Clinical Chemistry 43:10
1904–1912 (1997)

Lipids and Lipoproteins

Measurement of low-density lipoprotein particle size by high-performance gel-filtration chromatography

Peter G. Scheffer,[1*] Stephan J. L. Bakker,[2] Robert J. Heine,[2] and Tom Teerlink[1]

We describe a new technique for measuring LDL size by high-performance gel-filtration chromatography (HPGC). LDL was subjected to chromatography, and the column effluent was monitored at 280 nm. The retention time of the LDL peak was used to calculate the LDL diameter. We compared the HPGC method with gradient gel electrophoresis (GGE) on 2–10% nondenaturing polyacrylamide gels. In a group of 60 non-insulin-dependent diabetes mellitus patients, LDL size as measured by HPGC and GGE was highly correlated (r = 0.88, P <0.001). Good reproducibility, high precision, and the possibility of analyzing large series of samples are the main advantages of the automated HPGC method. Within-run and between-run CV for LDL size measured by HPGC were <0.1% and 0.2%, respectively. There was a significant inverse association between LDL size measured by HPGC and the logarithm of plasma triglycerides (r = −0.84, P <0.001), and a significant positive association with the LDL free cholesterol/protein ratio (r = 0.89, P <0.001).

LDL is the major carrier of plasma cholesterol in humans. Plasma LDL is a heterogeneous collection of particles varying in size, density, lipid content, and atherogenic potential. The diameter of LDL varies considerably from one individual to another. Smaller LDL particles are associated with high concentrations of plasma triglycerides (TG)[3] and low concentrations of HDL cholesterol *[1–4]*. Austin and Krauss *[5]* identified two LDL phenotypes: pattern A characterized by large buoyant LDL (>25.5 nm) and pattern B in which small dense LDL particles (<25.5 nm) predominate. Recently, Austin et al. *[4]* demonstrated that pattern B is a risk factor for the future development of non-insulin-dependent diabetes mellitus (NIDDM). According to other reports small LDL particles are more prevalent in coronary artery disease patients *[6]* and are associated with a threefold increased risk of myocardial infarction *[7]*. Several prospective studies demonstrated a relation between the presence of small, dense LDL particles and an increased risk of developing coronary disease *[8–10]*.

Several mechanisms have been postulated to explain the increased atherogenicity of small LDL. Studies on the interaction of LDL with cultured human fibroblasts indicate that small LDL has a reduced affinity for the LDL receptor compared with LDL of normal size, leading to an increased residence time in plasma *[11–13]*. This could lead to an enhanced interaction of small LDL with the arterial wall. Oxidation of LDL in the subendothelial space of the arterial wall is thought to play a key role in the pathogenesis of atherosclerosis *[14, 15]*. Recent studies have indicated that small, dense LDL is more readily oxidized than larger buoyant LDL *[16, 17]*. In addition, small LDL may more easily penetrate the arterial wall *[18, 19]* and has been shown to have increased interaction with arterial wall proteoglycans *[20]*.

A widely used technique for the measurement of LDL size is gradient gel electrophoresis (GGE) *[21, 22]*. However, this method is rather laborious. Ultracentrifugation *[23]*, dynamic light scattering *[24]*, and electron microscopy (EM) *[25]* can also be used for characterization of LDL particles by size and shape. These techniques are less suitable for larger series. To realize a high throughput of samples, an automated procedure is desirable.

Several reports discuss the use of gel-filtration chromatography to separate lipoprotein particles according to their size *[26–32]*. The column effluent is collected in fractions for subsequent cholesterol and TG measurements *[28, 31, 32]*, or lipoproteins are detected on-line

Departments of [1]Clinical Chemistry and [2]Endocrinology, Research Institute for Endocrinology, Reproduction and Metabolism, Academic Hospital Vrije Universiteit, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands.
* Author for correspondence. Fax 31-20-4443895.

[3] Nonstandard abbreviations: TG, triglyceride(s); CE, cholesterol ester(s); EM, electron microscopy; FC, free cholesterol; TC, total cholesterol; GGE, gradient gel electrophoresis; HPGC, high-performance gel-filtration chromatography; Lp(a), lipoprotein(a); NIDDM, non-insulin-dependent diabetes mellitus; PBS, phosphate-buffered saline; PL, phospholipid(s); TBE, Tris–boric acid–EDTA; apo, apolipoprotein.

Received April 4, 1997; revision accepted June 16, 1997.

after postcolumn derivatization with cholesterol reagent *[29, 30]*. Lipoprotein classes can thus be quantified.

Here we report on a procedure for LDL size measurement by high-performance gel-filtration chromatography (HPGC) after LDL isolation by ultracentrifugation. LDL was isolated from the plasma of 60 NIDDM patients with plasma TG in the range of 0.5–5.6 mmol/L. LDL particle diameter obtained by HPGC was compared with values obtained by polyacrylamide GGE. In addition, we present data on the relation between size and composition of the LDL particle.

Materials and Methods

PLASMA SAMPLES

Subjects were selected from NIDDM patients (31 men, 29 women) attending a diabetes center in a primary care setting in Amsterdam *[33]*. Selection was made to ensure a broad distribution of fasting plasma TG. Clinical characteristics of the study subjects are summarized in Table 1. Informed consent was obtained from all participants, and the study protocol was approved by our institutional ethics committee. Fasting blood samples (3 mL) were collected into evacuated tubes containing EDTA (final concentration ~4.1 mmol/L) as both anticoagulant and antioxidant *[34]*. Plasma was isolated by centrifugation at 1500*g* for 10 min at room temperature.

Plasma total cholesterol (TC), HDL cholesterol, and TG were determined on fresh samples. Plasma aliquots, containing sucrose (final concentration 6 g/L) as cryoprotectant, were stored at −70 °C for LDL size and composition measurements.

CHEMICALS

Thyroglobulin (T-1126), apoferritin (A-3641), and dithiothreitol (D-0632) were purchased from Sigma. Bovine serum albumin was from Pierce. All chemicals used in solvents and buffers were from Merck and were analytical grade.

