

A novel and simple method for quantification of small, dense LDL

Tsutomu Hirano,^{1,*} Yasuki Ito,[†] Haruhisa Saegusa,[†] and Gen Yoshino[§]

First Department of Internal Medicine,* Showa University School of Medicine, Tokyo, Japan; Research and Development Department,[†] Denka Seiken Co., Ltd., Tokyo, Japan; and Department of Laboratory Medicine,[§] Toho University School of Medicine, Tokyo, Japan

Abstract A preponderance of small, dense (sd) LDL is strongly associated with the development of coronary heart disease, but the method for the measurement of sd LDL is too laborious for clinical use. We report a simple method for the quantification of sd LDL that is applicable to an autoanalyzer. This method consists of two steps: first, to precipitate the lipoprotein of density (d) <1.044 g/ml using heparin-magnesium; and second, to measure LDL-cholesterol in the supernatant by the homogenous method or apolipoprotein B (apoB) by an immunoturbidometric assay. The cholesterol and apoB values obtained by the precipitation method (45 ± 26 and 33 ± 20 mg/dl, respectively) were similar to those obtained in the lipoprotein (d = 1.044–1.063) separated by ultracentrifugation (42 ± 22 and 31 ± 17 mg/dl, respectively), and there was an excellent correlation between the two methods for sd LDL-cholesterol ($y = 1.05X + 1$, $r = 0.88$, $n = 69$) and apoB ($y = 1.07X$, $r = 0.90$). Sd LDL values had a significant inverse correlation with LDL size. A high correlation was found between sd LDL-cholesterol and apoB values ($r = 0.94$). Sd LDL value was related to triglyceride, apoB, and LDL-cholesterol, but not to the buoyant LDL level. These results suggest that this precipitation method is a simple and rapid method for the measurement of sd LDL concentration.—Hirano, T., Y. Ito, H. Saegusa, and G. Yoshino. A novel and simple method for quantification of small, dense LDL. *J. Lipid Res.* 2003. 44: 2193–2201.

Supplementary key words cholesterol • apolipoprotein B • precipitation

LDL particles are heterogenous with respect to size and density (d) of lipid composition. Two distinct phenotypes based on LDL particles have been recognized: pattern A, with a higher proportion of large, buoyant LDL particles, and pattern B, with a predominance of small, dense (sd) LDL particles (1, 2, 3). It has been suggested that compared with buoyant LDL, sd LDLs are highly atherogenic as a result of their higher penetration into the arterial wall, their lower binding affinity for the LDL receptor,

prolonged plasma half-life, and lower resistance to oxidative stress (4, 5). Several studies have reported a 2- to 3-fold increase in coronary heart disease (CHD) risk among patients with pattern B (1, 2). We have also reported that sd LDL is highly associated with CHD events in Japanese, an ethnic group with lower LDL-cholesterol levels, compared with Western populations (6, 7). Therefore, sd LDL has been highlighted as a new potent risk marker for CHD.

LDL particle size is usually measured by gradient gel electrophoresis (GGE) using nondenaturing polyacrylamide according to the method of Krauss and Burke (8). However, this procedure requires a long assay time, i.e., overnight electrophoresis, staining, and destaining. Of course, this assay does not allow quantitative determination of sd LDL. Ultracentrifugation is the standard technique for the isolation of the sd LDL fraction (9, 10) and allows quantification of sd LDL. Griffin et al. (10) reported that LDL-III (equivalent to sd LDL, with d = 1.044–1.060 g/ml) concentration was significantly increased in CHD patients, and the relative CHD risk was increased 4.5-fold in individuals having LDL-III concentrations (protein plus lipid) >100 mg/dl, compared with those with lower concentrations. Their study suggests that in addition to measurement of LDL size, quantification of sd LDL is also useful for the assessment of CHD risk. However, the ultracentrifugation technique is too laborious for general clinical use, because it requires special equipment and a long running time.

It is well known that a combination of divalent cations and polyanions precipitates apolipoprotein B (apoB)-containing lipoproteins, which allows for the measurement of HDL-cholesterol. However, we found that the combination of heparin and Mg did not precipitate all of the apoB-containing lipoproteins; the denser part of LDL remained in the supernatant. Here we report a simple precipitation method for the direct measurement of sd LDL in serum

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¹ To whom correspondence should be addressed.
e-mail: hirano@med.showa-u.ac.jp

TABLE 1. Profiles of LDL size, serum lipid, and LDL subfractions determined by the ultracentrifugation method or the precipitation method in total subjects and in subjects with different LDL phenotypes (patterns A and B)

	Total Subjects (n = 69)	Range	Pattern A (n = 36)	Pattern B (n = 33)	P (Pattern A vs. Pattern B)
Mean LDL diameter (nm)	25.6 ± 0.9	22.0–27.0	26.2 ± 0.5	25.0 ± 0.9	<0.0001
Triglyceride (mg/dl)	176 ± 217	32–894	98 ± 39	261 ± 290	<0.002
LDL-cholesterol (mg/dl)	135 ± 45	31–264	129 ± 41	141 ± 48	ns
HDL-cholesterol (mg/dl)	58 ± 18	17–114	68 ± 16	47 ± 13	<0.0001
ApoA-1 (mg/dl)	139 ± 26	81–212	150 ± 22	127 ± 25	<0.0002
ApoB (mg/dl)	100 ± 28	41–188	90 ± 24	111 ± 27	<0.002
ApoE (mg/dl)	4.3 ± 1.8	2.4–15.0	3.9 ± 1.0	4.8 ± 2.4	<0.05
Sd LDL-C (ultracentrifugation) (mg/dl)	42 ± 22	13–133	31 ± 11	54 ± 24	<0.0001
Sd LDL-C (precipitation) (mg/dl)	45 ± 26	9–144	34 ± 17	57 ± 29	<0.0001
Sd LDL apoB (ultracentrifugation) (mg/dl)	31 ± 17	8–94	21 ± 7	42 ± 17	<0.0001
Sd LDL apoB (precipitation) (mg/dl)	33 ± 20	3–103	22 ± 11	45 ± 20	<0.0001
Lb LDL-C (ultracentrifugation) (mg/dl)	74 ± 39	8–167	86 ± 30	61 ± 23	<0.0005
Lb LDL apo (ultracentrifugation) (mg/dl)	44 ± 16	5–95	49 ± 16	38 ± 14	<0.005

