Analytical capillary isotachophoresis of total plasma lipoproteins: a new tool to identify atherogenic low density lipoproteins

Gabriele Bittolo-Bon and Giuseppe Cazzolato
Department of Internal Medicine and Metabolic Diseases, Pietro Avogaro Laboratory, Regional Center for Atherosclerosis, Venice General Hospital, C. po SS Giovanni e Paolo. 30122-Venice, Italy

Abstract Plasma low density lipoproteins from 20 patients were separated by capillary isotachophoresis (ITP). In each patient the apparent diameter of the predominant LDL peak on whole plasma was also determined by nondenaturing gradient gel electrophoresis. Furthermore the concentration of the more electronegatively charged in vivo oxidized LDL was accomplished using anion exchange high pressure liquid chromatography. By analytical capillary ITP of whole plasma lipoproteins, prestained with a lipophilic dye, LDL were separated into four subfractions. Usually, the predominant subfraction was the slow migrating LDL, followed by LDL, and then by the faster LDL and LDL. Slow migrating LDL correlated negatively with plasma triglycerides and LDL and positively with plasma high density lipoprotein (HDL) cholesterol and with the LDL diameter, while the faster LDL showed an inverse behavior. The LDL to LDL ratio showed a strong positive correlation with LDL concentration (r = 0.87; P < 0.001) and a highly significant inverse correlation with the LDL particle diameter (r = −0.74; P < 0.001). At least three highly atherogenic LDL that could be found in human plasma, namely oxidized, glycated and small-dense, are characterized by a greater electric charge. The LDL profile from capillary ITP and the relative prevalence of faster or slower migrating LDL fractions could indicate the presence of more atherogenic LDL: Bittolo-Bon, G., and G. Cazzolato. Analytical capillary isotachophoresis of total plasma lipoproteins: a new tool to identify atherogenic low density lipoproteins. J. Lipid Res. 1999. 40: 170-177.

Supplementary key words oxidized LDL • LDL size • lipoprotein subfractionation

Plasma low density lipoproteins (LDL) are a heterogeneous population of particles that vary in size, density, composition, and electric charge (1-4). One significant aspect of this variability, relevant to atherosclerosis, has been pointed out by the studies of Austin et al. (5) on the phenotypic patterns of LDL density and size distribution among individuals with and without coronary artery disease. Using nondenaturing gradient gel electrophoresis, various LDL subgroups were identified on the basis of size (6-8) and traditionally divided into three main phenotypes: pattern A, characterized by a predominance of large LDL particles; pattern B, with a predominance of small dense LDL; and an intermediate pattern (9). Individuals with lipoprotein profiles enriched in small dense LDL (pattern B) were found to be predisposed to coronary artery disease (5, 9, 10). Another aspect of LDL variability has been described by Avogaro, Bittolo-Bon, and Cazzolato (11), who identified a more electronegatively charged LDL (LDL), which is enriched in oxidized lipids and cytotoxic to cultured endothelial cells (11-14). Higher plasma levels of LDL have been found in patients with acute myocardial infarction, unstable angina, and thrombogenic carotid atherosclerosis (15, 16). It is of interest that LDL is found largely among the dense LDL fractions, and dense LDL particles also contain much greater amounts of lipid peroxides compared with total LDL or the more buoyant LDL fractions (17). The method of study of LDL, which uses ion exchange chromatography of LDL isolated by ultracentrifugation, requires particular care to avoid artifacts due to in vitro auto-oxidation of the LDL and is time consuming. Furthermore, the separation between LDL and normally charged LDL is quite arbitrary, depending on the profile of ionic gradient chosen for the chromatographic separation.

As previously shown by Schmitz, Borgmann, and Assmann (18), Nowicka et al. (19), and Schmitz and Mollers (20), a good method to separate plasma lipoproteins on the basis of its electric charge is capillary isotachophoresis (ITP). ITP is a high resolution electrophoretic technique, by which ionic sample components are separated according their net electric charge and without molecular sieve

Abbreviations: ITP, isotachophoresis; LDL, low density lipoproteins; HDL, high density lipoproteins; TC, total cholesterol; TG, triglycerides; Lp[a], lipoprotein[a]; CHD, coronary heart disease; LDL-C, LDL-cholesterol.

