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Current recommendations of the Adult Treatment Panel and the Children and Adolescents Treatment Panel of the National Cholesterol Education Program make the concentration of low-density lipoproteins cholesterol (LDL-C) in serum the basis for the classification and treatment of hypercholesterolemia. Numerous methodologies for the determination of serum LDL-C concentrations, in research and clinical laboratories, have been described. Here, we review the principles, performance, and limitations of major current methodologies for determining LDL-C concentrations. These methods include sequential and density-gradient ultracentrifugation, chromatographic and electrophoretic techniques, and precipitation methods. In addition, the advantages and disadvantages of estimating LDL-C concentration by the Friedewald equation, the most commonly used approach in clinical laboratories, are addressed.

Additional Keyphrases: heart disease · risk factor · apolipoproteins

Epidemiological and clinical studies have demonstrated a strong positive correlation between low-density lipoproteins cholesterol (LDL-C) concentrations in serum and the incidence of coronary heart disease (CHD) (1, 2).6 Furthermore, pathological studies have shown that increased LDL-C concentrations correlate highly with the extent of atherosclerotic lesions (3, 4). Decreasing the LDL-C concentration ameliorates the symptoms of CHD and can actually cause regression in the lesions (5).

The LDL class of lipoproteins is a heterogeneous population of spherical particles, with hydrophobic oily cores consisting of cholesteryl ester and triglyceride. These particles are coated with a native surfactant of phospholipids, free cholesterol, and apolipoproteins (6). On average, two-thirds of the total cholesterol in serum is carried by LDL. Each LDL particle contains one molecule of apolipoprotein B-100 (apo B-100), the main protein component of LDL. Other apolipoproteins such as apo E and apo CII are also present on LDL but in minute amounts.

By definition, LDL comprise the population of particles with hydrated density between 1.006 and 1.063 kg/L (7). This definition, referring to LDL separated by sequential density ultracentrifugation, or the so-called beta quantification method combining ultracentrifugation and chemical precipitation, has been the basis for measurement in most epidemiological studies. This wide-density LDL population is heterogeneous, including remnant particles of intermediate-density lipoproteins (IDL, 1.006–1.019 kg/L) and lipoprotein(a) [Lp(a), 1.050–1.080 kg/L]. The remaining LDL particles can be classified as light or heavy, the latter being considered more atherogenic (8). In practice, because all of the particles are atherogenic, the wide-density population of LDL (1.006–1.063 kg/L) is usually reported.

Production of LDL is the final stage of endogenous lipoprotein metabolism. Removal of these particles from circulation is accomplished by specific hepatic and extrahepatic receptors as well as by a nonspecific mechanism (9). A defect in the removal process of LDL or overproduction of very-low-density lipoproteins (VLDL), precursors of LDL, can result in increased LDL-C concentration in serum. The nonspecific non-receptor-mediated mechanism for removal is not saturable. Therefore, as the LDL concentration increases, more LDL are taken up from serum by this route. Ordinarily, an estimated two-thirds of LDL are degraded by the high-affinity receptor pathway, with the remainder being removed by the nonspecific nonreceptor pathway (6). Macrophages and other scavenger cells remove modified LDL and, as a result of excess accumulation of cholesteryl esters, these cells are converted to “foam cells,” considered the earliest components of the atherosclerotic plaque.

Because of its strong association with CHD, both the Adult Treatment Panel and the Children and Adolescents Treatment Panel of the National Cholesterol Ed-
ducation Program (NCEP) have made LDL-C the basis for the classification and treatment of hypercholesterolemia (10, 11). The LDL-C values used for the classification of children, adolescents, and adults for CHD risk are listed in Table 1. Several technologies have been described for determining the concentration of LDL-C in serum in research and clinical laboratories. Here we review the most important currently available methodologies for determining the cholesterol component of LDL: sequential and density-gradient ultracentrifugation, chromatographic and electrophoretic techniques, and precipitation methods. The advantages and disadvantages of estimating LDL-C concentration by using the Friedewald equation are also discussed.

Sequential and Density-Gradient Ultracentrifugation

Separation of the different classes of plasma lipoproteins has long been an important procedure for their study. Lipoproteins vary greatly in size, density, relative composition, and biological function. However, they share many compositional components, which make them difficult to evaluate or quantify without prior isolation. Various types of ultracentrifugation, taking advantage of the lipoproteins' differences in hydrated density, have been the primary means of making this separation.

The use of sequential differential ultracentrifugation to separate lipoprotein classes was first reported in the 1950s by De Lalla and Gofman (12) and by Havel et al. (7). Sequential ultracentrifugation provides a less complicated and relatively less expensive method than analytical ultracentrifugation, involving one or more successive ultracentrifugation steps, depending on the desired lipoprotein class separation (13). In routine analyses, generally only a single ultracentrifugation step is performed, separating the plasma at its own density of 1.006 kg/L. The VLDL and chylomicrons float to the top after ultracentrifuging at 109,000 × g (39,000 rpm in a Beckman 40.3 or 50.3 Ti rotor; Beckman Instruments, Palo Alto, CA) for 18 h; the IDL, LDL, and high-density lipoproteins (HDL) remain in the infranate. The supernatant and infranatant fractions are generally separated by a tube-slicing technique. The cholesterol concentrations of plasma, the 1.006 kg/L infranate, and the HDL supernate (obtained by chemical precipitation) are measured. VLDL cholesterol (VLDL-C) is calculated as plasma cholesterol minus infranatant cholesterol; LDL-C is calculated as infranatant cholesterol minus HDL cholesterol (HDL-C)

