methods

Measurement of cholesterol of major serum lipoprotein classes by anion-exchange HPLC with perchlorate ion-containing eluent

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Abstract **We have developed a high-performance liquid chromatography (HPLC) method for measurement of cholesterol in the major classes of serum lipoproteins, i.e., HDL, LDL, IDL, VLDL, and chylomicrons. Lipoproteins in serum were separated on a column containing diethylaminoethyl-ligand nonporous polymer-based gel by elution with a step gradient of sodium perchlorate concentration, and detected by post-column reaction with a reagent containing cholesterol esterase and cholesterol oxidase. The within-day assay and between-day assay coefficients of variation for cholesterol concentration in lipoproteins were in the ranges of 0.9–6.4% and 1.1–11.9%, respectively. The correlation coefficients between the values of HDL, LDL, IDL, VLDL, and chylomicron cholesterol measured by the HPLC method and those estimated by an ultracentrifugation method were 0.892, 0.921, 0.840, 0.930, and 0.873, respectively. Values of remnant-like particle cholesterol measured by an immunoseparation technique (Japan Immunoresearch Laboratories, Japan) were significantly correlated with VLDL and chylomicron cholesterol values measured by the HPLC method (r = 0.883 and r = 0.729, respectively). This rapid and accurate HPLC method was successfully applied to the analysis of plasma lipoproteins of patients with hyperlipidemia.**—Hirowatari, Y., H. Yoshida, H. Kurosawa, K-i. Doumitu, and N. Tada. **Measurement of cholesterol of major serum lipoprotein classes by anion-exchange HPLC with perchlorate ion-containing eluent. *J. Lipid Res.* 2003.** 44: **1404–1412.**

Supplementary key words hyperlipidemia • high-performance liquid chromatography • lipoprotein remnant-like particle

Hyperlipidemia is a risk factor for atherosclerotic events (1). LDL cholesterol plays a causal role in the development of atherosclerosis, and the guidelines adopted by the National Cholesterol Education Adult Treatment Panel in 1988 (ATP-I) recommended that normal values of LDL cholesterol are <3.36 mmol/l (2, 3). Updated guidelines released in 1993 (ATP-II) recognized HDL cholesterol as an independent risk factor for coronary artery disease (CAD), recommending that HDL cholesterol of <0.91 mmol/l be considered high risk for CAD, while ⩾1.56 mmol/l be considered protective against CAD (4). The latest guidelines adopted in 2001 (ATP-III) describe triglyceride (TG) as an independent risk factor, and recommend that TG values of <1.7 mmol/l are considered normal (5).

It is well known that the major classes of human lipoproteins are HDL, LDL, IDL, VLDL, and chylomicrons (6, 7). Numerous studies have investigated the relationship between IDL level and the risk of CAD (8–12). Krauss et al. (11) reported that IDL mass and ratios of HDL cholesterol to total cholesterol (TC) or LDL cholesterol were predictors for progression of CAD. Tarami et al. (8) reported that a high IDL cholesterol level was associated with a high frequency of CAD. Various methods for analysis of lipoproteins by ultracentrifugation (6, 7, 13), electrophoresis (14–16), gel-permeation chromatography (17, 18), and anion-exchange chromatography (19) have been reported. The cholesterol levels of all the major classes of lipoproteins in serum can be measured by ultracentrifugation, but it takes a long time to perform the analysis (6, 7, 13). The other methods have a poor ability to measure IDL cholesterol levels (14–19).

It is generally thought that remnant lipoproteins promote atherosclerosis. Remnant lipoproteins are products of partially catabolized chylomicrons and VLDL generated by lipoprotein lipase. Recently, an immunoseparation method was developed in order to determine serum

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Abbreviations: CV, coefficient of variation; CAD, coronary artery disease; RLP, remnant-like particle; TC, total cholesterol; TG, triglyceride.

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levels of cholesterol of remnant-like particle (RLP) (20). These RLP fractions consist of chylomicron remnants and a fraction of VLDL enriched in apolipoprotein E (apoE) (21–23). RLP cholesterol levels have been found to be high in sera of patients with CAD (24, 25), Type III hyperlipoproteinemia (26), and diabetic nephropathy (27).

We have developed a new method for lipoprotein analysis by anion-exchange chromatography on a diethylaminoethyl-ligand column. The use of a sodium perchlorate concentration gradient in the eluting solution allowed easy and rapid separation and determination of HDL, LDL, IDL, VLDL, and chylomicrons in serum. The obtained cholesterol levels of lipoproteins were compared with RLP cholesterol levels. This high-performance liquid chromatography (HPLC) method was confirmed as eligible for the rapid and accurate analysis and determination of the five major lipoprotein classes in hyperlipidemic sera.

MATERIALS AND METHODS

Materials and chemicals

The enzymatic cholesterol reagent for HPLC was the commercially available Cholesterol-E test Wako kit (Wako Pure Chemical Industries, Osaka, Japan). TC, TG, HDL cholesterol, and LDL cholesterol in samples were determined enzymatically using commercially available kits, Tcho-l, TG-LH (Wako Pure Chemical Industries), Cholestest N HDL, and Cholestest LDL (Daiichi Pure Chemicals Co., Tokyo, Japan), respectively. RLP cholesterol levels were also determined with a commercially available kit (Jimro-II, Japanese Immunoresearch Laboratories Co., Gunma, Japan).

