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Evaluation of an HPLC method for LDL-cholesterol determination in patients with various lipoprotein abnormalities in comparison with beta-quantification

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ABSTRACT

Background: The main purpose of this study was to evaluate an HPLC method for LDL-cholesterol determination in the presence of abnormal lipoproteins.
Methods: We compared LDL-cholesterol levels obtained by HPLC (HPLC-LDL), Friedewald (F-LDL), and β-quantification (BQ-LDL) methods on 47 healthy volunteers and 50 outpatients with lipid disorders, including apolipoprotein E2/2 phenotype, cholesteryl ester transfer protein deficiency and lipoprotein lipase deficiency.
Results: For the control group ($n=50$), the HPLC-LDL and the F-LDL correlated highly with the BQ-LDL ($r=0.984$ and 0.969, respectively), but the HPLC-LDL was lower than the BQ-LDL (mean bias: −4.0%, $P<0.001$). For the subjects with lipoprotein abnormalities, significant biases were found in HPLC-LDL for the hypertriglyceridemia (+25%, $n=17$, $P<0.01$), the hyper HDL (−15.2%, $n=10$, $P<0.01$) and the hyper lipoprotein(a) groups (−13.4%, $n=12$, $P<0.001$). The F-LDL was significantly higher than the BQ-LDL in the apolipoprotein E2/2 subjects (+92%, $n=8$, $P<0.001$), but not significantly different in other subjects with triglycerides <4000 mg/l.
Conclusions: There were several discrepancies in LDL-cholesterol levels determined by the HPLC and the BQ methods in samples with lipoprotein abnormalities. However, the HPLC method can be useful and informative for analysis of abnormal lipoproteins.

1. Introduction

The association between total cholesterol (TC) and risk of developing coronary heart disease (CHD) has been well established. Most of the cholesterol in circulation is carried by low-density lipoproteins (LDL), which has been conclusively shown by many prospective studies and randomized clinical trials to be primarily responsible for the association with CHD risk [1]. The Adult Treatment Panel (ATP) guidelines of the National Cholesterol Education Program consider LDL-cholesterol (LDL-C) the major indicator for initial classification of CHD risk status and identify lowering of LDL-C as the primary goal of therapy [2].

The most widely accepted reference method for LDL-C is a version of β-quantification (BQ) combining ultracentrifugation and heparin/$MnCl_2$ precipitation, as performed at the CDC [3]. The LDL-C values determined by the BQ method, which are calculated by subtracting HDL-cholesterol (HDL-C) from cholesterol concentration measured in the 1.006 kg/l bottom fraction obtained by ultracentrifugation, include intermediate-density lipoproteins (IDL; density, 1.006–1.019 kg/l) and lipoprotein(a) [Lp(a)] as well as LDL particles with a density of 1.019–1.063 kg/l. IDL and Lp(a) are also generally considered to be atherogenic. Therefore, LDL-C values by the BQ method can be considered to represent the cholesterol contained in several potentially atherogenic lipoproteins [3].

The most common approach to determining LDL-C in the clinical laboratory is the Friedewald calculation [4], but the calculated LDL-C can be inaccurate for serum triglyceride (TG) concentrations >4000 mg/l or in the presence of chylomicrons (CM) or type III hyperlipoproteinemia. Recently, a new generation of homogeneous methods capable of full automation has been introduced that uses various types of specific reagents to selectively expose and directly measure the cholesterol associated with LDL [5,6]. Such methods will

Abbreviations: TC, total cholesterol; CHD, coronary heart disease; LDL, low-density lipoprotein; ATP, Adult Treatment Panel; LDL-C, LDL-cholesterol; BQ, β-quantification; HDL-C, HDL-cholesterol; IDL, intermediate-density lipoprotein; Lp(a); lipoprotein(a); TG, triglyceride; CM, chylomicron; HPLC, high-performance liquid chromatography; apo, apolipoprotein; LPL, lipoprotein lipase; CETP, cholesteryl ester transfer protein; (CM+VLDL)-C, (CM+VLDL)-cholesterol; HPLC-LDL, LDL-cholesterol determined by the HPLC method; BQ-LDL, LDL-cholesterol determined by the beta-quantification or modified beta-quantification methods; F-LDL, LDL-cholesterol calculated by the Friedewald equation.