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VLDL-C = \text{total cholesterol} - \text{infranate cholesterol;}
\]

LDL-C = infranate cholesterol - HDL-C. This single-centrifugation step procedure, combined with Abell-Kendall cholesterol analysis and heparin-manganese precipitation of the 1.006 kg/L infranate, is called the beta-quantification method and has been adopted for use by the Lipid Research Clinics (14), other lipoprotein research laboratories, and some clinical laboratories, and has been accepted as the Reference Method for LDL-C quantification. Additional ultracentrifugation steps can be performed, after adjustments to increase the density of the 1.006 kg/L infranate to individually separate additional density classes (12, 13, 15). Density adjustment to 1.019 kg/L is used to float IDL; adjustment to 1.063 kg/L will float LDL; and adjustment to 1.21 kg/L isolates HDL. Further subfractionation can be accomplished with additional density adjustments. Ultracentrifugation is generally performed with fixed-angle rotors, the size of the rotors and tubes depending on the fraction volumes required. However, the density cut-points separate lipoproteins solely by their hydrated density and not specifically by their lipoprotein or apolipoprotein components. In addition, the particles, particularly HDL, have the potential for being disrupted during centrifugation (16). Some apolipoproteins, especially apolipoprotein A-I, appear to be easily removed from the particles and may constitute a portion of the quantity that can be found in the lipoprotein-free, 1.21 kg/L infranate after removal of all lipoproteins. Some of the apo A-I found in the infranate may occur naturally as unbound protein or nascent HDL.

Another method for lipoprotein separation involves discontinuous density-gradient ultracentrifugation (17-25). With this ultracentrifugation technique, different density solutions are carefully layered into each tube along with the sample. After ultracentrifugation to equilibrium, each of the lipoproteins will have migrated into its respective isopycnic density region. Nonequilibrium methods that separate by flotation rate have also been described. These approaches have the advantage of separating all lipoprotein classes in a single centrifugation step; the drawbacks include the time involved in layering the gradients, collecting the fractions after centrifugation, analyzing each of the (usually dilute) fractions, and performing the mathematical curve resolution procedures to correct for incomplete separation. The incomplete separation may introduce a certain amount of variability to the results; the dilution problem, however, can be overcome with the use of very sensitive assays (25).

The density-gradient ultracentrifugation methods, in turn, can be subdivided by rotor type: swinging bucket, vertical, and fixed angle. The swinging-bucket procedure provides excellent separation, but requires relatively long centrifugation times (17-66 h) (17, 18, 20, 21, 23, 25). The use of vertical rotors can shorten the

| Table 1. Serum Low-Density-Lipoproteins Cholesterol Concentrations Used to Classify Children, Adolescents, and Adults for Risk of Heart Disease | LDL-C, mg/L (mmol/L) |
|---|---|---|---|
| Class/Classification | Children & adolescents* | Adults |
| Desirable | <1100 (2.85) | <1300 (3.37) |
| Borderline high risk | 1100-1299 | 1300-1590 |
| High risk | (2.85-3.34) | (3.37-4.12) |
| | >1300 (3.37) | >1600 (4.14) |

* Children older than two years, and adolescents from families with hypercholesterolemia or premature cardiovascular disease.
section time considerably (45 min–3 h), owing to the decreased migration distance, with generally only a slight loss in separation characteristics (22, 24, 26). Fixed-angle rotors, primarily used for sequential ultracentrifugation, can also be used for density-gradient ultracentrifugation, at a centrifugation time only slightly longer than that for vertical rotors (25, 27).

For the diagnostic purposes of measuring LDL-C concentrations to assess lipoprotein abnormalities and CHD risk, both sequential and density-gradient ultracentrifugation methods are costly, labor intensive, and—except for the single-centrifugation step in the sequential procedure—may be more labor intensive than is justified for normal clinical assessment. Both require expensive ultracentrifuges, rotors, and tubes, and both can be performed on only a few samples per day. The density-gradient procedures have the advantage of shorter centrifugation times and smaller sample volumes; the sequential method has the advantage of requiring much less time for pre-centrifugation setup and post-centrifugation fraction collecting. Neither is ideal for handling the large number of samples currently being referred to clinical laboratories for lipoprotein testing.

Other modifications have been proposed to alleviate some of the drawbacks of conventional ultracentrifugation, e.g., the use of table-top, air-driven ultracentrifuges (Beckman Airfuge) (28–31). However, disadvantages of the Airfuge include (a) an increased potential for incomplete separation of VLDL and LDL (31), (b) the remixing of particles during deceleration (30), (c) the difficulty of aspirating fractions without tube slicing, and (d) the decreased precision because of the small sample volume (32).

The newer generation of table-top ultracentrifuges may offer advantages over conventional floor models. Small quantities of sample can be used, the centrifugation time is much less than for normal ultracentrifugation and similar to that for the Airfuge, and the lipoprotein separation may be better than with the air-driven units (33, 34). In addition, the costs of this equipment, although not trivial, are significantly less than those of the floor models. On the other hand, capacities are less than in conventional ultracentrifuges.