Chromatography

The HPLC system was composed of two pumps, an anion-exchange column, a post-column reactor, and a photometer. The column, which contained 2.5 μm of nonporous polymer-based gel with diethylaminoethyl ligands, was 4.6 mm ID × 20 mm in size. The column was replaced after 300 samples had been analyzed. Eluent A (50 mM Tris-HCl + 1 mM ethylenediamine tetraacetic acid, disodium salt, dihydrate, pH 7.5) and Eluent B (50 mM Tris-HCl + 500 mM sodium perchlorate + 1 mM ethylenediamine tetraacetic acid, disodium salt, dihydrate, pH 7.5) were used to separate the lipoproteins. We used a pump (CCPM-II, Tosoh Corp., Tokyo, Japan) for Eluents A and B, which are delivered through two pump heads for gradient elution. The flow rate was 0.8 ml/min. Eluents A and B were mixed on-line. The step gradient patterns for separation of the lipoprotein classes were 20.5% Eluent B for 0–3.5 min, 24.5% Eluent B for 3.5–7.0 min, 27.5% Eluent B for 7.0–9.0 min, 32.5% Eluent B for 9.0–12.0 min, 100% Eluent B for 12–13 min, and 20.5% Eluent B for 13–20 min. Therefore, it took 20 min to complete the assay of one sample. The eluent flowed into the photometer after 6 min. **Figure 1** shows a representative chromatogram of hyperlipidemic serum during the changes in the step gradient indicated by a hatched line. An auto sampler (AS-8021, Tosoh Corp.) was used. The temperature of the column was maintained at 25°C with a column oven (CO-8020, Tosoh Corp.). The eluate from the column was mixed with an enzymatic cholesterol reagent, which contained cholesterol esterase, cholesterol oxidase, peroxidase, 4-aminoantipyrine, *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline, and sodium salt. The flow rate of the enzymatic cholesterol reagent was set at 0.4 ml/min by using a pump (DP-8020, Tosoh Corp.). The mixed solution reacted at 37°C for 5 min in a Teflon coil (0.5 mm ID × 31 m). A reactor (CO-8020, Tosoh Corp.) was used for the postcolumn reaction. The eluate from the reactor was monitored at 600 nm using a photometer equipped with a flow cell (UV-8020, Tosoh Corp.). Chromatograms were recorded by a data processor (SC-8020, Tosoh Corp.). It was confirmed that the concentration of sodium perchlorate in Eluent B did not interfere with enzymatic activities in the cholesterol reagent (data not shown).

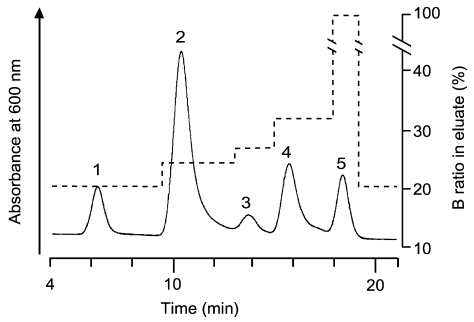

Fig. 1. Chromatogram of hyperlipidemic serum and pattern of step gradient. The patterns of lipoprotein profile and step gradient were indicated by a solid line and dotted line, respectively. Sample for chromatogram was serum of hyperlipidemic Patient 2. Retention times of Peaks 1, 2, 3, 4, and 5 were 6.15, 10.20, 13.60, 15.60, and 18.28 min, respectively.

Control serum (TC = 4.42 mmol/l, TG = 1.63 mmol/l, LDL cholesterol = 1.89 mmol/l, and HDL cholesterol = 1.50 mmol/l) was stored at −60°C. The cholesterol concentration of each lipoprotein peak in chromatograms of hyperlipidemic sera was calculated by the proportion of the peak area of each lipoprotein to the chromatogram's total area reflecting the TC level. TC levels of hyperlipidemic sera were calculated by the proportion of these chromatograms' total area of lipoprotein peaks of sample sera to the total area of chromatogram peak of the control serum with known concentration of TC.

Samples

Serum from a healthy subject (TC = 4.42 mmol/l, TG = 1.63 mmol/l, LDL cholesterol = 1.89 mmol/l, and HDL cholesterol = 1.50 mmol/l), described above as the control serum, and sera from two hyperlipidemic patients (Patient 1, TC = 5.87 mmol/l, TG = 4.16 mmol/l, LDL cholesterol = 1.99 mmol/l, and HDL cholesterol = 0.98 mmol/l; Patient 2, TC = 7.40 mmol/l, TG = 6.14 mmol/l, LDL cholesterol = 4.45 mmol/l, and HDL cholesterol = 0.91 mmol/l) were used for separation of HDL, LDL, IDL, VLDL, and chylomicrons by ultracentrifugation. Thirty-six hyperlipidemic sera were used to examine correlations between the data obtained by the HPLC method and that obtained by an ultracentrifugation method. The data for the hyperlipidemic sera were as follows: TC (mean = 6.59 mmol/l, SD = 1.10 mmol/l, max-min = 8.97–4.75 mmol/l), TG (mean = 5.42 mmol/l, SD = 1.99 mmol/l, max-min = 12.02–0.43 mmol/l), HDL cholesterol (mean = 2.60 mmol/l, SD = 0.98 mmol/l, max-min = 4.57–1.03 mmol/l), and LDL cholesterol (mean = 1.47 mmol/l, SD = 0.78 mmol/l, max-min = 4.12–0.72 mmol/l).