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Table 1
Lipoprotein characteristics of studied subjects

	Control	Hyper TG	Apo E2/2	Hyper HDL	Hyper Lp(a)
	(n=50)	(n=17)	(n=8)	(n=10)	(n=12)
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
	Min/max	Min/max	Min/max	Min/max	Min/max
TG[a,b]	1224±752	9799±5184†	2334±655†	660±235†	1093±329
	410/3320	4880/23,050	1448/2969	381/1202	410/1554
TC[b,c]	1917±343	2953±1028†	2105±419	2663±348†	2048±610
	1220/2720	2047/6518	1480/2798	2040/3182	1350/3332
(CM+VLDL)-C[b,c]	248±188	1766±972†	1000±397†	107±81†	191±41
	40/950	804/5003	484/1582	18/279	126/246
LDL-C[b,c]	1151±319	870±503§	581±174†	1263±387	1372±536
	573/1815	210/1724	359/790	633/2025	842/2635
HDL-C[b,c]	519±105	316±121†	525±121	1292±242†	485±222
	265/737	113/600	386/693	1003/1700	217/976
Lp(a)[b,d]	109±89	73±86§	108±90	137±55	850±402†
	10/390	16/210	20/290	50/220	436/1632
Apo A-I[b,d]	1460±229	1176±287†	1436±342	2144±223†	1323±439
	900/1900	290/1550	1100/1920	1712/2480	719/2030
Apo B[b,d]	910±246	1606±600†	654±183‡	862±148	1010±316
	420/1510	710/2620	430/950	660/1070	610/1564
Apo E[b,d]	42±18	141±39†	149±67‡	100±39‡	48±26
	17/101	92/248	64/276	55/190	26/118
VLDL size[e]	Not	42.0±2.1[f]	31.3±1.3	Not	Not
	observed	38.9/45.2	30.1/33.8	observed	observed
LDL size[e]	25.6±0.5	23.9±0.6†[g]	Not	25.4±0.6	25.8 0.4
	24.5/26.5	22.6/24.8	observed	24.7/26.9	25.1 26.4
HDL size[e]	10.7±0.3	10.1±0.3†	10.3±0.2†	12.5±0.6†	10.8±0.3
	10.1/11.2	9.4/10.7	10.0/10.6	11.8/13.6	10.4/11.6

†$P<0.001$, ‡$P<0.01$, §$P<0.05$ (compared to the control group).
[a] Automated enzymatic method.
[b] In mg/l.
[c] Reference method.
[d] Immunoturbidimetric method.
[e] Diameter in nm.
[f] $n=15$.
[g] $n=14$.

grow in use but still need careful examination on their reactivity to lipoprotein particles other than LDL [7,8]. We previously reported partial reactivity of 2 homogeneous LDL-C assay kits on small dense LDL and their nonspecific reactions to very-low density lipoproteins (VLDL) particles [9].

High-performance liquid chromatography (HPLC) with gel permeation columns is an alternative method for classifying lipoproteins on the basis on particles sizes [10,11]. We developed a new analytical tool of cholesterol levels in major and subclasses of lipoproteins from a small amount of serum or plasma within 16 min by gel permeation HPLC [12–14]. We defined 3 VLDL subclasses, 4 LDL subclasses, and 5 HDL subclasses by using Gaussian curve fitting technique [11,15,16]. Moreover, qualitative information about the particle sizes obtained from the observed peak detection time on HPLC patterns can be used for characterization of lipoproteins and a better understanding of lipoprotein metabolism [14]. In this study, LDL-C levels obtained by the HPLC, which were calculated from component peak area corresponding to particle size from 16 to 30 nm, were compared with those by the BQ method on samples from the subjects with lipid disorders.

2. Materials and methods

2.1. Serum samples

Blood samples were collected into glass tubes without anticoagulant after a fasting state of at least 8 h from 47 healthy male volunteers and 50 outpatients in Osaka University hospital including 9 patients with type V hyperlipidemia, 9 patients with hypertriglyceridemia, 8 patients with apolipoprotein (apo) E2/2 phenotype, 4 patients with low hepatic lipase activity, 2 patients with lipoprotein lipase (LPL) deficiency, 3 patients with cholesteryl ester transfer protein (CETP) deficiency, 2 patients with low CETP activity, 2 patients with apo E7/3 phenotype, 4 patients with familial hyperlipidemia, 1 patient with mild primary biliary cirrhosis, 1 patient with hypergammaglobulinemia, 2 patients with sleep apnea syndrome, 1 patient with hypertension, 1 patient with diabetes mellitus and 1 patient with gout. Among 8 patients with apo E2/2 phenotype, 4 patients represent type III hyperlipoproteinemia and 5 patients are under medication by lipid lowering drugs; 2 of them by bezafibrate and 3 of them by fenofibrate. The blood samples were allowed to clot at room temperature and were centrifuged at 2000 ×g for 15 min to obtain serum samples. All serum samples were stored at 4 °C and analyzed within 5 days after blood collection. All volunteers and outpatients gave informed consent to participate in this study, and this study was conducted according to the Osaka University Hospital ethics committees.

