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Characterization and quantitation of apolipoprotein B-100 by capillary electrophoresis

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Abstract The interaction of low density lipoproteins (LDL) with different surfactants was studied by capillary electrophoresis (CE) and sucrose density gradient ultracentrifugation as part of developing a method for quantitation of apoB-100 in serum. A mixture of surfactants consisting of 70% sodium dodecyl sulfate (SDS), 25% sodium myristyl sulfate, and 5% sodium cetyl sulfate was found to delipidate LDL particles more effectively than pure SDS or sodium decyl sulfate. The delipidation products of LDL [apolipoprotein B-100 (apoB-100) and lipids] were resolved as two distinct peaks by CE when using a 3.5 mM 70% SDS mixture, 20% (v/v) acetonitrile, 50 mM sodium borate, pH 9.1 buffer. This CE method was also used to characterize apoB-100 derived from samples of lipoprotein [a] and very low density lipoproteins (VLDL). A CE-based quantitation method for apoB-100 was developed utilizing the observed linear relationship between apoB-100 concentration and its corrected 214 nm absorbance peak area measured on-line by CE. Concentration values of apoB-100 in LDL and VLDL samples were determined by CE and found to be accurate when compared to values obtained by immunoturbidimetric analysis and the Lowry method. Capillary electrophoresis can be used as a precise, accurate, and specific on-line method for the qualitative and quantitative analysis of the apoB-100 component of VLDL and LDL-related lipoproteins.—**Cruzado, I. D., S. L. Cockrill, C. J. McNeal, and R. D. Macfarlane.** Characterization and quantitation of apolipoprotein B-100 by capillary electrophoresis. *J. Lipid Res.* 1998. **39:** 205–217.

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Plasma lipoproteins are water-soluble pseudo-micellar particles composed of lipids and one or more specific proteins called apolipoproteins. There are three main lipoprotein classes according to density: very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL)(1). These main lipoprotein classes can also be separated by particle size, electrophoretic mobility, molecular weight, and affinity chromatography (2). In addition, each lipoprotein class is highly heterogeneous and composed of several subclasses that vary in their composition, metabolism, and influence on atherogenesis (3).

Studies relating to the structure and metabolism of LDL are important because of the direct correlation between atherosclerosis and high LDL levels in human plasma (4). LDL is the end product of VLDL catabolism and is the major cholesterol-transporting lipoprotein in human plasma (1). The majority of LDL particles contain a single apolipoprotein called apoB-100 (4). After the elucidation of the role of apolipoproteins in the regulation of lipoprotein metabolism, it became apparent that improvements in the characterization of apoB-100 were needed to facilitate the development of the linkage between LDL and atherosclerosis (1).

ApoB-100 is a glycoprotein, synthesized in the liver, consisting of 4,536 amino acids and with a molecular mass of 512,932 (5–7). There are 14 cystine residues in apoB-100: two are free while twelve form six intramolecular disulfide bonds (1). ApoB-100 is also a component of VLDL and lipoprotein[a] (Lp[a]). A related apolipoprotein, apoB-48, which is synthesized in the intestine, is a component of chylomicrons (7). One of the analytical problems in characterizing apoB-100 is that, in the free form, it is insoluble in aqueous solutions and tends to form aggregates. Consequently, the most effective delipi-

Abbreviations: ACN, acetonitrile; apo[a], apolipoprotein[a]; apoB-100, apolipoprotein B-100; BCA, bicinchoninic acid; BSA, bovine serum albumin; CE, capillary electrophoresis; EDTA, ethylenediaminetetraacetic acid; EOF, electroosmotic flow; GFC, gel filtration chromatography; HDL, high density lipoprotein; HPLC, high performance liquid chromatography; LDL, low density lipoprotein; LDL-b, buoyant LDL; LDL-d, dense LDL; Lp[a], lipoprotein[a]; PMSF, phenylmethylsulfonyl fluoride; SBB, Sudan black B; SdeS, sodium 1-decanesulfate; SDS, sodium dodecyl sulfate; UC, ultracentrifugation; VLDL, very low density lipoprotein; μ_e, effective mobility.

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dation process is one where a water-soluble complex of apoB-100 is formed. Both non-delipidated and delipidated forms of apoB-100 are sensitive to oxidation and adventitious enzymatic cleavage (1).

A variety of separation techniques has been used in the analysis of non-delipidated apoB-100. Ultracentrifugation (UC) (3, 8–12) and polyacrylamide gel electrophoresis (9) are the two most common analytical methods used. Tadey and Purdy (13, 14) used capillary electrophoresis (CE) to study the electrophoretic behavior of non-delipidated apoB-100 and other apolipoproteins using different surfactants and determined that the interaction with sodium dodecyl sulfate (SDS) at pH 10 produced stable anionic complexes with high electrophoretic mobility. In the earlier studies, a key finding was that high SDS concentrations efficiently delipidated LDL forming water-soluble complexes consisting of apoB-100 and surfactant anions (15). Simons and Helenius (16) determined that an SDS concentration of 0.2 M is sufficient to displace all lipids from LDL. Equilibrium dialysis showed that apoB-100 binds 2.6 times its weight in SDS, which is a much higher uptake ratio than for other high MW proteins. Optical rotatory dispersion and circular dichroism studies have shown that when SDS interacts with LDL above its critical micellar concentration (8 mM), significant conformational changes in the structure of apoB-100 occur (16). These conformational changes probably facilitate the penetration of additional SDS into the interior of the spherical LDL particle (17). Gotto et al. (18) obtained partial delipidation of LDL when using sodium decyl sulfate (SdeS) with concentrations of 20–100 mM. By using UC, they found that the delipidation products consisted of free lipids (floating fraction) and partially delipidated apoB-100 (sedimenting fraction).

