Comparison of a New Method for the Direct and Simultaneous Assessment of LDL- and HDL-Cholesterol with Ultracentrifugation and Established Methods

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Background: Automated electrophoresis combined with enzymatic cholesterol staining might improve routine assessment of LDL- and HDL-cholesterol (LDLC and HDLC), as an alternative to the Friedewald equation and precipitation. A new method (Hydrasys; SEBIA) that adapts the cholesterol esterase/cholesterol oxidase reaction within urea-free gels was evaluated.

Methods: Fresh sera from 725 subjects (512 dyslipidemics) were analyzed by electrophoresis, in parallel with sequential ultracentrifugation, β-quantification, calculation, and precipitation.

Results: Electrophoresis was linear up to 4 g/L cholesterol, with a detection limit of 0.042 g/L cholesterol/band. Within-run, between-run, between-batch, and between-operator imprecision (CVs) were 1.6%, 2.0%, 1.5%, and 2.7% for LDLC, and 3.9%, 4.3%, 5.5%, and 4.9% for HDLC, and remained unchanged up to 6.3 g/L plasma triglycerides (TGs). Precision decreased with very low HDLC (<0.25 g/L). Serum storage for 3–7 days at +4 or −80 °C did not interfere significantly with the assay. Agreement with β-quantification was stable for LDLC up to 5.07 g/L (r = 0.94), even at TG concentrations >4 g/L (r = 0.91). Bias (2.88% ± 12%) and total error (7.84%) were unchanged at TG concentrations up to 18.5 g/L. Electrophoresis predicted National Cholesterol Education Program cut-points with <0.04 g/L error, exactly and appropriately classified 79% and 96% of the subjects, and divided by 2.4 (all subjects) and 5.8 (TGs >1.5 g/L) the percentage of subjects underestimated by calculation. One-half of the patients with TGs >4 g/L had LDLC >1.30 g/L. For HDLC, correlation was better with precipitation (r = 0.87) than ultracentrifugation (r = 0.76). Error (−0.10% ± 26%) increased when HDLC decreased (<0.35 g/L). Direct assessment of the LDLC/ HDLC ratio detected 45% more high-risk subjects than the calculation/precipitation combination.

Conclusions: Electrophoresis provides reliable quantification of LDLC, improving precision, accuracy, and concordance over calculation, particularly with increasing plasma TGs. Implementation of methods to detect low cholesterol concentrations could extend the applications for HDLC assessment.

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LDL and HDL are major transporters of cholesterol in human plasma (1). The proatherogenic role of LDL and the antiatherogenic role of HDL have found their clinical relevance through epidemiological evidence that circulating concentrations of LDL-cholesterol (LDLC) and HDL-cholesterol (HDLc), are high biological predictors of cardiovascular disease (2–4). More recently, clinical intervention on cholesterol-rich lipoproteins has been shown to slow or reverse the development of atherosclerosis and its morbidity complications (5). Therefore, measurement of these markers has been proposed as a primary tool for

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risk assessment and monitoring of patients with or at risk of developing cardiovascular disease (6, 7).

The different types of lipoproteins have been defined historically by ultracentrifugation (8). However, the method is costly and time-consuming, so that simplified methods have been implanted in routine laboratories. HDL usually is separated in liquid phase by precipitation of large lipoproteins with polyanion-divalent cation reagents. Despite their relative simplicity, precipitation methods may overestimate HDLC concentrations in hypertriglyceridemia (9). LDL is most commonly evaluated using the Friedewald formula (10): LDLC (g/L)\(^{10}\) = TC − HDLC − TG/5, where TC the total cholesterol and TG is the triglyceride concentration. However, this indirect evaluation may be altered by poor precision and accuracy, particularly when TG-rich lipoproteins (chylomicrons, VLDL, or intermediate-density lipoproteins) are present in plasma (11). Interference by TGs becomes significant at concentrations as low as 2 g/L,\(^{11}\) preventing usage of this formula when the TG concentration exceeds 4 g/L. A reference method, named \(\beta\)-quantification, based on the combination of ultracentrifugation and precipitation has been proposed to overcome limitations of sequential ultracentrifugation as well as those of the usual methods (12, 13). This method, used in clinical trials or for evaluation of methods for LDLC and HDLC measurement, remains restricted to a minority of laboratories. Therefore, there is a paradox between the clinical need for accurate methods of assessment of these important biological markers and the lack of satisfactory methods to measure LDLC and HDLC over the whole range of lipid disorders encountered in common practice.

Several liquid-phase chemical methods (immunoseparation and separation with polymeran surfactant/detergent combinations) as well as physical methods for separation of lipoproteins (e.g., electrophoresis, capillary isotachophoresis, chromatography) have been investigated (14, 15). Among these, automation combined with enzymatic staining of cholesterol within gels may overcome classical drawbacks of electrophoresis (16–19): imprecision attributable to manual steps, variable resolution depending on the nature of the gel, nonspecificity of lipid stains, and time-consuming procedures. Recent improvements of automated electrophoresis were developed by SEBIA (http://www.sebia.com) by adapting technical conditions to the cholesterol esterase-cholesterol oxidase (peroxidase) enzymatic determination of cholesterol in lipoprotein fractions within agarose gels. Data from preliminary precision and accuracy studies (20) prompted us to extend the exploration of the analytical performance of this new method to a larger sample of patients more representative of the lipid disorders commonly encountered in clinical practice. Our objectives were to (a) study the behavior of cholesterol-rich lipoproteins on this type of gel through interference studies and comparison with sequential ultracentrifugation, (b) evaluate analytical performances and accuracy vs \(\beta\)-quantification, and (c) compare analytical performances of the new method with those of long-standing methods used in routine laboratories: the Friedewald formula for LDLC and precipitation for HDLC.