Mean LDL particle diameter was determined by 2–16% nondenatured polyacrylamide gel electrophoresis according to the method of Nicolus, Krauss, and Musliner (15). Pattern A was defined as a diameter of ≥ 25.5 nm, and pattern B was defined as a diameter ≤ 25.5 nm. Data represent mean \pm SD. ns = not significant ($P > 0.05$). Small, dense (sd) LDL-cholesterol (LDL-C) or sd LDL apo B was measured by the heparin-Mg precipitation method as described in Materials and Methods. Large, buoyant (lb) LDL-C with $d = 1.019$ – 1.044 g/ml and sd LDL with $d = 1.044$ – 1.063 g/ml were separated by the ultracentrifugation method.

that does not require special equipment and that can be performed by an autoanalyzer. Our method can be readily applied to screening, clinical testing, and other purposes that require rapid analyses of a large number of samples.

MATERIALS AND METHODS

Subjects

Sixty-nine subjects, aged 20 to 72 years, with a wide range of serum lipid levels were enrolled; these included individuals with hyperlipidemia and type 2 diabetes. Their serum lipid and lipoprotein profiles are shown in **Table 1**. Two subjects had chylomicronemia due to lipoprotein lipase deficiency, and their fasting serum triglyceride levels were $>1,000$ mg/dl. We have reported the analysis of the lipoprotein lipase gene in one of these patients (11).

Biochemical analysis

Triglyceride-rich lipoprotein (TGRL, $d < 1.019$ g/ml), large, buoyant LDL ($d = 1.019$ – 1.044 g/ml), sd LDL ($d = 1.044$ – 1.063 g/ml), and HDL ($d = 1.063$ – 1.210 g/ml) were separated from serum by sequential flotation in an ultracentrifuge (himac CS120GX, Hitachi Koki Co., Ltd., Tokyo, Japan) with a S100AT6 rotor (Hitachi Koki Co., Ltd.) according to the method of Havel, Eder, and Bragdon (12). Triglyceride was measured by standard laboratory procedures. Serum apoB, apoA-I, and apoE were determined by an immunoturbidometric assay (Daiichi Chemicals, Tokyo, Japan). LDL-cholesterol was measured by direct homogeneous assay of the serum using detergents (LDL-EX, Denka Seiken, Tokyo, Japan) (13). This assay for LDL-cholesterol determination consists of two reagents. In the first step, hydrogen peroxide is produced from non-LDL-cholesterol (HDL-cholesterol in this study) by cholesterol esterase and cholesterol oxidase and then decomposed to water and oxygen by catalase. In the second step, the LDL-cholesterol remaining in the reaction mixture is subjected to enzymatic reaction by cholesterol esterase and cholesterol oxidase to produce hydrogen peroxide. As catalase is in-

hibited by sodium azide in the second step, the hydrogen peroxide from LDL-cholesterol results in color development in proportion to its concentration in the presence of peroxidase. The entire reaction is complete in 10 min. The actual procedure and accuracy of this homogenous method was described previously (13, 14). HDL-cholesterol was measured by direct homogeneous assay of the serum using detergents (HDL-EX, Denka Seiken). Mean LDL particle diameter was determined by 2–16% nondenatured polyacrylamide gel electrophoresis according to

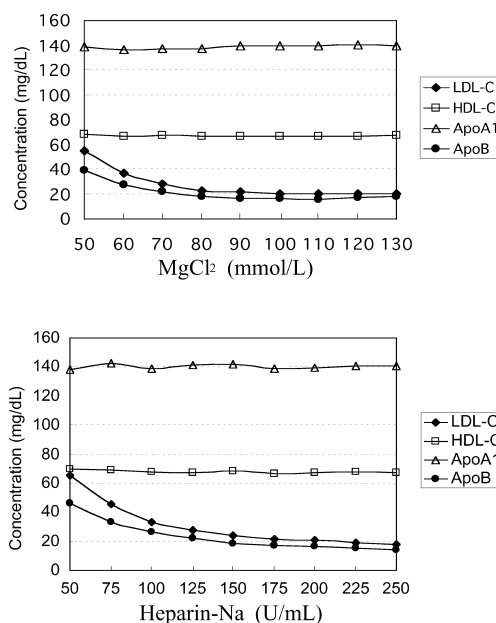


Fig. 1. The effects of various concentrations of heparin-sodium (Na) or $MgCl_2$ on HDL-cholesterol, apolipoprotein A-I (apoA-I), LDL-cholesterol, and apoB levels in the heparin-Mg supernatant of pooled sera containing 67 mg/dl HDL-cholesterol, 139 mg/dl apoA-I, 95 mg/dl LDL-cholesterol, and 65 mg/dl apoB. Constant concentrations of heparin-Na (150 U/ml) or $MgCl_2$ (90 mmol/l) were used when Mg or heparin concentrations were varied.

the method of Nichols, Krauss, and Musliner (15). Pattern A was defined as a diameter >25.5 nm, and pattern B was defined as a diameter ≤ 25.5 nm (15). The LDL band in serum or in the heparin-Mg supernatant was determined by 3% polyacrylamide disc gel (Lipophor Gel, Joko Co., Tokyo, Japan).

