Indian Journal of Clinical Biochemistry, 2005, 20 (2) 54-61

COMPARISON OF TWO METHODS OF ESTIMATION OF LOW DENSITY LIPOPROTEIN CHOLESTEROL, THE DIRECT VERSUS FRIEDEWALD ESTIMATION

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ABSTRACT

Current recommendations of the Adult Treatment Panel and Adolescents Treatment Panel of National Cholesterol Education Program make the low-density lipoprotein cholesterol (LDL-C) levels in serum the basis of classification and management of hypercholesterolemia. A number of direct homogenous assays based on surfactant/ solubility principles have evolved in the recent past. This has made LDL-C estimation less cumbersome than the earlier used methods. Here we compared one of the direct homogenous assays with the widely used Friedewald's method of estimation of LDL-C to see the differences and correlation. We used direct homogenous assay kit to estimate serum LDL - C and high-density lipoprotein cholesterol (HDL-C). Serum Triglyceride (TG) and Total Cholesterol (TC) was estimated and using Friedewald's formula LDL -C was calculated. The LDL-C levels obtained by both methods in 893 fasting serum samples were compared. The statistical methods used were paired t-test and Pearson's correlation.

There was significant difference in the mean LDL-C levels obtained by the two methods at the TG levels < 200 mg/dl (p<0.02) and TC levels >150 mg% (p<0.001). The correlation coefficient (r) between Friedewald's and direct assay estimation was 0.88. Friedewald's method classified 23.5 % of patients as high cardiac risk whereas there were 17.58% by direct assay.

Both had good correlation even though the serum triglyceride and total cholesterol levels affect the difference in LDL-C estimated by both methods. Taking into account the cost and performance, Friedewald's method is as good or even better for classifying and managing patients.

KEY WORDS

Direct assay, Friedewald's method, High-density lipoprotein cholesterol (HDL-C), Low-density lipoprotein cholesterol (LDL-C), Triglyceride (TG), Total cholesterol (TC).

INTRODUCTION

The Low Density Lipoproteins (LDL) is a heterogeneous population of spherical particles, with hydrophobic oily cores consisting of cholesteryl ester and triglycerides (TG). These particles are coated with a native surfactant of phospholipids, free cholesterol and apolipoproteins. On an average, LDL carries two thirds of the total cholesterol (TC) in serum. Each LDL particle contains one molecule of Apolipoprotein B-100 (apo B- 100), which is the main protein component of LDL, and the other minor apolipoproteins are apo E and apo C II (1).

By definition, LDL comprises the population of particles with hydrated density between 1.006 and 1.063 kg/l. This definition referring to LDL separated by sequential density ultra centrifugation, or the so-called beta quantification method combining ultra centrifugation and chemical precipitation, has seen the basis for measurement in most epidemiological studies (2). 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 (3). In practice, because all of the particles are

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atherogenic, the wide density population of LDL (1.006 - 1.063 kg/l) is usually reported.

Epidemiological and clinical studies have demonstrated a strong positive correlation between low-density lipoprotein cholesterol (LDL-C) concentrations in serum and the incidence of coronary heart disease (CHD) (4,5). Pathological studies have shown that increased LDL-C concentrations correlate highly with the extent of atherosclerotic lesions (6). A reduction of LDL-C decreases the risk and ameliorates the symptoms of CHD by causing a regression in the lesions (7, 8).

The diagnosis and management of adults with hypercholesterolemia are largely based on LDL-C concentration. In order to classify someone correctly into the National Cholesterol Education Program (NCEP) cut-points, LDL-C must be measured with a total error of = 12% (9). The serum LDL-C concentrations used to classify adults for high risk of heart disease are: Desirable <130 mg/dl, Borderline high-risk 130-159 mg/dl, High risk >160 mg/dl (2). The goal for subjects with two or more risk factor like diabetes, family history, hypertension, cigarette smoking, low High Density Lipoprotein Cholesterol (HDL-C) is to achieve LDL-C of 100 mg/dl (10). Therefore accurate and precise measurements of patients' LDL-C concentrations are necessary to appropriately identify individuals with hypercholesterolemia and to monitor the response to diet and drug treatments (11).