LDL ISOLATION

Ultracentrifugation was carried out in a Beckman Optima-TLX preparative ultracentrifuge with a fixed-angle type 100.4 rotor. Acceleration mode 6 and deceleration mode 6 were used. LDL was isolated from plasma between d 1.019 and 1.063 kg/L on the basis of the method described by Havel et al. *[35]* with some modifications. Briefly, the density of 0.9 mL of plasma was adjusted to d = 1.019 kg/L by the addition of concentrated salt solution. Then 2.2 mL of salt solution at d = 1.019 kg/L was gently layered on top of the density-adjusted plasma by use of a low-speed peristaltic pump (Pharmacia). After ultracentrifugation at 100 000 rpm (417 000g_{av}) for 1 h 40 min at 15 °C, the top layer containing VLDL and IDL was discarded. The infranatant was adjusted at d = 1.063 kg/L and ultracentifuged at 417 000g_{av} for 5 h at 15 °C. Subsequently, the LDL-containing top layer (~1 mL) was recovered and stored at 4 °C in the dark.

Table 1. Clinical and lipoprotein characteristics of the study group.

	Mean ± SD (range)
n	60
Sex, M/F	31/29
Age, years	63 ± 12 (36–85)
Plasma cholesterol, mmol/L	6.0 ± 1.0 (3.5–8.2)
HDL cholesterol, mmol/L	1.1 ± 0.4 (0.5–2.0)
Triglycerides, mmol/L	2.3 ± 1.2 (0.5–5.6)

ANALYTICAL METHODS, LIPOPROTEIN COMPOSITION

Samples were analyzed for TC, free cholesterol (FC), TG, and phospholipids (PL) in enzymatic, colorimetric assays with test kits from Boehringer Mannheim (cat. nos. 1489232, 310328, 816370, and 691844, respectively). The cholesterol ester (CE) concentration in LDL was calculated as the difference between TC and FC. Serum HDL cholesterol was measured after precipitation of the apolipoprotein B-100-containing lipoproteins with phosphotungstic acid/magnesium chloride. LDL protein was estimated by the method of Lowry et al. *[36]* with some modifications to simplify protein determination in lipoproteins samples *[37]*. Bovine serum albumin was used for calibration. The above-mentioned methods were adapted to the ELAN analyzer.

Lipoprotein(a) [Lp(a)] was measured by rate nephelometry with Beckman reagent (cat. no. 465360) on an Array analyzer.

LDL SIZE CALIBRATION SAMPLES

Measurement of the LDL particle size was carried out by comparing the migration distance (GGE) or retention time (HPGC) with two LDL calibration samples whose molecular diameters were determined by GGE. In addition, the sizes of the LDL calibrators were confirmed by EM.

LDL calibrator A (large, buoyant) was isolated by ultracentrifugation from the plasma of a young healthy female volunteer with normal plasma TG (0.6 mmol/L), and calibrator B (small, dense) was isolated from the plasma of a male NIDDM patient with increased plasma TG (5.8 mmol/L). Calibrators A and B were stored in aliquots at −70 °C, after the addition of sucrose and EDTA (final concentrations 6 g/L and 0.2 mmol/L, respectively).

The average particle diameters of the calibrator LDL samples were estimated by GGE from a linear calibration curve of the logarithm of the diameter vs the migration distance, with apoferritin (12.2 nm), thyroglobulin (17.0 nm), and thyroglobulin dimer (23.6 nm) as calibrators of known diameter. LDL calibrator A was estimated by GGE at 26.2 ± 0.2 nm and calibrator B at 24.0 ± 0.2 nm.

For EM measurements, LDL calibrators A and B were dialyzed against 10 g/L ammonium acetate buffer containing 0.1 mmol/L EDTA (pH 7.4), negatively stained with methylamine tungstate on copper mesh grids, and examined with a Philips CM 100 Bio electron microscope

at a magnification of 135 000 *[25]*. Several enlarged monitor images (final magnification 275 000) of electron micrographs were used to measure the diameter of at least 50 LDL particles. Aggregated particles and particles of abnormal shape were excluded. The diameter was calculated from the measured (by computer) circumference of the LDL particles. LDL calibrator A was estimated by EM at 26.2 nm (0.5 SE) and calibrator B at 23.9 nm (0.3 SE).

HPGC

The HPLC system consisted of a Model 616 pump, Model 486 UV detector, and Model 717 autosampler from Waters. To avoid deterioration of LDL samples, the sample compartment of the autosampler was cooled at 7 °C, and sample vials were capped. Millennium 2010 software (version 2.15) from Waters was used for instrument control and data acquisition and processing. The mobile phase was continually degassed with a Degasys Model DG2410 on-line degasser from Uniflows. Separations were performed on a Superose 6 column (30 × 1 cm) from Pharmacia. LDL (50 μL) was injected and eluted with phosphate-buffered saline (PBS; 0.1 mol/L $NaH_2PO_4 \cdot H_2O$, 0.2 mol/L NaCl, and 0.1 mmol/L disodium EDTA, adjusted to pH 7.4) at a flow rate of 0.5 mL/min. The pressure of the HPLC system was ~1725 kPa. Before use, PBS was filtered through a 0.45-μm filter. The pump was run in the SILK-mode to smooth the eluent flow by eliminating pressure pulses and surges. The effluent was monitored at 280 nm at a sampling rate of 2 Hz for data acquisition. LDL eluted between 20 and 26 min, and the total run time was 60 min. LDL calibrators A and B were assayed in each sample set. The retention time of the unknown LDL samples was used to calculate the LDL particle diameter by linear interpolation. Some LDL samples were slightly larger than calibrator A or smaller than calibrator B. The sizes of these LDL samples were calculated by linear extrapolation. A QC LDL sample stored at −70 °C was also included in every series of samples. After analysis of a series of samples the Superose column was washed with 0.1 mL/L Tween and stored in 200 mL/L ethanol.

GGE

Electrophoresis equipment and chemicals were obtained from Bio-Rad. LDL size was determined by electrophoresis as originally described by Krauss and Burke *[21]* and Nichols et al. *[22]*. Nondenaturing 2–10% polyacrylamide gradient gels, 10 lanes per gel, were cast in our laboratory according to the method of Lahdenperä et al. *[38]* with some modifications. The MiniProtean II multicasting chamber was used to cast eight 1-mm-thick gels simultaneously. The solutions were made immediately before casting the gradient. The light monomer solution consisted of 23.5 mL of water, 8.3 mL of 1.5 mol/L Tris-HCl (pH 8.3), 1.8 mL of 40% acrylamide/bisacrylamide (19:1), 47 μL of 6.6 mol/L tetramethylethylenediamine, and 135 μL of 50 g/L ammonium persulfate, and the heavy monomer solution contained 10.7 mL of water, 7.1 mL of Tris-HCl (pH 8.3), 7.3 mL of acrylamide/bisacrylamide, 25 μL of tetramethylethylenediamine, 70 μL of ammonium persulfate, and 3.7 mL of 400 g/L sucrose. Tetramethylethylenediamine and fresh ammonium persulfate were added immediately before pouring. We used 5 mL of the light solution as stacking gel. The gradient was generated within 10 min with a Model 385 gradient-former by use of a peristaltic pump. The gels were left undisturbed for 3 h for polymerization. For storage, the gels were moistened with TBE buffer (90 mmol/L Tris, 80 mmol/L boric acid, and 3 mmol/L EDTA, adjusted to pH 8.35) and kept in a tightly sealed container at 4 °C.

