

*East African Medical Journal Vol. 94 No. 3 March 2017*

## COMPARISON OF DIRECT AND PRECIPITATION METHODS FOR THE ESTIMATION OF MAJOR SERUM LIPOPROTEINS

J. M. Mulinge, BSc, MSc, Department of Medical Laboratory Sciences, School of Medicine and Health Sciences, Kenya Methodist University, P.O Box 45240-00100, Nairobi, Kenya, S. K. Waithaka, BSc, MSc, Department of Medical Laboratory Sciences, School of Health Sciences, Mount Kenya University, P. O. Box 342-01000, Thika, Kenya and S. N. Kaggia, MBChB, MMed, Department of Medical Medical Pathology, College of Health Sciences, Jomo Kenyatta University of Agriculture and Technology, P.O box 62000-00200, Nairobi, Kenya

Request for reprints to: J. M. Mulinge, Department of Medical Laboratory Sciences, School of Medicine and Health Sciences, Kenya Methodist University, P.O Box 45240-00100, Nairobi, Kenya

## COMPARISON OF DIRECT AND PRECIPITATION METHODS FOR THE ESTIMATION OF MAJOR SERUM LIPOPROTEINS

J. M. MULINGE, S. K. WAITHAKA and S. N. KAGGIA

### ABSTRACT

**Background:** There is increase in use of direct assays for analysis of high and low density lipoprotein cholesterol by clinical laboratories despite differences in performance characteristics with conventional precipitation methods. Calculation of low density lipoprotein cholesterol in precipitation methods is based on total cholesterol, triglycerides and high density lipoproteins, thus may cumulatively carry errors of individual methods. Adoption of direct assays is expected to decrease turnaround time and save on cost.

**Objectives:** To compare direct and precipitation methods for estimation of major serum lipoproteins.

**Design:** Cross sectional study.

**Setting:** Clinical Chemistry Laboratory, Kenyatta National Hospital, Nairobi, Kenya.

**Subjects:** Three hundred and eighty four (384) participants were recruited for the study.

**Results:** There was no significant difference in high density lipoprotein cholesterol estimated by direct and precipitation methods  $p=0.091$  as well as low density lipoprotein cholesterol estimated by direct method and Friedwald's formulae  $p=0.093$ .

**Conclusion:** Both direct and precipitation methods give similar results. Selection should be based solely on workload, availability and technical expertise.

### INTRODUCTION

High density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) are major lipoproteins of cholesterol in human plasma(1) and major transporters of cholesterol in human plasma, therefore measurements of these markers have been proposed as primary tools for risk assessment and monitoring of patients with risk of developing cardiovascular disease(2).

Total cholesterol in humans is distributed primarily among three major lipoprotein classes: very low density lipoproteins cholesterol (VLDL-C), low density lipoproteins cholesterol (LDL-C), and high density lipoproteins cholesterol (HDL-C). Smaller amounts of cholesterol are also contained in two minor lipoprotein classes: intermediate density lipoprotein (IDL) and Lipoprotein (a)(3). In normal individuals, the minor lipoprotein classes can be expected to contribute on average about 0.0621mmol/L to the

total cholesterol measurement. Several studies have indicated that there is an inverse relationship between the risk for coronary heart disease and the plasma concentration of high density lipoprotein (HDL-C) cholesterol(4). Possible mechanisms by which HDL-C might play a direct protective role have also been studied as well as conditions associated with elevated or depressed HDL-cholesterol levels. These investigations have led to an increased interest in the routine determination of HDL-cholesterol levels to aid in the assessment of risk for ischemic heart disease, as well as interest in further studies of the putative protective effect of HDL-C, and have stimulated efforts to understand better the capabilities and limitations of quantitative HDL-C methods (5). There is increase in use of direct assays for analysis of high and low density lipoprotein cholesterol by clinical laboratories despite differences in performance characteristics with conventional precipitation methods. Calculation of low density lipoprotein cholesterol in precipitation

methods is based on total cholesterol, triglycerides and high density lipoproteins, thus may cumulatively carry errors of individual methods. Adoption of direct assays is expected to decrease turnaround time and save on cost(6).