**Materials and Methods**

**Subjects**

Patients (\(n = 725\)) attending three medical departments, (Metabolic Disease, Cardiology, and Hepatology) at the University Hospital of Saint Antoine in Paris, were enrolled prospectively between January 1997 and January 1999. Patients, who were prescribed a fasting lipid profile for diagnosis or treatment follow-up purposes, gave consent to the study, which was approved by the local Committee of Ethics (Hôpital Saint Antoine, Paris). Blood samples were collected from resting patients after a 12-h overnight fast. Patients could receive any kind of medication, including lipid-lowering drugs (fibrates, statins, and resins); however, none were supplemented with ascorbic acid.

The study population size was designed to collect groups of 100–150 representative dyslipidemic and normolipidemic subjects (Table 1). Two TG cut-points were chosen: 1.5 g/L, which corresponds to a risk threshold for cardiovascular disease (21, 22); and 4 g/L, the concentration above which Friedewald formula is no longer applicable. Two subjects with type III hyperlipidemia were studied independently.

**Description of the Test Method**

Automated agarose gel electrophoresis of lipoproteins in alkaline buffer was adapted to allow the direct quantification of cholesterol in each lipoprotein fraction within the gel, using the cholesterol esterase/cholesterol oxidase reaction (SEBIA). The method has been improved by automation of several procedures on the HYDRASYS 1211 apparatus from SEBIA: sample application, electrophoretic migration, enzymatic reaction and staining, washing, drying of gels, and computer-assisted scanning of gels. Conditions such as current, temperature (controlled by Peltier effect), voltage, rate of fluid circulation, and time allocated for each reaction step were controlled to remain constant. Agarose gels (5 g/L) contained 1.5 and 8.2 g/L barbital at pH 9.4 ± 0.1. Electrophoresis of lipoprotein was performed for 20 min at 20 °C at 26V-h, in a buffer containing 3 g/L sodium azide, 4.4 g/L barbital,

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\(^{9}\) Nonstandard abbreviations: LDLC, HDLC, and VLDLC; LDL-cholesterol, HDL-cholesterol, and VLDL-cholesterol; TC, total cholesterol; TG, triglyceride; Lp(a), lipoprotein(a); LDL-e and HDL-e, LDL and HDLC by electrophoresis; NCEP, National Cholesterol Education Program; HDL-p, HDLC by precipitation; LDL-i, LDLC by the Friedewald formula; LDL-uc and HDL-uc, LDLC and HDLC by sequential ultracentrifugation; CI, confidence interval; apo, apolipoprotein; and LDL-u and HDL-u, LDLC and HDLC by \(\beta\)-quantification.

\(^{10}\) For conversion to mmol/L, multiply by 2.586. For conversion to mg/dL, multiply by 100.

\(^{11}\) For conversion to mmol/L, multiply by 1.129. For conversion to mg/dL, multiply by 100.
and 24.7 g/L sodium barbital at pH 9.3 ± 0.1. The cholesterol esterase/cholesterol oxidase colorimetry was performed at 50 °C for 15 min in a morpholinoethanesulfonate buffer (195.24 g/L) at pH 7.0 ± 0.3, using the orange chromogen, aminoethyl carbazole (1.25 g/L). Gels were washed for 10 min in 0.1 g/L sodium azide at pH 8.8 ± 0.3 and dried under hot air (75 °C) for 20 min. Densitometric scanning of electrophoretograms (HYRYS densitometer 1012; SEBIA) at 540 nm, using a green filter (or at 420 nm, using a blue filter), allowed quantification of colorimetric intensities of bands corresponding to LDLc, of an intermediate band corresponding to VLDL-cholesterol (VLDLc) and lipoprotein(a) [Lp(a)], and of a band corresponding to HDLc (Fig. 1). Serum LDLc and HDLc concentrations measured by this electrophoretic method (LDL-e and HDL-e) were calculated as the percentage of total plasma cholesterol represented by the relative intensity of each band. Except for the manual determination of cut-points defined at the nadir between lipoprotein fractions, the scanning process was fully au-

<table>
<thead>
<tr>
<th>n</th>
<th>Age, years</th>
<th>TC, g/L</th>
<th>TGs, g/L</th>
<th>LDLC, a g/L</th>
<th>HDLC, b g/L</th>
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<tr>
<td>Men</td>
<td>341</td>
<td>53.9 ± 13.6</td>
<td>2.32 ± 0.58</td>
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<td>Women</td>
<td>299</td>
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<td>2.37 ± 0.62</td>
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<td>2.21 ± 0.59</td>
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<td>0.18–18.34</td>
<td>0.41–4.63</td>
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</table>

a Data are given as mean ± SD.
b LDLC and HDLC were measured by sequential ultracentrifugation.
c Comparison between men and women: P < 0.0001.
d Normolipidemia: TC < 2 g/L; TGs < 1.5 g/L; HDLC > 0.35 g/L.
e Hypercholesterolemia: TC > 2 g/L; TGs < 1.5 g/L.
f Combined hyperlipidemia: TC > 2 g/L; 1.5 g/L < TGs < 4 g/L.
g Hypertriglyceridemia: TGs > 4 g/L.
h Hypoalphalipoproteinemia: HDLC < 0.35 g/L.
tomated and computer assisted. The system allowed up to 30 samples of 15 μL each to be analyzed within 1 h, and final results were obtained within 1.5 h. After scanning, gels were rinsed for 1 min in 450 mL/L ethanol for storage. Data from densitometry were stored electronically, allowing repeated readings.

LIPOPROTEIN ANALYSES
Venous blood (14 mL) was collected into evacuated tubes without anticoagulant and allowed to clot at room temperature. Sera were separated and processed immediately after blood centrifugation (1000g for 15 min at 10 °C) in a Jouan CR312 centrifuge (http://www.jouan.com). Cholesterol (as TC) and TG concentrations were measured on a Hitachi 704 automatic analyzer (http://www.hiitachi.com) using reagents, calibrators, and recommendations from Boehringer Mannheim (http://www.boehringer-mannheim.com). Cholesterol measurements were compared with the Abell–Kendall method under the protocol recommended by and performed at a reference laboratory belonging to the CDC Cholesterol Reference Laboratory Network (Dr. Feruccio Ceriotti, Istituto Scientifico H.S. Raffaele, Milan, Italy). Imprecision (as CV) was 1%, the correlation coefficient was r = 1, and the absolute total error was 7.5% for TC, meeting the National Cholesterol Education Program (NCEP) goal (total error was 7.5% for TC, meeting the National Cholesterol Education Program (NCEP) goal (5)).

