A Simple Economical Method for Assay of Atherogenic Small Dense Low-Density Lipoprotein-Cholesterol (sdLDL-C)

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Abstract In the present study, we report a simple and economical precipitation method for the quantitative determination of small, dense LDL-cholesterol (sdLDL-C) in serum that is considered to be an emerging risk factor for cardiovascular disease. This method consisted of precipitation of lipoproteins of density <1.044 g/ml using heparin-MnCl₂ and quantification of cholesterol existed in the supernatant using reagents for routine cholesterol assay instead of the costly direct low density lipoprotein-cholesterol assay kit. The supernatant contained sdLDL and high-density lipoprotein (HDL) that was confirmed by polyacrylamide gel electrophoresis. sdLDL-C concentration can be calculated by subtracting the HDL-C value from the total cholesterol concentration of the supernatant. sdLDL-C values obtained by this modified method were similar to those obtained by direct assay of sdLDL-C and there was significant correlation between the two methods. In conclusion, this method is highly economical, do not require special equipments and is useful to evaluate atherogenic risk.

Keywords Coronary heart disease · Lipoproteins · Small dense LDL · Atherosclerosis

Introduction

Coronary Heart Disease (CHD) is the leading cause of death and disability in developed nations and is increasing rapidly in the developing world [1]. In the past three

decades, heart disease rates in India have doubled in rural areas and tripled in urban areas due to rapid changes in life style consequent to economic development [2, 3]. One of the most important advances in medicine has been the identification of the major risk factors for CHD [4]. Earlier studies in Asian Indians have shown that classical risk factors do not always explain the excess of CHD seen in this ethnic group.

Lipoproteins play a pivotal role in atherogenesis. Low Density Lipoproteins-Cholesterol (LDL-C) is a major target in the guidelines for the prevention of CHD. However, plasma LDL-C levels are insufficient to identify individuals with incidence of CHD events, because approximately 50% of all CHD events occur in persons with normal or even low LDL-C concentrations [5]. LDL is composed of heterogeneous particles differing in density, size and chemical composition. It is reported that people with predominance of small, dense LDL (sdLDL) have a 3-fold increased risk of CHD. Recently, sdLDL has been highlighted as a new potent risk marker for CHD in Westerners and also in Japanese populations, which has relatively lower LDL-C levels [6]. Smaller LDL particles are more susceptible for oxidation *in vitro*, have lower binding affinity for the LDL receptors and lower catabolic rate, have a higher concentration of polyunsaturated fatty acids and potentially interact more easily with proteoglycans of the arterial wall and are better able to penetrate the intima and uptake by macrophages—the early steps of atherogenesis. The observed increase in risk forms the basis of the rationale in using LDL particle size as an adjunct to the standard proven means of risk assessment [7, 8].

LDL size is generally measured by gradient gel electrophoresis and ultracentrifugation techniques, which are laborious and time consuming and need special equipments. The objective of the present study was to standardize a

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simple precipitation method for the rapid quantitation of sdLDL-C using reagents for cholesterol assay and to demonstrate the prevalence of sdLDL phenotype in healthy subjects having different lipid levels.

Materials and Methods

The study consisted of 25 healthy volunteers both males and females from the age group of 20–60 years. Out of these, 20% were diabetic, 16% had hypertension and 20% were overweight (Body Mass Index > 25). Fasting serum samples were collected from all these subjects and the following biochemical parameters were done. Serum total cholesterol and triglyceride concentrations were estimated by standard enzymatic assay method [CHOD-POD and GPO-POD, respectively] using SPINREACT Kits (Spain) [9, 10]. HDL-C was quantitated by polyethylene glycol method as described by Izzo et al. [11]. LDL-cholesterol was calculated using the Friedewald's formula as LDL-C = [total cholesterol – (HDL-C + (TG/5))] mg%. For additional experiments, we have used Direct LDL and Direct HDL assay reagents from Olympus Life and Material Science, Germany. The entire assay was carried out using UV-VIS spectrophotometer (Shimadzu-1601 PC).

The sdLDL-cholesterol content in serum was quantitated according to the method described by Tsutomu Hirano et al. [12] with a minor modification. For this, precipitation of lipoproteins of density <1.044 g/ml, which include very low density lipoprotein (VLDL), Intermediate density lipoprotein (IDL), and large buoyant low density lipoprotein (IbLDL), were carried out using the precipitation reagent containing 40 U/ml heparin sodium salt and 30 mmol/l MnCl_2 . In brief, 0.1 ml of precipitation reagent was mixed with 0.1 ml of serum, incubated for 10 min at 37°C, and then placed in ice bath for 15 min. After which, the supernatant was collected by centrifugation at 15,000 rpm for 15 min at 4°C. The total cholesterol content in the supernatant (sdLDL and HDL) was quantitated using cholesterol assay kit, which permits the calculation of sdLDL as: sdLDL = (total cholesterol in the supernatant – HDL-C). Characterization of the lipoproteins was carried out by 3.5% polyacrylamide gel electrophoresis as described by Frings et al. [13].