To whom correspondence should be addressed.
effects. We have therefore evaluated whether this method could provide a new tool to identify more atherogenic plasma LDL.

MATERIALS AND METHODS

Subjects
Twenty patients (8 women and 12 men; age range 21 through 70 years; plasma cholesterol concentration 166 to 319 mg/dL; and plasma triglyceride concentration 36 to 283 mg/dL) were selected for the study. Candidates were chosen among the patients attending our lipid clinic each Monday for 4 consecutive weeks. The only exclusion criteria was the use of hypolipidemic drugs or plasma levels of Lp[a] higher than 5 mg/dL. Clinical data of the selected patients were reported in Table 1. As shown in the table, the majority of patients (15 out of 20) were dyslipidemic, according to the guidelines of the European Society of Atherosclerosis (LDL-C > 160 mg/dL in the absence of other known risk factors, LDL-C > 130 mg/dL in the presence of other risk factors, and/or plasma triglycerides > 200 mg/dL). Based upon criteria of clinical history, ECG, and ultrasound study of peripheral or carotid arteries, eight patients were identified as manifesting signs of ischemic heart disease, peripheral artery disease, or precranial artery disease. All these patients were on aspirin. Diabetics were well controlled with oral hypoglycemic agents, and hypertensive patients were treated mostly with ACE inhibitors or β-blockers. Two postmenopausal women were on hormone replacement therapy.

Blood samples
Venous blood from all subjects was collected after a 12-h overnight fast. Blood samples were drawn into EDTA-containing tubes, and plasma was promptly separated by a 5-min centrifugation at 3000 g. Plasma was kept at 4°C and analyzed within a few hours of sample collection.

Assays
Total cholesterol (TC) and triglyceride (TG) concentrations were determined enzymatically with specific test kits from Menarini (Milano, Italy). Proteins were assayed by the method of Lowry et al. (21) with human albumin as standard. High density lipoprotein cholesterol (HDL-C) was measured according to Kostner et al. (22). LDL cholesterol (LDL-C) was calculated according to the formula LDL-C = TC – (TG/5 + HDL-C). Lp[a] values were checked by rocket immunoelectrophoresis (23).

Lipoprotein preparation
Lipoprotein fractions were isolated from total plasma by preparative ultracentrifugation with a Beckman L 60-Optima ultracentrifuge equipped with an SW 41 rotor as previously described (13). In order to achieve faster preparation of the LDL (d 1.019-1.063 g/mL), a single-spin procedure was utilized (24). Briefly, 11 mL of plasma was adjusted to a density of 1.21 g/mL by the addition of solid KBr, in a Beckman OptiSeal (26 × 87 mm) polycarbonate tube. Samples were overlaid with a NaCl solution at d 1.019 g/mL, purged with nitrogen, and stabilized by means of filtration through a 0.2μm cellulose nitrate membrane. The LDL was then centrifuged at 50,000 rpm in a Beckman VTi 50 rotor for 1 h (with slow acceleration and deceleration). The LDL visible in the middle of the tube was immediately aspirated and dialyzed at 4°C in the dark against nitrogen-sparged and filtered 0.9% NaCl, pH 7.2, in the presence of 1 g/l Chelex 100 chelating resin (Sigma).

Nondenaturating gradient gel electrophoresis
Nondenaturating 2% to 16% polyacrylamide gel electrophoresis was performed on whole plasma, according to the procedures described by Krauss and Burke (6) and McNamara et al. (8). Gradient gels were prepared in our laboratory using an LKB 11300 UltraTaq gradient mixer under standard conditions. Seven μL of plasma was subjected to electrophoresis after diluting 4:1 with 50% sucrose and 0.01% bromophenol blue tracking solution. After 30 min pre-run at 100 V, the electrophoresis was performed at 4°C for 16 h: 1 h at 60 V, 10 h at 80 V, and 5 h at 200 V. Gels were then fixed, stained in Sudan black, and destained under standard procedures (25). The distribution profile of lipoprotein subfractions was finally obtained by densitometric scanning of the gels at 633 nm with a 2222-02 UltraTaq XL laser densitometer (LKB), equipped with an integrator that directly indicated the position of the predominant LDL peak. The apparent diameters of the separated lipoproteins were obtained by comparison with the migration of standards of known diameter, such as ferritin (diameter, 12.2 nm), thyroglobulin (diameter, 17 nm; Pharmacia High Molecular Weight Protein Calibration kit), two different carboxylated latex beads (diameter, 38.0 nm; Duke Scientific; diameter, 46.0 nm; Sigma) that were subjected to electrophoresis with the samples. Analyses of pooled plasma standards revealed that the identification of the major LDL peak was highly reproducible (coefficient of variation between runs <3%). Two plasma control samples were applied in each plate of the analyzing samples, together with the standards of known diameter.