Lipoprotein analyses from Boehringer Mannheim (http://www.boehringer-mannheim.com). Cholesterol measurements were compared with the Abell–Kendall method under the protocol recommended by and performed at a reference laboratory belonging to the CDC Cholesterol Reference Laboratory Network (Dr. Feruccio Ceriotti, Istituto Scientifico H.S. Raffaele, Milan, Italy). Imprecision (as CV) was 1%, the correlation coefficient was r = 1, and the absolute total error was 7.5% for TC, meeting the National Cholesterol Education Program (NCEP) goal (5). A modified β-quantification method for LDL (LDL-u) and HDLC (HDL-u) measurements was performed in a subgroup of 442 subjects, similarly to methods described previously (12, 13, 26). After separation of the VLDL/chylomicron fraction, an aliquot of plasma was collected, and its volume was adjusted with isotonic saline (0.15 mol/L NaCl). LDL was calculated as the difference between cholesterol in the infranatant before and after precipitation, measured as described above. This modified β-quantification procedure was well correlated with sequential ultracentrifugation (r = 0.95). Lipid measurements, electrophoresis, and ultracentrifugation were performed in parallel. Electrophoresis was performed blind of ultracentrifugation, Friedewald formula, or precipitation results.

INTERFERENCE STUDIES
For in vitro bilirubin interference studies, various amounts of a stock bilirubin solution (Horleco-Planstiehl), prepared as described (23, 26), were added to six sera at concentrations of 5–525 μmol/L total bilirubin, and the sera were then analyzed in duplicate. For in vivo interference studies, total and conjugated bilirubin in plasma from cholestatic patients were measured using reagents from Boehringer Mannheim on a Beckman CX4 analyzer. For hemolysis interference studies, a pellet of red blood cells was prepared by centrifugation (1000g for 10 min) from 5 mL of venous blood collected in heparin/lithium-coated tubes. A hemolysate stock solution containing ~50 g/L (2.9 mmol/L) hemoglobin, prepared as described (23), was added to six sera at concentrations of 0.25–3.5 g/L free hemoglobin, and the sera were then analyzed in duplicate. Serum hemoglobin was measured on a Beckman-Coulter automatic analyzer. Plasma Lp(a) concentrations were measured using an immunonephelometric method according to the manufacturer’s instructions (Dade Behring). ApoE isoforms were identified by isoelectric focusing (27). In type III patients, E2 homozygosity was confirmed by allele-specific oligonucleotide genotyping using reagents from Innogenetics.

STATISTICAL ANALYSES
Data were cross-read by two investigators and stored as Excel 97 (Microsoft) files. Analyses were conducted using SAS and STATVIEW 4.51 software (SAS Institute; http://www.statview.com). Comparisons of gaussian-distributed values were performed using the paired Student t-test in parallel with the nonparametric Wilcoxon test (significance set at P < 0.01). For TGs and VLDLC, log-transformed values were used for comparison. Mann–Whitney and Kruskal–Wallis nonparametric tests were used for comparison between qualitative groups of data. Linear regression and Bland–Altman residuals plots were performed as described (28). Predictions derived from linear regression equations were made according to Ar-
mitage and Berry (29). For concordance studies, the proportion of observed agreement, the $\kappa$ index, and intraclass correlation coefficients were computed on the SAS software as described (30, 31).

**Results**

**Linearity and Sensitivity.** Serial dilutions of a normotriglyceridemic serum were made with a solution of albumin in physiological saline to obtain a range of cholesterol values from 0.5 to 4 g/L. The concentration percentage for each dilution point, obtained after scanning of dried gels, was plotted against cholesterol concentrations, giving a regression coefficient of $R^2 = 0.09$ for LDLC and HDLC (not significant). When gels were read without previous drying, the regression coefficient increased ($R^2 = 0.59$); however, the increase was not significant. In hyperlipidemic sera (up to 6.3 g/L TGs), the method was linear ($R^2 = 0.10$; not significant) for LDLC and HDLC, as well as for VLDLC ($R^2 = 0.18$; not significant).

To determine how accurately small changes in cholesterol concentration would be detected by the test method, two normolipidemic sera with different LDLC/HDLC ratios (10.6 and 1.5, respectively) were mixed in various proportions ($n = 15$ points) and electrophoresed. The linear regression analysis of measured values as a function of predicted values gave a coefficient of $r = 0.96$ for LDLC and $r = 0.98$ for HDLC. The detection limit was 42 mg/L cholesterol per band. Comparable results were obtained when gels were scanned without previous drying ($r = 0.96$ for LDLC; $r = 0.989$ for HDLC). In sera with low (0.75 g/L), medium (2.64 g/L), or high (5.68 g/L) TG concentrations, the detection limits were similar for LDLC ($r = 0.974$), HDLC ($r = 0.950$), and VLDLC ($r = 0.970$).