All of the ultracentrifugation procedures will have limited use in clinical laboratories. Regardless of the approach used, centrifugation will probably remain a specialty procedure. With the NCEP emphasis on using LDL-C concentrations as a basis for classification and monitoring treatment of dyslipidemias, an important future priority will be to develop methods for reliably determining LDL-C concentration in large numbers of samples without the need for ultracentrifugation. At present, in laboratories where the ultracentrifugation technique is not available, LDL-C can be best estimated by the method of Friedewald et al. (35), provided that the patient’s triglyceride concentration is <4000 mg/L (<4.58 mmol/L) (36, 37). This method is discussed in detail later. Specimens with triglyceride concentrations >4000 mg/L should continue to be referred to laboratories with ultracentrifugation capabilities for accurate LDL-C determination until such time that reliable methods for measuring LDL-C, other than ultracentrifugation, become available.

Chromatographic and Electrophoretic Methodologies

Several chromatographic and electrophoretic techniques for the isolation of LDL have been described. Because of the great differences in size between the various lipoproteins, gel-filtration chromatography can be used to separate these complexes. Numerous chromatographic systems and columns have been used to separate the lipoprotein classes (38–43). Lipoprotein-containing fractions have been identified by measuring absorbance at 280 nm, and their cholesterol content has been subsequently determined. The continuous monitoring of the relative distribution by size permits evaluation of heterogeneity of the samples.

Agarose-column chromatography (38, 39) and Toyoda Soda high-pressure gel-filtration column chromatography (40, 42) have commonly been used. These procedures are gentle and nondestructive, being simultaneously preparative and analytical and capable of providing an adequate recovery of lipoproteins after separation. The recovery of LDL-C from agarose columns reportedly ranges between 80% and 98% (44). The major drawbacks of agarose column chromatography are the length of the procedure (24 h) and the inability to obtain homogeneous lipoprotein fractions (42, 45, 46). Multiple columns in series, each containing gels with various exclusion limits, could be used to more completely separate the lipoproteins. That will further lengthen the procedure, however, and make it more cumbersome.

The development of methodologies based on high-pressure liquid chromatography has alleviated some of these problems. High-pressure gel-filtration chromatography provides a relatively rapid isolation of lipoproteins, and the individual lipoproteins obtained have been shown to be intact (based on chemical composition) and relatively free of contamination from other lipoproteins, on the basis of electrophoretic mobility, apolipoprotein patterns, and immunological criteria (42). The isolation of apo B-100–containing lipoproteins by immunosaffinity chromatography was recently reported (47). The LDL fractions can be further separated from the other apo B-100–containing particles by either gel-filtration chromatography or ultracentrifugation.

The purification and characterization of bovine lipoproteins by heparin–Sepharose chromatography has been described (41). Heparin–Sepharose has a high affinity for apo B-100- and apo E-containing lipoproteins. Chylomicrons and VLDL are initially separated from LDL and HDL by gel-filtration chromatography. Because bovine HDL do not contain apo E, LDL fractions can then be separated from HDL by heparin–Sepharose. The lipoproteins of several species have already been characterized by heparin–Sepharose chromatography (41). The chromatographic methods are
powerful tools in the purification, isolation, characterization, and quantification of lipoprotein classes. However, the use of these techniques has been restricted to research laboratories because of the complexity of the procedures and instruments and the length of analysis time.

Unlike chromatography, electrophoresis, which separates lipoproteins according to their charge and size, has been popular in clinical laboratories. Because electrophoresis allows the visual examination of lipoprotein patterns, it was initially used for qualitative analysis, primarily in identifying lipoprotein phenotypes (48). Currently, lipoprotein electrophoresis of whole serum for the qualitative evaluation of the lipoprotein patterns is not recommended. The Lipid Research Clinics Program used electrophoresis of the lipoprotein fractions obtained after ultracentrifugation at density 1.006 kg/L (51) to detect variant lipoproteins: beta-VLDL, associated with Type III hyperlipoproteinemia (49), and sinking pre-beta, corresponding to the Lp(a) lipoprotein (50).

Electrophoretic detection of the beta-migrating VLDL, characteristic of Type III hyperlipoproteinemia, is still common in lipid specialty laboratories. Use of electrophoresis for assessment of Lp(a) is less common. The sensitivity of some systems may be marginal to detect the low Lp(a) concentrations.

Electrophoretic methods involve various support media, including paper (52), cellulose acetate (53), agarose (54-55), and polyacrylamide (59). Lipoproteins have been stained with lipophilic dyes such as Sudan black (59) and with enzymic (53, 55) or chemical precipitation (60) reagents on the gels. With recognition of the differential role of the lipoproteins in assessing risk of CHD, electrophoretic methods have been adapted for quantitative analysis, especially for HDL-C. Lipoproteins are quantified on the basis of the relative densities of their stained electrophoretic bands determined by scanning densitometry, with the measured cholesterol being allocated to the relative proportions.