LDL-C is accurately measured by ultra centrifugation as recommended by Lipid Research Clinic-Bioquantification (LRC-BQ) (12, 34). However, because it is costly, labor intensive, requires expensive ultracentrifuges, rotors, and tubes, is time consuming and can be performed only on a few samples a day hence its use in routine clinical laboratories is limited. Until a few years from now, LDL-C has been estimated from Friedewald's equation (13) for clinical purposes, based on three independent measurements: HDL- Cholesterol (HDL-C), Triglycerides (TGs) and total Cholesterol (TC). The Friedewald equation assumes that dividing the blood TG concentration by a factor of 5 can approximate the amount of cholesterol in VLDL. The Friedewald equation has shown to be relatively reliable and was recommended by the NCEP as a routine method for estimation of LDL-C (14), but it has its shortcomings: (a) combining three measurements increases analytical imprecision; and (b) it is unreliable at TG concentrations >400 mg% (15) and can be used only in the fasting state. Recently direct LDL-Cholesterol assay kits using novel surfactants (the homogenous methods) based on different principles have become commercially available and are widely used. It has been reported that these assays are suitable even for serum with high TG levels (16, 17). The aim of this study is to compare one of these homogeneous assays that has a synthetic polymer/ detergent (SPD) method from Daiichi with LDL-C obtained by Friedewald calculation.

MATERIAL AND METHODS:

Data was obtained from the lipid profile analysis performed in the Clinical Biochemistry Laboratory of Christian Medical College and Hospital, Ludhiana. The original serum samples were obtained by withdrawing venous blood after 10-12 hours of overnight fasting collected in plain vials. The serum was separated by centrifugation and the following parameters estimated.

1. Total Cholesterol (TC) by Enzymatic endpoint CHOD- PAP method (18)
2. Triglycerides (TG) by Enzymatic Glycerol Phosphate Oxidase/ Peroxidase method (19, 20).
3. HDL-Cholesterol (HDL-C) by Homogenous Enzymatic Direct Assay (21).
4. LDL-Cholesterol (LDL-C) by Homogenous Enzymatic Direct Assay (22)
5. LDL-Cholesterol (LDL-C) obtained by Friedewald calculation (13).

For measurement of serum HDL-Cholesterol, Cholestest N HDL reagent kit used was from Daiichi Pure Chemicals Co., Ltd. Tokyo, Japan. Accurex Biomedicals Pvt Ltd., Mumbai markets it in India. Cholestest N HDL has two liquid reagents that directly measures the HDL-C concentration by a direct homogenous assay method based on the selective solubilizing effect of propriety detergent to the different lipoproteins. A special detergent solubilizes only HDL; other lipoproteins such as LDL, VLDL and chylomicrons are not disrupted. After HDL is selectively disrupted, HDL cholesterol is measured enzymatically using cholesterol esterase, cholesterol oxidase and peroxidase (23).

The reagent kit for direct LDL-C assay (Cholestest-LDL) was obtained from Daiichi Pure Chemicals Co., Ltd. Tokyo, Japan, marketed in India by Accurex Biomedicals Pvt Ltd., Mumbai. Cholestest- LDL contains two ready to use stable liquid reagents that directly measures the concentration of LDL-C by homogenous method based on detergent technology. The detergent 1 in Reagent 1disrupts the structure of HDL, VLDL and chylomicrons and causes release of cholesterol. The cholesterol esterase releases free cholesterol. Cholesterol oxidase releases hydrogen peroxide from free cholesterol, which reacts with 4-aminoantipyrine in the presence of peroxidase to give a colorless product. The second step starts with the addition of Reagent 2. Detergent 2 in Reagent 2

specifically acts on LDL releasing cholesterol. With the action of Cholesterol esterase and Cholesterol oxidase, hydrogen peroxide is liberated from free cholesterol of LDL. The coloring agent N, N- bis (4-Sulfobutyl) m- toluidine disodium salt (DSBmT) reacts with hydrogen peroxide in the presence of Peroxidase to give a bluish purple product measured at 546nm main and 660 subsidiary. The intensity of color is proportional to concentration of LDL-C.