**Precision**

**Within- and between-run precision.** Within-run precision was tested on two sets of six fresh serum samples corresponding in pairs to three concentrations of low, medium, and high LDLC (<1.3, 1.3–1.6, and >1.6 g/L) or HDLC (<0.35, 0.35–0.60, and >0.60 g/L). Each serum was run 30 times on a single gel, using gels and reagents from the same batch. The within-run CV, $C_{V_w}$, was 1.6% ± 0.5% for LDLC and 3.9% ± 1.6% for HDLC. $C_{V_w}$ was unchanged (1.0–2.0%) as a function of LDLC concentrations, whereas $C_{V_w}$ increased within the recommended range (1.8–5.2%) with decreasing HDLC concentrations. Because LDL and VLDL fractions may overlap with increasing TG concentrations, sera with low (0.52 g/L), medium (2.35 g/L), and high (3.2 g/L) TG concentrations were analyzed. $C_{V_w}$ was unchanged: 1.2%, 1.5%, and 1.6% for LDL, and 5.4%, 4.1%, and 4.4% at low, medium, and high plasma TG concentrations. Between-run precision was tested on 15 sera run in duplicate on 10 different gels from the same batch. Each set was composed of sera with high, medium, and low LDLC and HDLC concentrations. The between-run CV, $C_{V_B}$, was 2.0% ± 1.0% for LDLC and 4.3% ± 1.7% for HDLC. The $C_{V_B}$ for LDLC was unchanged (1.9–2.1%) as a function of increasing LDLC concentrations, whereas $C_{V_B}$ tended to increase nonsignificantly (2.7–5.4%) for HDLC as a function of decreasing HDLC concentrations. In hypertriglyceridemic sera, $C_{V_B}$ was unchanged for LDLC (1.2–2.4%), whereas it increased within the recommended range for HDLC (4.2–7.4%) as a function of decreasing HDLC. Thus, $C_{V_w}$ and $C_{V_B}$ were within recommended limits for LDLC and HDLC, even in hypertriglyceridemic sera.

**Between-operator, -batch, and -laboratory precision.** Because cut-points between lipoprotein fractions were determined manually, between-operator and between-laboratory CVs were studied. The same set of 50 sera was tested in parallel by six independent operators, on the same batch of gels, in three laboratories (Evry, Nice, and Paris), each blind of the results from the other laboratories. Between-operator CVs were within the recommended ranges of 2.5–2.7% for LDLC and 3.9–5.5% for HDLC at low (1.2 ± 0.3 g/L), medium (2.0 ± 0.3 g/L), and high (2.8 ± 0.3 g/L) TC concentrations, respectively. The CVs were 2.3–3.3% for LDL and 3.9–5.2% for HDLC at low (0.7 ± 0.2 g/L), medium (1.4 ± 0.3 g/L), and high (3.3 ± 1.4 g/L) TG intervals. Between-batch reproducibility was analyzed on 15 fresh serum samples run in duplicate on three gels using reagents and gels from different batches. The between-batch CV was 1.5% ± 0.8% for LDLC, and 5.5% ± 2.0% for HDLC, remaining within the recommended range regardless of the LDLC or HDLC intervals. Between-laboratory variations were 5.3% for LDLC and 16% for HDLC, increasing frankly above the recommended limits for HDLC in sera with low HDLC (<0.25 g/L) regardless of the TG concentrations.

**Interferences**

**Storage experiments.** Biases were minor after storage for 3 days at 4 °C or 1 week at −80 °C (Table 2). Basal LDLC and HDLC values were well correlated with those after storage at 4 °C or freezing ($r = 0.95$ and $r = 0.94$, respectively), allowing us to predict that LDLC concentrations of 1.0, 1.5, and 2.0 g/L after storage would correspond to initial concentrations of 1, 1.45, and 1.90 g/L, respectively. Significant overestimation of LDLC and underestimation of HDLC increased with duration of freezing, temperature (−20 °C), and hypertriglyceridemia.

**Low-molecular weight heparin, bilirubin, and hemoglobin.** Heparin is known to interfere with electrophoretic migration of lipoproteins through in vivo activation of lipases (32). Bias for LDL-e vs LDL-u (6.55% ± 13% vs 3.01% ± 13%; not significant) and absolute bias (10.0% ± 10% vs 10.3% ± 9%; not significant) remained unchanged in patients treated with low-molecular weight heparin ($n = 36$) when compared with nonheparinized patients ($n = 639$), whereas the difference was highly significant ($P$
apoE and Lp(a). In a subgroup of 124 subjects, allelic frequencies of apoe isoforms were similar with those expected (not shown) (27). Interferences with apoE isoforms were analyzed in E2/E3 (n = 20), E3/E3 (n = 74), and E3/E4 (n = 25) carriers. No allelic differences were found, except for the VLDLC/TG ratio, an indicator of intermediate/apoE-rich lipoproteins, which was higher in E2/E3 carriers (0.199; P = 0.003) than in E3/E3 (0.155), or E3/E4 carriers (0.138). Biases (LDL-e vs LDL-uc) were positively correlated with the VLDLC/TG ratio in E2/E3 carriers (r = 0.485). This suggested that the behavior of some intermediate lipoproteins (β-VLDL) was to be found with LDL in the same fraction in electrophoresis. In keeping, in two subjects with type III hyperlipidemia (VLDLC/TG = 0.55 and 0.36; both E2/E2), intermediate lipoproteins overlapped most of the LDL fraction in electrophoresis, whereas these lipoproteins floated mainly with VLDL after sequential ultracentrifugation.

Plasma Lp(a) was increased (>0.3 g/L) in 30 subjects (0.54 ± 0.27 g/L). The bias for LDL-e vs LDL-uc in β-quantification was significantly negative in subjects with high Lp(a) (−77 vs 35 mg/L; P = 0.0006), whereas no difference was observed when LDL-e was compared to LDL-uc. Moreover, HDLC tended to be higher after sequential ultracentrifugation (0.62 vs 0.53; P = 0.024), whereas it was unchanged after electrophoresis or precipitation. This confirmed that Lp(a) did not overlap with LDL in electrophoresis, comigrating within the VLDL band.

