<td><strong>Age, yr</strong></td>
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Mean ± SD.

Taken together, our results suggest that the present precipitation method for simultaneously determining HDL- and LDL-cholesterol is readily adaptable for use in the routine clinical laboratory, and can be expected to facilitate the differential diagnosis of hyperlipoproteinemias.

**References**