The plasma concentrations of VLDL and LDL particles are important parameters in establishing a comprehensive cardiac risk profile. Two different approaches have been used to measure these concentrations. One approach focuses on the apoB–100 concentration because each of the VLDL and LDL particles contains a single apoB-100 species. Immunoassay techniques have been developed targeting specific epitopes on the surface of apoB-100 (19). A fundamental problem with this approach is variability in the antigen/antibody interaction within the population of VLDL and LDL particles. Other problems include variable dose-response working curves, lack of purified apoB-100 standards for calibration, and a coefficient of variance of 5–10% for within-laboratory analysis and up to 30% for inter-laboratory comparisons (20). A second approach is based on the interaction of lipoproteins with lipophilic dyes and absorbance measurements of the amount of dye incorporated (21). Problems encountered with this approach include variability in absorption of the dye due to the heterogeneity in the structures and lipid content within the VLDL and LDL populations (2).

The first objective of this work is to study the interaction of LDL with different surfactants and concentrations as a prelude to the apoB-100 quantitation work using CE and UC techniques developed in our laboratory. Previously, we have described the use of CE in the study of Lp[a] (22), the separation of HDL and LDL particles (23), and in the characterization and quantitation of apolipoproteins A-I and A-II from HDL fractions (24). We found that the best CE separation for lipoproteins and their apolipoproteins is achieved when using a 50 mM sodium borate, 3.5 mM SDS (70% pure), 20% (v/v) acetonitrile (ACN), pH 9.1 buffer. Coupled with these studies is the development of a fast UC technique that utilizes a self-generating sucrose density gradient for the separation of lipoproteins from human plasma and serum. This UC technique converts the UC pattern of the stained lipoproteins into a lipoprotein profile by using a combination of photography, digitization, and computer analysis. The second objective was to develop a CE-based quantitation assay for apoB-100 and to compare the performance of this assay method with immunoturbidimetric analysis and the Lowry method. (25)

MATERIALS AND METHODS

Materials

Acetonitrile (ACN; EM Science, Gibbstown, NJ), sodium borate (Fisher Scientific, Fair Lawn, NJ) and SDS (Sigma Chemical Co., St. Louis, MO) were used in the preparation of the CE buffers. Human purified LDL samples were obtained from Sigma and Calbiochem (San Diego, CA). Human Lp[a] was isolated by UC and purified by lysine affinity chromatography in our laboratory as described previously (21). Other reagents used were: sodium bromide (NaBr; Fisher Scientific), sucrose (Fisher Scientific), sodium azide (Aldrich Chemical Co., Milwaukee, WI), ethylenediaminetetraacetic acid (EDTA; J. T. Baker Inc., Phillipsburg, NJ), phenylmethylsulfonyl fluoride (PMSF; Sigma), Sudan black B (SBB; Sigma), dimethyl sulfoxide (Burdick and Jackson Laboratories Inc., Muskegon, MI), benzamidine (Sigma), SdeS (Aldrich), methanol (Mallinckrodt, Paris, KY), and 2-mercaptoethanol (Sigma). Deionized water was obtained from a Milli Q water purification system (Millipore, Bedford, MA) and used in the preparation of all solutions.

CE analysis of lipoprotein samples

CE separations were carried out using a Beckman P/ACE model 5510 unit (Beckman Instruments Inc.,

Fullerton, CA) equipped with a diode array detector and connected to a 80486DX IBM PS/2 computer. The System Gold Chromatography software (Beckman) was used to control the instrument functions and to analyze the electropherograms. An untreated fused silica capillary tube (Polymicro Technologies, Phoenix, AZ) with a 75 μm i.d., 375 μm o.d. was used. The total length of the capillary tube was 56.8 cm, and the distance from injection site to detector was 50.1 cm. The capillary was operated at 20°C. The UV absorbance was monitored at 214 nm. An applied voltage of 17.5 kV was used in all CE separations. All the CE buffers contained 50.0 mM sodium borate and had a pH value of 9.1. The electroosmotic flow (EOF) was measured for each run by making a 1 s injection of either water, methanol–water 1:1 or 1.2 mM benzyl alcohol (Mallinckrodt, Paris, KY) solution. The EOF direction was towards the cathode, and it was strong enough to carry the negatively charged particles to the negatively charged electrode. The effective mobility (μ_e) values for negatively charged analytes were calculated using the following equation:

$$\mu_e = \frac{L_c L_d}{U}\left(\frac{1}{t} - \frac{1}{t_{EOF}}\right)$$

where L_c is the total length of the capillary [cm], L_d is the length of the capillary from injector to detector [cm], U is the applied voltage [volts], t is the migration time of the sample component [s], and t_{EOF} is the migration time of the EOF marker [s]. All sample injections were carried out by applying pressure of 0.5 PSI for 4 s, unless indicated otherwise. All CE experiments were repeated at least three times to measure the precision of the μ_e values.

Separation of human serum lipoproteins by sucrose density gradient UC

Serum lipoproteins were separated by single discontinuous density gradient UC (12). Blood from the antecubital vein of the donor (12 h fasted) was drawn into 7 mL Vacutainer tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) containing no additives. The blood was allowed to clot at room temperature for 45 min, and the serum was collected after a 20-min spin at 2,000 rpm (161 *g*) and 4°C. For the UC analysis, a serum volume of 250 μL was first incubated with 10 μL of SBB in dimethyl sulfoxide (1% w/v) for 30 min at 37°C (Scientific Products TempCon Incubator, Baxter Diagnostics Inc., McGaw Park, IL). SBB served as a lipophilic stain for visualizing the lipoprotein bands after the UC separation. Then, 200 μL of the stained serum was mixed with 200 μL of water. This solution was then layered over 800 μL of 20% sucrose in a 1.5-mL polycarbonate open-top tube (Beckman). The tube was spun at 100,000 rpm (435,680 *g*) for 6 h at 20°C using a Beckman TL-100 ultracentrifuge equipped with a TLA100.2 rotor. The acceleration and deceleration steps were set to 2.5 and 3 min, respectively. After separation, the lipoprotein layers were removed by aspirations using a hypodermic syringe.