The gels were equilibrated in precooled TBE buffer at 100 V for 30 min in a cold room. LDL (30 μL) was mixed with 50 μL of sample buffer [4 mL of 1.5 mol/L Tris-HCl (pH 8.3), 0.5 mL of 1 g/L bromphenol blue, and 2 mL of glycerol], and 20 μL of 1 g/L thyroglobulin was added as internal reference. After a 10-min incubation at room temperature, 10 μL of this suspension (containing ~2 μg of LDL protein) was loaded on the gels. The two outer lanes were not used. LDL calibrators A and B were run on each gel to calibrate for particle size. A QC LDL sample stored at −70 °C was also included in every run.

Electrophoresis was performed by applying voltage to the chamber in the following sequence: 15 V for 15 min, 70 V for 20 min, and 125 V for 24 h. After electrophoresis, the gels were fixed in 100 g/L sulfosalicylic acid for 1 h and stained for protein for 3–4 h in 0.4 g/L Coomassie Brilliant Blue R-250 in 35 mL/L perchloric acid. Gels were destained in 50 mL/L acetic acid for at least 2 h. After destaining, gels were soaked overnight in 30 mL/L glycerol and dried between two Gelair Cellophane sheets.

The gels were scanned with a Model GS700 imaging computer-assisted scanning densitometer with Molecular Analyst software. The migration distance of each LDL band relative to thyroglobulin was measured. The LDL calibrators A and B were used to calculate the particle diameters of the unknown samples by linear interpolation *[39]*. LDL samples larger than calibrator A or smaller than calibrator B were calculated by linear extrapolation. Some subjects had several bands, but the size of the most predominant LDL subfraction was calculated.

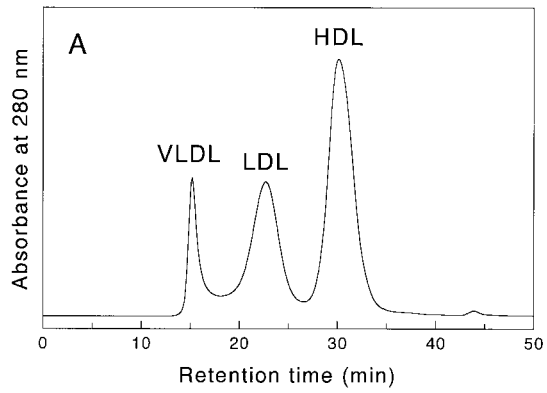
STATISTICS

Throughout the present study, results are expressed as mean ± SD. Pearson's correlation coefficients were calculated to describe the relationships between variables. Statistical difference was tested by paired Student's *t*-test. The value of statistical significance used in this study was $P < 0.01$. To reduce skewness, the plasma TG concentrations were transformed to their natural logarithms before statistical analyses.

Results

HPGC

HPGC was used in this study as a technique for LDL size measurement, but can also be used as an analytical tool to evaluate purity of the LDL preparations obtained by ultracentrifugation. After lipoprotein isolation by ultracentrifugation at d = 1.23 kg/L, the major lipoprotein classes are well resolved by HPGC, as demonstrated in Fig. 1A. A chromatogram of LDL isolated by dual-spin ultracentrifugation from a single plasma sample is shown in Fig. 1B. This chromatogram demonstrates the almost complete absence of VLDL and HDL lipoproteins. Because detection at 280 nm is not specific to lipoproteins and some serum proteins eluted in the same time-range as LDL, LDL was isolated by ultracentrifugation before chromatography. HPGC profiles of LDL calibrators A and B are shown in Fig. 2. These two samples, exhibiting a difference in retention time of ~0.8 min, were included in each series of samples. In addition, in each chromatographic sample set an LDL sample of intermediate size was included for QC purposes.



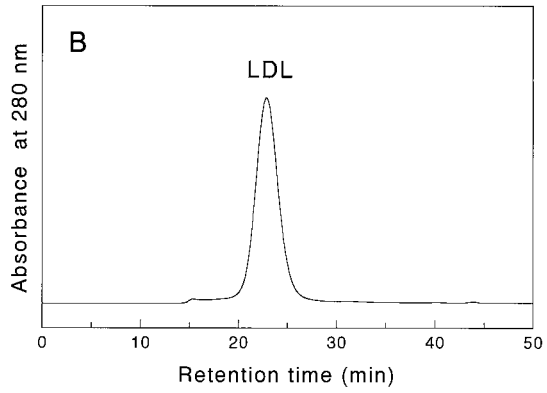


Fig. 1. HPGC lipoprotein profiles at 280 nm (*A*) and HPGC chromatogram of LDL isolated by dual-spin ultracentrifugation at 1.019 < d < 1.063 kg/L (*B*).

In *A*, the lipoproteins were obtained by ultracentrifugation at d = 1.23 kg/L and were separated by HPGC into three lipoprotein classes (VLDL, LDL, and HDL).

HPGC vs GGE

The LDL peak particle diameters of 60 NIDDM patients determined by HPGC were compared with values obtained by GGE. The mean LDL diameter of the NIDDM patients obtained by GGE and HPGC was 25.27 ± 0.81 and 25.48 ± 0.85 nm, respectively. As shown in Fig. 3A, the LDL particle sizes estimated by GGE and HPGC were highly correlated [$y = (0.93 \pm 0.07)x + (2.05 \pm 1.66)$; r = 0.88; $S_{y|x}$ = 0.41 nm]. However, the results obtained by HPGC were somewhat, but significantly (P <0.001), higher than those determined by GGE. To detect the between-method bias for the LDL size methods under comparison, the absolute difference was plotted in Fig. 3B against the mean for each pair of measurements *[40]*.