2.2. HPLC method

Serum lipoproteins were analyzed by HPLC, as previously described [11,14–16]. In brief, 5 μl of whole serum sample was injected into 2 connected columns (300×7.8 mm) of TSKgel LipopropakXL (Tosoh, Tokyo, Japan) and eluted by TSKeluent Lp-1 (Tosoh) on HPLC system consisting of an AS-8020 auto-injector, CCPS and CCPM-II pumps and a UV-8020 detector (Tosoh). The effluent from the columns was continuously monitored at 550 nm after an on-line enzymatic reaction with a commercial kit, Determiner L TC (Kyowa Medex, Tokyo, Japan).

We defined 3 VLDL subclasses, 4 LDL subclasses, and 5 HDL subclasses using 20 component peaks on the basis of lipoprotein particle size (diameter) [15]. Cholesterol levels in major classes were obtained by grouping of these subclasses as follows: CM (>80 nm), VLDL (30–80 nm), LDL (16–30 nm) and HDL (8–16 nm). The within-run precision of cholesterol measurement for major lipoprotein classes was very high and coefficient of variation (CV) values ($n=5$, normolipidemic and hyperlipidemic pooled sera) were 2.51% (208 mg/l on average) and 1.29% (400 mg/l) for VLDL-C, 0.60% (926 mg/l) and 0.81% (750 mg/l) for LDL-C and 0.20% (636 mg/l) and 0.78% (422 mg/l) for HDL-C, respectively [15].

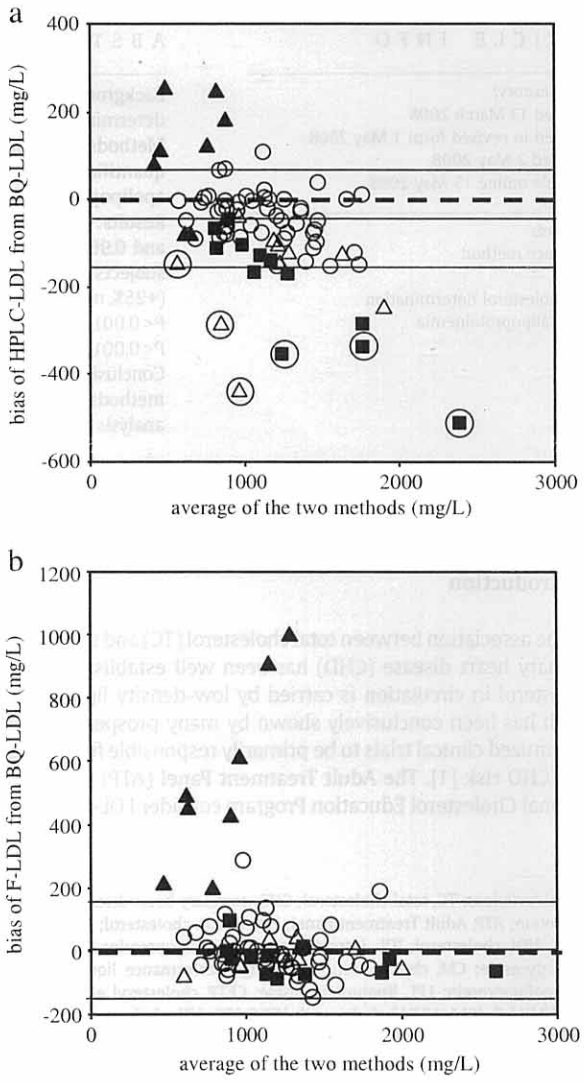

Fig. 1. Bland–Altman plots of biases of LDL-C levels by HPLC (HPLC-LDL) a) and Friedewald calculation (F-LDL) b) from those by BQ or modified BQ methods (BQ-LDL or mBQ-LDL) against average LDL-C levels by the two methods for controls (○, $n=50$) and subjects with apo E2/2 (▲, $n=8$), hyper HDL (△, $n=10$), and hyper Lp(a) (■, $n=12$). Dotted lines represent zero bias. Solid lines represent means of biases, means+2SD and means−2SD for the control subjects ($n=50$). Circled △, 3 subjects with CETP deficiency; Circled ■, 3 subjects with Lp(a) >1000 mg/l.