Separation of human plasma lipoproteins by NaBr density gradient UC

Blood from the antecubital vein of a donor (12 h fasted) was drawn into 7 mL EDTA-containing Vacutainer tubes. The plasma was separated from the red blood cells by a 20-min spin at 2,000 rpm (161 *g*) and 4°C. A preservative mixture of 1.0 M benzamidine (1 μL/mL plasma) and 0.2 M PMSF (5 μL/mL plasma) was then added to the plasma. A 200 μL aliquot was stained with 7 μL of 1% (w/v) SBB in dimethyl sulfoxide. The preparation of the ultracentrifuge medium involved a series of solutions layered in a 1.5-mL polycarbonate open-mouth tube in the following order: 250 μL of 1.26 g/mL NaBr solution, 200 μL of 1.10 g/mL NaBr solution, 207 μL of the stained plasma, and 600 μL of water. All of the layering solutions contained 0.01% EDTA and 0.01% sodium azide. The UC separation was carried out as described in the sucrose density gradient UC procedure with the exception that the samples were centrifuged for 3.5 h at 10°C.

An unstained LDL sample was obtained by centrifuging eight UC tubes containing unstained plasma along with two UC tubes of SBB-stained plasma under the conditions described above. After UC separation, the pale yellow LDL bands from the eight centrifuge tubes were extracted using as a location reference the position of the SBB-stained LDL layer in the other two UC tubes. Approximately 1 mL of unstained LDL was obtained by pooling the LDL fractions from the eight UC tubes and concentrating to a final volume of 100 μL by spinning the sample in a Centricon concentrator 100 (Amicon, Inc., Beverly, MA) for 1 h at 2,000 rpm (161 *g*) and 4°C. This concentrated unstained LDL sample was used in the preparation of a CE calibration curve for the quantitation of apoB-100.

Conversion of the stained lipoprotein UC profile into an intensity versus density plot

After the UC separation, each tube was photographed against the white background of an opalescent glass diffuser (Edmund Scientific, Barrington, NJ) using a Macro 5 SLR camera (Polaroid Corporation, Cambridge, MA) loaded with color Polaroid Spectra film and set to a 2× magnification. A 285 HV external flash unit (Vivitar, Santa Monica, CA) was synchronized to the camera shutter, positioned behind the glass diffuser, and used as the light source. Each photograph was digitized with a ScanJet 3p gray scale scanner (Hewlett-Packard,

Tokyo, Japan) and analyzed by SigmaGel software (Jandel Scientific Software, San Rafael, CA). This program measures the gray scale intensity along the vertical axis of the UC tube in the digitized photograph giving an intensity versus tube length plot. A gray scale intensity value of 0 corresponds to white and an intensity value of 255 corresponds to black. At a particular location along the vertical axis of the UC tube, the gray scale intensity is a measure of the concentration of SBB taken up by the lipoproteins with a specific density at that location in the final density gradient. The density gradient along the UC tube, measured by gravimetric analysis, was found to be highly reproducible when the UC conditions were kept constant. Using this scheme, a lipoprotein UC profile is converted into a SBB intensity versus particle density plot.

Study of the interaction of LDL with different surfactants by UC

Ten LDL samples from a normolipidemic human subject were isolated simultaneously by sucrose density gradient UC as described previously. The ten fractions were combined to yield a 2 mL volume sample of fresh, homogeneous LDL. Aliquots of 200 μL were mixed with 200 μL of solutions of known surfactant concentration. The surfactants used were SdeS, 99% pure SDS, and 70% pure SDS, and their final concentration in the LDL mixture varied from 0 to 100 mM. The LDL–surfactant mixtures were incubated for 30 min at 37°C, after which they were analyzed by sucrose density gradient UC. The UC results were converted into SBB intensity versus density plots as described above. Aliquots of the floating and sedimenting fractions were removed with a pipet and analyzed by CE.

Bicinchoninic acid (BCA) protein quantitation

The reactions involved in the BCA analysis are the reduction of Cu^{2+} to Cu^{1+} mainly by the tyrosine and tryptophan residues in proteins, and the formation of a purple complex (λ_{MAX} = 562 nm) when the Cu^{1+} ions complex with BCA. The Lowry method (25) is based on a similar principle: the reduction of Cu^{2+} ions followed by complexation with the Folin-Ciocalteu reagent which also produces an intense colored complex with Cu^{1+} (26). The weakness of both of these assays is that there is considerable variability in reactivity depending on the nature of the protein (26). In studies where it is not feasible to prepare protein standard solutions with known concentrations, as in the case of apoB-100, the strategy of linking the calibration to that obtained from a set of bovine serum albumin (BSA) solutions with known concentrations has proven to be a reasonable alternative that has been widely adopted.