Relations between LDL size and plasma lipids and LDL composition

The mean LDL size determined by HPGC and GGE and lipoprotein composition analyses are shown in Table 2. The correlation coefficients for the associations between LDL size and lipid variables are shown in Table 3. Correlation coefficients between lipid variables and LDL size as measured by HPGC and GGE were of the same magnitude, but correlation coefficients were slightly higher for the HPGC measurements. LDL size showed a positive correlation with HDL cholesterol and a strong inverse correlation with the logarithm of plasma TG. The association between LDL size estimated by HPGC and plasma TG is illustrated in Fig. 4. Small dense LDL particles (≤25.5 nm), according to HPGC, were found only in subjects with plasma TG ≥1.9 mmol/L. LDL size as measured by HPGC was strongly correlated with the FC content of LDL and to a lesser extent with the CE content. We also observed a positive correlation with LDL-PL, whereas LDL size was not correlated with the TG content of LDL (Fig. 5).

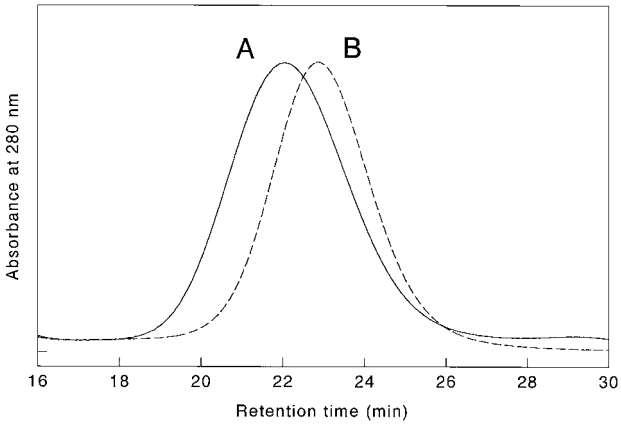


Fig. 2. HPGC lipoprotein profiles at 280 nm of LDL size calibrators A and B.

The mean particle diameter of these two samples was estimated by GGE to be 26.2 and 24.0 nm, respectively.

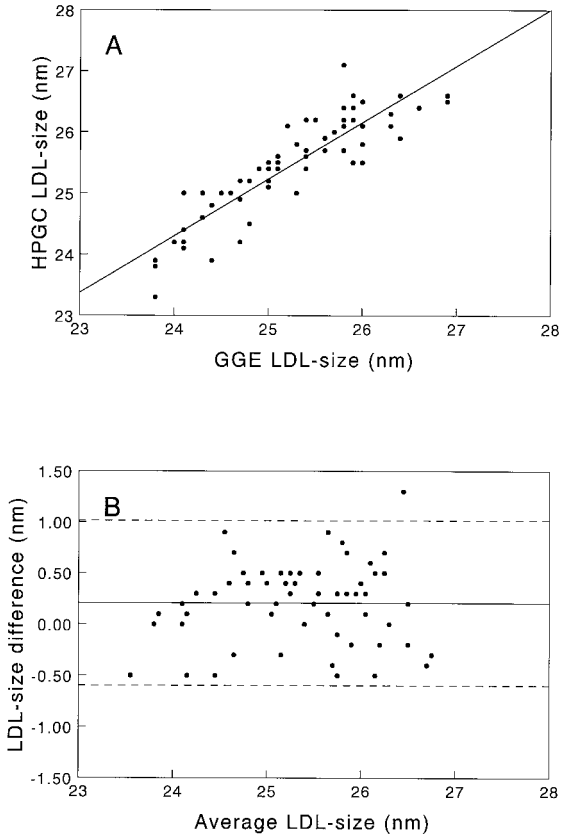


Fig. 3. LDL size analyzed by HPGC plotted against LDL size measured by GGE ($r = 0.88$, $P < 0.001$; $y = 0.93x + 2.05$) (*A*) and bias plot of absolute differences between LDL size determined with HPGC and GGE vs the average of the two methods, with the mean of the differences (*solid line*) and mean ± 2 SD limits (*broken line*) (*B*).

REPRODUCIBILITY

Between-run reproducibility for particle diameter was determined by repeatedly analyzing an isolated LDL sample stored in aliquots at −70 °C. GGE and HPGC reproducibility, expressed as CV determined over an 8-week period, were 0.6% (n = 14) and 0.2% (n = 12), respectively. Within-run imprecision for LDL size measurements was assessed only for the HPGC method. The CVs for two different samples were <0.1% (n = 10). To establish the influence of the HPLC pump on the HPGC reproducibility, we also tested another pump (Gynkotek, Model 480). With this pump, the within-run imprecision was also <0.1%.

Table 2. Chemical composition and particle diameter estimated by HPGC and GGE of LDL measured in 60 NIDDM patients.

	Mean ± SD (range)
LDL composition (mmol/g LDL-protein)	
FC	0.95 ± 0.16 (0.58–1.30)
CE	2.57 ± 0.27 (1.92–3.07)
TG	0.37 ± 0.09 (0.21–0.63)
PL	1.30 ± 0.21 (0.52–1.70)
LDL particle diameter (nm)	
HPGC	25.48 ± 0.85 (23.3–27.1)
GGE	25.27 ± 0.81 (23.8–26.9)

Table 3. Correlation coefficients (*r*) between LDL particle diameter measured by HPGC and GGE and plasma lipids and LDL chemical composition (expressed as mmol/g LDL-protein) in samples of 60 NIDDM patients.

	LDL size	
	HPGC	GGE
Plasma lipids		
TC	+0.07NS	−0.03NS
HDL cholesterol	+0.50	+0.48
Log TG	−0.84	−0.82
LDL composition		
FC	+0.89	+0.78
CE	+0.62	+0.47
PL	+0.36	+0.20NS
TG	−0.08NS	−0.16NS

Significant correlation ($P < 0.01$) unless stated. NS, not significant.

COLUMN-COLUMN VARIATION

We analyzed >500 samples with one Superose column (column I). To verify column variation, we measured LDL size of 20 samples on two separate HPGC columns. We compared column I with a brand new column (column II). There was no statistical difference ($P = 0.639$) between the LDL size measured on column I or II. The mean LDL size for column I was 25.38 ± 0.91 nm and for column II, 25.37 ± 0.95 nm [$y = (1.03 \pm 0.03)x - (0.76 \pm 0.83)$; $r = 0.99$; $S_{y|x}$ 0.13 nm].