Heparin-Mg precipitation

Figure 1 shows the effects of various concentrations of heparin-sodium (Na) or $MgCl_2$ on HDL-cholesterol, apoA-I, LDL-cholesterol, and apoB levels in the heparin-Mg supernatant of pooled sera containing 67 mg/dl HDL-cholesterol, 139 mg/dl apoA-I, 95 mg/dl LDL-cholesterol, and 65 mg/dl apoB. HDL-cholesterol and apoA-I concentrations remained constant even when the heparin or Mg concentrations varied widely. LDL-cholesterol and apoB levels in the supernatant decreased with an increase of the heparin concentration by 150 U/ml, but then showed a plateau, even when the heparin level was increased further. LDL-cholesterol and apoB levels in the supernatant also decreased with an increase of $MgCl_2$ of 90 mmol/l, and then showed a plateau. Thus, we used 150 U/ml of heparin and 90 mmol/l of $MgCl_2$ as the minimum concentrations for yielding maximal LDL precipitation.

Various precipitation reagents

We examined several combinations of polyanion and divalent cations that can selectively precipitate lipoproteins with $d < 1.044$ g/ml and that do not interfere with the direct LDL-cholesterol and apoB assay. Finally, phosphotungstate- Ca^{2+} (0.3%, 15 mmol/l), dextran sulfate- Mg^{2+} (1.5%, 40 mmol/l), heparin- Mn^{2+} (40 U/ml, 30 mmol/l), and heparin- Mg^{2+} (150 U/ml, 90 mmol/l) were nominated as candidates for precipitation reagent. Figure 2 shows the correlation between $d = 1.044$ – 1.063

g/ml lipoprotein cholesterol levels separated by ultracentrifugation and those values determined by the precipitation method using the indicated polyanion and divalent cation combinations. The precipitation method using phosphotungstate- Ca^{2+} , dextran sulfate- Mg^{2+} , heparin- Mn^{2+} , and heparin- Mg^{2+} had a significant association with the ultracentrifugation method; however, a combination of heparin and Mg^{2+} gave the best correlation with the ultracentrifugation method. Therefore, we decided to use heparin- Mg^{2+} as the precipitation reagent in subsequent studies.

Serum was stored at $4^\circ C$ and was used for the assay within 7 days of sampling. We observed that there was no significant change in the serum for the sample until day 7, but sera stored for more than 7 days, plasma, and frozen sera were not adequate for this assay.

Assay procedure

The precipitation reagent (0.1 ml) containing 150 U/ml heparin-sodium salt (Sigma H3393, St. Louis, MO) and 90 mmol/l $MgCl_2$ (catalog number 209-09, Nakarai, Tokyo, Japan) was added to each serum sample (0.1 ml), mixed, and incubated for 10 min at $37^\circ C$. The samples were placed in an ice bath and allowed to stand for 15 min, then the precipitate was collected by centrifuging at 15,000 rpm for 15 min at $4^\circ C$ (microcentrifuge MRX-150, Tomy Disital Biology Co., Ltd., Tokyo, Japan). The precipitate was packed tightly at the bottom of the tube, and the supernatant was clear. An aliquot of the supernatant was removed for LDL-cholesterol and apoB analyses. The LDL-cholesterol in the heparin- Mg^{2+} supernatant [containing sd LDL ($d = 1.044$ – 1.063 g/ml) and HDL] was directly and selectively measured by the homogenous method (LDL-EX, Denka Seiken). The LDL-cholesterol assay was performed according to the manufacturer's instructions (13). This direct LDL-cholesterol assay