A calibration curve based on the BCA method was first obtained using a set of BSA solutions with accurately known concentrations. This calibration curve was then used to standardize the unstained concentrated LDL sample isolated by NaBr density gradient UC in terms of [apoB-100] based on the BCA method. The standardized solution was then used to prepare a set of solutions with varying [apoB-100] by serial dilution for use in the CE calibration. In the application of the BCA quantitation method to the analysis of BSA and apoB-100, the standard working reagent was prepared in situ by mixing 50 mL of reagent A with 1 mL of reagent B. Reagent A was purchased from Pierce Chemical Company (Rockford, IL) and consisted of 1% BCA, 2% $Na_2CO_3 \cdot H_2O$, 0.16% sodium tartrate, 0.4% NaOH, and 0.95% $NaHCO_3$. Reagent B was made by preparing a 4% $CuSO_4 \cdot 5H_2O$ (Mallinckrodt, Paris, KY) solution. A set of BSA solutions with known concentrations was prepared. A 1-mL volume of the standard working reagent was mixed with 50 μL of each BSA solution. Then the solutions were incubated for 30 min at 37°C then equilibrated to room temperature for 15 min. The absorbance at 562 nm of each solution was measured using 500-μL volume quartz cuvettes with a 1.0-cm path length (Fisher Scientific) and a Perkin-Elmer Lambda 4B UV/Vis spectrometer (Norwalk, CT). A linear relationship was obtained between the absorbance at 562 nm of the Cu^{1+}–BCA complex and the BSA concentration (8–90 mg/dL): slope value was 0.0078 (± 0.0002), y-axis intercept value was 0.01 (± 0.01), and the linearity was 0.994. This calibration curve was then used for the standardization of the LDL stock solution in terms of [apoB-100]. Because of the high [apoB-100], it was necessary to dilute an aliquot of the stock a factor of 5 prior to analysis by the BCA method. From this measurement, it was determined that the stock solution had an [apoB-100] of 280 (±8) mg/dL.

The BCA method was also used as a sensitive qualitative test for the identification of lipid and protein fractions isolated by sucrose density gradient UC in the delipidation studies. In this application, 10 μL of the micro BCA reagent (26) was mixed with 10 μL of sample in a Falcon 3911 Microtest III flexible assay plate (Becton Dickinson Labware, Oxnard, CA). The mixture was then incubated at 37°C for 60 min. Samples testing positive for proteins showed a color change from light green to purple.

RESULTS AND DISCUSSION

Interaction of LDL with buffers of different composition studied by CE

In the first part of the study, we investigated how the electrophoretic properties of LDL are affected by the

use of different surfactants and an organic modifier in the CE background electrolyte. Earlier studies have shown that SDS binds to the apolipoproteins increasing their electrophoretic mobilities and thus improving their CE separation (13, 14). We have demonstrated that the separation of plasma lipoproteins is further improved when ACN is added to the SDS-containing buffer as an organic modifier (23). In addition, the use of a mixture of surfactants can enhance the resolution of a CE protein separation. In a recent work, we used a mixture of surfactants that consisted of 70% SDS (C_{12}-chain), 25% sodium myristyl sulfate (C_{14}-chain) and 5% sodium cetyl sulfate (C_{16}-chain), which proved to give a superior CE separation of proteins than pure SDS (24). This mixture of surfactants is referred in this paper as 70% SDS, and pure SDS as 99% SDS.

In the first measurement, a sample of LDL was adjusted to pH 9.1, and analyzed by CE in the absence of surfactant. The electropherogram showed a single symmetric peak. Next, the influence of the presence of the surfactants (70% and 99% SDS SdeS) in the CE buffer was studied without adding ACN. In each case, the surfactant concentration in the CE buffer was fixed at 3.5 mM. The electropherograms indicated significant interactions had taken place between the LDL particles and the surfactant in the CE buffer. The single peak observed in the absence of surfactant was transformed into two peaks with higher μ_e values than the original value measured for LDL in the absence of surfactant. **Table 1** summarizes the μ_e values observed for the different LDL components in each CE buffer. The two peaks observed are referred to as LDL-1 and LDL-2.

The formation of two distinct species with different μ_e values from LDL indicates that the surfactant has dissociated the LDL particles in the capillary forming two distinct groups of negatively charged particles with μ_e values depending on the nature of the surfactant. As shown in Table 1, the two species formed in the interaction with SdeS have higher μ_e values than LDL. The trend continues with 99% SDS and 70% SDS. Most likely, a component of the surfactant interaction is related to differences in the hydrophobicity of the negatively charged surfactants. SdeS is the less hydrophobic because it has the shortest carbon chain (C_{10}) and interacts with LDL less strongly than the 70% SDS, which appears to have an even larger interaction with LDL than 99% SDS. The effectiveness of the 70% SDS may be due to combined interaction of SDS to maintain solubility and its long chain components (C_{14} and C_{16}), which bind more strongly to the non-polar domains.

The influence of the addition of an organic modifier to the CE buffer (20% ACN) was then studied. The presence of the organic modifier increases the hydrophobicity of the buffer modifying the interaction of LDL with the surfactant as well as inducing conformational changes in LDL (27). With ACN present in the buffer but no surfactant, a single LDL peak was observed with a slightly higher mobility than when ACN is not present. The electropherogram of LDL in the SdeS–ACN buffer had a single LDL peak with a μ_e value close to what was observed in the absence of SdeS, indicating that there was no interaction of this surfactant with LDL. The other two surfactant anion systems (70% and 99% SDS) react with LDL in the presence of ACN resulting in dissociation and complex formation but to different degrees. The interaction of 99% SDS with LDL in ACN produced four families of dissociation products with distinct μ_e values higher than that for intact LDL while the 70% SDS gave the same pattern of two dissociation products similar to that observed in the absence of ACN but with lower μ_e values. The μ_e values are given in **Table 2**.

In the absence of ACN in the CE buffer, the surfactant anions interact with the LDL particle destabilizing its structure resulting in dissociation and delipidation. In the presence of ACN, SdeS does not partition into

TABLE 1. Effective mobility values of the LDL components separated by capillary electrophoresis in buffers containing no surfactants or 3.5 mM SdeS, 99% SDS, and 70% SDS

	Effective Mobility (μ_e)	
Surfactant	LDL-1	LDL-2
	$10^{-5}\ cm^2\ V^{-1}\ s^{-1}$	
None	−14.1 ± 0.3	
SdeS	−18.13 ± 0.04	−19.56 ± 0.03
99% SDS	−26.84 ± 0.04	−28.86 ± 0.08
70% SDS	−28.39 ± 0.02	−29.70 ± 0.03

The CE samples consisted of 2 μL of LDL (isolated by NaBr density gradient UC) mixed with 20 μL of the CE buffer. The applied voltage was 17.5 kV. The total length of the untreated fused silica capillary was 56.8 cm, and its length from injector to detector was 50.1 cm. The CE capillary was thermostatted at 20°C. Samples were injected by a 4 s 0.5 PSI pressure pulse. The buffer also contained 50 mM sodium borate adjusted to a pH of 9.1.