Studies suggest that many of the electrophoretic methods do not achieve acceptable precision (60). In addition, proficiency surveys (College of American Pathologists Comprehensive Proficiency Surveys) demonstrate substantial inaccuracy for the electrophoretic methods in common use in routine laboratories. Serious limitations to electrophoretic quantification have curtailed its use by routine laboratories. For example, electrophoretic separations do not correspond exactly to fractions obtained by ultracentrifugation or chemical precipitation. The Lp(a) lipoproteins fractionate with LDL by chemical precipitation, and with LDL and HDL by ultracentrifugation, but have pre-beta mobility, primarily corresponding to VLDL, by electrophoresis (50). The beta-VLDL fraction, as the name implies, has beta mobility on agarose gel but separates with VLDL by ultracentrifugation (49). Lipophilic dyes stain primarily the ester bonds of triglycerides and esterified cholesterol; free cholesterol and phospholipids do not bind the dyes. Patients with biliary cirrhosis could have a normal appearing electrophoretic pattern despite substantially increased free cholesterol values >10 000 mg/L (>25.9 mmol/L) (61). Lipoprotein electrophoresis should therefore be coupled with a quantitative method, such as determination of total cholesterol.

Exponential gradient acrylamide/agarose gel electrophoresis, in conjunction with immunoelectroblotting with anti-apo B-100 and anti-Lp(a) antisera, provides better resolution in the separation of lipoprotein classes than does agarose gel alone (62). This procedure has not been used in clinical laboratories because it is very complicated and time consuming. Newer automated electrophoretic methods have demonstrated improved precision and hold promise for routine electrophoretic quantification (unpublished observation, G.R.W.).

In summary, electrophoresis of unfraccionated serum to assign a lipoprotein phenotype is not recommended. Electrophoresis of isolated lipoprotein fractions to detect variant lipoproteins is valuable but for practical considerations is limited to specialty laboratories. Quantitative electrophoretic methods may be useful, especially for HDL-C, but must be validated for precision and accuracy.

Precipitation Methods

Three methods for selective chemical precipitation of LDL have been introduced. The principles, outlines, and performance of each method are presented below.

Heparin Precipitation

LDL are precipitated by heparin at pH 5.12, achieved by including sodium citrate buffer. LDL-C is determined by subtracting the cholesterol measured in the supernate from the total cholesterol measured. The specificity of the method depends on the absence of divalent cations (which cause coprecipitation of VLDL) and exact control of pH. The pH must be adjusted to a value at which LDL particles are the only positively charged lipoprotein species. The isoelectric points of VLDL and LDL are about 4.7 and 5.4, respectively. A pH of 5.12 ± 0.02 gives optimal recovery of LDL in the precipitate without coprecipitation of VLDL (64). Reagents for this procedure are commercially available as a kit (Merck Co., Rahway, NJ, or Genzyme, Cambridge, MA).

An evaluation by Wieland and Seidel (63) revealed that LDL are completely precipitated, and that HDL and VLDL do not coprecipitate, even in the presence of large amounts of LDL. The LDL-C values obtained by heparin precipitation agreed well with those determined by an established procedure (precipitation of LDL by heparin-MgCl2 treatment of the density fraction >1.006 kg/L). There was also satisfactory agreement with LDL-C results obtained by quantitative lipoprotein electrophoresis, but the correlation deteriorated somewhat when samples with triglyceride concentrations >4000 mg/L (>4.58 mmol/L) were analyzed. Preliminary results suggested that the VLDL remnants of Type III hyperlipoproteinemia were not coprecipitated with LDL, but later investigators found that VLDL remnants completely coprecipitated (64). Lp(a) lipoprotein coprecipitate with LDL.
Method was good, with a coefficient of variation (CV) of 3.5% at an LDL-C mean concentration of 1460 mg/L (3.83 mmol/L), measured over a period of six months. Other investigators, using various comparison methods, tested the effect of serum triglyceride concentration on the performance of the heparin method. There was generally good agreement with other methods at low serum triglyceride values, but discrepancies were noted at higher triglyceride concentrations, as itemized below:

**Comparison method: Ultracentrifugation at 1.006 kg/L, combined with phophotungstate-MgCl₂ treatment of whole serum (65).** When triglyceride concentrations were >2000 mg/L (>2.28 mmol/L) but <9540 mg/L (<10.9 mmol/L), the heparin method gave falsely low values: \( y \) (precipitation) = 0.75x (comparison method) + 1.2 mg/L (0.003 mmol/L), \( r = 0.69 \).

**Comparison method:** Density-gradient ultracentrifugation, with LDL defined as the lipoproteins isolated in the density range 1.019–1.063 kg/L (66). The heparin method showed a significant positive bias when total triglyceride values were >1750 mg/L (2.0 mmol/L).

**Comparison method:** Ultracentrifugation at 1.006 kg/L, plus treatment of whole serum with polyethylene glycol 6000 (PEG 6000) to precipitate LDL and VLDL (64). The heparin method agreed well for samples with triglyceride concentrations <7000 mg/L (8.0 mmol/L); \( y = 0.95x + 97 \) mg/L (0.28 mmol/L), \( r = 0.96 \).

Polyvinyl Sulfate (PVS) Precipitation

LDL are precipitated from serum by PVS in the presence of EDTA and polyethylene glycol methyl ether. The LDL-C concentration is calculated as the difference between total cholesterol and cholesterol in the PVS supernate, or is measured directly after dissolving the precipitate (70). Precipitation of LDL by PVS is believed to be based on nonionic interactions, and has been shown to be independent of pH (67). Consequently, precise pH adjustment is not necessary. EDTA must be present to complex divalent cations, which favor precipitation of VLDL. PEG methyl ether acts as an "accelerator" of precipitation and promotes formation of a compact pellet in a reasonable time period. Reagents for this procedure are available commercially as a kit (Boehringer-Mannheim Diagnostics, Indianapolis, IN).

