Comparison of methods for measurement of apolipoprotein B and cholesterol in low-density lipoproteins

Ljubica Vrga, Christine Contacos, Stephen C.H. Li, and David R. Sullivan

We describe a new method for the direct measurement of LDL-apolipoprotein (apo) B by using a commercial kit that isolates LDL by immunoseparation. We evaluated immunoseparation of LDL for apo B and cholesterol measurement in 46 dyslipidemic patients with LDL-cholesterol (LDL-C) between 1.5 and 8.2 mmol/L, 11 of whom had plasma triglyceride (TG) concentrations >4.0 mmol/L. There was a reasonable correlation (r = 0.94, n = 40) between LDL-apo B obtained after immunoseparation and d >1.006 kg/L apo B obtained after ultracentrifugation. LDL-C by the immunoseparation method also correlated well (r = 0.98, n = 46) with the d >1.006 kg/L cholesterol after ultracentrifugation. These results show that immunoseparation can be used to determine LDL-apo B, even in hypertriglyceridemic samples. This method may provide a quick and simple alternative for the identification of hyperapobetalipoproteinemia, even when TG concentrations are high.

INDEXING TERMS: ultracentrifugation • triglycerides • precipitin tests • immunologic techniques

Increased concentrations of LDL cholesterol (LDL-C) are regarded as a risk factor for coronary artery disease (CAD) [1, 2]. Sniderman et al. [3, 4] have suggested that increased concentrations of LDL-apolipoprotein (apo) B may identify patients with increased numbers of LDL in whom CAD risk is increased. These patients may not necessarily have increased concentrations of LDL-C. LDL-apo B has traditionally been measured by an immunodiffusion technique [5], which may be difficult to standardize between laboratories. On the other hand, direct measurement of LDL-apo B requires the use of one of several possible ultracentrifugation methods. All these procedures are labor intensive, time consuming, and expensive. This limits the use of direct LDL-apo B and LDL-C measurements in routine clinical management, restricting test availability to major referral laboratories or to research purposes.

In routine clinical practice, total apo B measurement and LDL-C estimated by the Friedewald equation [6] tend to be used, thereby avoiding ultracentrifugation. Total apo B may not distinguish between apo B48- and apo B100-containing particles. In addition, apo B methods that involve light-scattering techniques may be unreliable in hypertriglyceridemic samples. Estimation of LDL-apo B cannot be calculated by a process equivalent to the Friedewald formula, and the Friedewald equation is also inappropriate for samples from nonfasting individuals or those with plasma triglyceride (TG) concentrations >4.0 mmol/L [6].

We have assessed the use of a commercial immunoseparation kit as part of an alternative method for LDL-apo B quantification. The kit was developed for LDL-C measurement in samples from hypertriglyceridemic or nonfasting individuals that are unsuitable for LDL-C calculation by the Friedewald equation [7, 8].

Materials and Methods

Specimens

Venous blood was collected from 46 outpatients who attended the Lipid Clinic at the Royal Prince Alfred Hospital, Sydney, NSW, Australia. The study was undertaken in compliance with the Helsinki declaration and the Central Sydney Area Health Service Ethics Review Committee. Eleven subjects with TG concentrations >4.0 mmol/L were included to assess performance of the assays in situations in which the Friedewald equation is not recommended. Each subject was requested to fast...
overnight (12 to 16 h) before attending the clinic. Blood was drawn into Vacutette vacutainer tubes containing 1.8 g/L K₂EDTA (Greiner Laborteknick, Kremsmunster, Austria) and processed within half an hour of collection. Plasma was separated by low-speed centrifugation at 1500 g for 4 min at room temperature, then stored at 4 °C. This time and speed was sufficient to separate the plasma from cells. Samples were analyzed within 7 days. The ranges for plasma TG and total cholesterol (TC) were 0.8–14.6 mmol/L and 4.0–10.1 mmol/L, respectively.