ACCURACY

Direct LDLLC vs HDLC by ultracentrifugation or Friedewald equation. The mean bias for LDL-e vs LDL-uc was 2.88% ± 12.6% (Table 3), yielding a total analytical error of 7.84%, both within NCEP-recommended goals (<4% and ≤12%, respectively). In contrast, a negative bias (−6.72% ± 12.1%), and a stronger absolute bias (9.6% ± 10% vs 8.7% ± 8.2%; P < 0.0025; TGs <4 g/L; n = 410) were observed for LDL-f vs LDL-uc, indicating greater underestimation and variability for calculated LDL. The bias for LDL-e was a little more pronounced vs LDL-uc (3.01% ± 13.1%). Differences between mean LDL-u and LDL-e varied as a function of plasma TG concentrations, remaining nonsignificant up to TG concentrations <1.98 g/L, whereas differences were significant for LDL-f at all TG concentrations. Underestimation of LDL became excessive at TG concentrations as low as 2 g/L for LDL-f, whereas total error for LDL-e remained within the NCEP-recommended range (Fig. 2). These trends were also observed for absolute biases, except when TG concentrations were >4 g/L, where absolute bias, as the percentage, exceeded the goal, but in much lower proportions than were observed with LDL-f. Absolute bias for LDL-e was <12% in 73% of subject vs 69% for LDL-f.

LDLC measured with electrophoresis was compared by least-square linear regression analysis (Fig. 3) with LDL measured by β-quantification (95% CI for slope, 0.91–0.97; Syx = 0.175). LDL-e was also well correlated with LDL-uc (r = 0.93) and with LDL-f (r = 0.95). As opposed to LDL-f (r = 0.67), the relationship between LDL-e and LDL-uc was not much altered when TG concentrations were >4 g/L (r = 0.91). Biases were plotted as a function of LDL-u or TG concentrations (Fig. 4). A nonsignificant positive intercept (40 mg/L) and correlation (r = 0.013) were observed for LDL-e, indicating a negligible change in bias for LDL-u over a wide range of LDL concentrations (0.34–5.07 g/L), whereas the negative intercept (−89 mg/L; P < 0.0015) was significant for LDL-f. LDL-e bias and percentage of bias vs LDL-uc did

<table>
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<tr>
<th>Storage</th>
<th>TGs, mg/L</th>
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<th>ΔLDLC, mg/L</th>
<th>ΔLDLC, %</th>
<th>ΔHDL, mg/L</th>
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<tbody>
<tr>
<td>4 °C for 3 days</td>
<td>All</td>
<td>84</td>
<td>11 ± 118</td>
<td>0.7 ± 9</td>
<td>−4 ± 82</td>
<td>4 ± 29</td>
</tr>
<tr>
<td></td>
<td>&gt;1.5</td>
<td>26</td>
<td>14 ± 137</td>
<td>0.12 ± 12</td>
<td>−7 ± 76</td>
<td>8 ± 36</td>
</tr>
<tr>
<td>−80 °C for 1 week</td>
<td>All</td>
<td>79</td>
<td>75 ± 151</td>
<td>5.0 ± 9</td>
<td>−10 ± 82</td>
<td>−1.1 ± 23</td>
</tr>
<tr>
<td></td>
<td>&gt;1.5</td>
<td>68</td>
<td>88 ± 153</td>
<td>5.5 ± 9</td>
<td>−5 ± 75</td>
<td>−1.8 ± 23</td>
</tr>
<tr>
<td>−20 °C for 12–36 weeks</td>
<td>&lt;1.5</td>
<td>55</td>
<td>94 ± 100</td>
<td>7.2 ± 9</td>
<td>−96 ± 95</td>
<td>−12.6 ± 18</td>
</tr>
<tr>
<td></td>
<td>&gt;1.5</td>
<td>126</td>
<td>228 ± 197</td>
<td>15.0 ± 13</td>
<td>−101 ± 85</td>
<td>−19.0 ± 16</td>
</tr>
</tbody>
</table>

<0.0001) for LDL-f. HDL-e remained basically unmodified vs HDL-p (−7.5% ± 19% vs −5.3% ± 18%; not significant). Negative errors for LDLC and HDLC were more pronounced with increasing concentrations of bilirubin in vitro; however, they were less pronounced than for calculated LDLC (−7.9% vs −20.6%; P = 0.0001). Bias was acceptable (<4%) for total bilirubin concentrations up to 67 µmol/L for LDLC, whereas a 10% bias or less was observed for HDLC up to 50 µmol/L bilirubin (3N). In a group of 28 subjects with frank jaundice (total bilirubin, 165.3 ± 154 µmol/L; conjugated bilirubin, 121 ± 117 µmol/L), abnormal patterns of lipoprotein migration and the biases of LDL-e vs LDL-u were significantly higher (11.7% ± 18%) than in noncholestatic subjects (3.01% ± 13%; P = 0.0004). Accordingly, biases between HDL-e and HDL-p were increased in cholestatic patients (−14.5% vs −5.3%; P < 0.0001). Increasing amounts of hemoglobin up to 3.5 g/L (203 µmol/L) had no significant effect on LDLC (0.62 – 0.67), whereas these lipoproteins floated most of the LDL fraction in electrophoresis, whereas these lipoproteins floated mainly with VLDL after sequential ultracentrifugation. A significant bias (10%) was observed for HDLC above hemoglobin concentrations of 3.5 g/L (frank hemolysis).
not vary significantly as a function of increasing TG concentrations up to 18.5 g/L (r = 0.08; not significant). In contrast, the negative error was more pronounced for calculation with increasing TG concentrations ranging up to 4 g/L. However, bias increased significantly when the VLDL/TG ratio increased both for LDL-e (r = 0.264; P < 0.0001) and for LDL-f (r = 0.368; P < 0.0001). Because a practical issue is to use NCEP LDLC cut-points to start or monitor lipid-lowering therapy, we investigated from regression equations how these cut-points defined by LDL-u (Table 4) could be predicted either by LDL-e (for all TG concentrations) or by LDL-f (when applicable). At all cut-points, LDL-e gave closer predictions than LDL-f because of lower biases. In addition, among subjects with TG concentrations >4 g/L who were excluded from assessment by calculation, approximately one-half (27 of 42) had LDLC >1.30 g/L, approximately one-third (15 of 42) had LDLC >1.60 g/L, and approximately one-fourth (10 of 42) had LDLC >1.90 g/L, as detected by electrophoresis.