Assmann et al. (69) evaluated the PVS precipitation method and determined that HDL, VLDL, and LpX do not coprecipitate with LDL, but that Lp(a) completely coprecipitates. LDL-C results by PVS precipitation agreed well with those of a comparison method (a combination of ultracentrifugation at 1.006 kg/L and HDL-C determination in whole serum) for sera with triglyceride values <3680 mg/L (<4.2 mmol/L). In a separate experiment, specimens with triglyceride >4070 mg/L (>4.6 mmol/L) gave falsely low results by PVS precipitation relative to the comparison method, and the magnitude of the error increased with increasing triglyceride concentration. Between-run precision was good, with a CV of 2.4%. All evaluators of the PVS method reported good agreement with comparison methods when triglyceride values were low. High triglyceride concentrations produced variable effects on the PVS results, depending on the method used for comparison:

**Comparison method:** Ultracentrifugation at 1.006 kg/L, plus phophotungstate–MgCl₂ treatment of the density fraction >1.006 kg/L (67). For triglyceride concentrations >3940 mg/L (>4.5 mmol/L), Kersher et al. (67) state that the PVS method sometimes gave falsely low results, with the frequency of low results increasing with increasing triglyceride concentration.

**Comparison method:** Density-gradient ultracentrifugation, with LDL defined as the lipoproteins isolated in the 1.019–1.063 kg/L density fraction (66). For triglyceride concentrations >1750 mg/L (>2.0 mmol/L), the PVS method showed a significant positive bias.

**Comparison method:** Ultracentrifugation at 1.006 kg/L (with direct determination of VLDL in the <1.006 kg/L density fraction), plus PEG 6000 treatment of whole serum (64). For triglyceride concentrations <7000 mg/L (<8.0 mmol/L), agreement was satisfactory: \( y = 1.03x - 54 \) mg/L (0.14 mmol/L), \( r = 0.96 \).

**Bio-Merieux Kit Method**

LDL are precipitated from serum by unspecified amphiphatic polymers in imidazole buffer at pH 6.10 (Bio-Merieux, Charbonnieres les Bains, France). The precipitate is redissolved and its cholesterol content is measured directly or LDL-C is calculated as the difference between total cholesterol and the cholesterol concentration in the supernate.

This method was evaluated by Moss et al. (71), who reported a good correlation with ultracentrifugation and electrophoresis, but the effects of serum triglycerides, VLDL remnants, or other lipoproteins were not presented. Mainard and Madec (72) found that the components precipitated from serum by the bio-Merieux procedure contained different relative concentrations of cholesterol, phospholipid, and apo B-100 than did LDL isolated by sequential ultracentrifugation of serum, and concluded that the precipitated components were not truly LDL particles. Shaikh and Miller (73) reported that HDL do not coprecipitate, but that VLDL coprecipitate to some extent, even in normotriglyceremic sera.

In general, results of the bio-Merieux method correlate well with those of other methods when triglyceride concentrations are low. Effects of high triglyceride concentrations are summarized as follows:

**Comparison method:** Sequential ultracentrifugation, with LDL defined as the lipoproteins isolated in the 1.006–1.063 kg/L density fraction (73). For triglyceride concentrations <1750 mg/L (<2.0 mmol/L), agreement was satisfactory \( y = 0.99x + 50 \) mg/L (0.13 mmol/L), \( r = 0.94 \), although slight precipitation of VLDL was noted. For triglyceride values from 1750 to 10 680 mg/L (2.0–12.2 mmol/L) without increased chylomicrons, VLDL coprecipitated significantly, producing a large positive bias. For triglyceride values of 16.45–38.68 g/L (18.8–44.2 mmol/L) associated with hyperchylomicronemia and deficient lipoprotein lipase activity, results
were again high relative to the comparison method, but the discrepancy was not as severe as when all of the excess triglyceride was contributed by VLDL. Apparently, chylomicrons are less precipitable by the bio-Merieux reagents than are VLDL particles.

Comparison method: Density-gradient ultracentrifugation, with LDL defined as the lipoproteins isolated in the 1.019–1.063 kg/L density fraction (66). For triglyceride concentrations >1750 mg/L (>2.0 mmol/L), a large positive bias was observed.

Comparison method: Ultracentrifugation at 1.006 kg/L, plus PEG 6000 treatment of serum (64). For triglyceride <7000 mg/L (<8.0 mmol/L), agreement was considered good: y = 0.98x + 31 mg/L (0.08 mmol/L), r = 0.96.

Comparison method: Ultracentrifugation at 1.006 kg/L, plus phosphotungstate–MgCl₂ treatment of serum (65). For triglyceride concentrations ranging from 2000 to 9540 mg/L (2.28–10.9 mmol/L), the correlation was good but there was a bias: y = 0.9x + 181 mg/L (0.47 mmol/L), r = 0.94.

Comparison with centrifugation methods and the Friedewald calculation: Two studies included comparisons of all three precipitation methods (64, 66), not only with a centrifugation method but also with the Friedewald calculation (discussed in detail below).

In comparison with density-gradient ultracentrifugation (66), all three precipitation methods agreed well with either the ultracentrifugation method or the Friedewald formula, when serum triglyceride concentrations were <1750 mg/L (<2.0 mmol/L). However, when serum triglyceride concentrations exceeded that figure, the precipitation methods showed a significant positive bias, and the Friedewald formula correlated better with the ultracentrifugation method than did any of the precipitation methods.