Sera of nine healthy subjects and 19 hyperlipidemic patients were used for the comparison of lipoprotein cholesterol data obtained by the HPLC method and RLP cholesterol values. The 28 sera were obtained from venous blood samples drawn after a 12 h fast. The sera were stored at 4°C and analyzed within 3 days. In sera of the healthy subjects, TC and TG were less than 5.66 mmol/l and 1.68 mmol/l, respectively. The healthy group ex-

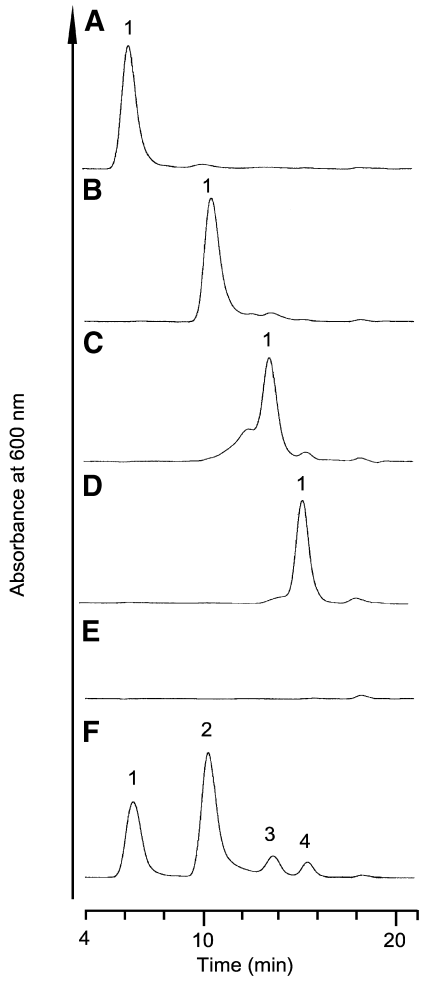

Fig. 2. Chromatograms of lipoproteins separated from healthy serum by ultracentrifugation. Samples for chromatograms A, B, C, D, and E were HDL, LDL, IDL, VLDL, and chylomicrons separated by the ultracentrifugation method, respectively. Chromatogram F is that of whole healthy serum. Retention times of Peak 1 in A, B, C, and D were 6.28, 10.48, 13.51, and 15.43 min, respectively. Retention times of Peaks 1, 2, 3, and 4 in F were 6.40, 10.26, 13.64, and 15.41 min, respectively.

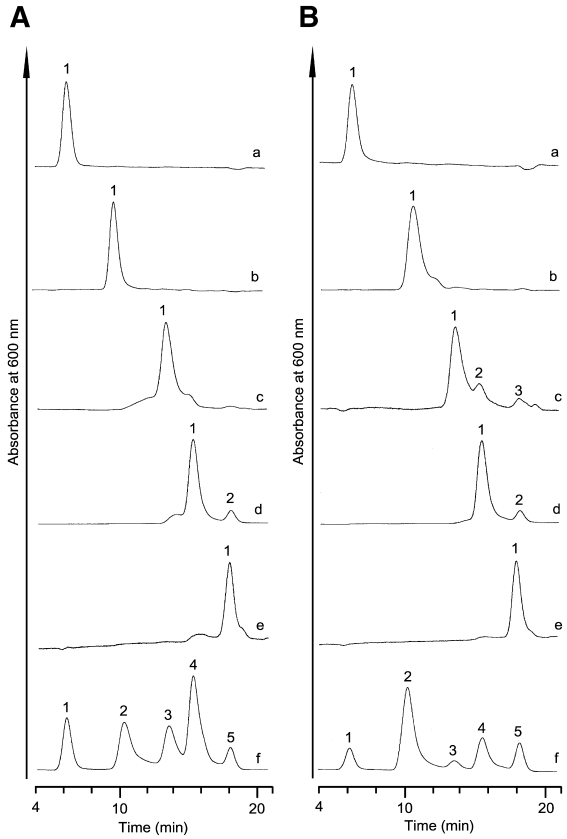

Fig. 3. Chromatograms of lipoproteins separated from hyperlipidemic sera by ultracentrifugation. A: Samples for chromatograms a, b, c, d, and e were HDL, LDL, IDL, VLDL, and chylomicrons separated from serum of hyperlipidemic Patient 1 serum by the ultracentrifugation method, respectively. Chromatogram f is that of the whole serum. Retention times of Peak 1 in a, Peak 1 in b, Peak 1 in c, Peaks 1 and 2 in d, and Peak 1 in e were 6.29, 9.95, 13.50, 15.45, 18.25, and 18.05 min, respectively. Retention times of Peaks 1, 2, 3, 4, and 5 in f were 6.13, 10.35, 13.65, 15.45, and 18.20 min, respectively. B: Samples for chromatograms a, b, c, d, and e were HDL, LDL, IDL, VLDL, and chylomicrons separated from serum of hyperlipidemic Patient 2 by the ultracentrifugation method, respectively. Chromatogram f is that of the whole serum. Retention times of Peak 1 in a, Peak 1 in b, Peaks 1, 2, and 3 in c, Peaks 1 and 2 in d, and Peak 1 in e were 6.27, 10.56, 13.61, 15.27, 18.12, 15.50, 18.22, and 18.04 min, respectively. Retention times of Peaks 1, 2, 3, 4, and 5 in f were 6.15, 10.20, 13.60, 15.60, and 18.28 min, respectively.

cluded subjects with diabetes mellitus, hypertension, heart disease, thyroid disorder, liver dysfunction, or renal dysfunction. The 19 hyperlipidemic patients had serum levels of TC > 6.21 mmol/l or TG > 1.70 mmol/l.

Ultracentrifugation method

Sequential ultracentrifugation of serum lipoproteins was performed by the method reported previously (6, 7). The flotation rates of chylomicrons and VLDL were set at >400 and 20–400, respectively, in a solution of 1.745 mol/l sodium chloride (d = 1.063 g/ml). Densities of IDL, LDL, and HDL were set as follows: 1.006 < d < 1.019 g/ml, 1.019 < d < 1.063 g/ml, and 1.063 < d g/ml, respectively. An SCP70H2 ultracentrifuge (Hitachi Koki Co., Tokyo, Japan) and an RP55T angle rotor (Hitachi Koki Co.) were used.