Ion exchange HPLC of LDL
Separation of LDL from unmodified LDL was accomplished using anion exchange high pressure liquid chromatography (Bio-Rad Gradient Module Model 700), as described previously (12). Briefly, isolated LDL was injected into the HPLC at an adjusted concentration of 0.5 mg cholesterol/ml. The eluate was monitored at 280 nm and peaks corresponding to defined/normal LDL (nLDL) or LDL were integrated. The concentration of LDL was expressed as % of total LDL. Chelex-treated buffers were used during all steps of the isolation procedure.

Coating procedure of glass electrophoretic capillary
In order to reduce the electroendosmosis and absorption of solutes, the capillary was coated using a modification of the method of Hjertén (26) procedure with some variation. Fused silica capillary (SGE Ringwood VIC, North Melbourne, Australia) of 75 μm internal diameter and 360 μm outside diameter was used; the total length was 50 cm, with 45 cm to the detection window, made by cutting off 2.5 mm of the polymide layer. The capillary was first etched with 1 m potassium hydroxide solution for 30 min, rinsed with water for 10 min, then flushed with 20% nitric acid to remove K ions from the wall to produce free silanol groups, washed again with water, and dried under nitrogen flow. The capillary was then silylated with 80 μL of γ-methacryloxypropyltrimethoxysilane (Acros Chimica Milano, Italy) in 20 mL water, adjusted to pH 3.5 with acetic acid. The silane solution was forced through the capillary under nitrogen pressure overnight. Subsequently the capillary was washed with water, and then dried under nitrogen flow. The capillary was then filled with a degassed 5% acrylamide (Bio-Rad Milano, Italy) solution containing 1 mg potassium persulfate and 1 μL TEMED per mL solution. After 2 h the unbound reagent was expelled by nitrogen pressure. To crosslink the linear-coated polyacrylamide, the capillary was next filled with a 37% solution of formaldehyde which was adjusted to pH 10 with 1 m NaOH. After 3 h the capillary was washed with water, dried under nitrogen, and was ready for use.
Analytical capillary isotachophoresis (ITP) of plasma lipoproteins

Analytical ITP of plasma lipoproteins was performed utilizing the method described by Nowicka et al. (19), with some modifications. Plasma was pretrained with a lipophilic dye. In order to evaluate the efficiency of the staining, three lipophilic dyes were utilized: Oil Red O (Serva), Sudan Black B (Sigma), and Fat Red 7B (Fluka). Each stain was dissolved in ethylene glycol to give a 1% solution, stirred in the dark at room temperature for 6 h, and filtered. These dyes have been tested at various amounts with the same concentration of plasma lipoproteins. Samples stained with Sudan Black B, Oil Red O, and Fat Red 7B were read respectively at 570, 620, and 519 nm. At the same ratio with plasma lipids, the three stains did not reveal significant differences in number and shape of the peaks. The higher photometric signal was, however, obtained with the Fat Red 7B. For this study, 25 μL of plasma was therefore incubated for 30 min at 4°C with 13 μL of a 1% solution of Fat Red 7B (Fluka) in ethylene glycol at 4°C in the dark. After incubation, the sample was diluted with 110 μL of spacers solution consisting of phenylalanine, histidine, serine, glycyl-glycine, glycine, alanyl-glycine, histidine-leucine, 3-methyl-histidine, glutamine, pseudouridine, methionine, glycyl-histidine, glycyl-sarcosine, and valyl-glycine (Sigma) at 5 mg/ml of each. The leading electrolyte consisted of 10 mm H₂PO₄ and 0.25% hydroxypropylmethyl cellulose (Sigma) adjusted to pH 9.0 with 1 m 2-amino-2-methyl-1,3-propanediol (Ammediol; Serva). The terminating electrolyte contained 100 mm valine and 1 m Ammediol to pH 9.2. The buffers were filtered through 0.2 μm cellulose nitrate filters (Amicon) and flushed under nitrogen. In order to assess the reproducibility of the migration time and the stability of capillary perfor-
and positively with HDL-C concentrations (r 0.642; P < 0.01). We also confirmed the presence of an inverse correlation between the LDL diameter and the concentration of LDL (r -0.659; P < 0.01). By analytical capillary ITP, whole serum can be separated into 11 subfractions (Fig. 1). In ten consecutive runs of different samples, the electropherogram profile started at 16.75 ± 0.68 min (SD) and completed at 23.55 ± 0.50 min; therefore the electropherogram mean time was 6.77 ± 0.38 min. Six runs of the same sample showed variation in peak times of less than 0.10 min, and the coefficient of variation of the areas under the peaks was <3%.