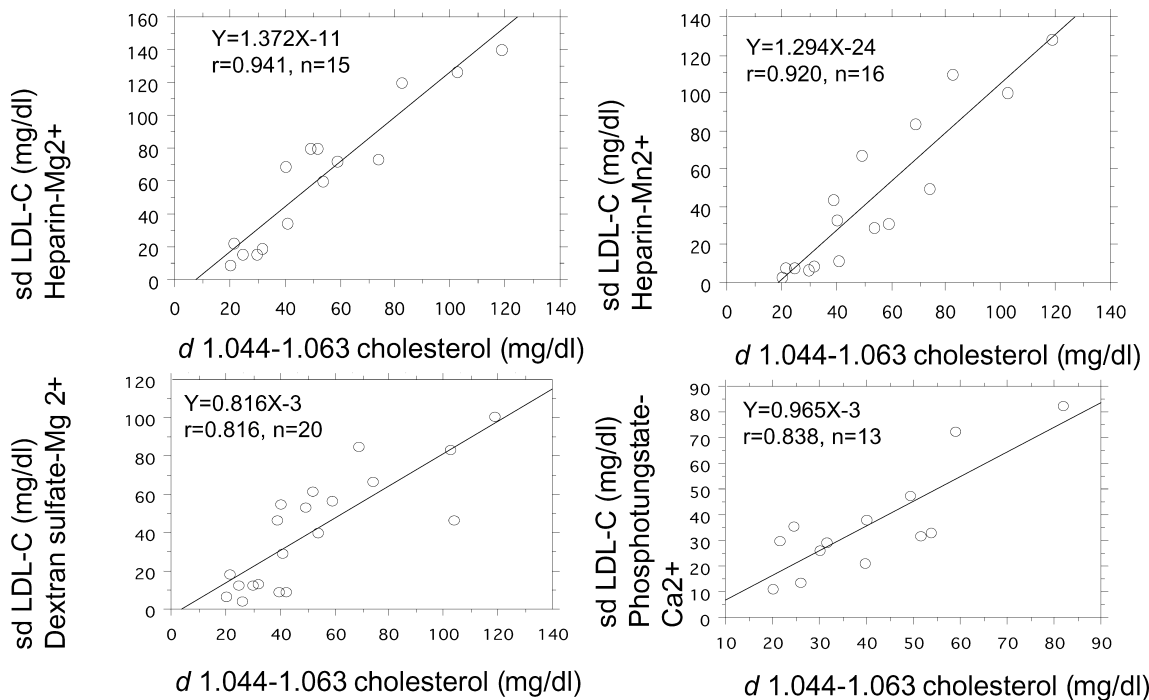


Fig. 2. Correlation between the ultracentrifugation and precipitation methods using various polyanion divalent cations for the measurement of small, dense (sd) LDL-cholesterol concentration. The lipoprotein with density (d) = 1.044–1.063 was separated by the ultracentrifugation method. In the precipitation method, 0.1 ml of heparin- Mg^{2+} (150 U/ml, 90 mmol/l), heparin- Mn^{2+} (40 U/ml, 30 mmol/l), dextran sulfate- Mg^{2+} (1.5%, 40 mmol/l), or phosphotungstate- Ca^{2+} (0.3%, 15 mmol/l) was added to 0.1 ml of serum. After precipitation, the supernatant was used for LDL-cholesterol assay using the homogenous method (13).

TABLE 2. Composition of the supernatant of the heparin-Mg precipitation method

	Supernatant	Serum	TGRL	Lb LDL	Sd LDL	HDL
			<i>mg/dl</i>			
Triglyceride	28 ± 17	105 ± 58	72 ± 50	12 ± 4	8 ± 5	14 ± 8
Cholesterol	114 ± 27	230 ± 38	56 ± 7	87 ± 29	56 ± 37	58 ± 19
HDL-cholesterol	50 ± 13	59 ± 14	1 ± 0	2 ± 0	1 ± 1	48 ± 19
apoA-I	134 ± 20	133 ± 20	0 ± 0	0 ± 0	0 ± 0	129 ± 36
apoB	45 ± 30	113 ± 33	12 ± 7	51 ± 16	42 ± 24	1 ± 1

TGRL, triglyceride-rich lipoprotein. Data represent mean ± SD of seven samples. The supernatant was taken after precipitation of lipoproteins with $d < 1.044$ g/ml by the heparin-Mg²⁺ precipitation method as described in Materials and Methods. TGRLs ($d < 1.019$ g/ml), large, buoyant LDL with $d = 1.019$ – 1.044 g/ml, sd LDL with $d = 1.044$ – 1.063 g/ml, and HDL with $d = 1.063$ – 1.210 g/ml were separated by the ultracentrifugation method.

was applied to an autoanalyzer (Hitachi type 7170, Hitachi Ltd., Tokyo, Japan). When the autoanalyzer was used, the assay time was 10 min. Sd LDL-apoB in the heparin-Mg²⁺ supernatant was measured by an immunoturbidometric assay (ApoB Auto.N “DAIICHI,” Daiichi Chemicals), which was also applied to an autoanalyzer. The coefficients of variation of inter- and intra-assay for the precipitation method were 4.1–7.5% and 4.3–6.4%, respectively.

Statistics

Pearson's single linear regression analysis was used to assess the correlation between two parameters.

RESULTS

We compared lipid and apolipoprotein concentrations between the heparin-Mg supernatant and lipoproteins isolated by ultracentrifugation (Table 2). Serum triglyceride was mainly recovered in the TGRL fraction; the heparin-Mg supernatant contained far less triglyceride. After subtracting HDL-triglyceride from the triglyceride in the supernatant (14 ± 15 mg/dl, mean ± SD), the difference corresponded to the value for triglyceride in the sd LDL separated by ultracentrifugation (8 ± 5 mg/dl). In contrast, a large amount of cholesterol existed in the heparin-Mg supernatant. When HDL-cholesterol was subtracted from the cholesterol in the supernatant (64 ± 37 mg/dl), the result was similar to the cholesterol level in the sd LDL separated by ultracentrifugation (56 ± 37 mg/dl). In addition, the apoB level in the supernatant (45 ± 30 mg/dl) was almost identical to that in the isolated sd LDL (42 ± 24 mg/dl). HDL-cholesterol and apoA-I levels in the supernatant corresponded to those in serum or in HDL separated by ultracentrifugation, indicating that HDL was not precipitated and was completely recovered in the heparin-Mg supernatant.

To ascertain whether the heparin-Mg precipitate only contained large, buoyant LDL, while sd LDL remained in the supernatant, we performed electrophoresis of the serum and its corresponding heparin-Mg supernatant on 3% polyacrylamide gel (Lipophor Gel, Joko Co.). The serum samples were obtained from individuals with different LDL phenotypes in whom the LDL diameter had been measured by GGE. As shown in Fig. 3, the LDL band was very faint in the heparin-Mg supernatant of pattern A

serum (mean particle diameter = 26.5 nm), whereas most of the LDL band remained in the supernatant of pattern B serum (mean particle diameter = 25.0 nm).

The sd LDL-cholesterol values obtained using the precipitation method (45 ± 26 mg/dl) were similar to those obtained by the ultracentrifugation method (42 ± 22 mg/dl) (Table 1). Similarly, apoB concentrations were also comparable for the two methods (33 ± 20 vs. 31 ± 17 mg/dl) (Table 1). We divided the subjects into a pattern A group and a pattern B group based on their LDL size determined by GGE. The pattern B group had higher levels of triglyceride, apoB, and apoE, but lower levels of HDL-cholesterol and apoA-I than the pattern A group. The pattern B group had higher levels of sd LDL-cholesterol and apoB, but lower levels of large, buoyant LDL-cholesterol and apoB than the pattern A group, irrespective of the method used for quantification of LDL. The levels of sd LDL-cholesterol and apoB, and large, buoyant LDL-cholesterol and apoB were identical when compared between the precipitation method and the ultracentrifugation method in both the pattern A group and the pattern B group.