Linearity and precision tests

To test for linearity, hyperlipidemic serum (TC = 6.65 mmol/l, TG = 13.11 mmol/l, LDL cholesterol = 3.13 mmol/l, and HDL cholesterol = 1.03 mmol/l) was used. The samples were diluted serially with 0.05% BSA solution, and 7 μl aliquots were injected. For the within-day assay and between-day assay precision tests, the hyperlipidemic serum was stored at 4°C until used. The injected volume was 3.5 μl.

Correlation test

HDL, LDL, IDL, VLDL, and chylomicron fractions from the 36 hyperlipidemic sera were separated by the sequential ultracentrifugation method. Cholesterol levels of each lipoprotein fraction and the whole serum were determined using an enzymatic cholesterol kit (Tcho-l, Wako Pure Chemical Industries) with an automated chemical analyzer (Model 7350E, Hitachi Koki Co.).

Statistics

The correlations between cholesterol values of each lipoprotein measured by the HPLC method and those estimated by an ultracentrifugation method or RLP cholesterol values were eval-

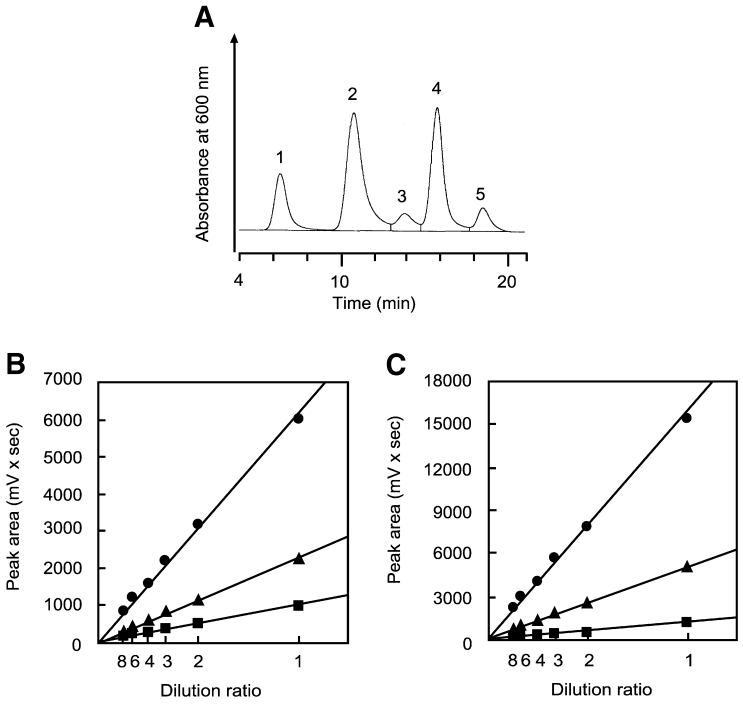

Fig. 4. Linearity of peak areas of each lipoprotein. A: A hyperlipidemic serum was analyzed. The retention times of Peaks 1, 2, 3, 4, and 5 were 6.38, 10.70, 13.77, 15.67, and 18.45 min, respectively. The sample diluted two times with 0.05% BSA solution was analyzed using an injection volume of 7 μl. B: The data are areas of Peaks 1 (triangle), 2 (circle), and 5 (square) from 7 μl samples diluted up to eight times. C: The data are areas of Peak 3 (square), Peak 4 (triangle), and total peaks (circle) in 7 μl samples diluted up to eight times.

uated in terms of Pearson product-moment correlation coefficients. Student's unpaired *t*-test was used for determining the statistical significance of differences ($P < 0.05$) between cholesterol values of each lipoprotein and RLP cholesterol values of the healthy group and those of the hyperlipidemic group.

RESULTS

Chromatogram of the HDL, LDL, IDL, VLDL, and chylomicron fractions separated by ultracentrifugation

Four peaks were identified in the chromatogram of serum from a healthy subject (**Fig. 2F**). These peaks were eluted at 20.5%, 24.5%, 27.5%, and 32.5% Eluent B, respectively, and were detected at 6.40, 10.26, 13.64, and 15.41 min, respectively (Fig. 2F). The HDL, LDL, IDL, VLDL, and chylomicron fractions of the serum were analyzed by the HPLC method to identify the four peaks. Figures 2A–E show the chromatogram patterns of the individual lipoprotein fractions. The peaks of HDL, LDL, IDL, and VLDL derived from the healthy serum were eluted at 6.28, 10.48, 13.51, and 15.43 min, respectively (Figs. 2A–D). Figures 2A–D and F indicate that the major components of Peaks 1, 2, 3, and 4 of the healthy serum are HDL, LDL, IDL, and VLDL, respectively. In the chromatogram, the lipoprotein peak of the chylomicron fraction was essentially absent, which is as expected, because the amount of chylomicrons in healthy serum is very small (Fig. 2E).

Five peaks were identified in chromatograms of hyperlipidemic sera (**Fig. 3**). These peaks were eluted at 20.5%, 24.5%, 27.5%, 32.5%, and 100% Eluent B, respectively.

TABLE 1. Precision data for assay of hyperlipidemic serum[a]

	Retention Time						Cholesterol Concentration					
	Between Assay			Within Assay			Between Assay			Within Assay		
Lipoprotein[b]	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
	min		%	*min*		%	*mmol/l*		%	*mmol/l*		%
HDL	6.47	0.070	1.08	6.42	0.030	0.47	1.00	0.017	1.72	0.98	0.017	1.76
LDL	10.61	0.084	0.79	10.58	0.048	0.46	2.79	0.108	3.78	2.73	0.033	1.20
IDL	13.81	0.050	0.36	13.76	0.045	0.33	0.37	0.037	9.75	0.35	0.015	4.19
VLDL	15.73	0.040	0.25	15.68	0.032	0.21	2.17	0.025	1.12	2.15	0.020	0.93
Chylomicrons	18.53	0.058	0.31	18.50	0.016	0.09	0.32	0.038	11.82	0.38	0.024	6.40
Total[c]	—	—	—	—	—	—	6.69	0.110	1.61	6.60	0.059	0.89

CV, coefficient of variation; SD, standard deviation.
[a] The sample used was the same as that for the linearity test (Fig. 3A).
[b] HDL, LDL, IDL, VLDL, and chylomicrons lipoprotein are Peaks 1, 2, 3, 4, and 5 in Fig. 3A, respectively.
[c] Total cholesterol concentration was calculated from the total peak area of lipoproteins.