LDL AGGREGATION DURING ISOLATION

Once isolated, LDL exhibits a tendency to aggregate. Aggregated LDL can easily be recognized by HPGC, as it elutes at the same time as VLDL. We stimulated LDL aggregation by vortex-mixing an isolated LDL sample. Vortex-mixing (30 s) of isolated LDL resulted in a dimin-

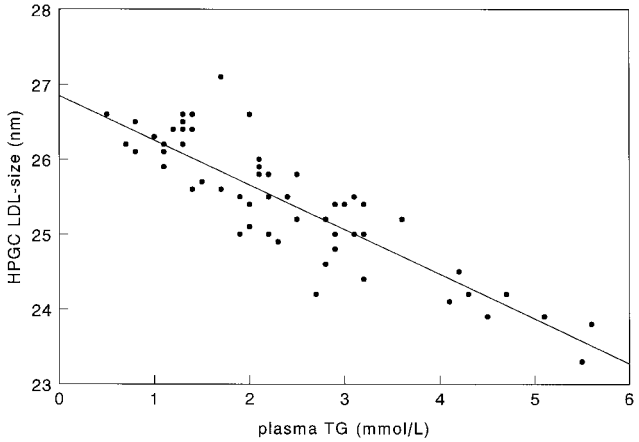


Fig. 4. LDL particle diameter estimated by HPGC vs the plasma TG in 60 NIDDM patients ($r = -0.88$, $P < 0.001$).

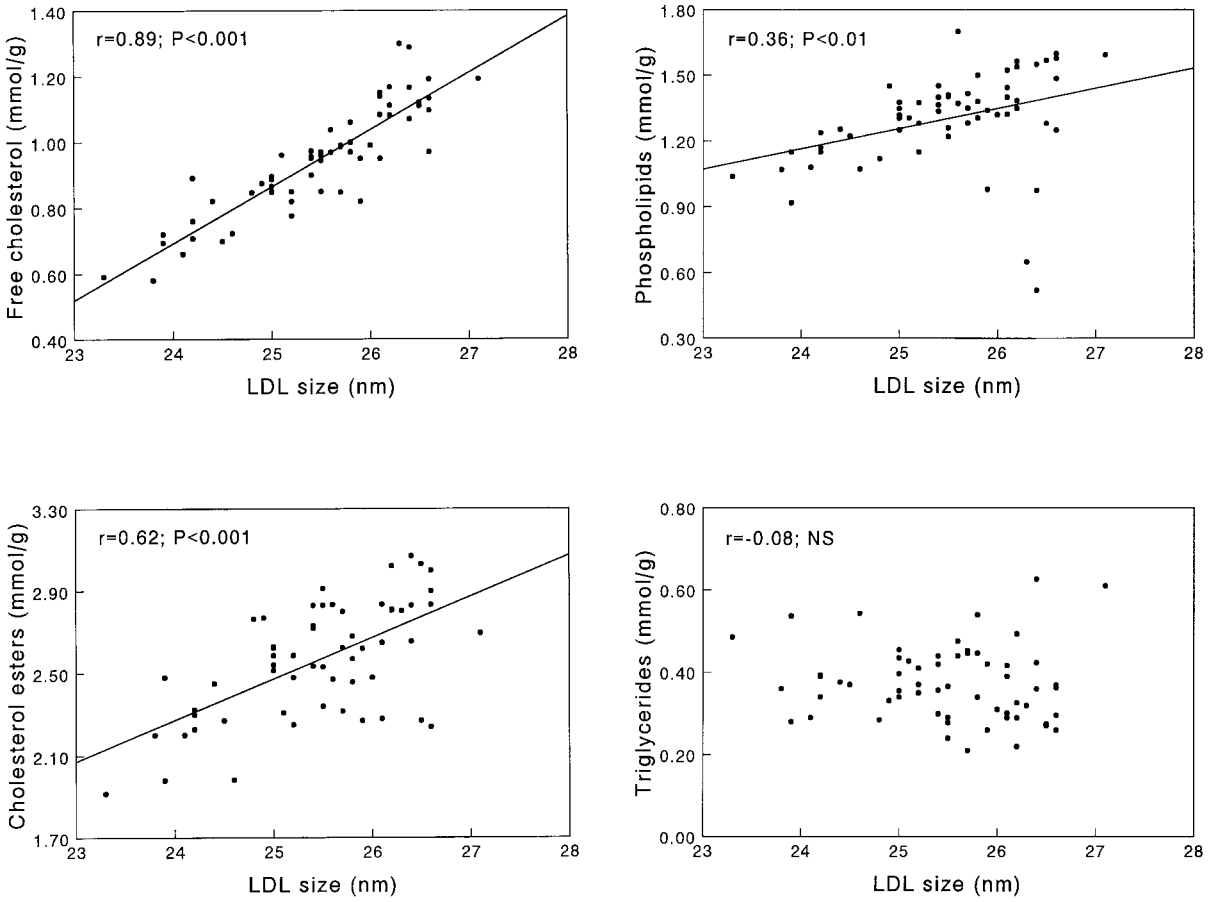


Fig. 5. LDL particle diameter measured by HPGC vs the lipid content of LDL (mmol/g LDL-protein) in a group of 60 NIDDM patients.

ished peak area of the main LDL peak eluting at 23 min, with a concomitant increase of the peak of aggregated LDL eluting at 15 min. The calculated LDL size of the native LDL (26.23 nm) was almost equal to the size of the aggregated LDL sample (26.21 nm), suggesting that mild LDL aggregation does not seriously affect size determination by HPGC.

INTERFERENCE BY IDL AND LP(A)

LDL used in this study was isolated by dual-spin ultracentrifugation. After the first ultracentrifuge run, the top layer contains VLDL and most of the IDL ($1.006 < d < 1.019$ kg/L). VLDL subjected to HPGC eluted between 14 and 17 min as illustrated in Fig. 1A. IDL elutes between VLDL and LDL (~17 min, data not shown). Hence, it is not likely that the remaining IDL in the second ultracentrifuge run will affect the position of the LDL peak in HPGC.

On the other hand, the density of Lp(a) spans both the LDL and HDL range, with most of the Lp(a) found between 1.050 and 1.100 kg/L. In patients with substantial amounts of Lp(a) it is likely that LDL isolated by ultracentrifugation between 1.019 and 1.063 kg/L is contaminated with Lp(a). To establish the retention time of Lp(a) in the HPGC system, Lp(a) was isolated by ultracentrifugation between $1.063 < d < 1.090$ kg/L from a plasma with a high Lp(a) content (720 mg/L). As shown in Fig. 6 the retention time of Lp(a) is considerably shorter than that of LDL. Fig. 6 also illustrates that addition of Lp(a) to LDL resulted in the appearance of a prominent shoulder on the leading edge of the LDL peak. However, this addition did not influence the retention time of the LDL peak. Visual inspection of the LDL HPGC profiles of all 60 NIDDM patients revealed four samples with a leading shoulder, although much less conspicuous than in Fig. 6. The presence of substantial amounts of Lp(a) was corroborated by Lp(a) measurements in the plasma of these four patients, ranging from 490 to 1030 mg/L. In contrast, the range of Lp(a) of four plasma samples with HPGC profiles without a shoulder was 20–45 mg/L. The disulfide bridge linking apolipoprotein(a) and apolipoprotein (apo) B-100 is cleaved when Lp(a) is incubated with 0.01 mol/L dithiothreitol for 1 h at room temperature, resulting in a smaller particle very similar to LDL *[41]*. Addition of dithiothreitol to the four Lp(a)-containing LDL samples before chromatography resulted in the disappearance of the shoulder in the LDL profiles. The retention time of LDL samples, with and without a shoulder, was not influenced by the addition of dithiothreitol.

