LDL-cholesterol (LDL-C) is the main marker in the evaluation and treatment of dyslipidemia (1). LDL-C is typically estimated after isolation by ultracentrifugation (2) or by calculation with the Friedewald formula (3):

\[
LDL-C = \text{total cholesterol} - \text{HDL-C} - \text{triglyceride}/2.17 \text{ (in mmol/L)}
\]

where HDL-C is HDL-cholesterol. Beta-quantification, the most frequently used ultracentrifugation-based method to estimate LDL-C, includes all cholesterol associated with LDL, as well as cholesterol associated with lipoprotein(a) [Lp(a)] and intermediate-density lipoprotein (IDL) particles (4). Because beta-quantification is cumbersome and requires sophisticated equipment, most clinical laboratories use the Friedewald formula, which also includes LDL-C, IDL-cholesterol, and Lp(a)-cholesterol in the estimation of LDL-C. Although chylomicronemia and increased concentrations of VLDL and IDL particles are known to interfere with the Friedewald formula, acceptable bias may also be found with triglyceride (Tg) concentrations <4.6 mmol/L (4,5). Therefore, direct methods that reliably measure LDL-C would be of great interest in clinical practice.

Since the first introduction of an immunochemically based method (6), other methods based on the selective solubilization of LDL particles have also been reported (7). When compared with beta-quantification, most of these methods show a certain bias (8–10), which could be caused by unequal reactivity of reagents with the broad range of plasma lipoproteins estimated as LDL by commonly used procedures. Thus, before a direct method can be introduced into clinical practice, the cholesterol fraction(s) measured and its equivalence with beta-quantification must be evaluated. In this study, we assessed LDL-C Plus, a direct, homogeneous method for the measurement of LDL-C, and analyzed which lipoproteins are recognized as LDL.

The LDL-C Plus assay (cat. no. 1985604; Roche Diagnostics) was used according to manufacturer instructions and calibrated with the calibrator provided by the manufacturer. The principle of the method has been described elsewhere (8–10). In the first step, a mixture of Mg\(^{2+}\), sulfated \(\alpha\)-cyclodextrin, and dextran sulfate was used to reduce the reactivity of chylomicrons and VLDL-cholesterol (VLDL-C) with the enzymes used in the final reaction.

Total cholesterol (TC) and TG concentrations were measured by standard enzymatic methods (CHOD-PAP and GPO-PAP; Roche Diagnostics). HDL-C was measured by a direct method, and Lp(a) concentrations were measured by immunoturbidimetry (both from Roche Diagnostics). All assays were performed in an Hitachi 911 analyzer.

LDL-C was determined by the Friedewald formula (\(LDL_f\)) and by modified beta-quantification (LDL\(_{BQ}\)). VLDL was separated at \(d < 1.006 \text{ kg/L}\) by ultracentrifugation (18 h; 105,000 × \(g\); 4 °C), and LDL-C was measured in the infranatant by the direct method (instead of precipitation). During the period of the study (October–December 1999), the inaccuracies of TC, Tg, direct HDL, and LDL\(_{BQ}\) measurements were controlled by a multilevel control from the Pacific Biometrics Research Foundation. Maximum inaccuracies were 2.2%, −4.4%, and −4.5% for TC, Tg, and direct HDL-C, respectively, whereas the inaccuracy for LDL\(_{BQ}\) was 1.6–7.5%. Lipoprotein fractions were isolated by sequential ultracentrifugation from pooled sera using the following densities: \(d < 0.95 \text{ kg/L}\) for chylomicrons, 0.95–1.006 kg/L for VLDL, 1.006–1.019 kg/L for IDL, 1.019–1.050 kg/L for LDL, 1.050–1.100 kg/L for Lp(a), and >1.100 kg/L for HDL and the lipoprotein-deficient serum fraction. The range 1.019–1.050 kg/L was selected for LDL to avoid the inclusion of Lp(a) particles in this fraction. TC content in these fractions was measured as indicated above.