Comparing the precipitation methods with a combination of ultracentrifugation at 1.006 kg/L and PEG 6000 treatment of serum (64) showed that, for sera with triglyceride concentrations <7000 mg/L (<8.0 mmol/L), the Friedewald formula agreed better with the ultracentrifugation method than did any of the precipitation methods: y = 1.02x – 38.6 mg/L (0.10 mmol/L), r = 0.98. For Type III sera, all of the precipitation methods and the Friedewald method showed large positive biases. For Type V sera, none of the precipitation methods or the Friedewald formula consistently agreed with the centrifugation method.

Comments

The task of reviewing published evaluations of LDL precipitation procedures is difficult because there is no universally accepted reference method for LDL-C measurement. Most investigators find that the precipitation methods are plagued with systematic errors when samples with high triglyceride values are analyzed. However, whether the bias observed is positive or negative depends on the method chosen for comparison. A variety of ultracentrifugation–precipitation combination methods have been used as comparison methods. The various precipitating agents do not all give exactly the same HDL-C values because they leave slightly different populations of HDL particles in solution. This in turn will result in different LDL-C values produced by the different methods. Nevertheless, LDL precipitation methods generally agree well or give low results compared with ultracentrifugation–precipitation combination methods, whereas precipitation methods generally give high LDL results compared with ultracentrifugation alone. The most convincing evidence that precipitation methods overestimate true LDL-C values when triglyceride concentrations are high comes from the finding that the bio-Merieux reagent precipitates not only LDL but also VLDL (73).

The three precipitation methods can be precise, and they produce LDL-C results that are accurate, relative to the Friedewald calculation (74) and to any ultracentrifugation method, when triglyceride values are low. However, because these precipitation methods exhibit the same shortcomings as does the Friedewald formula, they have been judged to be superfluous for laboratories that have accurate and precise assays for total cholesterol, triglycerides, and HDL-C (64). The precipitation methods give inaccurate results when serum triglyceride values are high (indeed, they seem to be worse than the Friedewald calculation in that regard) and they give falsely high results in the presence of VLDL remnants. The precipitation methods, like most routine methods, do not distinguish between Lp(a) and LDL. Some caution would be required in estimating cardiovascular risk from the current epidemiological database when LDL-C measurements do not include other atherogenic particles that are normally included in that database. Although some investigators advocate including Lp(a) cholesterol with LDL-C, there is evidence that the two lipoproteins do have different prognostic significance and it may be beneficial to quantify and report them separately (75).

Considering the importance of LDL-C with respect to the current clinical guidelines and the well-documented deficiencies of the common Friedewald formula (76, 77), methods for direct measurement of LDL-C would certainly be preferred. However, the chemical precipitation methods introduced to date do not appear to have substantial advantage over estimation, other than the fact that LDL-C can be obtained from two measurements rather than three. Specific immunoprecipitation methods, in which LDL particles may be separated not on the basis of buoyant density but rather on the basis of their characteristic apolipoprotein composition, offer promise for the future (67, 78).

Estimation of LDL-C by the Friedewald Equation

In 1972, William Friedewald, Robert Levy, and Donald Fredrickson published a landmark paper (35) for estimating the concentration of LDL-C in plasma without use of the preparative ultracentrifuge. This paper included a formula for estimating LDL-C that has been popularly called the Friedewald formula. LDL-C concentrations in fasted serum or plasma are estimated
from three measurements: total cholesterol, HDL-C, and total triglycerides. LDL-C is estimated from the Friedewald formula as follows:

$$\text{LDL-C} = \text{total cholesterol} - (\text{HDL-C} + \text{VLDL-C})$$

where VLDL-C = triglyceride/5, or triglyceride $\times 0.20$ (for mg/dL or mg/L LDL-C values), or

$$\text{VLDL-C} = \text{triglyceride}/2.22, \text{or triglyceride} \times 0.45 \quad \text{(for mmol/L LDL-C values)}$$

Comparison of estimated LDL-C values from this formula with LDL-C values determined with the ultracentrifugation method yielded correlation coefficients of 0.94 to 0.99, depending on the patient population compared (35). Accuracy of this formula depends on the observation that serum triglyceride concentrations are highly correlated with VLDL-C concentrations.

An important limitation of the Friedewald equation is the requirement to estimate LDL-C in fasting serum (79). The need for a fasting sample arises from transient changes in lipoprotein cholesterol concentrations that can occur as a consequence of compositional changes that accompany the metabolism of chylomicrons. Chylomicronemia will result in an overestimation of VLDL-C and an underestimation of LDL-C (79, 80). Chylomicrons can be easily detected in serum as a white, creamy layer of floating fat on top of serum stored in the refrigerator overnight. Similarly, the Friedewald equation tends to become less reliable for estimating LDL-C when plasma triglyceride concentrations increase, because the ratio of VLDL-C to serum triglyceride gradually changes as serum triglyceride concentrations increase (80). Thus the error in estimated LDL-C gradually increases as fasting serum triglyceride concentrations increase (35, 36, 77, 80–82). In some studies, the Friedewald equation tended to overestimate VLDL-C and underestimate LDL-C at higher triglyceride concentrations (80, 82); in other studies, there tended to be a similar amount of positive and negative errors at higher triglyceride values (36, 37). The apparent bias of the Friedewald equation may differ according to the completeness of recovery of the ultracentrifugal fractions—recoveries that potentially could differ in samples with low and high triglycerides. Because of increased errors in LDL-C estimation at high triglyceride concentrations, most investigators do not recommend using the Friedewald equation when triglyceride concentrations exceed 4000 mg/L (4.52 mmol/L). McNamara et al. (36), using data from dyslipidemic patients and adults from Cycle 3 of the offspring follow-up of the Framingham Heart Study, found that in 84% to 86% of the subjects, estimated LDL-C values were within 10% of the ultracentrifugally measured LDL-C when plasma triglyceride concentrations were $\leq 2000$ mg/L ($\leq 2.26$ mmol/L). When plasma triglyceride concentrations were between 2010 and 3000 mg/L (2.27–3.39 mmol/L), 77% of the estimated LDL-C values were within 10% of the measured value. This proportion decreased to 69% when triglyceride concentrations were between 3010 and 4000 mg/L (3.40–4.52 mmol/L) and to 41% when triglyceride concentrations were between 4010 and 6000 mg/L (4.53–6.78 mmol/L).