As documented by the behavior of the individual lipoprotein fractions obtained by ultracentrifugation and then subjected to capillary ITP, the first four peaks of total plasma capillary ITP correspond to HDL, peaks 5–7 correspond to VLDL and IDL, while LDL migrate in the last four peaks. The total area of HDL separated by capillary ITP correlated with HDL-C (r 0.70; P < 0.001), that of VLDL/IDL with TG (r 0.88; P < 0.01) and that of LDL with LDL-C (r 0.75; P < 0.001). We focused our attention, however, on LDL subfractions (Fig. 2). The relative contribution of each of the individual LDL subfractions to total LDL is reported in Table 2. Usually, the predominant subfraction is the slow migrating LDL1, followed by LDL3, and then by the faster LDL2 and LDL1. Table 3 represents the correlation of the four LDL subfractions with the various plasma lipid parameters, the apparent diameter of the LDL particle, or to post-secretory phenomena, like oxidation, are detectable by capillary ITP, and whether this method offers information that is applicable to clinical practice. For this purpose we selected subjects from a group of patients who were referred by their family physicians to our lipid clinic for the first time. Most are dyslipidemics or have a family history of dyslipidemia, but many do not use hypolipidemic drugs. Subjects with high values of Lp[a] were excluded from the study because of the possibility that Lp[a] could affect the capillary ITP and introduce an additional and variable component that could make interpretation of results more difficult. Preliminary data, however, suggested that no lipoprotein subfraction obtained by capillary ITP correlated with Lp[a] plasma concentration. It has also been previously reported by capillary ITP that Lp[a] is heterogeneous and is spread throughout the VLDL and LDL fractions (19).

Free flow and capillary ITP has been utilized for many years to study plasma lipoproteins (18, 19, 31). The discriminating principle is based on the net charge of lipoproteins. Analysis of lipoproteins by capillary ITP can be performed directly and rapidly on whole serum and plasma. Sample pretreatment is negligible and a only few nanoliters of sample are necessary for the analysis. Despite these advantages, there are as yet no applications of lipoprotein analysis by capillary ITP.

DISCUSSION

The results of this study agree with previous data showing that the abundance of small LDL particles, as measured by laser densitometric scanning of polyacrylamide gradient gels, correlate positively with plasma triglycerides and negatively with plasma HDL-C (5, 8, 27–30). It was also confirmed that small dense LDL are enriched of the more electronegatively charged in vivo oxidized LDL (17). The aim of this study was, however, to verify whether changes in electric charge due to different size, structure of the particle, or to post-secretory phenomena, like oxidation, are detectable by capillary ITP, and whether this method offers information that is applicable to clinical practice. For this purpose we selected subjects from a group of patients who were referred by their family physicians to our lipid clinic for the first time. Most are dyslipidemics or have a family history of dyslipidemia, but many do not use hypolipidemic drugs. Subjects with high values of Lp[a] were excluded from the study because of the possibility that Lp[a] could affect the capillary ITP and introduce an additional and variable component that could make interpretation of results more difficult. Preliminary data, however, suggested that no lipoprotein subfraction obtained by capillary ITP correlated with Lp[a] plasma concentration. It has also been previously reported by capillary ITP that Lp[a] is heterogeneous and is spread throughout the VLDL and LDL fractions (19).