Cholestest calibrator was used for calibration of both HDL-C and LDL-C. The same was used as a control for all tests done in lipid profile. All the four parameters were measured in a fully automated analyzer, RAXT (Technicon).

In 1972, Friedewald *et al.* (13) published a landmark report describing a formula to estimate LDL-C as an alternative to tedious ultra centrifugation. Because VLDL carries most of the circulating TGs, VLDL-C can be estimated reasonably well from measured TGs divided by 5 for mg/dl units. LDL-C is then calculated as Total Cholesterol minus HDL-C minus estimated VLDL-C.

We used paired t-test and Pearson's correlation coefficient to find the statistical significance.

RESULTS

The mean and standard deviation (SD) of LDL-C estimated by Direct and by Friedewald's formula showed a significant difference (p <0.02 and <0.01) at lower TG ranges of 1-100 and 101- 200 mg/ dl respectively (Table 1). When the means and SDs of Direct LDL-C was compared to Friedewald estimated LDL-C (Table 2), there was no significant difference at lower cholesterol range of 50- 99 and 100- 149 mg/dl (p >0.4 and >0.9 respectively). The difference in means and SDs were highly significant (p <0.001) at

Table 1. Mean and SDs (in mg/dl) of direct LDL-C and friedewald LDL-C at each category of triglyceride (TG)

TG range	n	Mean ± SD direct LDL-C	Mean ± SD friedwald LDL-C	p
1-100	204	84.91±34.5	92.94 ± 38.08	<0.02
101-200	478	101.09±31.38	107.83± 36.19	<0.01
201-300	163	118.28±34.97	121.68± 38.28	>0.4
301-400	34	112.71±27.7	111.76± 36.56	>0.8
≥ 401	14	115.5± 42.8	112.57± 46.27	>0.8

Table 1 shows the mean and SDs of LDL-C estimated by direct assay and Friedewald's method. They are grouped according to their TG levels. There was significant difference between the two methods at TG levels 1-100 and 101-200 mg/dl (p<0.02, <0.01 respectively). There was no significant difference at TG levels > 200 mg/dl.

Table 2. Mean and SDs (in mg/dl) of direct LDL-C and friedewald LDL-C at each category of total cholesterol (TC)

TC range	n	Mean ± SD direct LDL-C	Mean ± SD friedwald LDL-C	p
50-99	31	40.29± 15.23	37.65± 14.12	>0.4
100-149	180	68.06± 15.68	68.05 ± 16.26	>0.9
150-199	356	95.81± 17.06	100.01 ± 16.85	<0.001
200-249	241	122.17± 22.09	131.75 ± 19.34	<0.001
≥ 250	85	156.74± 35.39	175.76 ± 29.38	<0.001

This table shows the mean and SDs of LDL-C levels at different categories of Total Cholesterol. There was statistically significant difference in the means of LDL-C levels obtained by the two methods at TC levels >150 mg/dl (p<0.001). The difference was not seen at TC levels <150 mg/dl.

Table 3. LDL-cholesterol difference (direct-Friedewald estimation) at different triglyceride levels

S.No.	TG levels In mg/ dl	n	Mean ± SD	p
1	1- 100	205	-7.86± 17.95	<0.001
2	101- 200	477	-5.73± 27.45	<0.001
3	201- 300	163	-3.15± 18.21	<0.001
4	301- 400	34	0.94± 19.83	>0.1
5	^{3}401	14	3.36± 20.83	>0.1

Table 3, the difference in LDL-C obtained by direct assay and Friedewald estimation is shown. The mean difference ranged for - 7.86 to 3.36 mg% at various levels of TG. The difference was significant at TG levels 1-100, 101-200 and 201-300 whereas it was not significant at TG levels of 301-400 and > 401 (p>0.1).