ULTRACENTRIFUGATION
Lipoproteins of d <1.006 and d >1.006 kg/L were fractionated from whole plasma of all participants by ultracentrifugation. Plasma (3 or 4 mL) was dispensed into 6-mL capacity Quick-Seal centrifuge tubes (Beckman Instruments, Palo Alto, CA), tubes were filled with d = 1.006 kg/L solution (0.15 mol/L NaCl, 0.1 g/L NaN₃, and 0.1 g/L EDTA), and then centrifuged at 199 800 g for 16 h at 4 °C in a 50.4 Ti rotor and Optima L-80 ultracentrifuge (Beckman Instruments). The top fraction (d <1.006 kg/L), containing VLDL, and the bottom fraction (d >1.006 kg/L), containing IDL, LDL, and HDL, were recovered after tube slicing. Both fractions were restored to the original plasma volume with d = 1.006 kg/L solution. The mean (SD) cholesterol recovery, based on the sum of cholesterol concentrations in the two fractions relative to the total plasma cholesterol, was 99 (3)%.

PRECIPITATION
HDL were isolated from plasma and the d >1.006 kg/L plasma fraction after precipitation of apo B-containing lipoproteins with dextran sulfate:magnesium chloride [9].

IMMUNOSEPARATION
LDL were isolated from plasma by using the Direct LDL-C Immunoseparation reagent kit kindly provided by Genzyme Diagnostics (Genzyme Corp., Cambridge, MA). This procedure involves a suspension of polystyrene latex beads coated with goat polyclonal antibodies to human apo AI and apo E. Plasma (30 μL) and reagent (200 μL) were mixed in the inner tube of a dual-chamber centrifuge filter unit. After a 10-min incubation at room temperature, the unit was centrifuged at 1500 g for 5 min. Immunoprecipitated VLDL, IDL, and HDL remained in the inner tube, being trapped by a filter at its base. LDL passed freely through the filter, and an LDL-containing filtrate was recovered from the outer tube. CVs were determined by measuring cholesterol concentration in the filtrates of control samples provided (1.8 mmol/L and 5.3 mmol/L). The intrassay CVs were generated by repeated immunoseparation (n = 10) of both controls. These were 3.9% and 2.0%, respectively. The interassay CVs were generated by immunoseparation of the same controls on 10 separate runs. These were 10.0% and 3.5%, respectively.

LIPID, LIPOPROTEIN, AND APOLIPOPROTEIN QUANTIFICATION
TC and TG were measured by standard enzymatic techniques on an automated analyzer (BM/Hitachi 747, Tokyo, Japan) by using Boehringer Mannheim (Mannheim, Germany) reagents (CHOD-PAP for TC, GPO-PAP for TG). The interassay CVs were 2.5% and 4.8%, respectively, whereas external quality-assurance performance on Cycle 35 of the Australian Quality Assurance Programme (AQAP) revealed CV = 15%, bias = 0.13 mmol/L for TC and CV = 14%, bias = 0.23 mmol/L for TG.

HDL-C was measured by standard enzymatic methods on a centrifugal analyzer (Multistat III Plus; Instrumentation Laboratory, Lexington, MA) with Australian Scientific Enterprise (Sydney, Australia) reagents (CHOD-PAP). The interassay CV was 2.0% and AQAP performance was CV = 9.0% and bias = 0.21 mmol/L.

LDL-C was determined by three methods: (a) In plasma with TG ≤4.0 mmol/L, LDL-C (range 1.6–8.2 mmol/L, n = 35) was estimated by the Friedewald equation [6]; (b) in the d >1.006 kg/L fraction, LDL-C (range 0.9–8.5 mmol/L, n = 46) was measured by subtracting HDL-C from d >1.006 kg/L cholesterol; this measurement includes IDL-C (1.006<d<1.019 kg/L); (c) in the immunoseparated filtrate, LDL-C (range 1.5–8.2 mmol/L, n = 46) was measured directly as the cholesterol concentration of the ultrafiltrate.