Particle sizes of VLDL, LDL and HDL were determined from the elution time of HPLC patterns using a column calibration curve, a plot of logarithm of the particle diameter of standard samples as described previously [13,15]. The precision of particle size determination of LDL and HDL was very high and CV values (n=5, normolipidemic and hyperlipidemic pooled sera) were 0.27% and 0.56% for LDL and 0.36% and 0.45% for HDL, respectively [15].

2.3. CDC reference methods for TC, VLDL-C, LDL-C and HDL-C

The reference method for cholesterol is the Abell–Levy–Brodie–Kendall assay [17], as modified by the CDC, described previously in detail [18]. The reference method for HDL-C consists of a three step procedure involving ultracentrifugation, precipitation, and cholesterol analysis [19]. After ultracentrifugation of whole sera at a density of 1.006 kg/l, apo B-containing lipoproteins in the ultracentrifugal infranate were precipitated with heparin/$MnCl_2$, and the cholesterol in the heparin–$MnCl_2$ supernatant was measured by the Abell–Kendall method [18]. For a part of samples from control subjects, we used the designated comparison method for HDL-C assay and a modified BQ method for LDL-C assay [20,21], where a dextran-sulfate/$MnCl_2$ reagent and a enzymatic reagent (Determiner L TC II from Kyowa Medex) were used for precipitation and cholesterol determination, respectively.

LDL-C was calculated by subtracting HDL-C values from cholesterol concentration in the ultracentrifugal infranate at a density of 1.006 kg/l of serum samples. Cholesterol in CM and VLDL [(CM+VLDL)-C] was calculated by subtracting cholesterol concentration in the ultracentrifugal infranate at a density of 1.006 kg/l of serum samples from serum TC levels. These reference methods were carried out at the Lipid Research Laboratory in Osaka Medical Center for Health Science and Promotion.

2.4. Measurement of lipids, apolipoproteins and Lp(a)

Serum TG was determined enzymatically using commercial kits (Kyowa Medex), correcting for the presence of endogenous glycerol. Serum apo A-I, apo B and apo E, and Lp(a) concentrations were determined on a JCA-BM12 biochemical analyzer (Japan Electron Optics Laboratory, Tokyo, Japan) by immunoturbidimetric methods from Daiichi Chemical, Tokyo, Japan. LDL-C levels by Friedewald method (F-LDL) were calculated using the Friedewald equation; [serum TC]−[HDL-C]−[serum TG]×1/5 [4].

2.5. Statistical analysis

Data were analyzed with SPSS (ver. 10.0, SPSS Inc, Chicago, IL). Continuous measures are expressed as mean±SD, and judged by Student's t test. All the reported P values were 2-tailed, and those <0.05 were considered to be statistically significant. Correlations between various variables were presented as the Pearson correlation coefficient (r-value).

3. Results

3.1. Lipid, apolipoprotein, lipoprotein levels and lipoprotein profiles of studied subject groups

Excluding apo E2/2 subjects (n=8), we classified serum samples to 4 groups according to serum TG, HDL-C and Lp(a) levels ; hyper TG group (n=17) with TG ≥4000 mg/l, hyper HDL group (n=10) with HDL-C ≥1000 mg/l, hyper Lp(a) group (n=12) with Lp(a) ≥400 mg/l and control group (n=50) with TG <4000 mg/l, Lp(a) <400 mg/l and HDL-C <1000 mg/l. Mean levels of serum TG, serum TC, (CM+VLDL)-C, LDL-C, HDL-C, Lp(a), apo A-I, apo B and apo E are shown in Table 1.

Mean particle sizes of VLDL, LDL and HDL obtained from the peak detection time on HPLC profiles are also shown for each group in Table 1. Although the peaks of HDL were observed in all the subjects, no peak of VLDL was observed in all subjects of the control, hyper HDL and hyper Lp(a) groups, and no peak of LDL was observed in all the apo E2/2 subjects and 3 subjects of the hyper TG group. LDL particle size was significantly smaller (P<0.001) in the hyper TG group than the control group. HDL particle size was significantly smaller (P<0.001) in the hyper TG and the apo E2/2 groups, and larger (P<0.001) in the hyper HDL group, compared to the control group.

