Evaluation of Two Different Homogeneous Assays for LDL-Cholesterol in Lipoprotein-X-positive Serum

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Background: The purpose of this study was to evaluate the performance of two homogeneous assays for LDL-cholesterol (LDL-C), a polyethylene/cyclodextrin (PC) assay and a detergent (D) assay, which are based on different principles, in cholestatic serum.

Methods: We compared serum LDL-C concentrations determined by the two assays for healthy normolipidemic subjects (n = 42) and cholestatic patients (n = 51). LDL-C concentrations obtained with the homogeneous assays were also compared with those obtained by HPLC for patients’ sera. In the interference study, conjugated bile acids were added to normal serum, and their effects on the two assays were examined. The effects of lipoprotein-X (LP-X), intermediate-density lipoprotein (IDL), and apolipoprotein (apo) E-rich HDL on the LDL-C assays were also investigated by adding these lipoproteins to normal serum.

Results: The LDL-C concentrations obtained with the D assay were higher than those obtained with the PC assay in the serum with high LP-X. The bias for LDL-C vs LP-X in choledastic serum correlated with LP-X concentration (r = 0.582; P < 0.001; n = 51). In the interference study, no effect of bile acids on the LDL-C assays was observed. However, the D assay measured 51.0% of the cholesterol in LP-X, whereas no reactivity was observed for LP-X in the PC assay. In addition, the D assay and the PC assay measured IDL-cholesterol at 31.2% and 52.4%, respectively, and measured apo E-rich HDL-C at 7.6% and 17.8%, respectively.

Conclusions: Although both homogeneous LDL-C assays are suitable for most cases, the present study showed that each homogeneous assay has a different limitation for choledastic serum with gross alterations in lipoproteins.

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LCL-cholesterol (LDL-C) concentrations positively correlate with the incidence of coronary heart disease (1, 2), and a reduction of LDL-C decreases the risk of coronary heart disease (3, 4). Therefore, accurate and precise measurements of patients’ LDL-C concentrations are necessary to appropriately identify individuals with hypercholesterolemia and to monitor the response to diet and drug treatments. LDL-C cannot be measured by ultracentrifugation in routine testing laboratories because ultracentrifugation is a time- and money-consuming method. Until now, LDL-C has not been measured directly but was estimated from the Friedewald equation for clinical purposes, based on three independent measurements: HDL-cholesterol (HDL-C), triglycerides (TGs), and total cholesterol (TC) (5). The Friedewald equation assumes that the amount of cholesterol in VLDL can be estimated by dividing the blood TG concentration by a factor of 5. Although the Friedewald equation had long been shown to be relatively reliable and was recommended by the National Cholesterol Education Program as a routine method for estimation of LDL-C (6), it has shortcomings: (a) combining three measurements necessarily increases analytical imprecision; and (b) it is invalid at TG concentrations ≥4.5 mmol/L (7) and can be used only in the fasting state.

Recently, several homogeneous assays for LDL-C, based on different principles, have been developed, and the kits for these assays are currently available. It has been

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1 Nonstandard abbreviations: LDL-C and HDL-C, LDL- and HDL-cholesterol; TG, triglyceride; TC, total cholesterol; LP-X, lipoprotein-X; IDL-C, intermediate-density lipoprotein-cholesterol; and apo, apolipoprotein.
reported that these assays are reliable and suitable even for serum with high TG concentrations (8, 9). However, it has not yet been determined whether these assays are suitable for serum samples from patients with any pathologies. We found a discrepancy in LDL-C values between two assays, particularly in serum from patients with cholestasis. Bile acids, which accumulate in cholestatic serum, may affect the assays differently. Alternatively, lipoprotein-X (LP-X), which is an abnormal lipoprotein appearing in the serum of patients with cholestasis, may influence the homogeneous assays. Other lipoproteins such as intermediate-density lipoprotein (IDL) and apolipoprotein (apo) E-rich HDL may also affect the assays because IDL and apo E-rich HDL can be increased in cholestatic patients.

In this study, we determined serum LP-X concentration in cholestatic patients and examined whether the bias in LDL-C results between the homogeneous method and a HPLC method correlates with LP-X concentration. As an additional experiment, we added bile acids, LP-X, IDL, or apo E-rich HDL to normal serum and examined the effects of these substances on LDL-C measurements.