TABLE 2. Effective mobility values of LDL components separated by capillary electrophoresis in buffers containing 3.5 mM surfactant of three different types and 20% (v/v) ACN

	Effective Mobility (μ_e)			
Surfactant	LDL-1	LDL-2	LDL-3	LDL-4
	$10^{-5}\ cm^2\ V^{-1}\ s^{-1}$			
None	−15.3 ± 0.2			
SdeS	−14.8 ± 0.1			
99% SDS	−17.1 ± 0.03	−19.6 ± 0.2	−21.5 ± 0.2	−24.8 ± 0.1
70% SDS	−19.33 ± 0.09	−25.9 ± 0.2		

Capillary electrophoresis separation conditions were as described in Table 1.

LDL but SDS still interacts because the non-polar interaction with LDL is stronger due to the longer aliphatic chain. It would appear that the species formed in the interaction of 99% SDS in the presence of ACN are partial delipidation products while those formed in the 70% SDS interaction may be the end products of LDL delipidation but with fewer surfactant anions incorporated when ACN is present as evidenced by the lower μ_e values. To test this hypothesis, a sucrose gradient UC separation was used to separate the delipidation products of LDL produced by interaction with the surfactants and analyze them by CE.

UC analysis of delipidated LDL

UC density profiles of LDL were measured after treatment with SdeS, 99% SDS, and 70% SDS for a range of surfactant concentrations from 0 to 10 mM. The results obtained for SdeS showed only a slight increase in the density of the LDL band (1.015–1.033 g/mL) presumably due to loss of some lipids. The two peaks observed in the electropherogram are presumably due to minimally delipidated LDL particles that have retained most of their density.

In contrast, the influence of SDS on the LDL particle density distribution is substantial as shown in **Fig. 1**. In the 99% SDS, the intensity of the LDL peak decreases and a new peak appears with a density of 1.013 g/mL (Fig. 1a). (The peak labeled M is the location of the meniscus in the UC tube and serves as a reference point in the profile.) The intensity of this new peak increases with a corresponding loss in intensity of the LDL peak and a slight shift to higher density when 70% SDS is used (Fig. 1b). When the SDS concentration is increased to 7 mM and 10 mM, the LDL peak disappears, the intensity of the low density fraction increases, and a high density component appears near the bottom of the UC tube. The low density component is referred to as the floating fraction and the high density component, the sedimenting fraction.

The floating and sedimenting fractions were withdrawn from the UC tube and tested for protein using the BCA method. The floating fraction was found to contain no proteins while the sedimenting fraction had a high protein content.

The floating and sedimenting fractions were then analyzed by CE. The results are shown in **Fig. 2**. Fig. 2a is the electropherogram of the floating fraction and Fig. 2b, of the sedimenting fraction. The μ_e values for the floating and sedimenting fractions are the same as those obtained for LDL-2 and LDL-1, respectively (Table 2). Increasing the SDS concentration up to 100 mM did not change the electropherograms of these two fractions. We conclude that the sedimenting fraction is LDL-1 and is apoB-100 that has been fully delipidated and complexed with SDS. LDL-2 is the floating fraction and is a mixture of UV-absorbing lipid products of the

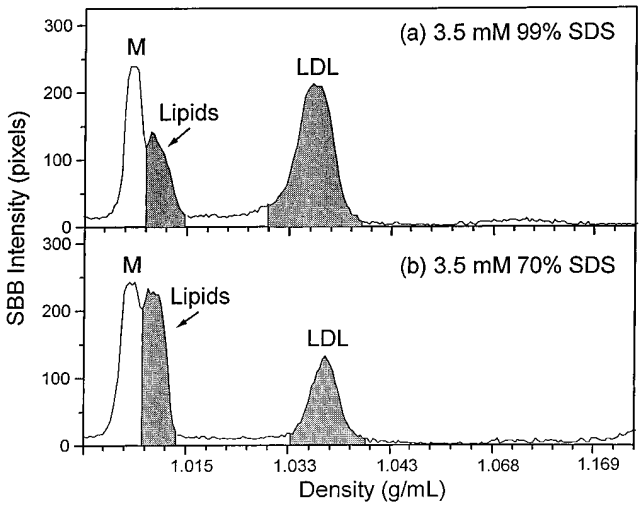

Fig. 1. UC profiles of LDL samples treated with (a) 3.5 mM 99% SDS, and (b) 3.5 mM 70% SDS. Peak: M, meniscus. The UC separation was carried in 1.5 mL open-mouth polycarbonate tubes. Each LDL sample (200 μL), previously stained with SBB and separated from serum by sucrose density gradient UC, was diluted with water to a final volume of 400 μL, and then layered over a 20% sucrose solution (800 μL) in each UC tube. The tubes were centrifuged for 6 h at 100,000 rpm (435,680 *g*) and 20°C.