With commercial controls containing two different LDL-C concentrations (Precinorm \(^*\) L and Precipath \(^*\) HDL/LDL; Roche Diagnostics), LDL-C Plus showed a run-to-run imprecision of <2.0%, which satisfied criteria established by the National Cholesterol Education Program (5). Recovery and serial dilutions of isolated LDL produced direct inaccuracy estimates of <5.1%. When LDL-C Plus was assessed in samples with chylomicrons and/or increased VLDL (\(n = 9\)), a positive difference was found (3.07 ± 1.73 mmol/L for LDL-C vs 2.71 ± 1.58 mmol/L for LDL\(_{BQ}\); \(P < 0.0001\), Wilcoxon \(t\)-test). However, when one sample with a serum VLDL-C/Tg ratio of 1.05 (in mmol/L) was excluded from analysis, the difference became negative (2.24 ± 0.93 mmol/L for LDL-C Plus vs 2.37 ± 1.00 mmol/L for LDL\(_{BQ}\); \(P < 0.0001\)). These results suggest a strong dependence of LDL-C Plus values on serum VLDL-C/Tg (i.e., on serum IDL particle content).

Passing-Bablok regression analysis (11) comparing LDL-C Plus and LDL\(_{BQ}\) assessed in 115 samples (TC, 3.38–10.56 mmol/L; Tg, 0.42–11.60 mmol/L; HDL-C, 0.52–2.32 mmol/L; LDL-C\(_{BQ}\), 0.69–8.28 mmol/L; 10 samples with >4.6 mmol/L Tg, 5 of which contained chylomicrons), produced the equation:

\[
LDL-C \text{ Plus} = -0.44 + 1.058 \times LDL-C\text{\(_{BQ}\)}
\]

The confidence interval of the slope included 1, but that of the y-intercept did not include 0. The regression analysis revealed a significant negative bias and lower concentrations of LDL-C Plus (3.15 ± 1.07 mmol/L) compared with
A significant positive association was found with the serum shown that these particles are recognized by the HDL-C modified mice with very high IDL content in hyperlipidemic patients, and we, in hyperlipidemic genetically

<table>
<thead>
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<th>VLDL-C</th>
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<th>LDL</th>
<th>Lp(a)</th>
<th>HDL+ LPDS</th>
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<td>7</td>
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<td>70</td>
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Both the Roche HDL and LDL direct assays essentially share the same mixture as first reagent (12, 15). Thus, both the previous and the present results demonstrate that this mixture does not eliminate the reactivity of IDL-C. This failure to eliminate the reactivity of IDL-C, which interferes in HDL-C measurement, allows the recognition of most IDL-C as LDL-C with the LDL-C Plus method, as occurs with the Friedewald equation and beta-quantification.

According to these data, LDL-C Plus mainly recognized cholesterol associated with LDL and IDL, but not with Lp(a); the latter being strikingly different from LDL_{HDL} and LDL_{V}. However, the contribution of Lp(a) to TC is <0.2–0.3 mmol/L in subjects with Lp(a) concentrations <300 mg/L (16, 17). Thus, the substantial differences found between LDL-C Plus and LDL_{HDL} are independent of which lipoprotein-cholesterol is recognized by the assay.

To elucidate the role of calibration in the negative bias of LDL-C Plus, we assessed 68 additional serum samples (TC, 3.42–8.90 mmol/L; Tg, 0.43–12.5 mmol/L; HDL-C, 0.55–2.51 mmol/L; LDL-C_{HDL} = 1.77–6.19 mmol/L; 11 samples with >4.6 mmol/L Tg, of which 6 contained chylomicrons) after adding 0.31 mmol/L (the difference between means observed in the first set of 115 samples) to the initial value of the calibrator and obtained the equation:

\[
LDL-C_{Plus} = 0.022 + 1.008 \times LDL_{HDL}
\]

The confidence intervals for the slope and y-intercept included 1 and 0, respectively. LDL-C Plus and LDL_{HDL} concentrations were not significantly different (3.63 ± 1.34 mmol/L for LDL-C Plus and 3.71 ± 3.30 mmol/L for LDL_{HDL}), but in the same samples, LDL_t was significantly lower (3.27 ± 1.47 mmol/L; \(P < 0.001\)). Of samples with <4.6 mmol/L and ≥4.6 mmol/L Tg, 81% and 45%, respectively, had a bias lower than 10% according to LDL-C Plus, whereas 79% and 18% showed this bias according to LDL_t. Thus, it is likely that the differences found between LDL-C Plus and LDL_{HDL} could be attributed to the calibration of the assay. This has also been suggested by two recent studies (9, 10), but our data show, for the first time, the reactivity of LDL-C Plus reagents with the broad range of lipoproteins estimated as LDL by currently used methods.