3.2. Comparison of LDL-C levels between HPLC, BQ or modified BQ methods and Friedewald calculation

We determined LDL-C levels by HPLC (HPLC-LDL) as a sum of component peaks 8 to 13. Although the control subjects were analyzed by BQ or modified BQ methods, combined data were used for statistical analysis because of no significant differences in mean biases of HPLC-LDL from LDL-C determined by the BQ or modified BQ methods (BQ-LDL).

Bland–Altman plots [22] of the biases of HPLC-LDL and F-LDL from BQ-LDL against the average LDL-C levels of the 2 methods for all subjects with TG <4000 mg/l are presented in Fig. 1a and b, respectively. Table 2 presents summary statistics for comparison of LDL-C levels obtained by three methods for each group. For the control group, both HPLC-LDL and F-LDL showed a very high correlation (r=0.984 and 0.969, respectively) with the BQ-LDL. The HPLC-LDL was significantly lower than the BQ-LDL (P<0.0001) but there was no significant difference between F-LDL and BQ-LDL. The biases for the HPLC-LDL against the BQ-LDL showed no significant correlations with serum TG, HDL-C and Lp(a) levels (results not shown).

In the hyper TG group (n=17), HPLC-LDL was significantly (P<0.01) higher than BQ-LDL in spite of a very high correlation, and their biases of HPLC-LDL from BQ-LDL were significantly (P<0.01) correlated with (CM+VLDL)-C levels both by the HPLC (r=0.605) and the reference methods (r=0.687).

For the subjects with apo E2/2, F-LDL was significantly (P<0.01) higher than BQ-LDL (mean bias: +533±293 mg/l), but no significant difference was found between HPLC-LDL and BQ-LDL (mean bias: +101±129 mg/l) as shown in Table 2.

Table 2
Comparison of the HPLC and the Friedewald methods with the BQ or the modified BQ methods for LDL-C determination

			Control	Hyper TG	Apo E2/2	Hyper HDL	Hyper Lp(a)
			(n=50)	(n=17)	(n=8)	(n=10)	(n=12)
LDL-C (mg/l)	BQ	Mean±SD	1151±319	870±503	581±174	1263±387	1372±536
		Min/max	573/1815	210/1724	359/790	633/2025	842/2635
	HPLC	Mean±SD	1111±301	981±464	683±197	1096±389	1171±409
		Min/max	570/1771	301/1741	450/966	484/1774	758/2127
	Friedewald	Mean±SD	1153±304	N.D.	1114±416	1238±398	1345±514
		Min/max	615/1959		568/1787	552/1967	869/2570
Correlation	BQ vs HPLC	r	0.984†	0.980†	0.765§	0.970†	0.992†
	BQ vs F	r	0.969†	N.D	0.808§	0.995†	0.996†
Bias (mg/l)	HPLC−BQ	Mean±SD	−40±57†	110±103†	101±129	−177±117†	−201±140†
		Min/max	−153/109	−44/305	−83/252	−440/−40	−508/−46
	F−BQ	Mean±SD	2.8±79.4	N.D.	533±293‡	−24.9±39.8	−27.7±51.2
		Min/max	−146/286		194/998	−81.0/38.6	−86.0/98.0
% Bias	(HPLC−BQ)/BQ	Mean±SD	−3.2±4.9†	24.6±26.2‡	21.0±26.4	−15.2±11.4‡	−13.4±5.4‡
		Min/max	−12.5/10.3	−3.6/71.7	−12.4/70.2	−38.8/−4.0	−25.0/−5.0
	(F−BQ)/BQ	Mean±SD	0.9±7.7	N.D.	92.2±38.8‡	−2.6±4.6	−1.5±4.8
		Min/max	−9.7/34.2		28.3/130	−12.8/3.0	−6.9/11.6

Bias and % bias are judged by the one-sample t test (test value: 0).
†P<0.001, ‡P<0.01, §P<0.05.
F: Friedewald.