Table 4. Classification of patients by the two methods of LDL-C measured

LDL-C levels	By direct assay (%)	By Friedewald estimation (%)
< 130 mg/dl	736 (82.4%)	683 (78.48%)
> 130 mg/dl	157 (17.58%)	210 (23.5%)
Total no. of patients	893 (100%)	893 (100%)

Patients were classified as low and high cardiac risk taking 130 mg% LDL-C as cut- off levels. By Direct assay, 17.58% of patients had high risk and 23.5% of patients by Friedewald's estimation.

Table 5. Percentage error of the means of LDL-C estimated by two methods

TG range	n	Mean of Friedewald LDL-C	Mean of direct LDL-C	% Error
1-100	204	92.94	84.91	8.64
101-200	478	107.83	101.092	6.25
201-300	163	121.68	118.276	2.79
301-400	34	111.76	112.706	-0.846
≥ 400	14	112.57	115.5	-2.60

The percentage error between Friedewald LDL-C and Direct ranged from 8.64% to -2.6% at different TG levels. The error was more at TG levels 1-100 and 101-200 mg%.

cholesterol levels of 150- 199, 200- 249 and ≥250-mg/ dl. The LDL-C estimated by direct homogenous method and by Friedewald calculation showed a mean of -7.86 to 3.36 (Table 3). At lower TG levels that are from 1- 300 mg/ dl Friedewald estimation was higher than direct method and the difference was highly significant (p <0.001). At TG levels >301 mg/ dl the direct method showed a higher value than Friedewald's estimation but the difference was not significant (p>0.1). Table 4 shows that there was a discrepancy of 53 patients classified as high or low cardiac risk groups by the two methods used. In our study 53 out of a total of 893 patients constitute 5.93%. The percentage error of the means of LDL-C estimated

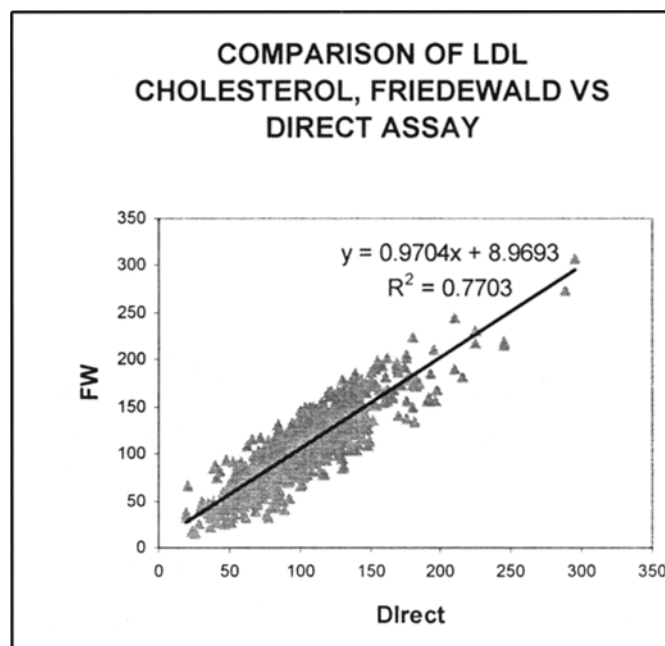

Fig. 1. Scatter plot of LDL cholesterol estimated by direct assay against Friedewald's estimation. There was a correlation of r^2 = 0.77 and r = 0.88

by two methods at different TG levels ranged from -2.60 to 8.64 % (Table 5). This is within the NCEP recommendation of = 12%. Figure 1 showed a good correlation between LDL-C levels obtained by Friedewald and direct methods, r2= 0.77, r= 0.88.