Precipitation method involves precipitation of Apo -b lipoproteins followed by centrifugation at 3000g, for 10 minutes, the supernatant is used for estimation of HDL-C using a method similar to that of total cholesterol(6). LDL-C is estimated by use of Friedwald's formulae which is based on total cholesterol (TC), Triglycerides (TG's) and High density lipoproteins cholesterol (HDL-C).

Measurements of HDL-C and LDL-C by direct methods offer the potential to improve both analytical and biological variability, since precision of HDL-C and LDL-C measurement does not depend upon the analytical variability in measurement of total cholesterol and low levels cholesterol in supernatants after precipitation(7). Capabilities and limitations of quantitative HDL-C methods is not known, the study intended to compare quantitative analytical methods used for estimation of the major serum lipoproteins.

In clinical practice, LDL-C is either estimated by the Friedewald's formula or directly measured with a homogeneous assay. Since the calculation is based on serum TG, TC, and HDL-C, it necessarily includes the accumulated errors in all the three measurements(8). Despite the widespread belief that the calculation or measurement of LDL or HDL cholesterol is standardized and reproducible, data indicates that results can vary significantly with methods from different manufacturers, and calculated LDL cholesterol may not agree with measured LDL cholesterol(9). Limitations of the Friedewalds equation were recognised early including the fact that calculation is not valid for specimens having triglycerides >4.52mmol/l for patients with Type III hyperlipoproteinemia or chylomicronemia, or with non-fasting specimens. In fact, the equation is increasingly inaccurate with TG from 2.28 to 4.56 mmol/l(9). Despite the continued efforts to standardize LDL-C and HDL-C analytical methods results have shown method to method variation. Calculated LDL-C may not agree with measured LDL-C cholesterol while direct HDL-C assays have also had reliability issues which relate to ambiguity in definition and heterogeneity of LDL-C and HDL-C particles(9).

Precipitation methods for HDL-C involves multiple pipetting and centrifugation steps which are dependent on experience of the technologist. Automated assays are bound to improve variability of results of serum lipoprotein measurements as it avoids the need to centrifuge and multiple pipetting. The aim of the study was to compare direct assays and precipitation methods used for estimation of serum lipoproteins.

## MATERIALS AND METHODS

*Samples:* Serum samples from 384 adults comprising of 192 males and 192 females received at Clinical Chemistry Laboratory, Kenyatta national Hospital, and Nairobi were used. Only those where a full fasting lipid profile was requested were considered. The specimen was collected from study participants after (8-12 hours) fasting and placed in plain vials. The serum was separated by centrifugation and was used to estimate various parameters using Mindray BS 800 Clinical Chemistry analyzer (Shenzhen-Mindray Biomedical and Electronics Company Limited China).

*Total cholesterol and Triglycerides assay:* Total cholesterol was estimated by Cholesterol Oxidase/Peroxidase Enzymatic Method (10). Using recommended procedure by Mindray-Shenzhen, China. Cholesterol ester was catalyzed cholesterol ester hydrolase (CHE) and cholesterol oxidase (CHO) to yield H<sub>2</sub>O<sub>2</sub>, which oxidated 4- Aminoantipyrine with phenol to form a colored dye of quinoneimine.

Triglycerides were estimated using Glycerol-3-Phosphate/ Peroxidase Enzymatic Method(11,12), using recommended procedures and reagents Mindray-Shenzhen, China. Through a sequence of enzymatic catalysis steps by lipase, Glycerol kinase and Glycerol phosphate dehydrogenase, triglycerides was catalysed to yield hydrogen peroxide which oxidize 4-Aminoantipyrine to yield a colored dye of quinoneimine.