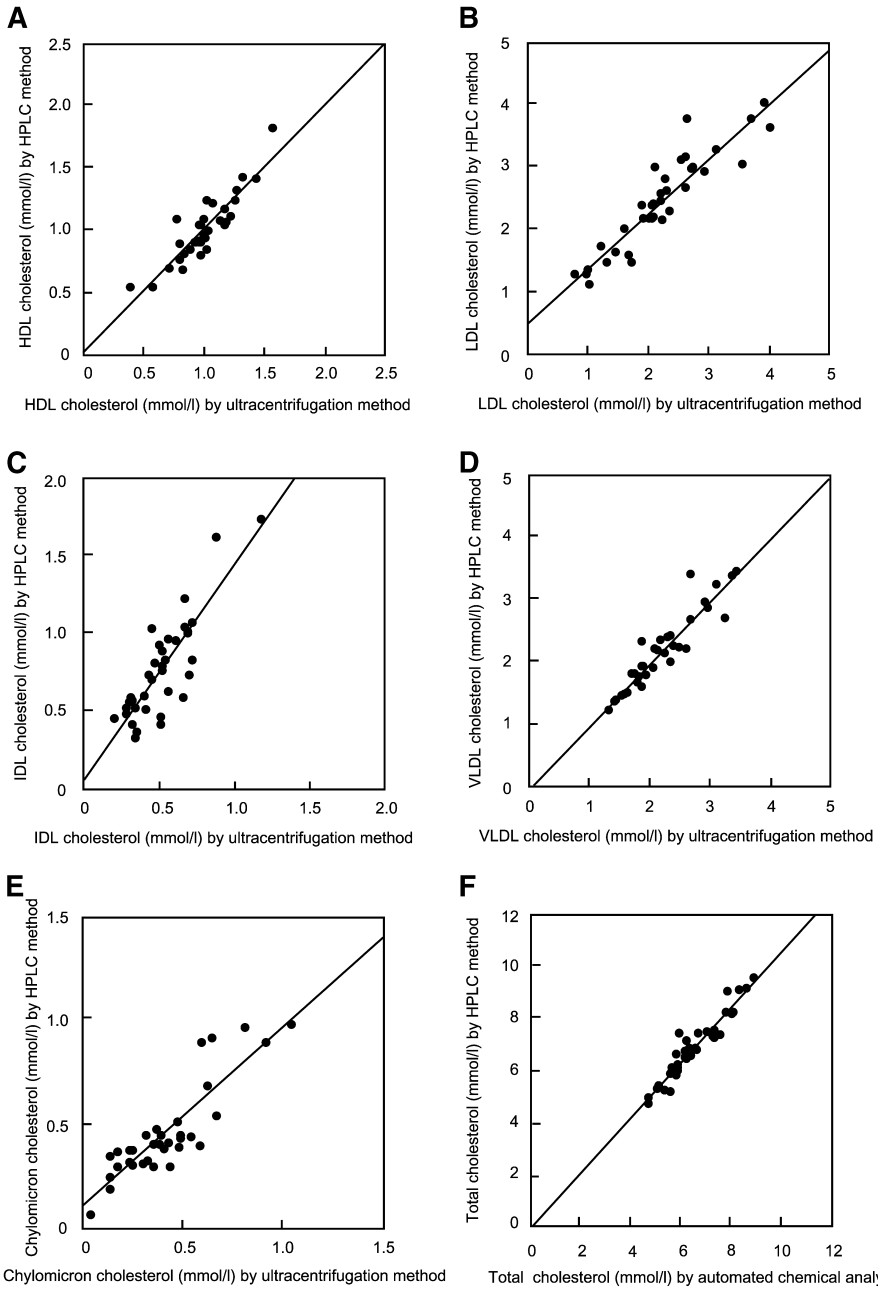

Fig. 5. Correlation of HDL cholesterol, LDL cholesterol, IDL cholesterol, VLDL cholesterol, chylomicron cholesterol, and total cholesterol (TC) values obtained by the high-performance liquid chromatography (HPLC) method with those obtained by using ultracentrifugation and an automated chemical analyzer. Aliquots of 3.5 μl of 36 hyperlipidemic sera were analyzed by the HPLC method, and the cholesterol concentration of each lipoprotein was determined. Each lipoprotein fraction in the 36 hyperlipidemic sera was separated by ultracentrifugation, and cholesterol levels in the lipoprotein fraction and the whole serum were measured using a commercial enzyme kit. A, B, C, D, E, and F show the correlations for HDL cholesterol, LDL cholesterol, IDL cholesterol, VLDL cholesterol, chylomicron cholesterol, and TC, respectively. Linear regression equations and correlation coefficients were as follows: A: HDL cholesterol ($y = 0.988x - 0.0012$, $r = 0.892$); B, LDL cholesterol ($y = 0.885x + 0.467$, $r = 0.921$); C, IDL cholesterol ($y = 1.369x + 0.0504$, $r = 0.840$); D, VLDL cholesterol ($y = 0.983x - 0.015$, $r = 0.930$); E, chylomicron cholesterol ($y = 0.856x - 0.091$, $r = 0.873$); F, TC ($y = 1.050x - 0.073$, $r = 0.954$).