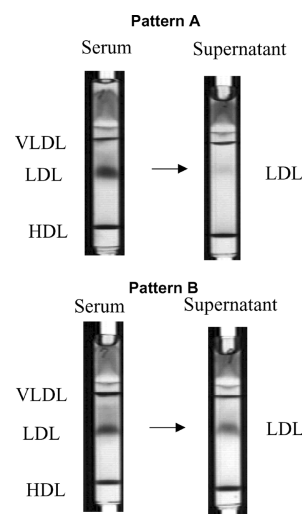


Fig. 3. LDL band in 3% polyacrylamide disk gel electrophoresis in serum and its corresponding heparin-Mg supernatant obtained from the typical subjects with pattern A, whose LDL diameter is 26.5 nm or the subjects with pattern B, whose LDL diameter is 25.0 nm.

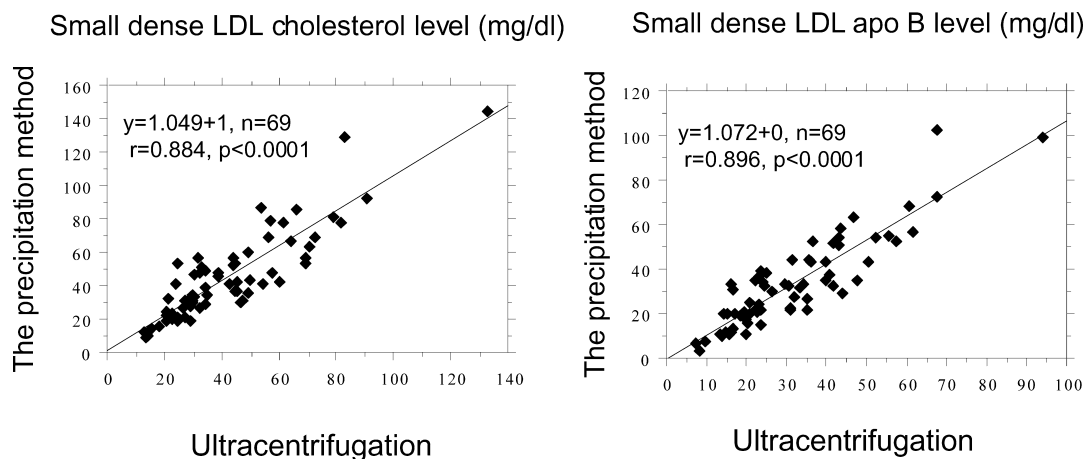


Fig. 4. Correlation between ultracentrifugation and the heparin-Mg²⁺ precipitation method for sd LDL-cholesterol and sd LDL apoB values.

There was an excellent correlation between the ultracentrifugation and heparin-Mg²⁺ precipitation methods for sd LDL-cholesterol values ($y = 1.049X + 1$, $r = 0.884$, $P < 0.0001$) (Fig. 4). An excellent correlation was also observed between these methods for sd LDL apoB values ($y = 1.072X + 0$, $r = 0.896$, $P < 0.0001$) (Fig. 4). There was a very close association between sd LDL-cholesterol and sd LDL apoB values in the precipitation method ($y = 1.240X + 4$, $r = 0.944$, $P < 0.0001$) (Fig. 5). Sd LDL-cholesterol and sd LDL apoB values determined using the precipitation method were not related to large, buoyant LDL-cholesterol ($d = 1.019$ – 1.044 g/ml) and apoB values determined using the ultracentrifugation method (Fig. 6).

Sd LDL-cholesterol and apoB values determined using the precipitation method were inversely associated with mean LDL particle diameter measured by GGE in total subjects (Fig. 7A, C), but data from two chylomicronemic subjects interfered with these associations. When these subjects were excluded, a marked inverse correlation was observed between LDL size and sd LDL-cholesterol ($r = -0.658$, $P < 0.0001$) (Fig. 7B) and sd LDL apoB values ($r = -0.713$, $P < 0.0001$) (Fig. 7D).

We examined whether the large, buoyant LDL fraction ($d = 1.019$ – 1.044 mg/dl) could be estimated by subtracting the sd LDL-cholesterol level determined by the precipitation method from the total LDL-cholesterol level. The large, buoyant LDL-cholesterol values estimated by this method correlated significantly with the values actually determined by the ultracentrifugation method ($y = 0.874X + 25$, $r = 0.858$, $P < 0.0001$) (Fig. 8).

Sd LDL-cholesterol level was significantly related to LDL-cholesterol and serum apoB levels (Fig. 9A, B). Similarly, sd LDL apoB level was significantly related to LDL-cholesterol and serum apoB levels (Fig. 10A, B). There was no correlation between serum triglyceride and sd LDL-cholesterol levels in total subjects (Fig. 9C). When two chylomicronemic subjects were excluded, this correlation became significant (Fig. 9D). There was a weak correlation between serum triglyceride and sd LDL B levels in total subjects (Fig. 10C). When two chylomicronemic subjects were excluded, this correlation became more

significant (Fig. 10D). HDL cholesterol and apoA-I levels were not associated with sd LDL-cholesterol or sd LDL apoB. Serum apoE levels were significantly associated with sd LDL apoB ($r = 0.403$, $P < 0.001$) but not with sd LDL-cholesterol levels ($r = 0.193$, ns) in total subjects. However, when two chylomicronemic subjects were excluded, these correlations became significant ($r = 0.594$ for sd LDL-cholesterol, and $r = 0.583$ for sd LDL apoB).