DISCUSSION

Although variation has been demonstrated in the TG/Cholesterol ratio in VLDL, it does not appear to influence the Friedewald formula as suggested by Lippi *et al.* (24). A previous study showed that variation in the TG/Cholesterol ratio affects the estimation of VLDL; however this variation is suggested to result in only a 7-10% error in the LDL-C estimation when the Friedewald formula is used (25). Thus the use of Friedewald formula has been compared to LDL-C determination in several studies involving large populations and samples with various TG concentrations. Apart from the time saved and the cost effectiveness of using the calculation instead of direct estimation, the conclusion was that the use of Friedewald formula for estimation of LDL-C is a reliable alternative when HDL-C and total cholesterol are determined. With increasing costs in the clinical laboratory, use of the Friedewald formula provides a cost saving and reliable estimate of LDL-C when appropriately used. In the opinion of Lippi *et al.*, deterred use of Friedewald formula on the basis of heterogeneity of VLDL is not recommended (24).

On comparing LDL-C values obtained from Friedewald's formula with that obtained by ultra centrifugation method yielded correlation coefficients of 0.94 to 0.99 depending on the patient population (13). In our study, on comparing Friedewald's estimation with direct homogenous assay for LDL-C showed a correlation coefficient of 0.88 (Fig 1). Comparison of homogenous methods for assay of LDL-C with ultra centrifugation showed high correlation of r= 0.95-0.98, however cross reactivity with IDL ranged from 31-64% for three methods compared, that was LDL-EX, Cholestest LDL and Determinor-L (26). Cholestest LDL that was used in our study had 47% cross reactivity with IDL (26).

In our study, we found significant difference in direct homogenous method and Friedewald's estimation for LDL-C, particularly in TG levels of <300 mg%. The difference was not significant at higher TG levels of >300 mg%. This is probably because the homogenous method, which uses unique detergent to selectively solubilize LDL-C, may mask or remove LDL-C species during VLDL and chylomicrons exclusion steps (27). Rifai N. et al in a similar study demonstrated that homogenous assays tend to have a negative bias (17) at the low TG concentrations. As a result it tends to misclassify hypercholesterolemic subjects into lower risk category (27). Despite the limitations, direct assays demonstrate adequate specificity that make them useful in following subjects with established

hypercholesterolemia, in nonfasting samples obtained from children and Type 1 Diabetes Mellitus (27). Its use is justified in situations when the Friedewald method is not suitable or type 3 dysbetalipoprotieinemia and in fasting subject with hypertriglyceridemia (28).

The surfactant method seems to underestimate LDL-C with the bias being independent of LDL-C concentration (9,17, 27). Nevertheless total analytical error was within the NCEP goals of 12% at all LDL-C medical decision points (17) because the excellent precision compensates for the bias (9). The NCEP Expert Panel observed in experienced and well standardized lipid laboratories that total analytical variability in calculated LDL-C averaged 4.0% ranging between 2.7% and 6.8% for LDL-C concentrations between 100- 225 mg/dl (29). In routine laboratories, the variability was much higher, that was averaging 12%. In order to achieve the requisite analytical performance using Friedewald calculation, The Panel recommends development of more precise direct methods or adoption of fully automated homogenous methods for HDL-C estimation, which is expected to improve imprecision, including the contribution to calculated LDL-C (29).

The availability of the homogenous methods for LDL-C, which are capable of full automation and well suited for workflow patterns in the modern clinical laboratory, raises questions, about their appropriate use. In spite the technical disadvantages of the Friedewald method it is firmly entrenched in routine practice and will likely be displaced only when the homogenous methods can demonstrate clear advantages in performance and overall cost effectiveness. Three out of five homogenous methods have shown to give the results comparable to the Friedewald calculation and appear to meet NCEP performance criteria (26, 30, 31).