*HDL-C assays:* HDL-C was estimated by precipitation method(13,14) using cholesterol liquid colour test kit manufactured by human diagnostics, Gesellschaft für Biochemical and Diagnostic mbH, Wielsbaden-Germany and distributed in Kenya by Chemlabs (E.A) Limited. The method is Based on selective precipitation of Very low density lipoproteins(VLDL), low density lipoproteins(LDL) and Lipoprotein(a) (LP(a) by phosphotungstic acid /magnesium chloride(MgCl<sub>2</sub>), sedimentation of precipitant by centrifugation and subsequent enzymatic analysis of high density lipoproteins (HDL) as residual cholesterol remaining in clear supernatant by CHO-PAP Method for total cholesterol.

HDL-C was measured directly in serum (15) using manufacturer recommendations by Mindray, Shenzhen- China. HDL-C particles were protected by surfactant as LDL-C, VLDL-C and chylomicrons were removed by cholesterol esterase (CE), and cholesterol oxidase (CO). HDL-C particles in the presence of second surfactant and peroxidase, generated hydrogen peroxide which reacts with 4-amino-antipyrine and HSDA to form a purple-blue dye. The color intensity of the dye was directly proportional to concentration of HDL-C and was measured photometrically at 585nm.

**LDL-C assays:** LDL-C estimated directly (16) using recommendations by Mindray Diagnostics, Shenzhen-China of protecting agent that protected LDL-C from enzymatic reactions. LDL-C was then catalyzed by cholesterylesterase (CE) and cholesteryl oxidase (CHO) to cholestenone and hydrogen peroxide which reacted of 4-aminoantipyrin in presence of peroxidase to form a coloured dye quinoemine.

Low density lipoprotein cholesterol (LDL-C) was estimated using the Friedewald formulae (18), which states that  $LDL-C = TC - (TG/2.2 + HDL-C)$ .

**Quality control:** An internal quality control for each parameter was included in each analytical session throughout the study period. Quality control results for the analysed parameters were within the specific assigned QC range.

**Statistical approach:** Paired t test was used for comparison of mean difference at an alpha value of  $p=0.05$  to assess significant difference using SPSS version 20. Any  $p$ -value  $\geq 0.05$  was considered not statistically significant while  $p$ -value  $\leq 0.05$  was

considered to be statistically significant.

**Ethical approval:** The study was cleared by Kenyatta National Hospital and University of Nairobi ethics and research committee (approval no P497/08/2014)

## RESULTS

Result of the 384 samples analysed by precipitation methods of HDL-C ranged from 0.35 to 2.86 mmol/l (mean 1.51) and LDL-C from 0.18 to 5.80 mmol/l (mean 2.89).

In direct method, the HDL-C ranged from 0.38 to 2.90 mmol/l (mean 1.52 mmol/l) and LDL-C from 0.1 to 5.80 mmol/l (mean 2.89).

The paired t test showed there was no statistical significant difference between precipitation and direct methods of HDL-C  $p=0.76$ , there was no significant difference between precipitation (Friedewald estimation) and direct methods LDL-C  $p=0.89$  as shown in table 1 below.

**Table 1**

*Results of HDL-C and LDL-C by Precipitation and Direct methods*

	Precipitation	Direct method	p-value
Total samples	384	384	384
HDL-C	1.52 ± 0.47	1.51 ± 0.47	0.76
LDL-C	2.89 ± 1.02	2.88 ± 1.01	0.89

There was no significant difference in Low density lipoproteins estimated by direct and Friedewald's formulae at different triglyceride ranges with  $p=0.89$  at triglyceride (TG) levels  $<1.14$  and  $p=0.97$  at TG levels  $>3.42$  mmol/l as shown in table 2 below.

**Table 2**

*Comparison of serum LDL-C by direct and Friedewald's formulae*

TG* (mmol/L)	N*	LDL-C(D)* (mmol/L)	LDL-C(FF)* (mmol/L)	p-value
<1.14	88	2.86 ± 0.94	2.88 ± 0.96	0.89
1.15-2.28	215	2.88 ± 0.99	2.90 ± 0.01	0.77
2.29-3.42	71	2.81 ± 0.07	2.82 ± 1.06	0.93
>3.42	10	3.48 ± 1.47	3.50 ± 1.48	0.97

TG\*-Triglycerides, N\*-Study population, LDL-C (D)\*-Low density lipoprotein cholesterol by direct method, LDL-C (FF)\*-Low density lipoprotein cholesterol by Friedewald's formulae.