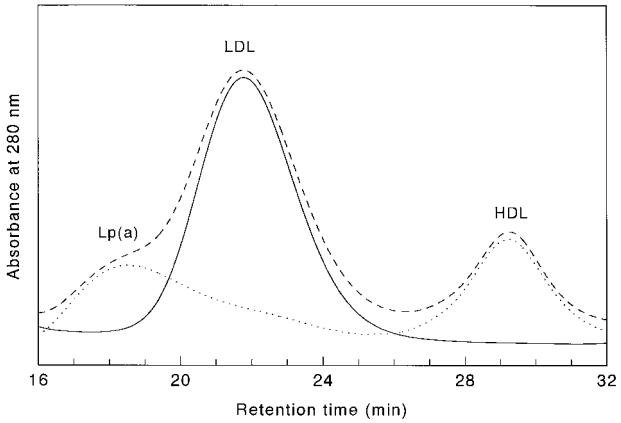


Fig. 6. HPGC of LDL isolated by ultracentrifugation between 1.019 and 1.063 kg/L (——), Lp(a) isolated between 1.063 and 1.090 kg/L (·····), and a mixture of LDL and Lp(a) (– – –).

INFLUENCE OF STORAGE CONDITIONS OF ISOLATED LDL SAMPLES

Isolated LDL samples were tested for stability during storage at 4 °C and −70 °C. The LDL sizes measured by HPGC of 9 fresh LDL samples were compared with those in the same samples after storage for 4 weeks at 4 °C. The mean LDL sizes were 25.37 ± 1.02 and 25.36 ± 0.99 nm (P = 0.814) before and after storage, respectively. A QC sample stored in aliquots at −70 °C demonstrated no change in size over a 4-month period.

Discussion

Human plasma LDL exhibits heterogeneity. LDL particles vary in density, chemical composition, and particle diameter. LDL size is usually estimated by GGE *[21, 22]*. However, this method is rather laborious and technically demanding. We have developed another technique for LDL size measurement based on HPGC. To validate the HPGC procedure for LDL size measurement, we compared the HPGC method with GGE. LDL size was measured by both methods, and the chemical composition of the isolated LDL was analyzed. A strong correlation between the LDL size of the most predominant subfraction in a GGE gel and the LDL size obtained by HPGC was found (r = 0.88; Fig. 3A). As shown in Fig. 3B the difference between the measurements by the two methods is not related to the magnitude of the measurement.

Column-to-column variation for the HPGC method was examined by measuring a series of samples on two different columns. The conclusion was that the LDL size did not depend on the HPGC column.

Within-run CV of LDL size as measured by HPGC was <0.1%. Because LDL size is linearly related to retention time, this means that retention time was reproducible within 0.1%, equivalent to 1.4 s for a retention time of 23 min. To attain this high precision, several instrumental and methodological variables must be carefully controlled. First, the acquisition rate of the data system must be high enough to allow sufficient resolution on this time scale. We routinely use an acquisition rate of 2 Hz, equivalent to a resolution of 0.5 s. Second, a fixed injection volume should be used during the analysis of a series of unknown and calibration samples. Increasing the injection volume leads to an apparent increase in retention time, caused by the sample being introduced on the column as a plug. A 50-μL sample will take 6 s to be flushed from the sample loop onto the column at a flow rate of 0.5 mL/min. With a 100-μL injection volume this time is increased to 12 s, leading to an increased retention time and thus substantial underestimation of LDL size. Finally, pump flow imprecision should be <0.1%. We tested two different pumps that were both sufficiently precise. However, pumps should be tested regularly for flow reproducibility. Between-run CV of the GGE method was 0.6%, in accordance with published values from other laboratories (0.7–3.5%) *[3, 4, 21, 38, 42]*. Between-run CV for the HPGC method was <0.2%. Therefore, the reproducibility of the HPGC method is at least 3 times better than the GGE method.

As mentioned by Tallis et al. *[32]*, the Superose column has a long lifetime and was in their hands capable of analyzing 300 filtered plasma samples. Loading the HPGC column with isolated LDL, as we did in this study, we observed no deterioration of column performance after >500 injections.

In some patients, LDL shows several peaks (polydisperse LDL) on polyacrylamide GGE. We calculated the LDL size of the predominant GGE subfraction. However, with the HPGC technique, LDL eluted as a single peak (Figs. 1 and 2), so polydispersity was not observed. LDL size as measured by HPGC thus represents the weighted average of LDL subfractions in polydisperse LDL. Because the meaning of polydispersity is not really understood, we do not consider this as a disadvantage of HPGC. It may in part explain small differences in LDL size as measured by both methods.

Several studies have demonstrated that LDL size is inversely associated with plasma TG and positively associated with HDL cholesterol *[1–4]*. The results (Table 3 and Fig. 4) of our selected NIDDM patients are consistent with these studies, showing significant correlations between LDL size estimated by HPGC and plasma TG and HDL cholesterol (r = −0.84 and +0.50, respectively).

The mechanism by which plasma TG affects LDL size is hypothesized by Lagrost et al. *[3]*. In hypertriglyceridemia, LDL-CE is exchanged for VLDL-TG, mediated by cholesteryl ester transfer protein. Subsequent hydrolysis of LDL-TG by hepatic lipase or lipoprotein lipase results in small, dense LDL particles. Our observation that LDL-TG in subjects with increased plasma TG (2.0–5.6 mmol/L; n = 34) is inversely associated with LDL-CE (r = −0.56, P <0.001) strengthens this hypothesis.