DISCUSSION

Lipoproteins can be selectively precipitated with combinations of particular polyanions and divalent cations (e.g., heparin sulfate, dextran sulfate, or sodium phosphotungstate in combination with Mn²⁺, Mg²⁺, or Ca²⁺). We found that the combination of heparin and Mg does not precipitate all of the apoB-containing lipoproteins, but leaves sd LDL in the supernatant, although the exact mechanism involved remains unknown. We examined the best combination of polyanion and divalent cations to selectively precipitate the lipoproteins with $d < 1.044$ g/ml,

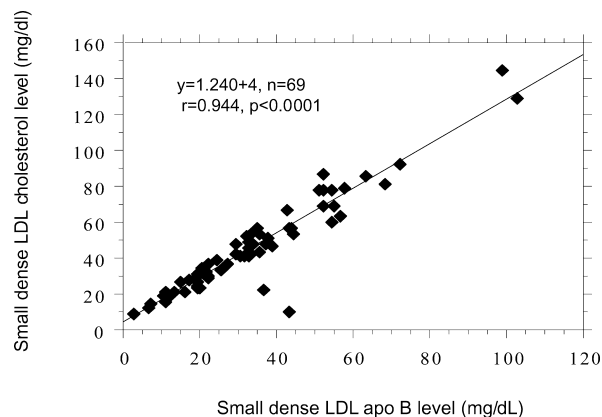


Fig. 5. Correlation between sd LDL-cholesterol and sd LDL apoB values in the heparin-Mg²⁺ precipitation method.

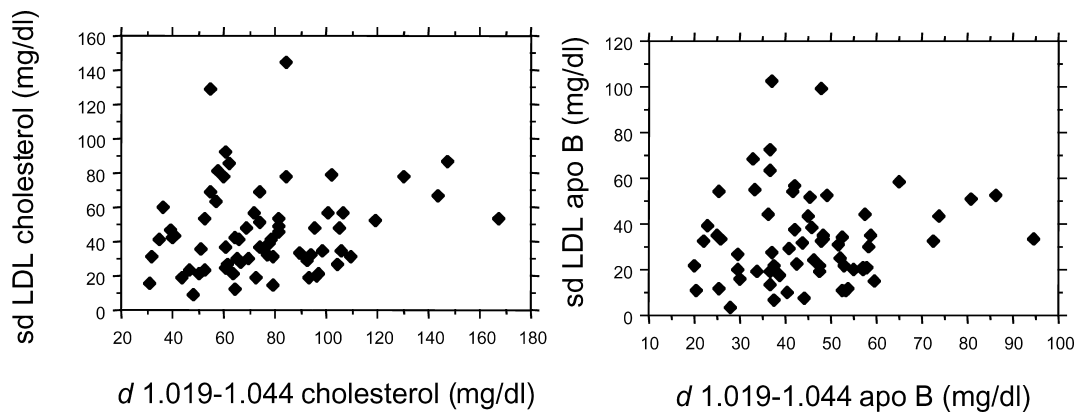


Fig. 6. Correlation between sd LDL-cholesterol and apoB values determined by the precipitation method and large, buoyant LDL-cholesterol ($d = 1.019\text{--}1.044$ g/ml) and apoB values determined by the ultracentrifugal method.

which include the VLDL, IDL, and large, buoyant LDL fractions, and finally found the combination of 150 USP unit/ml of heparin and 90 mmol/l of Mg^{2+} to be the best reagent for this specific precipitation. After precipitation, the supernatant contains lipoproteins with $d > 1.044$ g/ml, which include sd LDL and HDL. LDL-cholesterol can be directly measured by the homogenous method. We previ-

ously reported that the homogenous method using the LDL-EX assay (Denka Seiken) selectively measures the narrow-cut LDL with $d = 1.019\text{--}1.063$ g/ml, showing only a weak cross-reaction with IDL (14). Therefore, we could selectively measure sd LDL-cholesterol in the heparin- Mg^{2+} supernatant without the influence of other lipoproteins. ApoB determination is another option for selectively mea-