Apo B measurements were carried out on fractions from 40 subjects because sample volumes from 6 patients were insufficient. A Behring nephelometer and commercially available antibodies (Behringwerke, Marburg, Germany) were used. Apo B was determined in both the d >1.006 kg/L fraction, which includes IDL-apo B (range 0.52–2.24 g/L, n = 40), and the immunoseparated LDL filtrate (range 0.58–2.14 g/L, n = 40). The interassay CV was 4.0% and AQAP specimens based on an IFCC calibrator revealed CV = 2.8% and bias = 0.038 g/L.

STATISTICAL ANALYSES
Pearson correlation coefficients were computed to assess the association between parameters. All statistical tests were performed with Fig.P software (Fig.P Software Corp., Durham, NC).

Results
Apo B measured in immunoseparated LDL (y) correlated reasonably well with that measured in the d >1.006 kg/L plasma fraction (x) (y = 0.85x + 0.15; r = 0.94; n = 40). The 95% confidence intervals of the slope and intercept were 0.75–0.95 and 0.01–0.30 respectively. However, apo B concentrations in the ultracentrifuged sample tended to be higher than those measured in the immunoseparated filtrate, especially at mean LDL-apo B concentrations >~1.0 g/L (Fig. 1).

In samples with plasma TG ≤4.0 mmol/L (n = 35), LDL-C estimated by the Friedewald calculation (y) corre-
lated well with cholesterol measured directly in the \( d > 1.006 \text{ kg/L} \) fraction after ultracentrifugation \((y = 0.99x + 0.07; r = 0.98; n = 34)\). The 95% confidence limits for the slope and intercept were 0.92–1.06 and −0.29–0.43 respectively. Similar correlations were found between LDL-C obtained by immunoseparation and ultracentrifugation \((y = 0.87x + 0.71; r = 0.98; n = 46)\), and between immunoseparation and Friedewald calculation \((y = 0.88x + 0.61; r = 0.96; n = 35)\). The 95% confidence limits for slope and intercept were 0.81–0.93 and 0.42–1.00 respectively for the correlation between immunoseparation and ultracentrifugation, whereas those for the correlation between immunoseparation and the Friedewald equation were 0.79–0.97 and 0.12–1.10 respectively.

To detect the between-method bias for the LDL-C methods under comparison, the absolute difference was plotted against the mean for each pair of measurements. The comparison between immunoseparation and ultracentrifugation (Fig. 2A) and the comparison between immunoseparation and the Friedewald calculation (Fig. 2B) both show a positive bias in favor of immunoseparation at lower LDL-C concentrations, but a negative bias at higher LDL-C concentrations.

**Discussion**

A kit that has been developed by Genzyme Diagnostics isolates LDL from other plasma lipoproteins by selective immunoprecipitation \([7, 8]\). We have analyzed the LDL-containing filtrate obtained by this immunoseparation method for apo B as well as cholesterol. LDL-apo B measured by this method correlates reasonably well with direct measurement of apo B in the \( d > 1.006 \text{ kg/L} \) fraction isolated by ultracentrifugation. The immunoseparation technique removes IDL, but the ultracentrifugation technique used in this study retains IDL as well as LDL. This may account for the negative bias noted in Figs. 1 and 2. Although other centrifugation techniques that isolate the \( 1.019 < d < 1.063 \text{ kg/L} \) fraction would avoid this problem, they are more cumbersome and, as a consequence, have been used less frequently for the analysis of clinical samples.