Materials and Methods

SERUM SAMPLES
Forty-two serum samples from healthy normolipidemic subjects (ages, 25–56 years) and 51 serum samples from patients with cholestasis (ages, 45–71 years) were used for this experiment. Serum samples from patients were ordered to analyze lipids and liver function. We used pooled serum from other healthy normolipidemic subjects for in vitro studies.

Informed consent was obtained from all participants, and the study was approved by the Ethics Committee in Gifu University School of Medicine.

DETERMINATION OF SERUM CHOLESTEROL AND APO E CONCENTRATIONS
Serum TC (T-CHO Reagent·KL; International Reagent Co.) was measured enzymatically using an automated analyzer (Hitachi 736; Hitachi), and apo E was determined by ELISA as reported previously (10).

HOMOGENEOUS ASSAYS FOR LDL-C

Two commercial reagent kits, based on different assay principles, for the homogeneous LDL-C method were used for this experiment. Kyowa LDL, hereafter designated the polyethylene/cyclodextrin (PC) assay, was obtained from Kyowa Medex (Tokyo, Japan). This assay utilizes a nonionic surfactant, polyoxyethylene-polyoxypropylene block polyether and a sodium salt of sulfated cyclic maltotriose, α-cyclodextrin sulfate. Because polyoxyethylene-polyoxypropylene block polyether and α-cyclodextrin sulfate quench HDL-C, chylomicron-cholesterol, and VLDL-C, respectively, enzymatic reaction for cholesterol occurs only for LDL (8). The other kit used was Cholestest LDL, hereafter designated the detergent (D) assay, was obtained from Daiichi Pure Chemicals (Tokyo, Japan). The enzymatic reactions of this assay are as follows. Non-LDL lipoproteins are disrupted by a detergent, and the released cholesterol is hydrolyzed by cholesterol esterase. The free cholesterol thus formed reacts with cholesterol oxidase, generating hydrogen peroxide. After hydrogen peroxidite is consumed by a peroxidase in the presence of 4-aminoantipyrine to generate a colorless product, another added detergent releases the cholesterol from LDL particles. A similar enzymatic reaction to that described above occurs, except that hydrogen peroxide reacts with \( N,N'\text{-bis-(4-sulfobutyl)-m-toluidine disodium salt} \) to generate a colored product (9).

HPLC

HPLC separates lipoproteins on the basis of their sizes and generates lipoprotein profiles. It has been demonstrated that LDL-C concentrations determined by HPLC highly correlate with values obtained by ultracentrifugation (11, 12). We measured cholesterol by HPLC using an online postcolumn enzymatic reaction, as described previously, with minor modifications (13). HPLC analysis with photometric detection was performed using a SC-8010 HPLC system (TOSOH). A 25-μL sample was injected, and lipoproteins were separated on a Superox 6 HR10/30 column (Pharmacia Fine Chemicals) eluted with 10 mmol/L Tris-HCl, 0.15 mol/L NaCl, 1 mmol/L EDTA (pH 7.4) containing 1 g/L sodium azide at a flow rate of 0.4 mL/min. For cholesterol measurements, the effluent was mixed with enzymatic cholesterol reagents (Kyowa Medex), using a HPLC pump at 0.2 mL/min. The enzymatic postcolumn reaction was carried out in the reaction coil (cat. no. 018063; TOSOH) at 45 °C. The cholesterol in the postcolumn effluent was then detected selectively by the absorbance of 550 nm in an online system. LDL was eluted between 24 and 36 min, and the total run time was 60 min. LDL-C was calculated by multiplying the TC value by the percentage of LDL area on the HPLC pattern.

CONJUGATED BILE ACIDS

We examined the effect of bile acids on LDL-C measurements in vitro. A CBA (conjugated bile acids) kit was obtained from Sigma, and sodium salts of glycodeoxycholic acid, glycocholic acid, taurochenodeoxycholic acid, and taurocholic acid were used for this study. Each bile acid was dissolved in 0.15 mol/L NaCl and added to pooled serum prepared from healthy subjects (final concentrations of bile acids, 0, 25, 50, 100, and 200 μmol/L). Serum samples were incubated for 2 h at 37 °C to distribute the bile acids uniformly, and LDL-C in the samples was measured by the homogeneous assays.

