Performance of a direct LDL-cholesterol method compared to beta quantification

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Abstract   Low-density lipoprotein cholesterol is currently estimated by clinical laboratories using the Friedewald formula which requires fasting specimens and is subject to error with increasing triglyceride levels. We describe a rapid method for isolating low-density lipoproteins by immunoseparation for subsequent measurement of cholesterol by enzymatic assay. The Direct LDL™ Immunoseparation Reagent meets current guidelines for precision with intra-assay and interassay coefficients of variation of <3%. The results are highly correlated to the beta quantification reference method (r=0.980). The results are generally not affected by increasing levels of triglycerides or high-density lipoprotein cholesterol and patient fasting is not required for accurate analysis. The Direct LDL Immunoseparation Reagent overcomes limitations of the Friedewald formula and appears to be suitable for accurate quantitation of low-density lipoprotein cholesterol in the routine laboratory.

INTRODUCTION

Elevated serum levels of low density lipoprotein cholesterol (LDL-C) are an important risk factor for coronary heart disease (1,2). Increased LDL-C concentrations highly correlate with atherosclerotic lesions (2,3), and intervention to decrease LDL-C can improve the symptoms of coronary heart disease and result in regression of the lesions (4-7). Because of this strong and positive link between LDL-C and coronary heart disease the Adult Treatment Panels and the Children and Adolescents Treatment Panel of the U.S. National Cholesterol Education Program (NCEP) and the European Atherosclerosis Society have focused on LDL-C as the primary basis for classification and treatment of hyperlipidemia (8-10).

Currently, several indirect methods for measuring LDL-C in the clinical laboratory are employed. The first is the Friedewald equation which estimates LDL-C concentration from total cholesterol(TC), high density lipoprotein cholesterol (HDL-C) and triglyceride levels according to the equation:
LDL-C = TC - (HDL-C) - (triglycerides/2.2) (11). The Friedewald equation is limited to use with specimens from fasting individuals, with triglycerides < 4.52 mmol/L, and without Type III dyslipidemia (11). The second indirect method is polyanion precipitation of LDL-C using such reagents as polyvinyl sulfate and heparin (12). In this procedure the LDL-C value is calculated as the difference between the TC level of the serum and the cholesterol remaining in the supernatant after precipitation. Evaluations suggest that these precipitation methods can be accurate when triglyceride levels are low, but become increasingly inaccurate with higher triglyceride levels. Beta quantification (ßQ) is accepted as the reference method for measurement of LDL-C (13). It is a multistep procedure that combines separations by ultracentrifugation and chemical precipitation. ßQ is technique sensitive, labor intensive, and requires expensive equipment and therefore has remained a specialty procedure generally not available in routine laboratories. Due to the inadequacy of the current procedures, the NCEP identified the need for the development of direct methods for measurement of LDL-C suitable for the clinical laboratory. (14)

We present an evaluation of the Direct Immunoseparation Reagent that separates low density lipoproteins for subsequent direct measurement by enzymatic cholesterol assay.

MATERIALS AND METHODS

LDL-Cholesterol Immunoseparation Reagent. The Direct LDL-Cholesterol Reagent (Genzyme Diagnostics Cambridge, MA) utilizes latex beads coated with affinity purified goat antibodies to human apolipoproteins A-I and E. The antibodies are bound to separate populations of beads formulated in a suspension containing phosphate buffer, pH 7.0, with 0.1% sodium azide as

preservative. The antibody coated beads selectively bind chylomicrons, very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), and HDL from serum while LDL remains unbound. A dual chamber microcentrifuge filter unit consisting of a smaller inner tube with a filter, and a larger outer tube with cap is used to separate the bound fraction from the unbound fraction.

Procedure for Direct LDL Immunoseparation. Direct LDL Reagent (200 µL) and sample (30 µL) were pipetted into the inner tube of the separation device and mixed immediately by vortexing. The tubes were incubated for 5 to 10 minutes at room temperature, and then centrifuged at 12,000 g for 5 minutes. The filtrate collected in the outer tube was assayed for cholesterol using an enzymatic method and the results were corrected for dilution of the sample by the reagent using a multiplication factor.