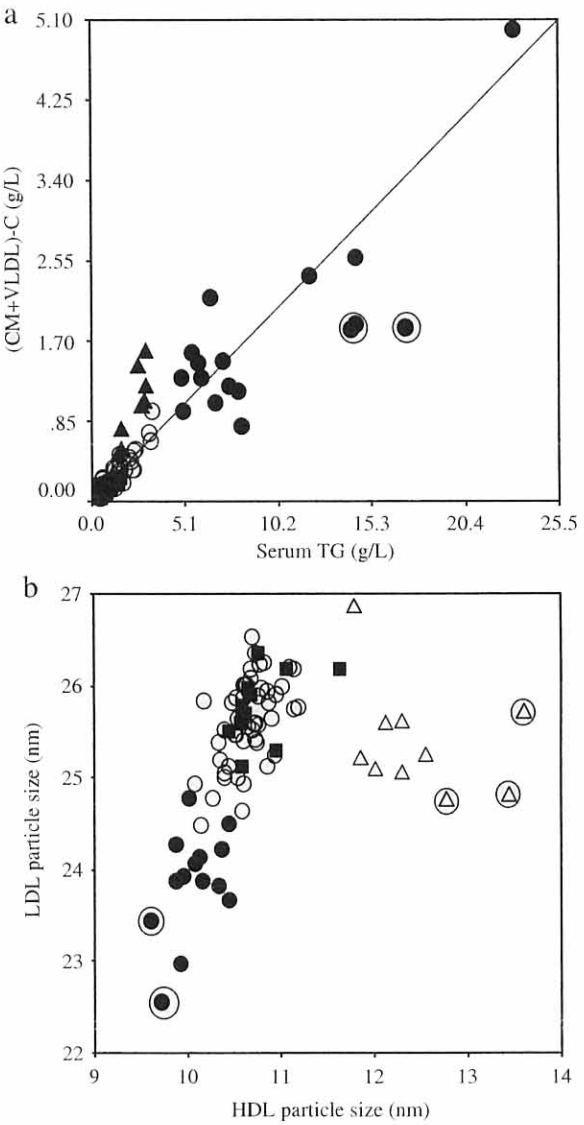

Fig. 2. a) Relationship between (CM+VLDL)-C levels and serum TG levels for controls (○, $n=50$) and subjects with hyper TG (●, $n=17$), apo E2/2 (▲, $n=8$), hyper HDL (△, $n=10$), and hyper Lp(a) (■, $n=12$). A solid line represents a relation of (CM+VLDL)-C=1/5×serum TG. Circled ●, two subjects with LPL deficiency. b) Relationship between LDL particle size and HDL particle size for controls (○, $n=50$) and subjects with hyper TG (●, $n=14$), hyper HDL (△, $n=10$), and hyper Lp(a) (■, $n=12$). Circled ●, two subjects with LPL deficiency; circled △, 3 subjects with CETP deficiency.

In the hyper HDL group, HPLC-LDL was significantly lower ($P<0.001$) than BQ-LDL, and their biases (HPLC−BQ) were significantly correlated with the ratio of apo E to TG ($r=0.780$, $P<0.01$, $n=10$). The large negative bias of HPLC-LDL in the hyper HDL subjects (mean bias: −177±117 mg/l), especially patients with CETP deficiency (encircled open triangles in Fig. 1a), was obtained.

In the hyper Lp(a) group, HPLC-LDL (1171±409 mg/l) was significantly lower ($P<0.001$) than BQ-LDL (1372±536 mg/l), and their biases (HPLC−BQ) were significantly correlated with serum Lp(a) levels ($r=-0.912$, $P<0.0001$, $n=12$). Since Lp(a) is a larger particle than LDL and a part of Lp(a) is excluded from LDL by the HPLC, high Lp(a) subjects >1000 mg/l become outliers as shown in Fig. 1a (encircled closed square).

3.3. Comparison of lipid and lipoprotein profiles among lipid disorder groups

The scattered plot of (CM+VLDL)-C determined by the CDC method against serum TG levels is shown in Fig. 2a. Large deviations from the relation of VLDL-C=1/5×TG (solid line in Fig. 2a) were observed in the hyper TG and the apo E2/2 groups. In the case of 2 patients with LPL deficiency (encircled closed circle), the deviation was in the opposite direction to that of apo E2/2 subjects. The ratio of (CM+VLDL)-C to TG in the apo E2/2 group (0.42±0.09, $n=8$) was significantly ($P<0.0001$) higher than other subjects (0.19±0.07, $n=89$). The ratio of HDL-C to apo A-I in the hyper HDL group (0.60±0.08, $n=10$) was significantly ($P<0.0001$) higher than other subjects (0.34±0.07, $n=87$), and highly correlated with HDL size ($r=0.865$, $n=97$, $P<0.0001$).