In two large studies consisting of 10,000 participants compared calculated LDL-C with LRC-BQ ultracentrifugation method and found encouraging results. With NCEP decision points of 130, 130-160 and 160 mg/dl, 86-88% of the participants were classified correctly (15, 32). Only 5% were classified one medical decision point low, 6% as one medical decision point high and 0.4% were misclassified two medical decision points high. In a Finnish study, only 2% were classified over two medical decision point levels (12).

The Adult Treatment Panel III recommendation to include TC, HDL-C, TG and LDL-C in screening all adults does not favor estimation of LDL-C with direct homogenous method (10). A thorough assessment of the cost effectiveness would also require consideration not only of the actual measurement cost, but also of the less tangible costs of measurement errors leading to inappropriate treatment decisions.

According to NCEP, the desirable LDL-Cholesterol limit is < 130-mg/dl. In our study 53 patients more were found to be at high risk by Friedewald estimation or 53 patients out of a total of 893 were at no cardiac risk by direct homogenous LDL-C measurement. If those 53 patients as seen by Friedewald estimation were treated as high risk and were given cholesterol lowering agents that would be of no harm but have long term benefits. However, if those patients as seen by direct assay were classified as low risk and not actively managed, they might later present with serious irreversible complication of coronary heart disease. Apart from the cost of treatment, it increases the morbidity and mortality rate. Yu *et al.* (27) had concluded that direct LDL-C assays have limited diagnostic utility and should not be used in screening.

Nauck *et al.* (33) in their review on LDL-C direct assays compared with calculated LDL-C concluded that there is evidence which supports recommending the homogenous assays for LDL-C to supplement the Friedewald calculation in those cases where calculation is unreliable, eg; triglycerides > 4000 mg/l. They also said that before the homogenous assays can be confidently recommended to replace the calculation in routine practice, more evaluation is needed (33). Miller (34) reported that the homogenous LDL-C results do not improve the performance of LDL-C calculated by Friedewald equation at triglyceride concentrations of <4000 mg/l. This is consistent with our work where we have got significant difference in LDL-C levels at lower TG levels (Table. 1). The Third Adult Treatment Panel (ATP III) of the NCEP has recommended the use of non-HDL cholesterol as a secondary target of lipid lowering, after achieving adequate control of LDL-C and if TGs are elevated (^{3} 200 mg/dl) (10). Because of its simple calculation, the non-HDL cholesterol level is easily available to the clinician with every lipid profile ordered, thus eliminating any additional costs (35). Whiting et al have reported that the error of an immunoseparation technique for direct LDL-C as a function of hypertriglyceridemia in diabetic patients is greater than that of Fridewald calculation. In contrast, the non-HDL Cholesterol level of the hypertriglyceridemic patient would still be available to the clinician, and could potentially be more accurate than either the directly measured or the calculated LDL-C level (35). Lu *et al.* (37) highlights the predictive value of non-HDL Cholesterol for CHD and the role that it may play in the management of Diabetic dyslipidemia. We have thus taken up a study to evaluate the predicative value of LDL-C and non-HDL Cholesterol in our center.

A reliable method for the measurement of serum LDL-C concentration suitable for routine use in the Clinical Laboratory is definitely needed. However, until then,

clinical chemists should recognize the limitations of the currently available methodologies.

In conclusion, the Friedewald's estimation for LDL-C can be used for screening of patients as high or low cardiac risk groups. The direct LDL-C assay is an expensive method and is going to turn out more expensive as it classifies more patients as low risk and hence prevents their early management. Direct assay should be used in patients where Friedewald's estimation is limited, as in non fasting serum (38), subjects with established hypercholesterolemia and when Friedewald method is invalid as when TG levels are >400 mg/dl. More work is needed to validate the direct assays and explain the low values obtained as compared to Friedewald's estimation.

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