ßQ. Serum was centrifuged at a density of 1.006 kg/L at 109,000 x g for 18 hours in a Beckman 40.3 or 50.3 Ti rotor (Beckman Instruments, Palo Alto, CA). Chylomicrons and VLDL were recovered in the d < 1.006 kg/L (top) fraction by a tube slicing technique. LDL and HDL were recovered in the d > 1.006 kg/L (bottom) fraction. HDL cholesterol was determined in an aliquot of unfractionated serum. LDL-C was calculated as that in the d > 1.006 kg/L fraction minus HDL-C. VLDL-C was calculated as the TC of the specimen minus that in the d > 1.006 kg/L fraction.

Determination of Serum Lipids. All cholesterol determinations were made using the Total Serum Cholesterol Assay Reagent (Diagnostic Chemicals Limited, Oxford, CT). HDL-C was measured by the dextran sulfate-Mg^{2+} precipitation procedure (15) with DextraLip® 50, (Genzyme Diagnostics). Triglycerides were measured enzymatically with glycerol blanking using reagents from Miles-Technicon (Tarrytown, NY). TC, HDL-C, ßQ LDL-C and triglyceride analyses were all performed on an Abbott Spectrum (Abbott Diagnostics, Irvine, TX) while Direct LDL filtrates were analyzed on a Cobas FARA (Roche Diagnostics, Montclair, NJ).

Precision. Intra-assay and interassay precision were determined on three fresh serum pools containing <3.36 mmol/L, 3.36 - 4.11 mmol/L, and ≥4.14 mmol/L LDL-C, reflecting the LDL-C cut points of the NCEP guidelines (8). For intra-assay precision, 20 aliquots of a serum pool were processed simultaneously and analyzed in one tray. For interassay precision, each serum pool was processed by the Direct LDL Reagent twice per day in duplicate for 5 days. The filtrates were analyzed on the day the sample was processed.

Method Comparison Study. ßQ and Direct LDL testing were performed on 218 fasting specimens (184 with triglycerides < 4.52 mmol/L and 34 with triglycerides ≥ 4.52 mmol/L) and the values were compared by linear regression analysis.

VLDL and HDL Capacity Studies. To evaluate the binding capacity of the Direct LDL Reagent for VLDL, 15 sera with VLDL-C concentrations between 1.19 mmol/L and 12.36 mmol/L were examined. First, the LDL-C in each specimen was determined using the Direct LDL Reagent. Then the VLDL-C fraction of each specimen was removed by ultracentrifugation, as previously described, and the LDL-C in the d > 1.006 kg/L fraction was measured by the Direct LDL Reagent. The difference between these two direct LDL-C measurements (the LDL-C in the specimen minus the LDL-C in the d > 1.006 kg/L fraction) represents the amount of VLDL-C not removed from the specimen by the reagent. The HDL-C binding capacity of the Direct LDL Reagent was determined using a purified HDL-C concentrate (Creative Lab Products, Indianapolis, IN) which was diluted in a phosphate buffer to produce ten specimens with HDL-C concentrations ranging from 0.65 mmol/L to 6.54 mmol/L. Each sample was processed with the Direct LDL Reagent or a buffer blank and the HDL-C content of the filtrates was measured as described.

Fasting and Nonfasting Comparison. Fifty-three subjects were divided into two groups. One group had blood drawn after a 12-hour fast; the other had blood drawn two hours after breakfast or lunch. Two weeks later, the fasting status of the two groups was reversed, and blood was drawn again from the volunteers. Paired Student's *t*-test analyses were used to compare the lipid profiles of fasting and non-fasting specimens.

Frozen Samples. Forty-two serum samples were assayed for LDL-C by ßQ and the Direct LDL Reagent. The samples were then divided into aliquots and frozen at -70°C. Testing was repeated at 0.5 month, 1 month, 3 months, 6 months, and 12 months.