As shown in Table 1, no peak of LDL was observed in the HPLC pattern of all the apo E2/2 subjects and 3 subjects of hyper TG group. The particle sizes of VLDL in the 3 subjects in hyper TG group without LDL peak (42.5±2.7 nm, $n=3$) were significantly ($P<0.01$) larger than those of the apo E2/2 group (31.3±1.3 nm, $n=8$). Therefore, it might be possible that dysbetalipoproteinemia is identified by HPLC pattern profiles; no LDL peak and the presence of VLDL particles less than 34 nm.

For the subjects with both LDL and HDL peaks on a chromatographic pattern, the scattered plot of LDL size against HDL size is presented in Fig. 2b. Positive correlation was observed between LDL and HDL sizes in all the subjects ($r=0.338$, $P<0.001$, $n=86$), and the correlation of coefficient was markedly increased ($r=0.492$, $P<0.0001$, $n=83$) by excluding 3 CETP deficient subjects (encircled triangle in Fig. 2b).

3.4. TC, (CM+VLDL)-C and HDL-C levels by HPLC and CDC reference methods

By the HPLC method, TC, (CM+VLDL)-C and HDL-C can be measured from total peak area (sum of component peaks 1 to 20), sum of component peaks 1 to 7 corresponding to (CM+VLDL) particle size and sum of component peaks 14 to 20 corresponding to HDL particle size, respectively. TC, (CM+VLDL)-C and HDL-C levels by HPLC were compared with those by the CDC reference method for the five groups (Table 3). There were very high correlations between HPLC and CDC methods for TC determination and no significant differences were obtained in all subjects except for the hyper TG group, as described in our previous study [13]. Except for hyper Lp(a) group, there was very high correlation between HPLC and CDC methods for (CM+VLDL)-C levels. (CM+VLDL)-C levels by the HPLC were significantly ($P<0.01$) lower in the hyper TG group and significantly higher ($P<0.0001$) in the hyper Lp(a) group than those by the CDC method. HDL-C levels by the HPLC were significantly higher than those by the CDC method in all groups although very high correlations were obtained between HPLC and CDC methods and these results are consistent with our previous report [13].

4. Discussion

In this study, we determined LDL-C levels by the HPLC and the BQ methods, and the Friedewald equation for healthy volunteers and outpatients with lipid disorders, and the results were compared between these methods in the 5 separated groups classified according to lipid disorder profiles. For the control group, there was no significant difference between the F-LDL and the BQ-LDL, but significantly negative mean bias was found in the HPLC-LDL. One explanation for this negative bias is that Lp(a), which is co-precipitated with apo B-containing lipoproteins by precipitation methods, is included in both F-LDL and BQ-LDL, and that a part of Lp(a) is estimated not to be contained in the HPLC-LDL since Lp(a) is larger than LDL and might correspond to small VLDL subclass by HPLC. These facts are confirmed by the result that (CM+VLDL)-C levels of the hyper Lp(a) group were overestimated by the HPLC and did not correlate to those by the reference method (Table 3), resulting in the underestimation of LDL-C by the HPLC in the hyper Lp(a) group (Table 2). Another reason of the negative bias for the HPLC-LDL is a significant overestimation of HDL-C by HPLC as presented in Table 3. As previously reported, the HPLC method overestimates HDL-C levels,

Table 3
Comparison of TC, (CM+VLDL)-C and HDL-C levels between the HPLC and the reference methods