LP-X DETERMINATION AND ITS ISOLATION FROM SERUM

It is well known that an abnormal lipoprotein designated LP-X appears in the plasma of patients with cholestasis (14). An important characteristic of LP-X is its electrophoretic mobility toward the cathode on a bacto-agar gel.
The serum LP-X concentration was measured using a precipitation method (15). Briefly, 50 μL of reagent I (80 g/L phosphotungstic acid and 10 mmol/L EDTA in 0.2 mol/L MES buffer, pH 5.3) was added to 50 μL of serum. After 10 min at room temperature, the mixture was centrifuged for 10 min at 1500g; 50 μL of the supernate was pipetted into a test tube, and 0.1 mL of reagent II (20 mmol/L MgCl₂ in 0.1 mol/L Tris-HCl buffer, pH 9.0) was added and mixed well. After 10 min, the mixture was centrifuged at room temperature at 1500g for 10 min. The phospholipid content in the precipitated fraction was measured, and the LP-X concentration was calculated by multiplying the phospholipid concentration by a factor of 1.5 because the content of phospholipid was estimated to be constantly ~67% in LP-X (14). The assay was linear in the range 30–3500 mg/L, and the detection limit was 15 mg/L. The average within-run CV was 3.5%. The concentration in normal serum was <15 mg/L.

To isolate LP-X from icteric serum, we used a precipitation method, as described above. The second precipitate mentioned above was washed with a 1:1:4 (by volume) mixture of reagent I, saline, and reagent II. Washed precipitate was dissolved in 30 g/L NaHCO₃ and used for the specificity study of the assay and for the observation of enzymatic reaction by the homogeneous assay. We confirmed that LP-X isolated by this method migrated toward the cathode in bacto-agar electrophoresis. The cholesterol concentration in the isolated LP-X fraction was determined using a kit for TC (T-CHO Reagent · KL).

Preparation of IDL and apo E-rich HDL
IDL (1.006 < d < 1.019 kg/L) and apo E-rich HDL (1.063 < d < 1.125 kg/L) were isolated from serum by ultracentrifugation after its density was adjusted with NaBr solution according to the method of Hatch and Lees (16). IDL and apo E-rich HDL were prepared from hypertriglyceridemic samples and cholestatic samples, respectively. Cholesterol concentrations in these isolated lipoprotein fractions were determined using a kit for TC (T-CHO Reagent · KL).

Statistical analyses
The means and SDs were calculated with Microsoft Excel, Ver. 5.0 (Microsoft). Least-squares linear regression analysis was performed using the Sigma Plot statistics program (Jandel Scientific).

Results
Comparison of LDL-C concentrations from homogeneous assays
We compared the LDL-C concentrations determined by two homogeneous assays in the sera of healthy normolipidemic subjects (n = 42) and cholestatic patients (n = 51). A significant correlation was observed for LDL-C values between the two assays in both groups. However, the correlation coefficient (r) in patients (r = 0.927) was lower than that in the normolipidemic subjects (r = 0.984), indicating that the enzymatic reaction for cholesterol may differ between the two assays in these patient samples. We compared the LDL-C concentrations in patient samples measured by two homogeneous assays with the concentrations obtained with the HPLC method. A stronger correlation was observed between the D assay and the HPLC method (r = 0.946) than between the PC assay and the HPLC method (r = 0.894).

Interference studies
We tested the effects of possible interferences on these assays in pooled serum using a kit (Interference Check-A; International Reagent Co.) (17). The effects of chylomicrons (turbidity) and hemoglobin on both assays were negligible. Conjugated and unconjugated bilirubin at concentrations up to 400 mg/L appeared to have little influence on either homogeneous assay (data not shown). Additionally, the effects of the bile acids tested were also negligible, at least up to the concentration of 200 μmol/L in both assays (Fig. 1). These observations were compatible with the finding that no significant correlation was found between the LDL-C concentration and the concentration of total bilirubin or total bile acids in serum (data not shown).

Effects of LP-X on homogeneous assays
Serum LP-X concentrations in patients were 30–1870 mg/L, and we compared LP-X concentrations with the difference in LDL-C measurements between the PC and D assays (Fig. 2). A significant correlation was found between the two assays, indicating that the bias between the two assays increases with the concentration of LP-X in serum.

Figs. 3 (PC assay) and 4 (D assay) show changes with time in the absorbance at appropriate wavelengths (600 nm for the PC assay and 546 nm for the D assay) during the reaction of LDL-C in normal serum, normal serum with added LP-X, and LP-X alone in the two homogeneous assays. Figs. 3 and 4 clearly show that the cholesterol in LP-X reacted in the D assay but not in the PC assay.