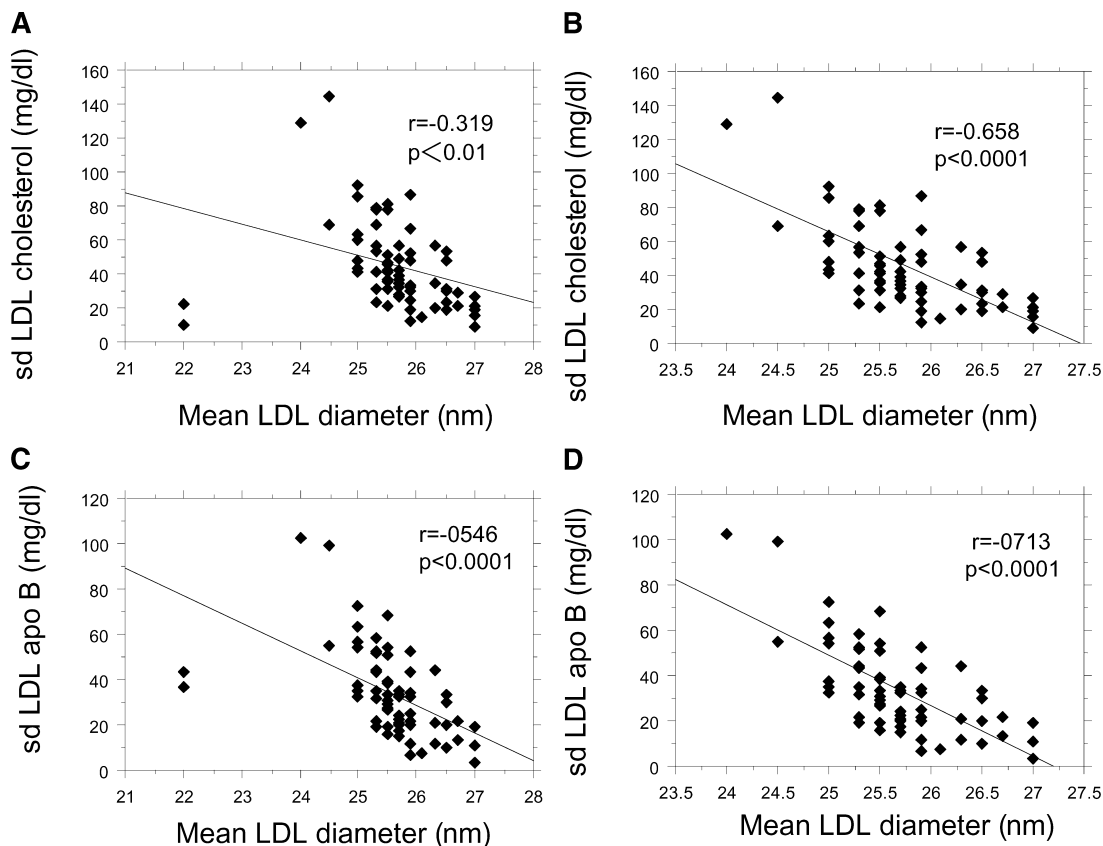


Fig. 7. Correlation between LDL size and sd LDL-cholesterol and sd LDL apoB values determined by the precipitation method. Mean LDL particle diameter was measured by gradient gel electrophoresis (15). A and C: total subjects; B and D: two chylomicronemic subjects were excluded.

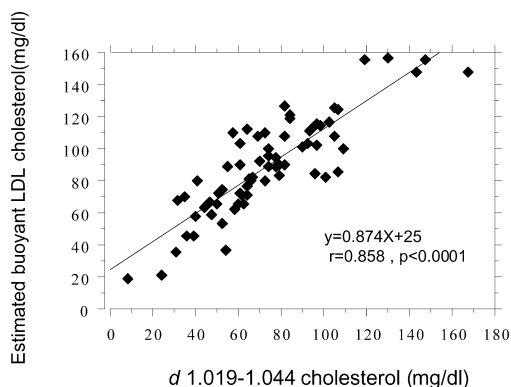


Fig. 8. Correlation between estimated large, buoyant LDL-cholesterol and the lipoprotein with $d = 1.019\text{--}1.044$ mg/dl cholesterol determined by the ultracentrifugation method. The large, buoyant LDL fraction was estimated by subtracting the sd LDL-cholesterol levels determined by the precipitation method from the total LDL-cholesterol level.

asuring sd LDL concentration excluding the influence of HDL in the heparin-Mg²⁺ supernatant. The accuracy of the precipitation method was estimated by comparing with ultracentrifugally separated sd lipoprotein with $d = 1.044\text{--}1.063$ g/ml. As shown in Fig. 2, we observed a markedly high correlation between these two methods.

Because sd LDL particles are cholesterol depleted (9, 16), we initially thought that sd LDL apoB or protein would precisely reflect the atherogenic lipoprotein mass but that sd LDL-cholesterol would not. However, we found a strikingly close correlation between cholesterol and apoB values, suggesting that cholesterol measurement is sufficient for evaluating the sd LDL mass, and that protein data are not indispensable.

As shown in Table 2, LDL lipid and apoB levels in the heparin-Mg supernatant were similar to those in sd LDL separated by ultracentrifugation. In addition, we demonstrated by polyacrylamide gel electrophoresis that the heparin-Mg precipitate only contained large, buoyant LDL while sd LDL remained in the supernatant (Fig. 3). However, we failed to demonstrate that the heparin-Mg reagent only precipitates $d < 1.044$ g/ml lipoprotein and not $d > 1.044$ lipoprotein isolated by ultracentrifugation (data not shown), probably because the isolated lipoprotein is not an adequate sample for the polyanion precipitation technique. Nevertheless, there is no doubt that the heparin-Mg supernatant contained sd LDL, because of similar absolute LDL values and an excellent correlation between the LDL levels in the heparin-Mg supernatant and in the $d = 1.044\text{--}1.063$ g/ml lipoprotein isolated by ultracentrifugation.

The accuracy of our method was also confirmed by comparison with the measurement of LDL size by GGE.