Direct HDLC vs HDLC by ultracentrifugation or precipitation. HDLC assessed with electrophoresis was closer to that measured with sequential ultracentrifugation than with the ultracentrifugation/precipitation method (bias, −0.10% ± 26% for HDL-e vs HDL-uc compared with

![Fig. 2. Mean percentage of bias for LDL-e and LDL-f as a function of TG intervals.](image)
-2.31% ± 28% for HDL-e vs HDL-u), remaining within the NCEP-recommended goal of 5% for bias. However, total analytical errors of 11.5% vs HDL-uc and 13.7% vs HDL-u, respectively, were closer to the limits of the goal (<13%). HDL-e tended to behave similarly to HDL-p vs HDL-uc, although with a less pronounced tendency to overestimate HDLC (Table 5). Biases tended to be more pronounced with decreasing HDLC concentrations, exceeding NCEP goals at concentrations <0.35 g/L for HDL-e, whereas they were already beyond this goal at an HDLC concentration of 0.60 g/L for HDL-p. Although mean absolute biases were in the ranges of those observed with LDLC in g/L (mean = 0.10 ± 0.09 g/L), the
magnitude of absolute biases as percentages (−20.1% ± 16% for HDL-e vs HDL-uc; −19.5% ± 22% for HDL-p vs HDL-uc; −13.7% ± 12% for HDL-e vs HDL-p) underlined the variability between methods for HLDC measurement. Overall relationships appeared weaker than for LDLC. HDL-e was better correlated with HDL-p (95% CI for slope, 0.93–1.013; Syx = 0.101 g/L), as shown in Fig. 5, than it was with ultracentrifugation (95% CI for slope, 0.85–0.98; Syx = 0.132 g/L). In particular, several outliers between HDL-e and HDL-p were observed in subjects with high plasma TG concentrations, whereas discrepancies with HDL-uc were observed in subjects with high Lp(a). Bland-Altman plotting showed a trend to overestimate high concentrations and to underestimate low concentrations of HDLC both for HDL-e (slope, −0.086; P < 0.005) and for HDL-p (slope, −0.200; P < 0.0001). Underestimation of HDLC was more pronounced when the VLDL/TG ratio increased (r = 0.153; P < 0.0001), suggesting interferences with subtypes of TG-rich lipoproteins as well.

Concordance. Subjects classified according to low-, moderate-, or high-risk LDL-c concentrations are shown in Table 6. The proportion of observed agreement (concordance) was 0.79. The k index, weighing this percentage for the proportion of random agreement, was k = 0.66 (95% CI, 0.61–0.72). Concordance was 0.76 and k = 0.63 (95% CI, 0.57–0.69), respectively, for LDL-e. When subjects were classified according to LDL-c medical decision cut-points of 1.00, 1.30, 1.60, and 1.90 g/L, concordance and k were 0.68 and 0.58 for LDL-e vs 0.65 and 0.55 for LDL-f, suggesting overall better classification for LDL-e. Moreover, 87% of misclassified subjects with LDL-e were classified into the next category, so that 95.7% subjects Table 5. HDLC measured by electrophoresis (e), ultracentrifugation (uc), or precipitation (p), and biases between different methods.a

<table>
<thead>
<tr>
<th></th>
<th>All</th>
<th>HDLC &gt;0.60 g/L</th>
<th>0.35 g/L &lt; HDLC &lt; 0.60 g/L</th>
<th>HDLC &lt;0.35 g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>640</td>
<td>197</td>
<td>376</td>
<td>67</td>
</tr>
<tr>
<td>HDL-uc, g/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.54 ± 0.17</td>
<td>0.74 ± 0.13</td>
<td>0.47 ± 0.68</td>
<td>0.29 ± 0.40</td>
</tr>
<tr>
<td>HDL-e, g/L</td>
<td>0.53 ± 0.20</td>
<td>0.71 ± 0.20</td>
<td>0.47 ± 0.13</td>
<td>0.31 ± 0.10</td>
</tr>
<tr>
<td>HDL-p, g/L</td>
<td>0.56 ± 0.18b</td>
<td>0.72 ± 0.18</td>
<td>0.51 ± 0.12a</td>
<td>0.36 ± 0.12b</td>
</tr>
<tr>
<td>Bias e-uc, g/L</td>
<td>−0.01 ± 0.13</td>
<td>−0.02 ± 0.16</td>
<td>−0.00 ± 0.12</td>
<td>0.02 ± 0.10</td>
</tr>
<tr>
<td>Bias p-uc, g/L</td>
<td>0.03 ± 0.13c</td>
<td>−0.02 ± 0.15</td>
<td>0.04 ± 0.11c</td>
<td>0.08 ± 0.13c</td>
</tr>
<tr>
<td>Bias e-p, %</td>
<td>−0.10 ± 26.21</td>
<td>−2.93 ± 22.04</td>
<td>2.15 ± 26.18</td>
<td>6.41 ± 35.32</td>
</tr>
<tr>
<td>Bias p-p, %</td>
<td>7.15 ± 28.32d</td>
<td>−2.18 ± 20.80</td>
<td>8.38 ± 23.43d</td>
<td>27.69 ± 51.57a</td>
</tr>
<tr>
<td>Bias e-uc, g/L</td>
<td>0.10 ± 0.09</td>
<td>0.13 ± 0.09</td>
<td>0.09 ± 0.08</td>
<td>0.08 ± 0.06</td>
</tr>
<tr>
<td>Bias p-uc, g/L</td>
<td>0.10 ± 0.09</td>
<td>0.12 ± 0.09</td>
<td>0.09 ± 0.08</td>
<td>0.08 ± 0.12</td>
</tr>
</tbody>
</table>

a Data are given as mean ± SD.

b–e Not significant unless specified: b P < 0.0001 vs HDL-uc and HDL-e; c P = 0.0001 vs bias e-uc, g/L; d P < 0.0001 vs bias e-u, %; e P < 0.0004 vs bias e-u, %.