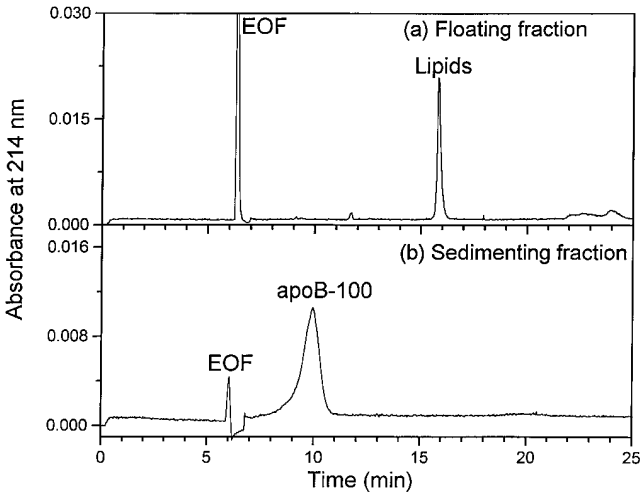

Fig. 2. Electropherograms of (a) floating and (b) sedimenting fractions of LDL sample treated with 7.0 mM 70% SDS and separated by sucrose density gradient UC. Background electrolyte: 50 mM sodium borate, 20% (v/v) ACN, 3.5 mM 70% SDS, pH 9.1. Samples were not diluted for CE analysis. EOF marker, water. μ_e values: apoB-100, -17.97×10^{-5} $cm^2V^{-1}s^{-1}$; and LDL lipids, -25.85×10^{-5} $cm^2V^{-1}s^{-1}$. Other CE conditions were as described in the legend to Table 1.

dissociation of LDL and complexed with SDS giving a distribution of anionic lipid complexes with a narrow charge/volume distribution. The UC results are consistent with those obtained by Gotto et al. (18). The LDL-1 peak observed for LDL delipidated by 99% SDS in ACN is fully delipidated apoB-100; LDL-2,3 are probably partially delipidated LDL and LDL-4 is due to free lipids.

The LDL particle density distribution is not affected by the other components of the CE buffer: 50 mM sodium borate and 20% ACN. Some delipidation of LDL occurs when the [SdeS] exceeds 50 mM but at 100 mM the delipidation is not complete. The LDL-to-surfactant concentration ratio influences the degree of delipidation.

The key finding serving as the basis for the quantitation study is that when the CE buffer is 3.5 mM 70% SDS and 20% (v/v) ACN, LDL is fully delipidated and the apoB-100 is separated from the lipids during the CE separation.

Factors influencing the delipidation of LDL by SDS

The delipidation of LDL by the anion of SDS is initiated by attachment of the anion to the surface of the particle with subsequent diffusion into the interior and disruption of the pseudomicellar structure. At high LDL concentrations in the absence of other lipoproteins, LDL forms aggregates that delipidate at a lower rate when reacted with SDS (28). This feature is an important consideration in developing a CE-based quantitation method for apoB-100 because delipidation by the CE buffer is a component of the analysis. This property of LDL places restrictions on the amount of LDL that can be injected into the capillary. To study this effect, electropherograms of LDL were obtained using different effective initial concentrations. In the first experiment, an aliquot of the concentrated LDL stock solution (280 mg/dL) was injected into the capillary using a 4 s duration pressure pulse. The sample was analyzed using the 70% SDS-ACN CE buffer. **Figure 3a** shows the electropherogram obtained. In contrast to what was observed for the more dilute LDL samples where an apoB-100 and a lipid peak were identified, only a single peak was observed which is presumably non-delipidated LDL ($\mu_e = -.20.75 \times 10^{-5}$ cm^2 V^{-1}s^{-1}). By reducing the injection time to 2 s, the concentration of LDL sample in the capillary is effectively reduced by a factor of 2. Figure 3b shows the electropherogram obtained. The intensity of the LDL peak decreases due to the reduced LDL concentration and two small peaks appear with μ_e values corresponding to delipidated apoB-100 and the lipid peak indicating that a small fraction of the LDL was delipidated at the lower concentration. Figure 3c is the electropherogram obtained after further dilution by a factor of 4. Under these conditions, the SDS interacts more effectively with LDL resulting in delipidation of approximately 80% of the LDL particles based on the relative peak intensities of the apoB-100 and lipid peaks. when an LDL sample is fully delipidated, the LDL peak is absent in the electropherogram and this feature is used to detect incomplete delipidation. When the LDL fraction from the UC tube is diluted by a factor of at least two with the 3.5 mM 70% SDS, 20% (v/v) ACN buffer, the electropherograms show complete delipidation. If the LDL sample is analyzed immediately after UC separation, delipidation occurs within 30 min when the LDL sample is incubated with the CE buffer. Samples stored at 4°C for more than 1 day require 2–4 h incubation for complete delipidation. It may be that apoB-100 assumes a conformation at 4°C that has a weaker interaction with SDS than its conformation at room temperature. Another possibility is that LDL forms aggregates after isolation that are more difficult to delipidate (28). The correlation between storing time at 4°C and increased time for full delipidation has only been observed for LDL but not the other lipoprotein fractions when analyzed by CE.

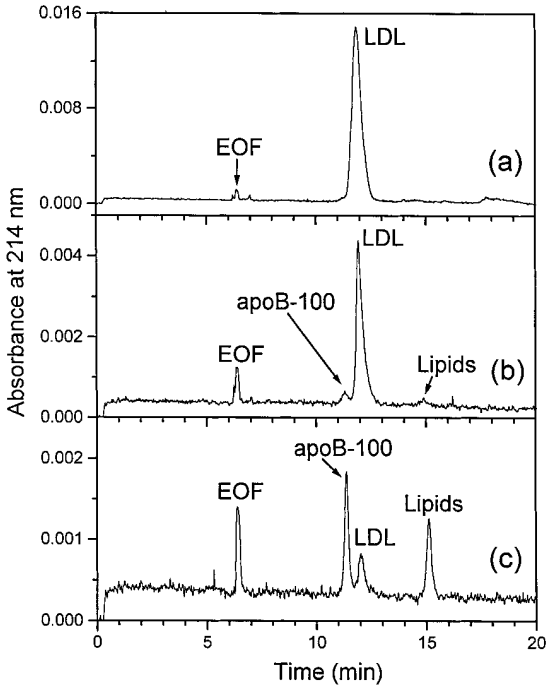

Fig. 3. Electropherograms of a concentrated LDL sample using a (a) 4 s, (b) 2 s, and (c) 1 s pressure injection. Background electrolyte: 50 mM sodium borate, 20% (v/v) ACN, 3.5 mM 70% SDS, pH 9.1. Sample: 2 μL of LDL (isolated by NaBr density gradient UC) mixed with 20 μL CE buffer, except for (c): 1 μL LDL mixed with 20 μL CE buffer. EOF marker, water. Other CE separation conditions were as described in the legend to Table 1.