Statistical analysis was carried out for the comparison of mean differences between groups. For this, Students 't' test and correlation co-efficient 'γ' were employed and a value of $P < 0.05$ was considered statistically significant.

Results and Discussion

Using heparin- MnCl_2 precipitation method, lipoproteins with density <1.044 g/ml (VLDL, IDL, IbLDL) can be

selectively precipitated. The supernatant contains lipoproteins with densities >1.044 g/ml, (sdLDL and HDL). To ascertain whether heparin- MnCl_2 precipitate contains only IbLDL, while sdLDL remains in the supernatant, we performed electrophoresis of serum and its corresponding heparin- Mn^{2+} supernatant on 3.5% polyacrylamide gels (Fig. 1). As shown in the figure, LDL band (sdLDL) was somewhat faint in the supernatant of serum compared to original serum pattern, which contained all the LDL particles. HDL-C in the supernatant corresponded with the original serum indicates that the HDL was not precipitated

Fig. 1 Electrophoretogram of serum lipoproteins and corresponding supernatant with sdLDL after precipitation of lipoproteins of density <1.044 g/ml with heparin- MnCl_2

Fig. 2 Electrophoretogram of serum lipoproteins and corresponding supernatant with sdLDL <10 mg%, after precipitation of lipoproteins of density <1.044 g/ml with heparin- MnCl_2

Table 1 Serum lipids and sdLDL-cholesterol concentrations in control subjects with different types of lipidemias and in subjects with known coronary heart disease

	Normal lipids LDL-C < 130; TG < 150	High LDL-C > 130; TG < 150	High TG > 150; LDL-C < 130	Combined hyperlipidemia LDL > 130; TG > 150	Subjects with CHD
Sample size (<i>n</i>)	6	9	5	5	6
TGs (mg/dl)	92 ± 18	133 ± 12*	204 ± 95*	183 ± 52*	216 ± 97*
HDL-C (mg/dl)	56 ± 23	42 ± 10	39 ± 11	39 ± 8	36 ± 8*
LDL-C (mg/dl)	113 ± 14	165 ± 32*	106 ± 8	187 ± 36*	183 ± 41*
sdLDL-C (mg/dl)	32 ± 10	48 ± 28	27 ± 16	71 ± 32*	102 ± 42*
% sdLDL-C	28 ± 7	29 ± 15	25 ± 15	38 ± 11	56 ± 19*
Total cholesterol (mg/dl)	188 ± 31	233 ± 40*	186 ± 31	263 ± 47*	263 ± 42*

Values are the mean ± SD; * $P < 0.01$ – 0.05 normal vs. lipidemic

and was completely recovered in the heparin-MnCl₂ supernatant. But, no band corresponding to VLDL was recovered in the supernatant indicating its complete precipitation. Further in few cases, where the sdLDL-C was comparatively low (<10 mg/dl), we could not observe any corresponding sdLDL band in the β -migrating position of LDL band as shown in Fig. 2.

sdLDL-C values were calculated by subtracting the value of HDL-C from the total cholesterol existed in the heparin-MnCl₂ supernatant. The concentrations of sdLDL-C and other lipids and lipoproteins were presented in Table 1. Subjects were divided into distinct categories based on their lipid profile. The upper limits of normal levels of LDL-C and TG were defined according to the criteria of National Cholesterol Education Program. For comparison, lipid profiles of few subjects with known CHD were also included. In normolipidemic subjects, the mean value of sdLDL-C observed by this method was 32 ± 10 mg/dl. This value is almost similar to that reported by Hirano et al. [14] for normolipidemic subjects [sd LDL-C 31 ± 13 mg/dl]. For those subjects having combined hyperlipidemia, the mean level of sdLDL-C was found to be significantly higher than that of normolipidemic subjects ($P < 0.01$). In the present study, we have also observed that in one control subject, despite having normal LDL-C and TG levels, the sdLDL-C concentration was substantially increased (50 mg/dl). Further, the mean sdLDL-C level was found to be 3-fold higher in CHD patients irrespective of the levels of total cholesterol or LDL-C when compared to those control subjects having combined hyperlipidemia.