Increased concentrations of LDL-apo B may identify patients with increased numbers of LDL in whom CAD risk is increased. LDL-C concentrations in these patients may not necessarily be increased. The original immunodiffusion technique for LDL-apo B measurement \([5]\) has been difficult to standardize between laboratories and has not been widely adopted. Many clinical laboratories use total apo B concentrations as an indication of hyperapo-betalipoproteinemia. The inclusion of VLDL and interference due to turbidity associated with \( d < 1.006 \) lipoproteins is likely to confound this approach, whereas the presence of apo B48-containing lipoproteins is also a theoretical concern. Nevertheless, the direct measurement of LDL-apo B in immunoseparated LDL avoids all of these confounding factors. We believe that the measurement of apo B by immunoturbidimetry is reliable after the removal of \( d < 1.006 \text{ kg/L} \) lipoproteins because this removes apo B48-containing particles and minimizes problems with turbidity. However, we acknowledge that optimization of the direct measurement of LDL-apo B may involve alternative methods for apo B quantification.

The role of LDL-C has become more important in the assessment of CAD risk and is the basis of some public health guidelines for the management of patients with dyslipidemia \((\text{National Cholesterol Education Program II}) [10]\). It could be argued that quantification of atherogenic lipoproteins should also include IDL because they are also extremely atherogenic. In cases of dysbeta-

![Fig. 1. Absolute differences between immunoseparated LDL-apo B and apo B in the \( d > 1.006 \text{ kg/L} \) plasma fraction. I, immunoseparated; U, ultracentrifuged.](image)

![Fig. 2. Absolute differences between immunoseparated LDL-C with ultracentrifugation LDL-C (A) and Friedewald LDL-C (B). I, immunoseparated; U, ultracentrifuged; F, Friedewald.](image)
immunoseparation technique provides important additional measurement of both cholesterol and apo B by using light-scattering techniques are used. The only methods available to assess LDL-C directly involve one of several ultracentrifugation procedures, all of which are relatively cumbersome, labor intensive, time consuming, and expensive. Friedewald et al. derived an equation to estimate the LDL-C so as to avoid this problem [6]. Our results on samples with plasma TG ≤4.0 mmol/L confirm that the calculated Friedewald estimation correlates very well with LDL-C measured after ultracentrifugation with minimal inaccuracy. The disadvantages are that calculation of Friedewald LDL-C in samples that have TG concentrations >4.0 mmol/L is inappropriate. Furthermore, it is inconvenient to require patients to fast for 14 to 16 h before blood collection, and samples from nonfasting individuals will be inaccurate. The new technique was not tested with postprandial samples, but it is unlikely that it would be subject to interference from postprandial TG-rich lipoproteins. Finally, the Friedewald equation does not provide information about other aspects of LDL composition such as its apo B concentration. The new method is simple and provides the extra benefit of allowing the assessment of LDL composition (e.g., LDL cholesterol:apo B ratio), which may reflect LDL size and density. LDL composition differs between individuals with differing LDL subclass phenotype [11], and increased concentrations of LDL-apo B may identify individuals with increased CAD risk despite nonincreased LDL-C concentrations due to increased numbers of small dense LDL [3]. Other methods for the assessment of LDL size and density include gradient gel electrophoresis, which is qualitative rather than quantitative, and sequential ultracentrifugation, which is extremely laborious.

In conclusion, immunoseparation offers a convenient technique for the direct estimation of not only LDL-C, but also LDL-apoB, even in hypertriglyceridemic samples. It requires only 30 µL of sample as compared with much larger volumes required for ultracentrifugation, and effectively eliminates cholesterol-containing lipoproteins other than LDL and lipoprotein(a). The removal of the TG-rich lipoproteins may reduce interference due to turbidity if light-scattering techniques are used. The measurement of both cholesterol and apo B by using the immunoseparation technique provides important additional clinical information because LDL composition reflects its size and density. Freezing and storage before immunoseparation causes a negative bias [7, 8], so this technique appears unsuitable for retrospective analysis of LDL-apo B in stored frozen samples. However, the convenience of this new technique may warrant the inclusion of this potentially useful measurement in clinical care and research.

This research was supported in part (to C.C.) by the National Heart Foundation of Australia (grant no. G94S4050).

References