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protein capillary ITP in clinical practice. As shown by other authors (18, 19), each lipoprotein fraction can be further divided into two or more subfractions, depending on the net charge. Similar results were obtained in this study. Our attention was focused on LDL, which is separated by capillary ITP into four discrete subfractions, each of which could have a different pathophysiologic role.

Despite overwhelming evidence that LDL is an atherogenic lipoprotein, the precise mechanism whereby LDL promotes atherosclerosis remains uncertain. Most investigators believe that LDL particles must undergo modification before they become pathologic. Several postulated modifications include oxidation (32, 33), glycation (34, 35), and enzymatic degradation (36, 37). A final answer as to which of these modifications imparts atherogenicity to LDL awaits further research, although it is plausible that each may contribute to atherosclerosis through a convergent mechanism, despite arising from different processes. It is remarkable, however, that each of these modifications induces changes in LDL electric charge. Another characteristic that is linked to the atherogenicity of LDL is the presence of smaller and denser particles. Several retrospective surveys (5, 10, 38) suggest that small dense LDL confers increased risk for CHD. More recently, a prospective study upheld this hypothesis (39), showing that high levels of small LDL are associated with increased risk of subsequently developing CHD in men and that this associated risk is partly independent of other lipoprotein abnormalities. The precise mechanisms that underlie the production of dense LDL remain largely undefined. Several processes include elevated production rates for apoB-containing lipoproteins (40), modified intravascular lipolysis of VLDL precursors (41), direct production of dense LDL (42), diminished rates of transfer and exchange of neutral lipids between VLDL, LDL, and HDL (43), and altered rates of catabolism of LDL by the LDL receptor pathway (44). These processes may be loosely grouped with those that potentially contribute to production of dense LDL subspecies and those that could enhance reduction in their catabolism. Together such processes would favor accumulation of dense LDL in plasma. The small dense LDL particles exhibit both high susceptibility to oxidation

TABLE 3. Correlation coefficients among total plasma cholesterol (TC), triglycerides (TG), LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C), percentage contribution of LDL- to total LDL and apparent diameter of the major plasma LDL subfraction as determined by polyacrylamide gradient gel electrophoresis with LDL subfractions obtained by capillary isotachophoresis

<table>
<thead>
<tr>
<th>Fraction</th>
<th>TC</th>
<th>TG</th>
<th>LDL-C</th>
<th>HDL-C</th>
<th>% of LDL-</th>
<th>LDL Diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL₁</td>
<td>−0.26</td>
<td>0.69a</td>
<td>−0.41b</td>
<td>−0.56b</td>
<td>0.88a</td>
<td>−0.71a</td>
</tr>
<tr>
<td>LDL₂</td>
<td>0.04</td>
<td>0.55b</td>
<td>−0.09b</td>
<td>−0.18b</td>
<td>0.65b</td>
<td>−0.69b</td>
</tr>
<tr>
<td>LDL₃</td>
<td>0.03</td>
<td>0.19b</td>
<td>−0.04b</td>
<td>−0.16b</td>
<td>−0.14b</td>
<td>−0.41b</td>
</tr>
<tr>
<td>LDL₄</td>
<td>0.11</td>
<td>−0.70a</td>
<td>0.24</td>
<td>0.54a</td>
<td>−0.76a</td>
<td>0.87a</td>
</tr>
</tbody>
</table>

ₐ P < 0.001.  
ₘ P < 0.01.  
ₘₐ P < 0.05.
tion (11–14). LDL

LDL, and has a modified apoB-100 composition (11–14). LDL shows a lower binding affinity to LDL receptors on human skin fibroblasts and endothelial cells, is a poor ligand for scavenger receptors (11), and is cytotoxic to cultured endothelial cells (13). In this study we observed a strong correlation between LDL− plasma concentration and the presence of the faster LDL subfractions in capillary ITP, particularly LDL1. Even though quantitative comparisons are not possible, due to the use of different methods, these findings further suggest that the more electronegatively charged LDL− is indeed present in the human blood stream and does not substantially arise from auto-oxidation or other artifacts during the isolation procedures. Because capillary ITP utilizes total plasma and does not require manipulation or fractionation of samples, artifacts arising from in vitro or isolation techniques are virtually nil.