Correlation studies with LDL size estimated by GGE and HPGC and the LDL lipid composition gave comparable results (Table 3). HPGC associations with LDL lipids are even somewhat stronger. The present study also is in

agreement with the findings by Capell et al. *[42]* in showing a significant positive correlation between LDL size estimated by HPGC and LDL-FC, LDL-CE, and LDL-PL and the absence of a significant relationship with LDL-TG (Table 3). The correlation between LDL size and LDL-PL (Fig. 5) is strongly influenced by two single samples with extremely low LDL-PL concentrations (LDL-PL: 0.52 and 0.65 mmol/g LDL-protein), resulting in a less significant correlation. In contrast, the LDL-FC concentrations of these two samples were remarkably high (1.30 and 1.29 mmol/g LDL-protein). FC and PL are primarily located at the surface of the LDL particle. It can be speculated that in these two samples the low PL content was by some mechanism compensated by increased FC concentrations, resulting in a partial conservation of the total amount of surface lipids.

The relationship between GGE migration distance and particle diameter is gel-specific. Therefore, in each gel two LDL calibration samples and one control sample were included. The two outer lanes were not used, so only 5 places remain for unknown samples. In contrast, the automated HPGC procedure allows the unattended analysis of large series of samples. For example, we routinely analyze series of 64 samples during the weekend.

As mentioned above, LDL calibrators A and B were run on each GGE gel and in every HPGC sample set to calibrate for particle size. Marker proteins, e.g., apoferritin and thyroglobulin, and calibrated latex beads are frequently used in GGE for calibration. To transform the migration distance into the LDL size, lipoprotein particles are assumed to exclude from the gel by the same criteria as the (protein) calibrators *[39]*. Calibration of the HPGC column with these proteins resulted in calculated LDL diameters ranging from 20 to 23 nm. Apparently, the shape of the stretched proteins differs too much from the spherical LDL. This phenomenon has been observed by others as well. For example, in a study by Van Gent and Van Tol *[28]* a Superose 6 column was calibrated with four proteins of known molecular mass. The molecular mass of LDL was estimated by them at 1.0×10^6 Da. However, the average molecular mass of LDL, calculated by dividing the molecular mass of apo B protein *[43]* by the protein content (weight percent) is $\sim 2.6 \times 10^6$ Da *[24]*. Attempts to calibrate our HPGC column with small (<50 nm) polystyrene latex beads (Duke Scientific) failed because these particles aggregated in PBS and clogged the column.

Lp(a) is structurally very similar to LDL, with an overlapping density and a somewhat greater size *[41]*. Addition of Lp(a) to LDL in various amounts did not influence the retention time of LDL. Furthermore, cleavage of the disulfide bond between apo(a) and apo B in Lp(a) did not alter the retention time of Lp(a)-contaminated LDL samples. Hence, LDL size estimated by HPGC is not affected by Lp(a) in patients with substantial amounts of Lp(a).

Carroll and Rudel *[44]* showed that varying the elution buffer concentration altered the elution profile of lipoproteins separated by HPGC. They suggested that at low buffer concentration, ion-exclusion effects may prevent lipoprotein particles from entering the pores in the column packing. We obtained comparable results by varying the ionic strength at low buffer concentrations. We found that an ionic strength of 0.4 mol/L is sufficient to obtain stable retention times. The ionic strength of the PBS buffer in the present study was 0.46 mol/L.

We have developed a HPGC-based technique for determination of the mean LDL particle diameter. Values measured by HPGC were in close agreement with values obtained by GGE. Although the HPGC method requires isolation of LDL by ultracentrifugation, we do not consider this as a major drawback, because in clinical studies LDL isolation is frequently required for other measurements as well, e.g., lipid composition and susceptibility to oxidation. In comparison with GGE the main advantages of the HPGC method are its high precision, good reproducibility, and the possibility for the automated analysis of large series of samples.

We are grateful to S. Lahdenperä and M.R. Taskinen (Department of Medicine, University of Helsinki, Helsinki, Finland) for sharing their expertise on GGE. We also thank I.L. Schadee-Eestermans and E.C.M. Hoefsmit (Department of Cell Biology, Medical Faculty, Vrije Universiteit, Amsterdam, The Netherlands) for EM analysis, L.P. Colly (Amsterdamse Thrombosedienst en Artsenlaboratorium, Amsterdam, The Netherlands) for her cooperation in collecting plasma samples, and A. Kok for performing Lp(a) measurements.

References

1. McNamara JR, Jenner JL, Li Z, Wilson PWF, Schaefer EJ. Change in LDL particle size is associated with change in plasma triglyceride concentration. Arterioscler Thromb 1992;12:1284–90.
2. Coresh J, Kwiterovich PO Jr, Smith HH, Bachorik PS. Association of plasma triglyceride concentration and LDL particle diameter, density and chemical composition with premature coronary artery disease in men and women. J Lipid Res 1993;34:1687–97.
3. Lagrost L, Cambert P, Lallemant C. Combined effects of lipid transfers and lipolysis on gradient gel patterns of human plasma LDL. Arterioscler Thromb 1994;14:1327–36.
4. Austin MA, Mykkänen L, Kuusisto J, Edwards KL, Nelson C, Haffner SM, et al. Prospective study of small LDLs as a risk factor for non-insulin dependent diabetes mellitus in elderly men and women. Circulation 1995;92:1770–8.
5. Austin MA, Krauss RM. Genetic control of low-density-lipoprotein subclasses. Lancet 1986;ii:592–5.
6. Campos H, Genest JJ Jr, Blijlevens E, McNamara JR, Jennner JL, Ordovas JM, et al. Low density lipoprotein particle size and coronary artery disease. Arterioscler Thromb 1992;12:187–95.
7. Austin MA, Breslow JL, Hennekens CH, Buring JE, Willett WC, Krauss RM. Low-density lipoprotein subclass patterns and risk of myocardial infarction. JAMA 1988;260:1917–21.
8. Gardner CD, Fortmann SP, Krauss RM. Association of small