To confirm the accuracy of this method, we performed another set of experiment using fresh fasting sample ($n = 10$) having mean total cholesterol 167 ± 48; Triglycerides 103 ± 42; HDL-C 45 ± 9; and compared the concentration of sdLDL-C obtained by the above method with that of direct LDL-C assay [12]. The sdLDL-C calculated by this method (27.4 ± 16.6) was similar to that obtained by direct LDL-C assay (28.6 ± 16.9) and there

was an excellent correlation ($\gamma = 0.997$) between the two methods. Further, sdLDL-C calculated alternatively (29 ± 16.2) by subtracting the HDL-C of the supernatant obtained by direct HDL assay, from the total cholesterol content of the supernatant was found to be similar to the above values of sdLDL-C. As HDL-C was completely recovered in the heparin-Mn²⁺ supernatant, sdLDL-C can be calculated by simply subtracting serum HDL-C from the cholesterol content of the supernatant that gives similar results to that of direct LDL assay.

sdLDL particles seem to be the one that cause more atherogenic risk, while the larger LDL particles are much less of a problem. Certain constituents of lipid metabolism, i.e., lipoprotein lipase, hepatic lipase activity, cholesterol ester transfer protein and TG have been shown to contribute to the formation of sdLDL particles. Although ~30% of the subjects with premature CHD have normal lipid values, there has been increasing interest in lipoprotein particle size and composition as additional risk factors for atherosclerosis.

In conclusion, this study suggests that quantification of sdLDL-C can more clearly indicates the overall atherogenic potential than the assessment of LDL-C or total cholesterol. As the present modified method gives results in consistent with that reported by the original one, this simple and economical technique can be easily adopted even in a small clinical laboratory for the rapid quantification of atherogenic sdLDL-C using routine cholesterol assay reagents. We plan to apply this simple method in a large sample size to determine the prevalence of sdLDL and its association with established risk factors for CHD to confirm the atherogenic potential of sdLDL.

References

- Enas EA, Garg A, Davidson MA, Nair VM, Huet BA, Yusuf S. Coronary heart disease and its risk factors in first-generation immigrant Asian Indians to the United States of America. *Indian Heart J.* 1996;48:343–53.

2. Reddy KS, Shah P, Prabhakaran P, Shah B, Prabhakar A, Shrivastava U, et al. Coronary heart disease risk factors in an industrial population in North India. *Indian Heart J.* 1997;49:659.
3. Dhawan J, Bray C. Are Asian coronary arteries smaller than Caucasian? A study of angiographic coronary artery size in estimation during life. *Int J Cardiol.* 1995;49:267–9.
4. Hackam DG, Anand SS. Emerging risk factors for atherosclerotic vascular disease. *JAMA.* 2003;290:932–40.
5. Grundy SM. Role of low-density lipoproteins in atherogenesis and development of coronary heart disease. *Clin Chem.* 1995;41:139–46.
6. Rizzo M, Berneis K. LDL size and cardiovascular risk assessment. *Q J Med.* 2006;99:1–14.
7. Dejager S, Mietus-Synder M, Pitas RE. Oxidized low-density lipoprotein bind to the scavenger receptor expressed by rabbit smooth muscle cells and macrophages. *Arterioscler Thromb Vasc Biol.* 1999;13:371–8.
8. Rajman I, Maxwell S, Cramb R, Kendall M. Particle size: the key to the atherogenic lipoprotein? *Q J Med.* 1994;87:709–20.
9. Naito HK. Cholesterol. In: Kaplan A, editor. *Clinical Chemistry.* St Louis: The C. V. Mosby Co; 1984.
10. Kaplan A. Triglycerides. In: *Clinical chemistry.* St Louis: The C. V. Mosby Co; 1984.
11. Izzo C, Grillo F, Murador E. Improved method for determination of HDL-C, isolation of HDL by use of PEG-6000. *Clin Chem.* 1981;27:371–4.
12. Hirano T, Ito Y, Saegusa H, Yoshino G. A novel and simple method for quantification of small, dense LDL. *J Lipid Res.* 2003;44:2193–201.
13. Frings CS, Foster LB, Cohen PS. Electrophoretic separation of serum lipoproteins in polyacrylamide gel. *Clin Chem.* 1971;17:111–3.
14. Hirano T, Ito Y, Koba S, Toyoda M, Ikejiri A, Saegusa H, et al. Clinical significance of small dense low-density lipoprotein cholesterol levels determined by the simple precipitation method. *Arterioscler Thromb Vasc Biol.* 2004;24:558–63.