The paired t test for comparison of mean differences was not significant at  $p=0.093$  for HDL-C and  $p=0.091$  for LDL-C as shown in table 3 below.

**Table 3**  
*Paired Samples Test for comparison of HDL-C and LDL-C by direct assay and precipitation/friedwald's formulae*

Analyte	Paired Differences		S.E* of Mean	95% C.I of the Difference		t	df	Sig. (2-tailed)
	Mean(mmol/L)	SD*(mmol/L)		Lower	Upper			
	HDLC	0.016		0.187	0.010			
LDLC	0.015	0.179	0.009	.034 .002	-1.697	383	0.091	

SD\*-standard deviation, S.E\*-Standard error, C.I\*- Confidence interval

## DISCUSSION

The study compared direct assays with precipitation /friedewald's formulae for the estimation of major serum lipoproteins. LDL-C/HDL-C ratio increase by 1 unit increases the risk of myocardial infarction by 53(16).

The paired t-test on comparison of LDL-C by direct method and friedewald's formulae showed no statistically significant difference at  $p=0.091$ , The results differed with findings of a study by Chartejee, C. *et al.*, (2011) whose results were statistically significant at  $p<0.001$ , hence depicting that there was a significant difference in LDL-C measurements by direct and Friedewald's equation at  $p=0.01$ (17). Friedewald's formula was first developed in 1972(18), 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 2.2 for mmol/l units. LDL-C was then calculated as Total Cholesterol minus HDL-C minus estimated VLDL-C.

The mean difference in HDL-C concentration by direct assay and precipitation method was not statistically significant  $P=0.093$ , in agreement with other studies. Arranz-Pena *et al.*, in their study showed close correlation of direct assay and several precipitation methods(19), Nauck, M. *et al.*, also showed that homogeneous assays produce precise and accurate HDL cholesterol concentration even for hypertriglyceridemia samples up to 10.26 mmol/l, the use for patients with higher triglycerides provides an advantage for use of direct techniques in non-fasting samples(20). In the current study direct and

precipitation assays were found to be reliable, this is consistent with study by Jabar *et al.*,(2006) who found both Precipitation method and direct method precise and accurate in estimation of HDL-C and LDL-C, however direct assays have an advantage of time saving and are less labour intensive(21). Use of direct assays can improve reliability of the results of lipoprotein testing because it avoids the precipitation and centrifugation steps which depend on technical skills and experience of the laboratory technologist. The precipitation method for HDL-C testing is highly dependent on pipetting skills of the technologist, the centrifuge speed and hence can lead to variabilities related to personnel hence direct method can provide a better alternative.

In conclusion, results obtained by direct and precipitation/calculated methods of serum lipoprotein measurement are comparable and the methods can be used alternately. The choice of the technique should depend on amount of workflow, technical competence. Despite this direct assays have the capacity to improve precision in laboratory analysis of HDLC and reduce errors due to inaccurate pipetting when performing precipitation assays.

## REFERENCES

1. Gregory, L.C. Lipids and lipoproteins. In: Christenson, R.H., Gregory, L.C. and Johnson, L.J. Appleton and Lange's outline review clinical chemistry USA McHill 2001; pp. 27-50.
2. Benlian, P., Cansier, C., Hennache, G. *et al.* Comparison of a new method for the direct and simultaneous assessment of LDL and HDL-cholesterol with ultracentrifugation and established methods *Clin. Chem.* 2000; **46**:493-505.