The individual lipoprotein fractions of the hyperlipidemic sera were analyzed by the HPLC method to identify the five peaks. Figures 3Aa–e and 3Ba–e show the chromatogram patterns of the individual lipoprotein fractions. The peaks of HDL, LDL, IDL, and chylomicrons in the serum of hyperlipidemic Patient 1 were eluted at 6.29, 9.95, 13.50, and 18.05 min, respectively (Figs. 3Aa–c, 3Ae). In the serum of hyperlipidemic Patient 2, the lipoprotein peaks were eluted at 6.27, 10.56, and 18.04 min, respectively (Figs. 3Ba–b, 3Be). The major peaks of VLDL in the

TABLE 2. Characteristics of healthy subjects and hyperlipidemic patients

	Healthy Subjects n = 9	Hyperlipidemic Patients n = 19
Age (years)	49.9 ± 21.6	60.2 ± 12.6
Sex (male/female)	7/2	10/9
Total cholesterol (mmol/l)	4.77 ± 0.76	5.97 ± 1.05[b]
Total triglyceride (mmol/l)	0.95 ± 0.36	3.01 ± 0.91[c]
HDL cholesterol (mmol/l)	1.49 ± 0.24	1.15 ± 0.42[a]
LDL cholesterol (mmol/l)	2.94 ± 0.90	3.36 ± 1.08
IDL cholesterol (mmol/l)	0.21 ± 0.10	0.31 ± 0.16[a]
VLDL cholesterol (mmol/l)	0.33 ± 0.13	0.91 ± 0.30[c]
Chylomicron cholesterol (mmol/l)	0.073 ± 0.023	0.163 ± 0.07[c]
RLP cholesterol (mmol/l)	0.13 ± 0.04	0.27 ± 0.09[c]

Values are presented as mean ± SD. Total cholesterol and triglyceride were determined enzymatically using the commercial kits. HDL, LDL, VLDL, and chylomicron cholesterol were determined by the high-performance liquid chromatography method. Data compared with those of healthy subjects by Student's unpaired *t*-test.

[a] $P < 0.05$.

[b] $P < 0.01$.

[c] $P < 0.001$.

serum from hyperlipidemic Patient 1 and IDL and VLDL in the serum from hyperlipidemic Patient 2 were eluted at 15.45, 13.61, and 15.50 min, respectively (Figs. 3Ad, 3Bc–d). Figure 3Aa–f and 3Ba–f indicates that the major components of Peaks 1, 2, 3, 4, and 5 of the hyperlipidemic sera are HDL, LDL, IDL, VLDL, and chylomicrons, respectively.

In the IDL fraction of serum from hyperlipidemic Patient 2, minor peaks were found at 15.27 and 18.12 min (Fig. 3Bc). The peaks of IDL observed in the healthy serum and the serum of hyperlipidemic Patient 1 were broader than the peaks of HDL and LDL (Figs. 3Aa–c, 2A–C), probably because IDL is heterogeneous. In the VLDL fraction of sera from hyperlipidemic Patients 1 and 2, minor peaks were found at 18.25 and 18.22 min, respectively (Fig. 3Ad, 3Bd). The minor peak was not found in the VLDL fraction of the healthy serum (Fig. 2D).

Linearity and precision of the HPLC method

Figure 4A shows a chromatogram of the hyperlipidemic serum used for performing the linearity test. Linear relationships were found between the peak area of each lipoprotein class (Peaks 1, 2, 3. 4, and 5) and total peak area and dilution ratio in the range of up to eight times (Fig. 4B, C). **Table 1** shows the precision of this HPLC method applied to a hyperlipidemic serum. The values of within-day assay and between-day assay coefficients of variation (CVs) of cholesterol concentration of each lipoprotein class were less than 6.4% and 11.9%, respectively. The reproducibility was satisfactory. Within-day assay and between-day assay CV values of retention time were less than 1.1%, which is excellent.

The good linearity of the relationships between peak area of each lipoprotein class and dilution ratio, in addition to the high precision, indicates that the cholesterol levels of each lipoprotein class and total lipoproteins in sera can be reliably determined by this HPLC method.

Correlations between cholesterol concentrations of serum lipoproteins obtained by the HPLC method and those estimated by an ultracentrifugation method

Correlations between the values of HDL, LDL, IDL, VLDL, and chylomicron cholesterol and TC in 36 hyperlipidemic sera, measured by the two methods, are shown in **Figs. 5A–F**. The cholesterol concentrations were calculated from the peak areas of the lipoprotein classes. TC was calculated from total peak area of the chromatogram. The linear regression equations and the coefficients of correlation between values of HDL cholesterol, LDL cholesterol, IDL cholesterol, VLDL cholesterol, chylomicron cholesterol, and TC found by the HPLC method and those estimated by using ultracentrifugation and an automated chemical analyzer were $y = 0.988x - 0.0012$ ($r = 0.892$), $y = 0.885x + 0.467$ ($r = 0.921$), $y = 1.369x + 0.0504$ ($r = 0.840$), $y = 0.983x - 0.015$ ($r = 0.930$), $y = 0.856x - 0.091$ ($r = 0.873$) and $y = 1.050x - 0.073$ ($r = 0.954$), respectively. The satisfactory correlations between the results of the two different methods support the usefulness of our HPLC method for determination of cholesterol levels in HDL, LDL, IDL, VLDL, chylomicrons, and total lipoproteins.