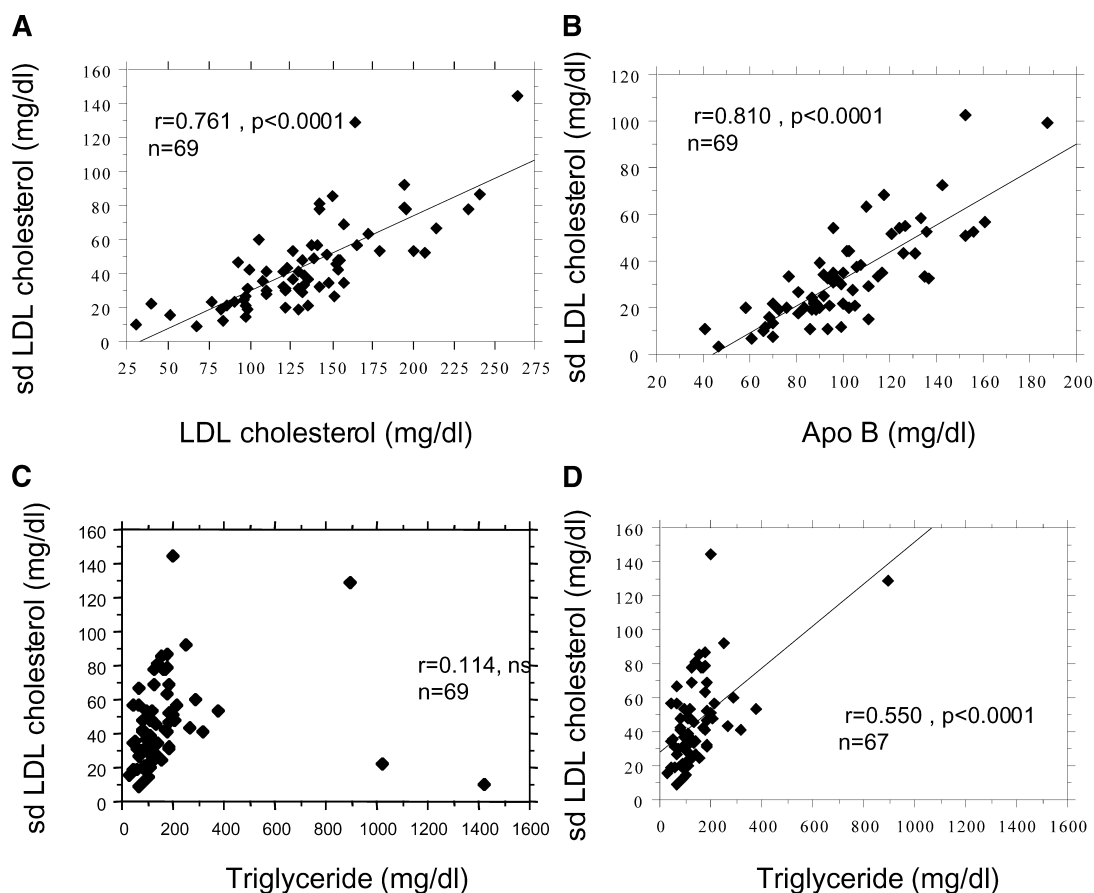


Fig. 9. Correlation between sd LDL-cholesterol and LDL-cholesterol (A), serum apoB (B), and triglyceride levels (C). D: two chylomicronemic subjects were excluded.

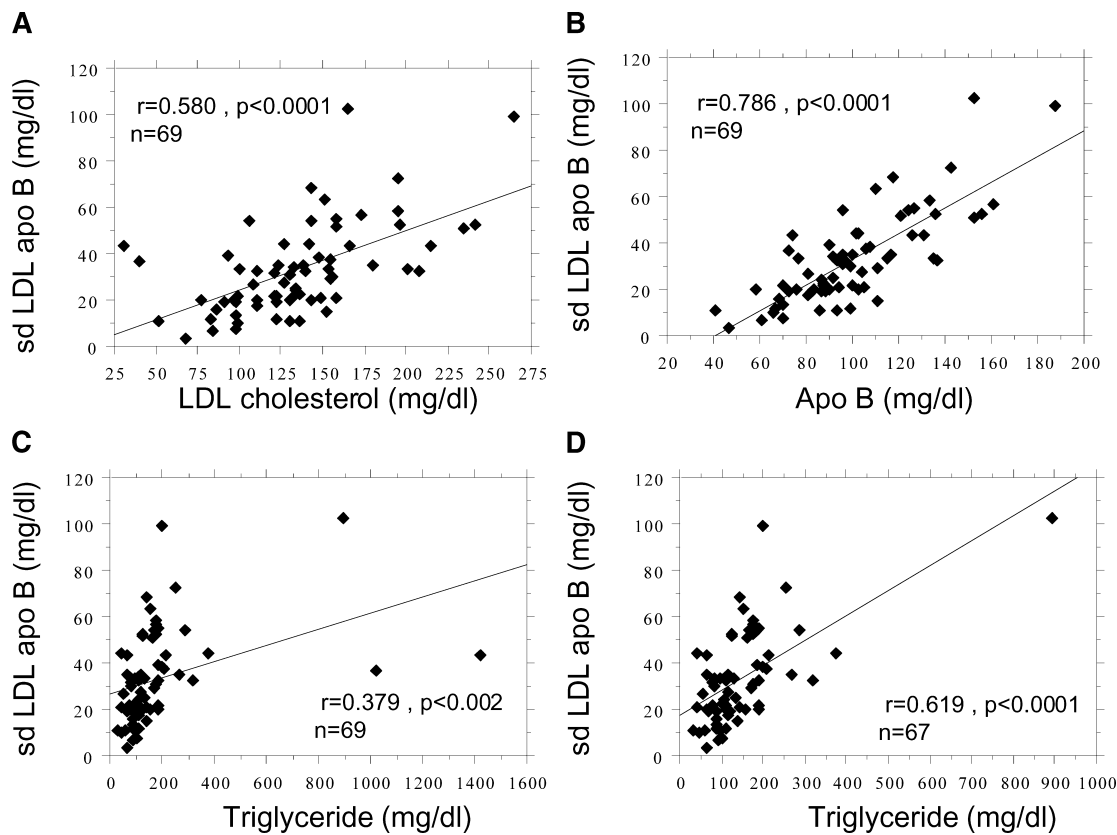


Fig. 10. Correlation between sd LDL apoB and LDL-cholesterol (A), serum apoB (8B), and triglyceride levels (8C). D: two chylomicronemic subjects were excluded.

There was a close association between mean LDL particle diameter and quantity of sd LDL determined using our precipitation method. Thus, our precipitation method may be used to indicate a preponderance of small-sized LDL particles. In this study, two chylomicronemic subjects with serum triglyceride levels $>1,000$ mg/dl were included. Because LDL size is inversely associated with serum triglyceride level (2, 3), they had very small-sized LDL. However, their LDL-cholesterol levels were very low, because the conversion from VLDL to LDL was impaired by the deficiency of lipoprotein lipase (11), which may explain the disproportionately low level of sd LDL-cholesterol levels versus LDL size. The atherogenic potency would increase with increased numbers of atherogenic lipoprotein particles. Even when LDL size is small, the atherogenic potential is reduced if the particle numbers are small. Therefore, quantification of sd LDL would be superior to measurement of LDL size for evaluating overall atherogenic risk. LDL size does not correlate with LDL-cholesterol level, but sd LDL level had a strong correlation with LDL-cholesterol, the most powerful risk factor for CHD, also suggesting that quantification of sd LDL could more clearly indicate overall atherogenic potential than could measurement of LDL size.

In conclusion, we have developed a novel and simple method for the quantification of sd LDL in serum using heparin-Mg²⁺ precipitation. This precipitation method is

applicable to an autoanalyzer that permits rapid measurement of a large number of samples. [Fig.](#)

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