Fig. 5. Linear regression analysis for HDLC measured by electrophoresis (HDL-e) vs precipitation (HDL-p; left) or ultracentrifugation (HDL-uc; right).
Equations for the lines: (left), HDL-e = 0.012 + 0.97(HDL-p); r = 0.87; (right), HDL-e = 0.041 + 0.914(HDL-uc); r = 0.76.
were adequately classified. In keeping, the intraclass correlation coefficient ($r = 0.94$ ($P < 0.0001$) for LDL-e suggested that the majority of estimations with LDL-e were close to the equality line with LDL-u. At TG concentrations $1.5 \text{ g/L}$, concordance remained nearly unchanged for LDL-e ($0.78$; $k = 0.62$; $n = 165$), whereas it lowered for LDL-f ($0.71$; $k = 0.53$; $n = 136$). In addition, the proportion of underestimated measurements was 2.4-fold greater for calculated LDLC ($129$ of $411$, $31\%$) than for LDL-e ($58$ of $440$, $13\%$), and $5.8$-fold greater ($42.6\%$ vs $7.3\%$) in subjects with moderate TG concentrations. On the other hand, the proportion of overestimation was greater for LDL-e ($20\%$ vs $5\%$).

**Table 6. Classification by number and percentage of subjects with electrophoresis (LDL-e) or calculated (LDL-f) with Friedewald equation (TGs $< 4 \text{ g/L}$) for LDLC as a function of NCEP medical cut-points defined by $\beta$-quantification (LDL-u) in g/L.**

<table>
<thead>
<tr>
<th>LDL-u, g/L</th>
<th>LDL-e cut-points*</th>
<th>LDL-f cut-points*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$&lt; 1.30 \text{ g/L}$</td>
<td>$1.30-1.60 \text{ g/L}$</td>
</tr>
<tr>
<td>$&lt; 1.30$</td>
<td>n = 85; 19.4%  (10.9%)</td>
<td>n = 21; 4.8%  (5.5%)</td>
</tr>
<tr>
<td>$1.30-1.60$</td>
<td>n = 19; 4.3%  (3.0%)</td>
<td>n = 76; 17.4%  (18.2%)</td>
</tr>
<tr>
<td>$&gt; 1.60$</td>
<td>n = 4; 0.9%  (1.2%)</td>
<td>n = 17; 3.9%  (1.8%)</td>
</tr>
<tr>
<td>Totalb</td>
<td>108 (25)</td>
<td>114 (42)</td>
</tr>
</tbody>
</table>

* Percentage of subjects with TGs $> 1.5 \text{ g/L}$ in parentheses.

**Discussion**

Electrophoresis was one of the earliest methods to discern heterogeneity of human plasma lipoproteins (33), but it has been confined since then to the qualitative rather than the quantitative assessment of lipoproteins. This was because of the poor precision and specificity of manual methods based on nonspecific lipid staining. Automation and enzymatic colorimetry of cholesterol within gels might change this perception. These methods have been shown to be linear up to $4 \text{ g/L}$ in plasma cholesterol, with a detection limit, $0.05 \text{ g/L}$ per lipoprotein band, regardless of whether gels contain urea and divalent cations, as observed here. Agarose gels enriched in urea and divalent cations have been developed to better resolve all lipoprotein fractions, particularly Lp(a) from VLDL (16–19). However, on the REP System, the run times for these gels were twice as long ($40$ vs $20 \text{ min}$), and the analytical precision was weaker. Here, the focus was to quantify LDLC and HDLC more precisely. We observed similar within-run CVs; however, between-run reproducibility
was improved (CV_b = 2.0% vs 3.7%) for LDLC and (CV_b = 4.3% vs 12%) for HDLC. Moreover, although the decision cut-points were determined manually, the rule of the nadir kept between-operator CVs within recommended ranges, regardless of any overlap between TG-rich lipoproteins and LDL. Cholesterol was quantified by the cholesterol esterase/cholesterol oxidase enzymatic reaction, a method used routinely (as opposed to cholesterol dehydrogenase) in parallel for TC quantification (34). These modifications might have contributed to the overall greater analytical precision observed here as compared with reported total errors of 13.3% for LDLC and 27.3% for HDLC (18). However, as mentioned previously (19), precision tended to decline at low HDLC concentrations, ranges at which staining is at its lower limits of detection. The behavior of cholesterol-rich lipoproteins could be analyzed. Electrophoresis withstood interferences at moderate degrees of hemolysis, or in patients treated with low-molecular-weight heparin. LDLC and HDLC measurements were stable during short-term storage at 4 °C, and remained within the limits of biological variation after short-term freezing at −80 °C, offering interesting applications for shipment and clinical trials. However, freezing/thawing procedures may disrupt the structure of lipoprotein particles, particularly of TG-rich lipoproteins, whereas sucrose (a commonly used cryopreservative) interferes with electrophoretic migration, thereby rendering electrophoresis more sensitive to freezing compared with other methods. A classical drawback in nonspecifically stained gels is the overlap between TG-rich lipoproteins and LDL. Here, LDL could be resolved in most samples from TG-rich lipoproteins in hypertriglyceridemic sera. However, apoE phenotyping suggested that intermediate-density lipoproteins (β-VLDL), contributed to the overlap between LDL and VLDL/Lp(a) fractions. Because the E2 allele is defective in LDL-receptor binding, apoE2-containing lipoproteins are enriched in apoE and cholesterol, behaving like intermediate-density lipoproteins even in normolipidemic plasma (35). Therefore, the LDLC fraction measured here may include subpopulations of atherogenic lipoproteins, which are part of LDLC measured by reference and usual methods. Conversely, the electrophoretic LDL fraction did not include Lp(a). Although LDLC might then depart from reference parameters, the gain in accuracy and concordance (see below) largely compensated for this disadvantage. Moreover, the difference remains minor because Lp(a) represents on average a small proportion of LDLC (7%). Upcoming standardization will allow independent and direct quantification of plasma Lp(a) (36).