RESULTS

Precision. The precision of the Direct LDL Immunoseparation Reagent was examined using normal, borderline and elevated LDL-C serum pools. The intra-assay and interassay precision profiles are summarized in Table 1. The mean intra-assay coefficient of variation (CV), from 20 determinations of the three serum pools was 2.03%. The mean interassay CV from 20 determinations over five days of the three serum pools was 1.63%.

TABLE 1. Precision of the Direct LDL Immunoseparation Reagent

	Intra-assay (n=20) LDL-C mmol/L			Interassay (n=20) LDL-C mmol/L		
	Mean	SD	CV%	Mean	SD	CV%
Serum Pool 1	2.48	0.06	2.43	2.43	0.03	1.42
Serum Pool 2	3.67	0.06	1.60	3.65	0.06	1.62
Serum Pool 3	4.99	0.10	2.07	5.02	0.09	1.86

SD = Standard deviation; CV% = coefficient of variation (%).

Method Comparison Study. The lipid distributions of the 218 specimens tested were: 3.09-13.55 mmol/L for TC, 0.36-2.43 mmol/L for HDL-C, 0.52-11.07 mmol/L for ßQ LDL-C and 0.36-47.19 mmol/L for triglycerides.

There was no significant difference between LDL-C values determined by ßQ or the Direct LDL Reagent (3.65±1.39, 3.73±1.43 mmol/L, respectively P=0.55). Furthermore, there was no significant difference between the methods when the sample population was divided according to triglyceride level. ßQ and Direct LDL values for the population with triglycerides levels < 4.52 mmol/L were 3.91 ± 1.32 and 3.96 ± 1.37 mmol/L, respectively, while LDL-C values for the population of specimens with triglycerides ≥ 4.52 mmol/L were 2.24 ± 0.84, and 2.44 ± 1.06 mmol/L, respectively. The linear regression analysis pairing Direct and ßQ LDL-C values for all specimens yielded a between method comparison of Direct LDL-C = 1.01(ßQ LDL-C) + 0.04 mmol/L and a correlation coefficient (r) of 0.980 (Fig. 1). Similar linear regression equations for the Direct LDL Reagent and ßQ were obtained for samples with triglycerides <4.52 mmol/L:
(y = 1.02x - 0.03 mmol/L, r=0.98, n=184) and samples with triglycerides ≥4.52 mmol/L:
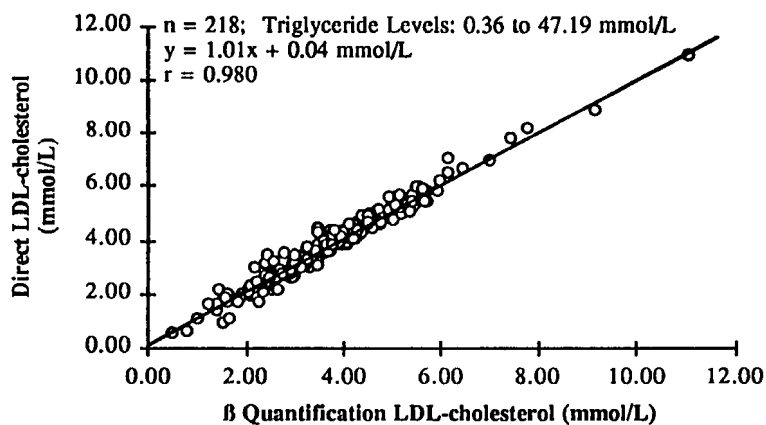(y = 1.18x - 0.19 mmol/L, r=0.94, n=34).

Fig. 1 Linear regression plot for Direct LDL-C results from 218 subjects measured by beta quantification (x-axis) and the Direct LDL Immunoseparation Reagent (y-axis).