A correlation between small dense LDL and lipoperoxide-enriched LDL− (17) suggests that denser LDL represents an older population of circulating particles that have also experienced more contact with oxidants. Oxidative mechanisms such as phospholipid peroxidation, followed by the action of phospholipases and degradation of damaged phospholipid, could contribute to changes in the LDL and production of smaller LDL (50, 51) and LDL−. The origin of LDL− remains a matter of speculation, but growing evidence suggests that it represents a population of older particles that are not sufficiently modified to be cleared from the circulation. Under these circumstances peroxides gradually accumulate either by radical propagation reactions, and/or by deposition of peroxidized lipids from dietary sources or generated by cells. In the latter case, the vessel wall has been shown to generate modified LDL as a component of the LDL oxidized in the interstitial spaces (32). Being minimally modified, LDL− may escape clearance by phagocytic cells or receptor-mediated uptake and egress back into the circulation in a manner similar to normal LDL (46). Whatever its origin, LDL− seems to behave as a more atherogenic lipoprotein or possibly a marker of active atherosclerosis (15, 16).

It is remarkable that at least three of the recognized highly atherogenic LDL that could be found in human plasma, namely oxidized, glycated, and small dense, are characterized by a greater electric charge. The LDL profile from capillary ITP and the relative prevalence of faster or slower migrating LDL fractions could, therefore, indicate the presence of more atherogenic LDL.

![Fig. 3.](image)

Scatterplot showing correlation between the ratio faster to slower LDL subfractions, obtained by capillary isochromatography (LDL1 + LDL2)/(LDL3 + LDL4), and the apparent diameter of the major plasma LDL subfraction as determined by polyacrylamide gradient gel electrophoresis of total plasma (upper panel). The (LDL1 + LDL2)/(ILDL3 + LDL4) ratio was calculated utilizing the integrated areas of each LDL peak detected at 519 nm. The lower panel shows the correlation between the (LDL1 + LDL2)/(ILDL3 + LDL4) ratio and the concentration of the more electronegatively charged LDL− measured by ion exchange HPLC of total LDL.

(45–47) and low affinity for the LDL receptor (44, 48), suggesting a higher atherogenic potential for these particles. The variation in biological function of small dense LDL is probably due to differences in apoB-100 conformation that alter the interaction with the cellular LDL receptor (49). Quantitative electrophoretic measurements of LDL subfractions with different densities indicated that the small dense LDL are the most negatively charged particles (49). These results indicate that variations in apoB-100 conformation and surface charge among the LDL sub-species are major determinants of their catabolic fate. The more net negatively charged particles in small dense LDL reduce binding to the LDL receptor and prolong their plasma residence time. Taken together, these key features underlie the elevated atherogenicity of small dense LDL.

A more electronegative LDL subfraction (LDL−) has been isolated from human plasma by ion exchange chromatography (11, 12). This lipoprotein represents an in vivo oxidized lipoprotein subpopulation that is enriched in lipid hydroperoxides and other peroxidation products, contains lower vitamin E levels than in unmodified or normal LDL (nLDL), and has a modified apoB-100 composition (11–14). LDL− shows a lower binding affinity to LDL receptors on human skin fibroblasts and endothelial cells, is a poor ligand for scavenger receptors (11), and is cytotoxic to cultured endothelial cells (13). In this study we observed a strong correlation between LDL− plasma concentration and the presence of the faster LDL subfractions in capillary ITP, particularly LDL1. Even though quantitative comparisons are not possible, due to the use of different methods, these findings further suggest that the more electronegatively charged LDL− is indeed present in the human blood stream and does not substantially arise from auto-oxidation or other artifacts during the isolation procedures. Because capillary ITP utilizes total plasma and does not require manipulation or fractionation of samples, artifacts arising from in vitro or isolation techniques are virtually nil.

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