In conclusion, we demonstrate that the LDL-C Plus

Fig. 1. Percentage of LDL (measured by LDL-C Plus) over TC (measured by CHOD-PAP) concentrations in lipoprotein fractions of pooled sera.

\(|A|_{\text{pool A, which contains high cholesterol and Tg, low HDL-C, and undetectable Lp(a) concentrations.}}\)

\(|A|_{\text{pool B, which contains high cholesterol and Lp(a) concentrations.}}\)

\(>700 \text{ mg/L} \text{ concentrations.} \)

TC, VLDL-C, TG, and Lp(a) concentrations were higher, whereas HDL-C was lower in pool A. For details, see text. ND, not detectable; LPDS, lipoprotein-deficient serum.
assay measures cholesterol associated with IDL and LDL but not with Lp(a) particles. The significant negative bias observed may be attributed to suboptimal assay calibration. After readjusting the calibration, we found a close relationship between LDL-C Plus and LDL_{ho} values, and LDL-C Plus became a reliable alternative to beta-quantification and a better approach for LDL-C measurement than the Friedewald formula.

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References


Effect of Specimen Anticoagulant and Storage on Measurement of Serum and Plasma Fatty Acid Ethyl Ester Concentrations, Raneem O. Salem, Joanne E. Cluette-Brown, Ali Hasaba, and Michael Laposata* (Division of Laboratory Medicine, Department of Pathology, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114; * Address correspondence to this author at: Room 235, Gray Bldg., Massachusetts General Hospital, Boston, MA 02114; fax 617-726-3256, e-mail mlaposata@partners.org)

Fatty acid ethyl esters (FAEES) are cytotoxic nonoxidative metabolites of ethanol that are produced by esterification of alcohol and fatty acids (1–3). After ethanol intake, FAEES are found mainly in the liver, pancreas, heart, and brain, which are the major organs adversely affected by ethanol intake (4, 5). FAEES are detectable in the blood for up to 24 h after consumption of ethanol to at least 600 mg/L (6–8), making the presence of FAEES in blood a useful marker for ethanol intake. This investigation presents the results of studies on the effects of collection tube, storage time, and storage temperature on FAEE concentrations in blood, with the goal of creating a reliable clinical assay for serum and plasma FAEE quantification.

Four volunteers participated in this study. Each subject was given a weight-adjusted amount of 100-proof vodka mixed with fruit juice in a 1:3 ratio (8). The vodka-juice beverage was divided into nine equal aliquots, which were administered every 10 min over a 90-min time period. After the last aliquot was drunk, blood samples were collected in four different 5-mL Vacutainer Tubes from each subject to assess the influence of the collection tube on the blood FAEE concentration. The tubes included a red-top tube with no anticoagulant, a purple-top tube with 150 g/L EDTA, a green-top tube with 72 US units of heparin, and a blue-top tube with 32 g/L sodium citrate. The tubes were kept on ice and processed within 30 min. FAEES were then isolated and quantified as described below. To study the effect of storage time and temperature on FAEE concentrations in whole blood, 40 mL of blood was collected from each of the same four subjects in red-top vacuum tubes (no anticoagulant). The 40 mL of blood was divided into two equal aliquots that were incubated at 25 and 37 °C for 0, 2, 4, 6, 24, and 48 h. FAEES were then isolated from the samples. To accomplish this, serum or plasma was first separated from the blood cells by centrifugation at 3420g at 4 °C for 20 min. FAEES were then isolated using an acetone-hexane (2:8 by volume) extraction followed by solid-phase extraction (9), and measured using gas chromatography–mass spectrometry as described previously (10). The within-assay CV for the entire method was 17%. The CVs for the individual stages were as follows: FAEE isolation via liquid extraction, ~13%; solid-phase extraction, ~9.7%; and quantification by gas chromatography–mass spectrometry, ~2.2%.

The effect of the collection tube on the amounts of FAEE in plasma or serum is shown in Table 1. For the four