VLDL and HDL Capacity Studies. Figure 2 shows the percentage of VLDL removed by the Direct LDL Reagent from 15 specimens with VLDL-C concentration between 1.19 and 12.36 mmol/L. The Direct LDL Reagent removed 95%±4% of the VLDL-C from the specimens. There was no relationship between the amount of VLDL-C in the specimen and the percent of VLDL-C removed. This study included specimens with VLDL-C levels much greater than the level representing the 95th percentile as derived from the Lipid Research Clinics population studies (13). The maximum HDL-C capacity of the Direct LDL-C Reagent, shown in Fig. 3, is between 2.59 to 3.23 mmol/L which is in excess of the normal range of HDL-C in sera. The Lipid Research Clinics population studies report the 95th percentile for HDL-C levels to be 2.33 mmol/L.
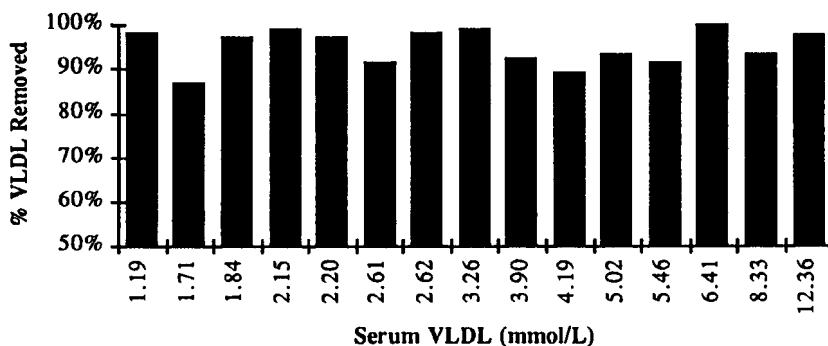
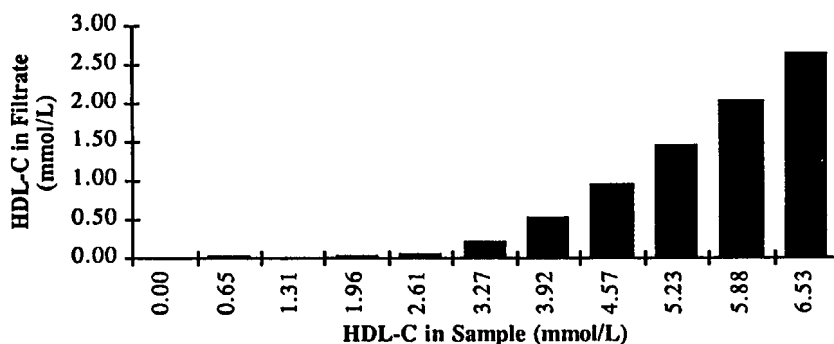Fig. 2 Percent VLDL removed from specimens by the Direct LDL Reagent

Fig. 3 HDL capacity of the Direct LDL Reagent

Fasting and Nonfasting Comparison. The means and SD for LDL-C, triglycerides and TC for the 53 fasting and nonfasting serum specimens are given in Table 2. The results for fasting and nonfasting specimens did not differ significantly in TC and LDL-C value (P>0.2). In addition, LDL-C measurement by ßQ or by Direct LDL Reagent was the same. Thus, use of the Direct LDL Reagent, like ßQ, was not affected by the fasting status of the subject. As expected, triglyceride values were significantly greater postprandially (P<0.05).

Frozen Samples. To determine the effect of freezing on LDL-C levels measured by ßQ and the Direct LDL Reagent, samples were studied fresh and following storage at -70°C. LDL-C values measured by the Direct LDL Reagent on frozen specimens decline with time of storage up until 3 months (-12%) and then stabilize from that point to at least 12 months. The 3 month Direct LDL values were significantly different (P<0.05) from the fresh values (Table 3). ßQ LDL-C values also decrease following storage at -70°C storage however, the differences were not significant (3 mo., P=0.12).

CONCLUSION

Elevated LDL-C is known to be a major risk factor for coronary heart disease (1,2). Therefore, the accurate and precise measurement of LDL-C is particularly important in classifying individuals at risk for CHD and for subsequent clinical management of these patients. ßQ which relies on ultracentrifugation to separate lipoprotein particles according to hydrated density, has been the accepted reference method for LDL-C quantitation. However, this method is time consuming and requires specialized, expensive equipment and highly trained personnel. Use of ßQ is limited to specialized lipid laboratories.