3. Bachorik, P.S. and Ross, J.W. National cholesterol education program recommendations for measurement of low density lipoprotein cholesterol: executive summary. *Clin. Chem.* 1995; **41**:1414-1420.
4. Grundy, S.M., Cleeman, J.I., Merz, C.N. *et al.* Implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III Guidelines. *J. Am. Coll. Cardiol.* 2004; **44**:720-732.
5. Miller, W.G., Myers, G.L., Sakurabayashi, I. *et al.* (seven direct methods for measuring HDL and LDL cholesterol compared with ultracentrifugation reference measurement procedures. *Clin. Chem.* 2010; **56**:977-986.
6. Warnick, G.R., Nauck, M. and Rifai, N. Evolution of methods for measurement of HDL-cholesterol: from ultracentrifugation to homogeneous assays. *Clin Chem* 2001; **47**:1579-96.
7. National cholesterol education programme-Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults Adult Treatment Panel III. *JAMA* 2001; **285**:2486-2497.
8. Baruch, L., Agarwal, S., Gupta, B., *et al.* Is directly measured low-density lipoprotein clinically equivalent to calculated low-density lipoprotein? *J. Clin Lipidol.* 2010; **4**:259-264.
9. Contois, J.H., Warnick, G.R. and Sniderman, A.D.. Reliability of low-density lipoprotein cholesterol, non-high density lipoprotein cholesterol, and apolipoprotein B measurement. *J. Clin. Lipidol.* 2011; **5**:264-272.
10. Meattini, F., Prencipe, L., Bardelli, F., *et al.* The hydroxybenzoate/ 4- aminophenazone chromogenic system used in the enzymatic determination of serum cholesterol. *Clin. Chem.* 1978; **24**:2161-2165.
11. Bucolo, G. and David, H. Quantitative determination of serum triglycerides by use of enzymes. *Clin. Chem.* 1973; **19**:476-482.
12. Fossati, P. and Prencipe, L. Serum triglycerides determined calorimetrically with an enzyme that produces hydrogen peroxide. *Clin. Chem.* 1982; **28**: 2077-2080.
13. LA, Pesce A.J. Clinical chemistry, theory, analysis, correlation. 3rd ed. New York: Mosby, 1996; pp. 640-81.
14. Khan F.A. and Dilawar, M. Lipids and lipoproteins. Karamat, A.K., Anwar, M. Butt, T., *et al.* *Manual of Laboratory Medicine.* 2nd ed, Pak: AFIP, 2003; pp.403-6.
15. Isezaki, M., Shirahata, K., Seto, H. *et al.* *J. Clin. Lab. Inst. Reag.* 1996; **19**:349-353.
16. Stampfer, M.J., Sacks, F.M., Salvini, W.C. *et al.* A prospective study of cholesterol, apolipoproteins and the risk of myocardial infarction. *N. Engl. J. Med.* 1991; **325**:373-81.
17. Chatterjee, S. and Mendez, D. A comparative and correlative study of direct LDL assay with friedewald's formula in rural Kolar population *Clin. Biomed. Sci.* 2011;(4).
18. Friedewald, W.T., Levy, R.I. and Fredrickson, D.S. Estimation of the concentration of low density lipoprotein cholesterol in plasma, without the use of preparative centrifuge. *Clin. Chem.* 1972; **18**:499-502.
19. Arranz, P., Maria, L., Juan, T.M., *et al.* Comparison of two homogeneous assays with precipitation method and an ultracentrifugation method for the measurement of HDL-cholesterol. *Clinical Chemistry* 1998; **44**:122499-2505.
20. Nauck, M., Marz W. and Wieland, H. New immunoseparation-based homogeneous assay for HDL cholesterol compared with three homogeneous and two heterogeneous methods for HDL cholesterol. *Clin. Chem* 1998; **44**: 7 p.1443-1451.
21. Jabbar, J., Siddiqui, I. and Raza, Q. Comparison of two methods (Precipitation Manual and Fully Automated Enzymatic) for the Analysis of HDL and LDL Cholesterol. *Journal Pak Med. Ass.* 2006; **56**:2 p59-61.