McNamara et al. (36) compared estimated LDL-C values with LDL-C values determined by the Lipid Research Clinics method (beta-quantification) and calculated the percentage of samples that were properly classified for LDL-C according to NCEP guidelines (Table 1). When plasma triglyceride concentrations were $\leq 4000$ mg/L ($\leq 4.52$ mmol/L), 86% of their subjects were correctly classified into NCEP risk categories through utilization of their estimated LDL-C value. When the measured LDL-C was $< 1300$ mg/L ($< 3.37$ mmol/L) or $> 1900$ mg/L ($> 4.92$ mmol/L), 92% to 94% of the subjects were correctly classified. However, if the subject had a measured LDL-C concentration between 1300 and 1890 mg/L (3.37–4.90 mmol/L), only 76–79% of subjects were classified into the correct NCEP risk group, based on their estimated LDL-C. Furthermore, subjects with measured LDL-C concentrations $< 100$ mg/L ($< 0.26$ mmol/L) different from an NCEP decision value were correctly classified only 69% of the time. On the other hand, subjects with measured LDL-C concentrations $\geq 100$ mg/L ($\geq 0.26$ mmol/L) different from a decision value were correctly classified 96% of the time. Others (37, 77) have reported similar findings.

Another pitfall of the Friedewald equation arises when it is used in patients with Type III hyperlipoproteinemia. These patients have a VLDL that is abnormally enriched with cholesterol relative to triglyceride. One of the diagnostic criteria for Type III is a VLDL-C/ serum triglyceride ratio $> 0.3$, when values are expressed in mg/L, or 0.675, when values are expressed in mmol/L. Another diagnostic criteria for Type III is the presence of beta-migrating VLDL on electrophoresis. Both of these diagnostic tests require ultracentrifugal isolation of VLDL. It is also possible clinically to use whole serum to detect Type III hyperlipoproteinemia. Electrophoretic analysis of whole serum by both polyacrylamide gel and agarose electrophoresis has been successful in detecting Type III and excluding non-Type III patients (83, 84). However, a clinician could miss the Type III diagnosis if LDL-C is estimated. The abnormal VLDL composition in Type III patients causes an underestimation of VLDL-C and an overestimation of LDL-C. This can cause a Type III patient to be misdiagnosed as having Type IIb hyperlipoproteinemia—a problem, because treatments for these disorders differ. Fortunately, Type III is a relatively rare condition in the general population: about 1–2 individuals per 1000 people (85). The misclassification rate will be higher in patients referred for hyperlipidemia.

Since the Friedewald formula was first published in 1972, several other investigators have explored refinements for improving its accuracy for estimating LDL-C. These refinements include using regression equations with non-zero intercepts (37, 81, 86–88); alternative slopes or triglyceride multipliers besides 0.20, when values are in mg/L, or 0.45, when values are in mmol/L (36, 37, 80, 82, 89–91); and age-, gender-, triglyceride-, and total cholesterol-specific equations (81, 87). In gen-
eral, the regression equations with nonzero intercepts vary between laboratories and populations studied and perform only marginally better than the Friedewald formula. However, most investigators in these reports failed to compare their regression equations with the Friedewald formula in populations different from the one from which the Friedewald equation was derived. Under these conditions, the regression equation with a nonzero intercept would naturally give slightly better estimates than the Friedewald formula. Many investigators have focused on determining alternative triglyceride multipliers. Table 2 summarizes the mean VLDL-C/serum triglyceride ratios reported by other investigators (35–37, 80, 82, 90, 91) for estimating LDL-C in both normal and hyperlipidemic populations. Delong et al. (82), who proposed an alternative triglyceride multiplier of 0.158, when values are in mg/L, or 0.366, when values are in mmol/L, based their proposal on data from a random recall group in the Lipid Research Clinics Prevalence Study. They noted a striking difference in the triglyceride multipliers used at the participating 10 clinics: the clinic-specific multipliers ranged from 0.112 to 0.194 (for values in mg/L).