Most laboratories estimate LDL-C using the Friedewald equation (11), which assumes that VLDL-C can be estimated by dividing triglycerides by 2.2(mmol/L). It is well accepted that this assumption results in several deficiencies. The Friedewald formula can only be used in fasting sera, and when

TABLE 2. Comparison of Fasting and Nonfasting Serum Specimens

	Mean±SD mmol/L n=53			
	ßQ LDL-C	Direct LDL-C	Triglycerides	Total Cholesterol
Fasting	3.62±1.04	3.72±1.17	1.64±1.08	5.64±1.22
Nonfasting	3.54±0.97	3.70±1.19	2.16±1.44	5.59±1.18

SD = standard deviation

TABLE 3. Effect of Frozen Samples on ßQ and Direct LDL-C

Mean ± SD mmol/L
Storage Intervals (months)

	0	0.5	1	3	6	12
Direct LDL-C	133±33	133±33	126±32	117±31*	120±31	121±31
ßQ LDL-C	139±34	134±32	135±33	128±32	127±32	126±31

SD = standard deviation,   *P<0.05

triglyceride levels are less than 4.52 mmol/L. Furthermore, the precision of the Friedewald formula relies on the accuracy and precision of the three measurements used in the calculation, namely TC, HDL-C and triglycerides.

Another method used to measure LDL-C is indirect polyanion precipitation (16). Recent studies conclude that these precipitation methods correlate less well with ßQ than does the Friedewald calculation and also become less accurate as triglyceride levels increase due to co-precipitation of VLDL with LDL.

The Direct LDL Immunoseparation Reagent employs an immunochemical approach to the isolation of LDL-C for cholesterol measurement. Lipoproteins containing apolipoprotein A-I and/or apolipoprotein E are bound and removed from the specimen prior to cholesterol analysis. This includes, HDL, VLDL and chylomicrons. The Direct LDL method was found to be in good agreement with ßQ (y = 1.01x + 0.04 mmol/L, r=0.980). Moreover, the Direct LDL-C values were highly correlated with ßQ LDL-C for both normotriglyceridemic (r=0.980) and hypertriglyceridemic (r=-0.940) populations. Capacity studies indicate that the Direct LDL Reagent is capable of removing HDL-C and VLDL-C at levels greater than the 95th percentiles for these lipoproteins, according to population studies (13). Comparison of specimens from individuals in the fasting and nonfasting state revealed no effect on the Direct LDL-C results. The ability to achieve an accurate LDL-C determination, without the requirement for patient fasting may provide a more useful tool for the clinician and greater convenience for the patient.

Specimens which have been frozen show a significant negative bias compared to fresh specimens, when tested by the Direct LDL-C Reagent indicating that only fresh samples are appropriate. Freezing may cause aggregation of LDL particles with subsequent trapping by the latex particles and/or separation device filter. There was also a negative trend in the recovery of ßQ LDL-C values in frozen specimens, though this was not statistically significant.

In summary, the Direct LDL-C Immunoseparation Reagent is an accurate method for determining serum LDL-C (17-20). This applies equally to samples from patients who are normotriglyceridemic (< 4.52 mmol/L), as well as patients who are hypertriglyceridemic (≥ 4.52 mmol/L) or are in the nonfasting state, where the Friedewald equation is inaccurate. Furthermore, the use of Direct LDL-C Reagent has the potential for reducing analytical variability in LDL-C measurements, as it is based on one direct measurement rather than two indirect measurements in the case of polyanion precipitation, or three indirect measurements in the case of the Friedewald equation. The Direct LDL Immunoseparation Reagents overcomes major limitations of current routine LDL-C methods. It is appropriate for use in the clinical laboratory for screening and especially for monitoring patient response to therapy without the need for repeat analysis and cost of the three separate tests needed to calculate LDL-C or the fasting requirement.

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