low-density lipoprotein particles with the incidence of coronary artery disease in men and women. JAMA 1996;276:875–81.
9. Stampfer MJ, Krauss RM, Ma J, Blanche PJ, Holl LG, Sacks FM, Hennekens CH. A prospective study of triglyceride level, low-density lipoprotein particle diameter, and risk of myocardial infarction. JAMA 1996;276:882–8.
10. Lamarche B, Tchernof A., Moorjani S, Cantin B, Dagenais GR, Lupien PJ, Després JP. Small, dense low-density lipoprotein particles as a predictor of the risk of ischemic heart disease in men. Circulation 1997;95:69–75.
11. Jaakkola O, Solakivi T, Ylä-Herttuala S, Nikkari T. Receptor-mediated binding and degradation of subfractions of human plasma low-density lipoprotein by cultured fibroblasts. Biochim Biophys Acta 1989;1005:118–22.
12. Galeano NF, Milne R, Marcel YL, Walsh MT, Levy E, Ngu'yen TD, et al. Apoprotein B structure and receptor recognition of triglyceride-rich low density lipoprotein (LDL) is modified in small LDL but not in triglyceride-rich LDL of normal size. J Biol Chem 1994;269: 511–9.
13. Campos H, Arnold KS, Balestra ME, Innerarity TL, Krauss RM. Differences in receptor binding of LDL subfractions. Arterioscler Thromb Vasc Biol 1996;16:794–801.
14. Parthasarathy S, Rankin SM. Role of oxidized low density lipoprotein in atherogenesis. Prog Lipid Res 1992;31:127–43.
15. Esterbauer H, Gebicki J, Puhl H, Jürgens G. The role of lipid peroxidation and antioxidants in oxidative modification of LDL. Free Rad Biol Med 1992;13:341–90.
16. De Graaf J, Hak-Lemmers HLM, Hectors MPC, Demacker PNM, Hendriks JCM, Stalenhoef AFH. Enhanced susceptibility to in vitro oxidation of the dense low density lipoprotein subfraction in healthy subjects. Arterioscler Thromb 1991;11:298–306.
17. Tribble DL, Holl LG, Wood PD, Krauss RM. Variations in oxidative susceptibility among six low density lipoprotein subfractions of differing density and particle size. Atherosclerosis 1992;93:189–99.
18. Nielsen LB. Transfer of low density lipoprotein into the arterial wall and risk of atherosclerosis. Atherosclerosis 1996;123:1–15.
19. Björnheden T, Babyi A, Bondjers G, Wiklund O. Accumulation of lipoprotein fractions and subfractions in the arterial wall, determined in an in vitro perfusion system. Atherosclerosis 1996;123: 43–56.
20. Anber V, Griffin BA, McConnell M, Packard CJ, Shepherd J. Influence of plasma lipid and LDL-subfraction profile on the interaction between low density lipoprotein with human arterial wall proteoglycans. Atherosclerosis 1996;124:261–71.
21. Krauss RM, Burke DJ. Identification of multiple subclasses of plasma low density lipoproteins in normal humans. J Lipid Res 1982;23:97–104.
22. Nichols AV, Krauss RM, Musliner TA. Nondenaturing polyacrylamide gradient gel electrophoresis. Methods Enzymol 1986;128: 417–31.
23. Swinkels DW, Hak-Lemmers HLM, Demacker PNM. Single spin density gradient ultracentrifugation method for the detection and isolation of light and heavy low density lipoprotein subfractions. J Lipid Res 1987;28:1233–9.
24. Chatterton JE, Schlapfer P, Bütler E, Gutierrez MM, Puppione DL, Pullinger CR, et al. Identification of apolipoprotein B100 polymorphisms that affect low-density lipoprotein metabolism: description of a new approach involving monoclonal antibodies and dynamic light scattering. Biochemistry 1995;34:9571–80.
25. Forte TM, Nordhausen RW. Electron microscopy of negatively stained lipoproteins. Methods Enzymol 1986;128:442–57.
26. Holmquist L, Carlson LA. Subfractionation and characterization of native and incubation enlarged human plasma high density lipoprotein particles by high performance gel filtration. Lipids 1985;20:378–88.
27. Williams MC, Kushwaha RS, McGill HC Jr. Quantification of baboon lipoproteins by high performance gel exclusion chromatography. Lipids 1987;22:366–74.
28. Van Gent T, Van Tol A. Automated gel permeation chromatography of plasma lipoproteins by preparative fast protein liquid chromatography. J Chromatogr 1990;525:433–41.
29. Kieft KA, Bocan TMA, Krause BR. Rapid on-line determination of cholesterol distribution among plasma lipoproteins after high-performance gel filtration chromatography. J Lipid Res 1991;32: 859–66.
30. März W, Siekmeier R, Scharnagl H, Seiffert UB, Gross W. Fast lipoprotein chromatography: new method of analysis for plasma lipoproteins. Clin Chem 1993;39:2276–81.
31. Van den Maagdenberg AMJM, Hofker MH, Krimpenfort PJA, De Bruijn I, Van Vlijmen B, Van der Boom H, et al. Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. J Biol Chem 1993;268:10540–5.
32. Tallis GA, Shephard MDS, Whiting MJ. Lipoprotein profiling by high performance gel chromatography. Clin Chim Acta 1994;228: 171–9.
33. De Sonnaville JJJ, Colly LP, Wijkel D, Heine RJ. Good glycaemic control in type II diabetic patients in general practice: results of a shared care system. Diabetologia 1993;36:A35.
34. Yoshida Y, Furuta S, Niki E. Effects of metal chelating agents on the oxidation of lipids induced by copper and iron. Biochim Biophys Acta 1993;1210:81–8.
35. Havel RJ, Eder HA, Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J Clin Invest 1955;34:1345–53.
36. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
37. Markwell MAK, Haas SM, Tolbert NE, Bieber LL. Protein determination in membrane and lipoprotein samples: manual and automated procedures. Methods Enzymol 1981;72:296–303.
38. Lahdenperä S, Puolakka J, Pyörälä T, Luotola H, Taskinen MR. Effects of postmenopausal estrogen/progestin replacement therapy on LDL particles; comparison of transdermal and oral treatment regimens. Atherosclerosis 1996;122:153–62.
39. Williams PT, Krauss RM, Nichols AV, Vranizan KM, Wood PDS. Identifying the predominant peak diameter of high-density and low-density lipoproteins by electrophoresis. J Lipid Res 1990;31: 1131–9.
40. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1986;8:307–10.
41. O'Neal D, Grieve G, Rae D, Dragicevic G, Best JD. Factors influencing Lp(a)-particle size as determined by gradient gel electrophoresis. J Lipid Res 1996;37:1655–63.
42. Capell WH, Zambon A, Austin MA, Brunzell JD, Hokanson JE. Compositional differences of LDL particles in normal subjects with LDL subclass phenotype A and LDL subclass phenotype B. Arterioscler Thromb Vasc Biol 1996;16:1040–6.
43. Knott TJ, Pease RJ, Powell LM, Wallis SC, Rall SC Jr, Innerarity TL, et al. Complete protein sequence and identification of structural domains of human apolipoprotein B. Nature 1986;323:734–8.
44. Carroll RM, Rudel LL. Lipoprotein separation and low density lipoprotein molecular weight determination using high performance gel-filtration chromatography. J Lipid Res 1983;24:200–7.