For all of its simplicity and limitations, the Friedewald formula probably remains the single best equation for estimating LDL-C in clinical laboratories. Because (a) the use of alternative triglyceride multipliers gives only marginally better LDL-C values and may not apply equally to all populations anyway, and (b) the Friedewald equation triglyceride multiplier seems to distribute the error about equally on both sides of zero, we conclude there is little advantage in using a modified Friedewald equation. Until a direct method for measuring LDL-C is developed that does not require ultracentrifugation, laboratory clinicians and scientists should probably continue to use the Friedewald formula on routine clinical samples. Samples from patients with triglyceride concentrations >4000 mg/L (>4.52 mmol/L), chylomicronemia, or suspected Type III hyperlipoproteinemia should be referred to one of the specialized laboratories for lipoprotein measurement by ultracentrifugation.

In conclusion, a wide variety of methodologies have been used for the determination of serum LDL-C concentration. Many of these techniques are cumbersome, time consuming, and require specialized instrumentation, which limits their use in the clinical laboratory. For example, the beta quantification method, which has been widely used in the specialized lipid laboratories, involves a combination of ultracentrifugation and chemical precipitation; nonetheless, this method has been used in most epidemiological studies and is usually considered the reference procedure. On the other hand, techniques that are more suitable for routine use have certain other limitations.

Currently, most clinical laboratories estimate LDL-C concentration by using the Friedewald equation. With

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Table 2. Mean VLDL-C/Triglyceride Ratios Reported for Estimating LDL-C

<table>
<thead>
<tr>
<th>VLDL-C/triglyceride</th>
<th>mg/dL or mg/L</th>
<th>mmol/L</th>
<th>n</th>
<th>Population studied</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.20</td>
<td>0.450</td>
<td>448</td>
<td></td>
<td>Normal and hyperlipidemic patients</td>
<td>35</td>
</tr>
<tr>
<td>0.20</td>
<td>0.450</td>
<td>4583</td>
<td></td>
<td>FHS follow-up, hyperlipidemic patients and research subjects, triglyceride ≤4000 mg/L (≤4.52 mmol/L)</td>
<td>36</td>
</tr>
<tr>
<td>0.23</td>
<td>0.518</td>
<td>4736</td>
<td></td>
<td>NWLRC database</td>
<td>37</td>
</tr>
<tr>
<td>0.22</td>
<td>0.495</td>
<td></td>
<td>b</td>
<td>NWLRC database, triglyceride ≤5000 mg/L (≤5.65 mmol/L)</td>
<td>37</td>
</tr>
<tr>
<td>0.21</td>
<td>0.472</td>
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<td></td>
<td>NWLRC database, triglyceride ≤5000 mg/L (≤5.65 mmol/L)</td>
<td>37</td>
</tr>
<tr>
<td>0.20</td>
<td>0.450</td>
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<td>b</td>
<td>NWLRC database, triglyceride ≤10 000 mg/L (≤11.3 mmol/L)</td>
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</tr>
<tr>
<td>0.196</td>
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<td>4131</td>
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<td>Normal patients</td>
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<tr>
<td>0.166</td>
<td>0.374</td>
<td>724</td>
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<td>Type I or V</td>
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<tr>
<td>0.213</td>
<td>0.479</td>
<td>2745</td>
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<td>Type IIA heterozygotes</td>
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<td>0.307</td>
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<td>Type IIA homozygotes</td>
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<tr>
<td>0.194</td>
<td>0.436</td>
<td>1552</td>
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<td>Type III</td>
<td>80</td>
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<tr>
<td>0.421</td>
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<td>Type III</td>
<td>80</td>
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<tr>
<td>0.171</td>
<td>0.385</td>
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<td>Type IV</td>
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<tr>
<td>0.158</td>
<td>0.356</td>
<td>6531</td>
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<td>LRC random recall group</td>
<td>82</td>
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<tr>
<td>0.180</td>
<td>0.405</td>
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<td>LRC high cholesterol and triglyceride</td>
<td>82</td>
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<tr>
<td>0.182</td>
<td>0.364</td>
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<td>LRC high cholesterol</td>
<td>82</td>
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<tr>
<td>0.182</td>
<td>0.364</td>
<td>964</td>
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<td>LRC high triglyceride</td>
<td>82</td>
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<td>0.682</td>
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<td>Normolipidemic Japanese</td>
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<tr>
<td>0.183</td>
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<tr>
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<td>Japanese Americans, impaired glucose tolerance</td>
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<td>0.423</td>
<td>78</td>
<td></td>
<td>Japanese Americans, Type II diabetes</td>
<td>91</td>
</tr>
</tbody>
</table>

* VLDL-C determined with ultracentrifugation.
* Unspecified n.

LRC, Lipid Research Clinics; NWLRC, Northwest Lipid Research Center; FHS, Framingham Heart Study.
this approach, total cholesterol, triglyceride, and HDL-C concentrations must all be determined accurately, and triglyceride concentrations must be <4000 mg/dL (<4.52 mmol/L) for LDL-C concentrations to be reliable. The presence of chylomicron or Type III also precludes reliable estimation. Failure to comply with these criteria will often result in inaccurate determination of LDL-C concentration. Given that the Adult Treatment Panel and the Children and Adolescents Treatment Panel of the NCEP recommend the use of LDL-C concentration in the diagnosis and treatment of hypercholesterolemia, erroneous LDL-C values can cause misclassification and mismanagement of patients.

A reliable method for the measurement of serum LDL-C concentration suitable for routine use in the clinical laboratory is definitely needed. However, until such an assay is available, clinical chemists should recognize the limitations of the current methodologies.

We thank Joen A. Waletzky and Kanta Kube for their contributions.

References