Influence of LDL particle density on the CE of delipidated LDL

The electropherogram of delipidated LDL using the 70% SDS–ACN buffer is characterized by a peak corresponding to apoB-100 complexed with SDS and a well-defined lipid/SDS peak each with reproducible μ_e values that have been found to be invariant for LDL samples from a large population of donors. Some of these donors had a significant dense LDL component in their particle density profile, a component recognized as a risk factor for atherosclerosis (3). A CE analysis was carried out for buoyant and dense LDL obtained from a donor with documented heart disease. **Figure 4** is the particle density profile of the plasma sample showing the two LDL bands: a buoyant component (LDL-b) and a dense component (LDL-d). The two shaded areas in the LDL region represent the locations where the samples were withdrawn from the UC tube. Electropherograms of the two fractions are shown in **Fig. 5**. In both electropherograms, the apoB-100 peak has the characteristic μ_e value of -19.1×10^{-6} cm^2 V^{-1} s^{-1} and the lipids peak, -25.5×10^{-5} cm^2 V^{-1}s^{-1}. Integrated peak area ratios for the apoB-100 and the lipids peak were measured to be 1.3 for the buoyant form (Fig. 5a) and for the dense form, the lipids peak is somewhat narrower (Fig. 5b) with a ratio of 1.9 which is consistent with the buoyant form having a higher lipid content.

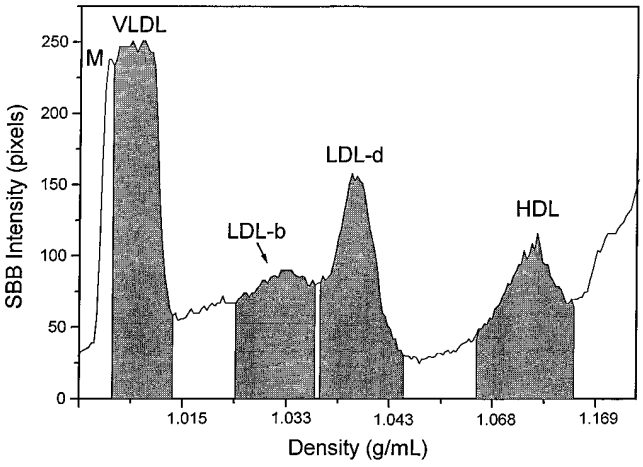

Fig. 4. UC lipoprotein profile of a donor with heart disease. Peaks: M, meniscus; LDL-b, buoyant LDL; and LDL-d, dense LDL. The plasma lipoproteins were separated by NaBr density gradient UC. These solutions were layered in a 1.5 mL open-mouth polycarbonate tube and in the following order: 250 μL of 1.26 g/mL NaBr solution, 200 μL of 1.10 g/mL NaBr solution, 207 μL of SBB-stained plasma, and 600 μL of water. The tube was then centrifuged for 3.5 h at 100,000 rpm (435,680 *g*) and 10°C.

Identification of apoB-100 in delipidated Lp[a] and VLDL

Using the 3.5 mM 70% SDS/20% (v/v)ACN borate buffer at pH 9.1 as the reference delipidating and CE buffer, the μ_e value for the apoB-100 has a sample-to-sample variation of ±2% which means that apoB-100 can be identified in the electropherogram of a mixture derived from fractions with densities close to VLDL and LDL. This feature was applied to the analysis of Lp[a]. The structure of Lp[a] is similar to that of LDL except that apolipoprotein [a](apo[a]) is attached to apoB-100 by disulfide bridges (29). An individual identified as having an elevated plasma Lp[a] level by an immunoassay screen donated blood for the analysis. Lp[a] was isolated by the lysine/Sepharose method (21) and analyzed by CE. **Figure 6a** shows the electropherogram obtained for the delipidated sample. Two peaks were observed with μ_e values, in units of 10^{-5} cm^2 V^{-1} s^{-1}, of −15.9 and −26.1. The peak with the larger mobility has a μ_e value corresponding to the lipids peak but the peak with the lower μ_e value does not correspond to apoB-100. Treatment with 4 mM 2-mercaptoethanol followed by boiling for 7 min at 100°C cleaved the disulfide bonds between apoB-100 and apo[a]. Analysis by CE showed four peaks (Fig. 6b), referred to as peaks 1–4, with μ_e values of −10.5, −14.5, −19.6, and -26.1×10^{-5} cm^2 V^{-1} s^{-1}, respectively. Peak #1 has been identified as mercaptoethanol (ME) by measurement of a pure sample. Peak #2 is a product of the reduction of Lp[a] and has been assigned to apo[a]. The third peak