We next examined the effect of LP-X on LDL-C measurements. LP-X-cholesterol at final concentrations of 135, 270, 405, and 540 mg/L was added to pooled serum, and LDL-C was determined by the two homogeneous assays. The LDL-C result obtained by the D assay increased with increasing LP-X-cholesterol concentration, whereas the LDL-C result obtained by the PC assay was unaltered (Fig. 5A). We estimated how much of the cholesterol in LP-X reacted with the reagents in these assays. The increase in LDL-C from the initial value (no added LP-X) was divided by the final concentration of LP-X-cholesterol added at each point. The average increases for the four concentration points were 51.0% in the D assay and 0% in the PC assay.

Effects of IDL and apo E-rich HDL on homogeneous assays
We examined the effects of IDL and apo E-rich HDL on the homogeneous assays by adding these lipoproteins to
pooled serum. We confirmed that the cholesterol elution profile of isolated IDL and apo E-rich HDL showed single peaks with retention times of 25.0 min and 36.2 min, respectively, on HPLC. IDL-cholesterol (IDL-C) at final concentrations of 152, 303, 455, and 607 mg/L was added to pooled serum, and LDL-C was determined by the two assays. The findings showed that reagents in both assays reacted with the cholesterol in IDL (Fig. 5B). The IDL-C concentration measured by the PC assay and the D assay was 52.4% and 31.2%, respectively. The effect of apo E-rich HDL on the assays was also examined. apo E-rich HDL-C at final concentrations of 212, 425, 637, and 850 mg/L was added to pooled serum (Fig. 5C). The apo E-rich HDL-C concentrations measured by the PC assay and the D assay were, on average, 17.8% and 7.6%, respectively.

Discussion

Although several homogeneous assays for LDL-C have been developed recently, it is necessary to examine whether these assays are suitable for all samples from patients with pathologies. In the present study, a significant correlation was observed between the two LDL-C
assays in both healthy, normolipidemic subjects and cholestatic patients, but the correlation coefficient ($r$) in cholestatic patients was lower than that in normolipidemic subjects, which indicates that the reactivity to LDL may be somewhat different between the two assays in samples from patients. It was further shown that the LDL-C concentration measured by the D assay was higher than that by the PC assay in terms of the regression line in patients: $y = 0.986x - 59.7$, where $x$ is the D assay, and $y$ is the PC assay. We thus examined the effects of the substances that accumulate in cholestatic serum on these assays. Bilirubin showed little influence on both assays as reported previously (8, 9). We next measured bile acids because serum bile acid concentrations are markedly increased in patients with cholestasis (18) and because bile acids have a detergent activity (19, 20). We could not, however, find any effects of bile acids on either homogeneous assay.

We studied the effects of LP-X because this abnormal lipoprotein appears in the serum of patients with cholestasis but not in the serum of normolipidemic subjects. The LDL-C concentration as measured by the D assay was relatively high compared with that measured by the PC assay in LP-X-positive sera. The correlation of LDL-C between the homogeneous assays and the HPLC method...
suggests that the $r$ value in the D assay is higher than that in the PC assay because LDL-C measured by HPLC includes LP-X-cholesterol to some extent on the basis of the elution profile and because the D assay measures a part of the cholesterol in LP-X. In addition, we found a weak but significant correlation between LP-X concentration and a bias for LDL-C between the two assays. These findings suggest that the presence of LP-X in serum affects the LDL-C assay. As an additional experiment, we compared LDL-C values between the two assays after LP-X isolated from patient serum was added to pooled normal serum. The results show that 51% of the cholesterol in LP-X was measured by the D assay but not by the PC assay. In the present study, the PC assay did not measure LP-X-cholesterol, but the D assay measured a part of it, indicating that LP-X cannot be completely distinguished from LDL in the D assay. However, the D assay in turn reacted less with IDL and apo E-rich HDL than the PC assay. Because these lipoproteins are eluted adjacent to LDL on HPLC, the specificity of these homogeneous assays does not depend on particle size alone. Although more complicated mechanisms may be associated with the specificity, it was beyond the scope of this study to define them.

In the present study, the D assay reacted less with IDL than the PC assay. However, this does not necessarily indicate a superiority of the D assay over the PC assay because IDL is also an atherogenic lipoprotein (21). LDL-C measured by the PC assay might be rather useful from the clinical point of view when LDL-C cannot be measured separately. We believe that both homogeneous LDL-C assays are suitable for most cases, but the present study shows that there is a specific limitation for each homogeneous assay for serum samples with gross alterations in lipoproteins. We should thus evaluate the LDL-C value while understanding the characteristics of each assay.

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References