Comparison of cholesterol levels of each lipoprotein measured by the HPLC method and RLP cholesterol level

Samples used for this comparison were sera from nine healthy subjects and 19 hyperlipidemic patients. **Table 2** shows the characteristics of the two groups. There were seven males and two females in the healthy group and 10 males and nine females in the hyperlipidemic group. Mean LDL cholesterol levels were similar. Hyperlipidemic patients had significantly lower levels of HDL cholesterol and higher cholesterol levels of IDL, VLDL, chylomicrons, and RLP cholesterol than did healthy subjects (Table 2). The correlation between the cholesterol levels of the five lipoprotein classes obtained by the HPLC method and the RLP cholesterol levels was examined. The correlation coefficients of HDL, LDL, IDL, VLDL, and chylomicrons were 0.493, 0.127, 0.166, 0.833, and 0.729, respectively (**Fig. 6**). Cholesterol concentrations of VLDL and chylomicrons obtained by the HPLC method were significantly correlated with RLP cholesterol ($P < 0.00001$ and $P < 0.00001$, respectively).

DISCUSSION

We showed that the five major classes of lipoproteins (HDL, LDL, IDL, VLDL, and chylomicrons) in serum can be separated within 20 min by means of a novel anion-exchange HPLC procedure involving elution with stepwise concentration changes of perchlorate ion. The sequential flotation ultracentrifugation method has the ability to separate major classes of lipoproteins and, moreover, subfractions of LDL, IDL, and VLDL can be separated by using a cumulative flotation ultracentrifugation method (13); however, it takes 4 days to separate the five major classes of lipoproteins by sequential flotation ultracentrifugation (13). Several HPLC methods for separation of lipoproteins have

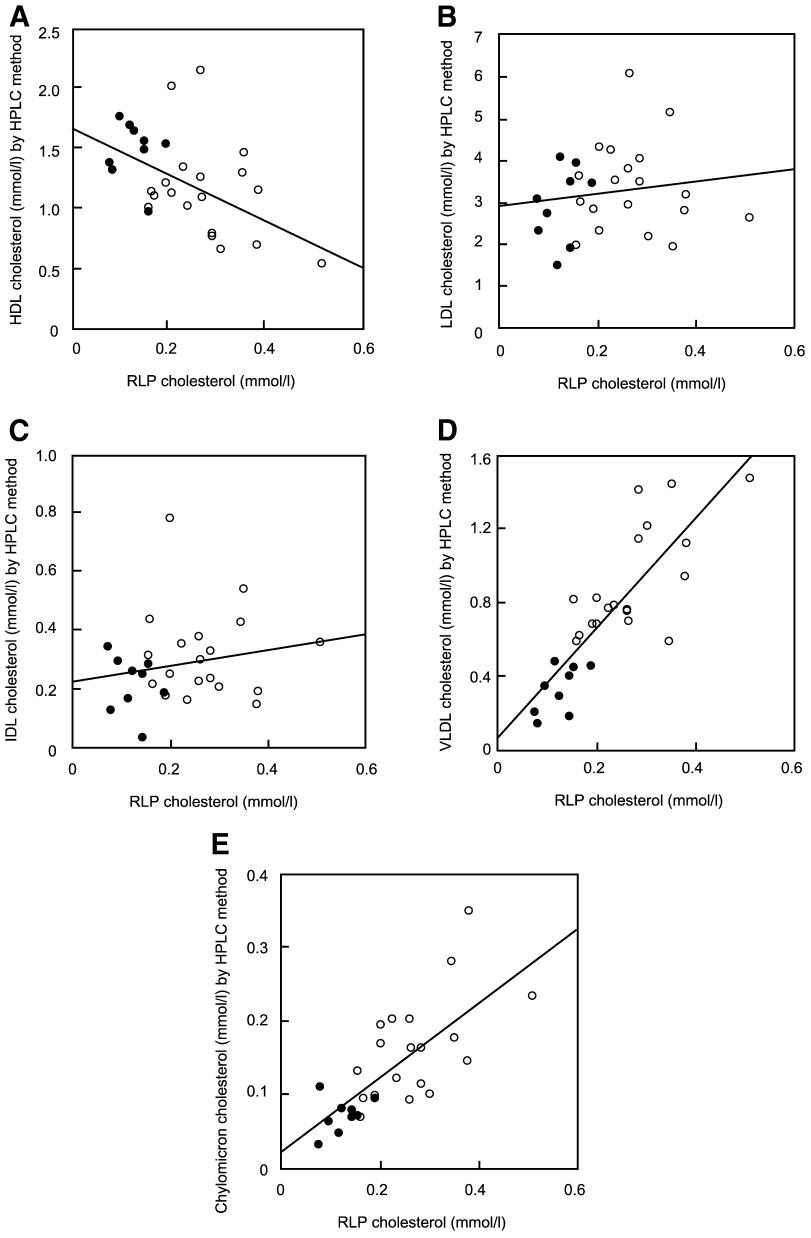

Fig. 6. Correlation of HDL cholesterol, LDL cholesterol, IDL cholesterol, VLDL cholesterol, and chylomicron cholesterol values obtained by the HPLC method with RLP cholesterol values. Aliquots of 3.5 μl of 28 sera were analyzed by the HPLC method, and the cholesterol concentration of each lipoprotein was determined. RLP cholesterol values in these sera were determined by using a commercial kit (Jimro-II, Japanese Immunoresearch Laboratories Co.). The data obtained for nine healthy sera and 19 hyperlipidemic sera are represented by closed circles and open circles, respectively. A, B, C, D, and E show the correlations for HDL cholesterol, LDL cholesterol, IDL cholesterol, VLDL cholesterol, and chylomicron cholesterol, respectively. Linear regression equations and correlation coefficients were as follows: A, HDL cholesterol ($y = -1.883x + 1.682$, $r = 0.493$); B, LDL cholesterol ($y = 1.250x + 2.944$, $r = 0.127$); C, IDL cholesterol ($y = 0.234x + 0.230$, $r = 0.166$); D, VLDL cholesterol ($y = 3.005x + 0.049$, $r = 0.833$); E, chylomicron cholesterol ($y = 0.507x + 0.020$, $r = 0.729$).

been reported (16–19). Okazaki et al. developed a separation method of plasma lipoproteins using gel permeation chromatography, with which analysis of one sample takes 16 min (17). In this method, the separation between HDL and LDL was sufficient, but the separation between LDL and VLDL was apparently not sufficient for measurement of the cholesterol levels of each lipoprotein (17). VLDL, IDL, and LDL in plasma from a patient with Type III hyperlipidemia, in which IDL is a major lipoprotein class, formed one broad peak (17). Haginaka et al. reported that HDL, LDL, and VLDL in plasma were completely separated within 20 min using an anion-exchange HPLC method with step-gradient elution (19); however, they did not examine the separation of IDL in plasma (19).