The most interesting results came from accuracy studies on LDLC by electrophoresis. As mentioned previously (16–20), the combination of automation and specific cholesterol staining provides estimations close to those obtained with the reference β-quantification method. Here, these characteristics were reproduced in a sample of 640 patients representative of a wide spectrum of lipid profiles encountered in clinical practice, including 204 diabetics, who will be reported elsewhere (37). In particular, we could observe a sustained accuracy over a wide range of plasma cholesterol (0.78–5.96 g/L) and TG (0.18–18.34 g/L) concentrations. Although ultracentrifugation and electrophoresis are very different methods in their physicochemical principles, they agreed remarkably for LDL separation. At all medical decision cut-points, even in the presence of increased plasma TGs, reliable estimations of LDL were obtained with a bias not exceeding 0.04 g/L. Because our calculations combined analytical errors of three independent methods, the bias was 2- to 3-fold higher. Considering that the majority of patients require a reduction in LDL-concentrations of 20–35% for appropriate monitoring around these cut-points (5–7), even a minor decrease in precision and accuracy may give rise to patient mismanagement. The analytical error of the Friedewald formula has been estimated to be 7% (38), which combined with a LDLC biological variability of 9.5% (39) requires iterative measurements. Therefore, the gain in precision and accuracy provided by the direct method makes LDL estimation more cost-effective and reliable for patient monitoring over the long term.

As opposed to electrophoresis, we observed that the accuracy for calculated LDL decreased significantly when TG concentrations increased, as underlined previously (11, 14). In addition, whereas most subjects misclassified with electrophoresis were by overestimation, these were underestimated in greater amplitude and proportions with calculation despite the constant recovery of cholesterol after ultracentrifugation. We observed that calculated LDL became excessively underestimated at concentrations as low as 1.5 g/L despite the fact that we controlled for directly measured VLDLC, as suggested (40). This underestimation was not unexpected because for constant TC and HDLC, a fixed ratio of TG/5 (in g/L) would lead to underestimation of LDLC as a function of increasing TG concentrations. Moreover, incomplete precipitation of apoB-rich lipoproteins may lead to overestimation of HDLC (9). Indeed, we observed this phenomenon. Therefore, although the Friedewald equation gave overall concordant estimations with the reference method, electrophoresis yielded more accurate results, particularly when plasma TG concentrations increased moderately. In addition, because it is not always feasible to send fresh serum samples with plasma TG concentrations >4 g/L to a reference laboratory, most patients with high plasma TGs may remain unexplored. We could detect a significant number of patients with LDLC in the high-risk categories by electrophoresis or ultracentrifugation, who would have otherwise remained unrecognized.

Overall, electrophoretic assessment of LDL appeared reliable for 96% of patients regardless of their lipoprotein profile. This is of particular importance in practice because the primary objective in cardiovascular prevention is to lower excessive LDLC. This goal may be achieved when plasma TG concentrations are normal. However,
underestimation and unpredictable increases in the variability of calculations might limit risk assessment of moderately hypertriglyceridemic patients. This may have clinical and public-health consequences because hypertriglyceridemia has been identified as an individual risk factor of cardiovascular disease (41, 42). TG concentrations may reflect accumulation of atherogenic lipoproteins in plasma (43), particularly when they cluster with increased LDLC (2, 3) or decreased HDLC (21, 22). Moreover, despite a consensus threshold of 2 mmol/L for plasma TGs (6, 7), the risk of cardiovascular events seems to be partitioned by a TG threshold of 1.5 g/L (22), if not 1.0 g/L (44). Thus direct assessment of HDLC may limit the number of otherwise undertreated high-risk patients with moderate to high TGs.

Because indirect assessment of LDLC by calculation is still widely used and recommended, stress has recently been put on precision and accuracy goals for direct HDLC assessment (12, 13). In agreement with previous reports, the analytical performance of electrophoresis for determining HDLC was weaker (18, 19), mainly resulting from decreased precision at low cholesterol concentrations. Overall, HDLC by electrophoresis yielded results similar to precipitation vs ultracentrifugation, except for a less pronounced overestimation of low HDLC. This again would be more desirable because high-risk patients are in the low HDLC ranges. However, ways to implement the detection of low cholesterol concentrations are needed to provide broader clinical applications to the method. The LDLC/HDLC ratio is a high predictor of cardiovascular risk (2, 4). In the PROCAM study, it predicted premature coronary events with a risk ratio of 6.1 (3), whereas in the Physician’s Health Study, each increase by 1 unit of the LDLC/HDLC ratio increased risk of myocardial infarction by 53% (45). Here, despite performances comparable to the calculation/precipitation combination, electrophoresis no longer underestimated the LDLC/HDLC ratio, improving sensitivity to detect 45% more high-risk patients.

Liquid-phase and chemical methods have been developed for the direct assessment of HDLC or LDLC (14, 15, 26, 46). These methods appear attractive for their simplicity and their potential high throughput and analytical performance because of full automation. However, some may be sensitive to matrix effects (47), to between-batch reagent instability, to TG-rich lipoproteins (48), or to specific clinical situations (49, 50), whereas interferences in agarose gel electrophoresis are more predictable, based on long-standing experience. As opposed to electrophoresis, which is low in cost of reagents (~$1 US), chemical methods may require expensive reagents (48–50) and time doubling to obtain results for both markers. Despite an apparent longer time for processing, 100 samples may be run routinely per day. An additional gain in throughput may be obtained by independent gel processing and reading.

In conclusion, provided that the within-gel detection of low concentrations of cholesterol is implemented for HDLC assessment, quantitative electrophoresis appears to be a simple, reliable, predictable method low in reagent costs for the direct assessment of LDLC over a wide spectrum of patients at risk of cardiovascular disease.

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