Variable	Group	Reference method (mg/l)		HPLC method (mg/l)		Correlation r	Bias (mg/l)	% Bias
		Mean±SD	Min/max	Mean±SD	Min/max		Mean±SD	Mean±SD
TC	Control (n=50)	1917±343	1220/2720	1909±340	1204/2680	0.994†	−8±39	−0.4±1.9
	Hyper TG (n=17)	2953±1028	2047/6518	2805±938	1474/5722	0.968†	−148±263§	−4.9±9.0§
	Apo E2/2 (n=8)	2105±419	1480/2798	2098±422	1557/2776	0.986†	−7±70	−0.3±3.9
	Hyper HDL (n=10)	2663±348	2040/3182	2647±343	2097/3193	0.975†	−15±77	−0.5±2.9
	Hyper Lp(a) (n=12)	2048±610	1350/3332	2073±620	1371/3364	0.998†	25±39	1.2±2.0
(CM+VLDL)-C	Control (n=50)	248±188	40/950	253±166	32/793	0.945†	6±62	17.0±55.1§
	Hyper TG (n=17)	1766±972	804/5003	1486±733	607/3895	0.965†	−280±326‡	−13.5±13.3†
	Apo E2/2 (n=8)	1000±397	484/1582	858±408	309/1328	0.971†	−141±98‡	−16.9±13.6§
	Hyper HDL (n=10)	107±81	18/279	106±55	42/205	0.961†	−2±32	21.4±49.0
	Hyper Lp(a) (n=12)	191±41	126/246	372±129	209/668	0.435	181±117†	100.0±74.8†
HDL-C	Control (n=50)	519±105	265/737	545±107	276/764	0.987†	26±17†	5.3±3.4†
	Hyper TG (n=17)	316±121	113/600	338±113	120/578	0.982†	22±24‡	8.6±8.9‡
	Apo E2/2 (n=8)	525±121	386/693	558±126	394/698	0.980†	33±25‡	6.5±5.2‡
	Hyper HDL (n=10)	1292±242	1003/1700	1448±307	1140/2096	0.951†	156±107‡	11.9±7.1†
	Hyper Lp(a) (n=12)	485±222	217/976	534±223	280/1045	0.993†	49±27†	12.3±8.5†

Bias and % bias are judged by the one-sample t test (test value: 0).
†P<0.001, ‡P<0.01, §P<0.05.

compared with a precipitation method [12,13], and this trend is pronounced in the case of hyper HDL subjects with a large amount of apo E rich HDL.

In the present study, underestimation of LDL-C by the HPLC was more strongly associated with hyper HDL subjects (mean bias: −177 mg/l) than the controls (mean bias: −40 mg/l). On the other hand, the bias of F-LDL from BQ-LDL was small, because of the use of the same HDL-C values determined by the precipitation method for LDL-C calculation. Therefore, apo E rich HDL is estimated as LDL-C by both methods and this calls the question whether apo E rich HDL should be included in HDL-C measurement, especially in the case of patients with CETP deficiency [23].

For the subjects with TG >4000 mg/l or with apo E2/2, the Friedewald calculation was not accurate, mainly because of inaccurate estimation of VLDL-C from serum TG levels [4,7,8], and remarkable heterogeneity of VLDL particles [24,25], as demonstrated in this study (Fig. 2a). Therefore, the F-LDL in the apo E2/2 group was significantly higher than the BQ-LDL, affected by the presence of cholesteryl ester rich VLDL. In order to obtain accurate LDL-C levels in the subjects with apo E2/2, ultracentrifugal separation of VLDL might be required. Although the subjects with TG ≥4000 mg/l are identified easily in clinical laboratory, identification of dysbetalipoproteinemia is very difficult without detailed examinations of lipid, apolipoproteins, and a ratio of VLDL-C to serum TG or detection of broad β band by agarose gel electrophoresis [26]. Agarose gel electrophoresis has been widely available to detect lipoprotein abnormalities in clinical laboratories, including Lp(a), lipoprotein-X, and apo E rich HDL. Contois et al. compared the cholesterol levels in Lp(a) by enzymatic cholesterol staining after electrophoresis with the accepted Lp(a) mass assay, and obtained a significant correlation between Lp(a)-cholesterol and Lp(a) mass [27]. Although our HPLC method with TSKgel lipopropak columns cannot detect Lp(a) as a single separated peak, the identification of dysbetalipoproteinemia was easily performed by HPLC pattern profiles, in which no LDL peak and the presence of small VLDL particle size were observed.

The HPLC-LDL was significantly higher than the BQ-LDL in the hyper TG groups, but not significantly different in other four groups. These results indicate that a part of VLDL is not separated from LDL by the HPLC method using the TSKgel columns as described previously [14].

The positive correlation of LDL and HDL particle sizes is considered as the results of the normal function of CETP, and this is confirmed by the large deviation for 3 patients with CETP deficiency as presented in Fig. 2b. Moreover, particle sizes of HDL were highly correlated with the ratio of HDL-C to apo A-I.

In conclusion, there were several discrepancies in LDL-C levels determined by the HPLC and the BQ methods in samples with abnormal lipoproteins. However, this does not necessarily indicate an inferiority of the HPLC method, because HPLC pattern profiles can be informative and useful as a new analytical tool for analysis of abnormal lipoproteins.

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