In a previous paper, we reported an HPLC method for serum lipoprotein using a cation-exchange column with magnesium ion-containing eluents (28). It was shown that HDL, LDL, IDL, and VLDL in hyperlipidemic serum were eluted in order from the column with a linear concentration gradient of magnesium nitrate, and that IDL did not form a distinct peak, being in part included in both the LDL and the VLDL peaks (28). Furthermore, IDL did not form a distinct peak in elution with a step gradient of magnesium nitrate concentration (data not shown). In contrast, we were able to separate HDL, LDL, IDL, VLDL, and chylomicrons in hyperlipidemic sera by using an anion-exchange column eluted with a step gradient of sodium perchlorate concentration, obtaining distinct peaks. However, the serum lipoproteins were not separated with a step gradient of sodium chloride, ammonium nitrate, or sodium sulfate concentration (data not shown). It is known that chaotropic ions such as perchlorate, iodide, and thiocyanate disrupt and decrease hydrophobic bonds (29). It is likely that the weak hydrophobic interaction between the lipoproteins and the gel surface in the column was disrupted by the eluent containing perchlorate ion, a chaotropic ion. Additionally, lipoproteins in a hyperlipidemic serum were separated by using the reported anion-exchange HPLC system with a linear gradient of sodium perchlorate concentration. In the 0–155 mM linear gradient of sodium perchlorate, two HDL peaks, one broad LDL peak, one IDL peak, and one broad VLDL peak were separated (data not shown). In the eluent containing 500 mM sodium perchlorate, chylomicrons were eluted from the column. The two HDL peaks and the broad forms of LDL and VLDL might reflect the subclasses of these lipoproteins, but this remains to be established.

The analysis of hyperlipidemic sera using agarose gel electrophoresis demonstrated that the chylomicrons were immobile (16). Therefore, chylomicrons do not carry a negative charge. In the present work, the chylomicrons in hyperlipidemic sera were bound to the gel and eluted by 100% Eluent B containing a high concentration of perchlorate ion (Fig. 3Af, 3Bf). The chylomicrons were probably bound more strongly than the other lipoproteins to the hydrophobic part of the gel.

Previous reports have shown that LDL, IDL, and VLDL in hyperlipidemic sera are heterogeneous (30–35). We observed one major peak and two minor peaks in the IDL fraction of hyperlipidemic Patient 2 (Fig. 3Bc), and serum from hyperlipidemic Patient 1 showed a broad peak of IDL (Fig. 3Ac). In addition, the LDL fraction showed tailing (Figs. 2b, 3Bb), and the VLDL fraction showed a slight leading portion (Figs. 2d, 3Ad). Therefore, the IDL peak appears to contain small amounts of LDL and VLDL, and this may be the reason why the IDL cholesterol concentrations estimated by our HPLC method were larger than those measured by ultracentrifugation (Fig. 5C).

In the VLDL fraction of hyperlipidemic sera, minor peaks were detected at ~18 min (Fig. 3Ad, 3Bd). The chylomicron cholesterol concentrations confirmed by this HPLC method were similar to those estimated by the ultracentrifugation method. This result suggests that chylomicrons do not account for the minor peak observed in the VLDL fraction. Conceivably, serum lipoproteins were partially disrupted during separation by ultracentrifugation, thereby resulting in generation of these minor peaks detected from the VLDL isolated by ultracentrifugation, but this remains to be examined.

It has been found that the cholesterol levels of VLDL and chylomicrons found by the HPLC method were positively correlated with RLP cholesterol levels (Fig. 6). Leary et al. reported that RLP cholesterol was correlated more highly with VLDL cholesterol measured by an ultracentrifugation method than with IDL cholesterol (22). Their results are consistent with ours. RLP contains chylomicron remnants and VLDL enriched in apoE (21–23). It was reported that VLDL enriched in apoE has a higher cholesterol level and smaller particle size compared with the average values of the VLDL fraction (34, 35). These findings are consistent with the strong correlation between VLDL cholesterol and RLP cholesterol. It is known that chylomicron remnants are products of partially catabolized chylomicrons in which some TGs have been hydrolyzed by lipoprotein lipase, and most of the cholesteryl esters of chylomicrons are retained in chylomicron remnants (36). Conceivably, most of the cholesterol esters of the chylomicron fraction in hyperlipidemic sera are included in chylomicron remnants. If so, this would support the correlation between chylomicron cholesterol and RLP cholesterol (Fig. 6), but this remains to be examined.

In conclusion, this study showed that the five major classes of lipoproteins in human sera can be separated by a novel anion-exchange chromatography procedure involving step-gradient elution with an eluent containing chaotropic ions, and that cholesterol levels in each lipoprotein can be determined. We validated the HPLC method by examining its linearity and precision, and the correlation of values measured by the HPLC method with data estimated by a sequential flotation ultracentrifugation method. The results suggest that the method presented here is suitable for rapid and accurate evaluation of cholesterol levels of HDL, LDL, IDL, VLDL, and chylomicrons in human hyperlipidemic sera.

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