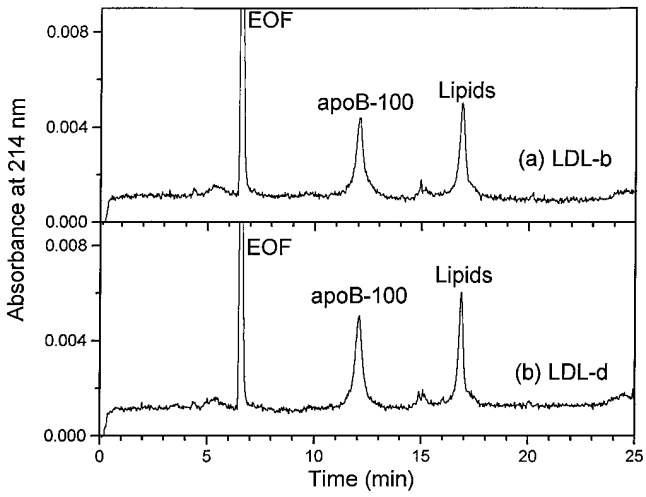

Fig. 5. Electropherograms of (a) buoyant LDL and (b) dense LDL. Background electrolyte: 50 mM sodium borate, 20% (v/v) ACN, 3.5 mM 70% SDS, pH 9.1. Samples: 2 μL of each LDL sample (from Fig. 4) was mixed with 20 μL CE buffer. EOF marker, water. Other CE separation conditions were as described in the legend to Table 1.

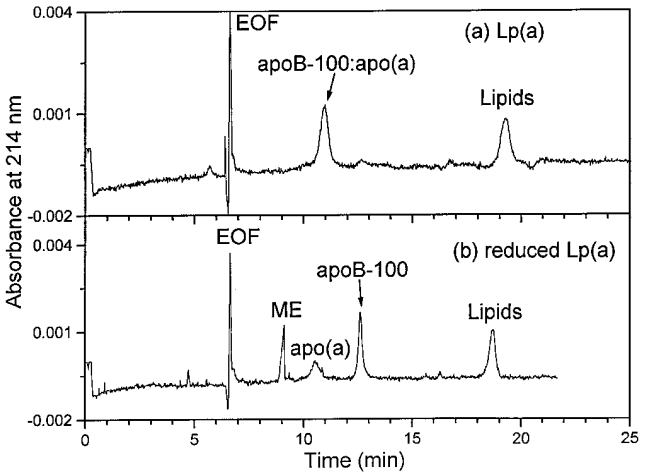

Fig. 6. Electropherogram of (a) Lp[a] and (b) reduced Lp[a]. Background electrolyte: 50 mM sodium borate, 20% (v/v) ACN, 3.5 mM 70% SDS, pH 9.1. Samples: (a) 2 μL of Lp[a] mixed with 20 μL CE buffer; and (b) 2 μL of Lp[a] mixed with 0.5 μL 0.09 M 2-mercaptoethanol and 10 μL CE buffer (5 s and 1 s pressure injections, respectively). EOF marker, 1.2 mM benzyl alcohol. Peaks: ME, 2-mercaptoethanol; apo[a], apolipoprotein [a]; and apoB-apo[a]; apoB-100 bound to apo[a]. Other CE separation conditions were as described in the legend to Table 1.

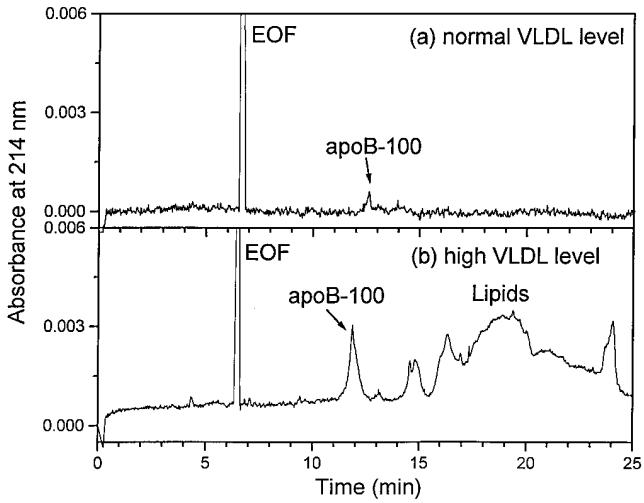

Fig. 7. Electropherograms of VLDL samples from (a) normolipodemic and (b) hypertriglyceridemic donors. Background electrolyte: 50 mM sodium borate, 20% (v/v) ACN, 3.5 mM 70% SDS, pH 9.1. Samples were isolated by NaBr density gradient UC and analyzed by CE after dilution by a factor of 2 with the CE buffer. EOF marker, water. Other CE separation conditions were as described in the legend to Table 1.

is due to apoB-100 and the fourth peak is the lipids peak based on μ_e values. In studying Lp[a] from other donors, considerable donor variability was found in the μ_e values for both delipidated apoB-100:apo[a] and apo[a] which is attributed to the different phenotypes of Lp[a]. A measurement of the μ_e value for delipidated Lp[a] is a potential CE-based marker for classifying Lp[a] phenotypes.

The detection by CE of apoB-100 as a product of delipidated VLDL was also investigated. The VLDL fraction from the UC separation of the serum from a normolipidemic donor was analyzed by CE using the same conditions as for the LDL study. **Figure 7a** is the electropherogram obtained. A weak intensity peak was identified with a μ_e value of -19.3×10^{-5} cm^2 V^{-1} s^{-1} which corresponds to apoB-100. There was no evidence for a lipid peak. Figure 7b is the electropherogram obtained for a hypertriglyceridemic donor. The apoB-100 peak is considerably higher in intensity and a broad complex pattern is also present which is due to anionic complexes of lipids with SDS with a considerably greater spread of mobility values than observed for LDL. Recent unpublished studies using a neutral surfactant for delipidation and the 70% SDS/ACN buffer for CE analysis confirmed that the broad peak in the electropherogram is due to coupling of free lipids to SDS during delipidation. S. L. Cockrill, L. W. Watkins, and R. D. Macfarlane, unpublished results).

Quantitation of apoB-100 by CE

The area of the apoB-100 peak in the electropherogram is proportional to the concentration of apoB-100 in solution and this is the basis for using CE to quantitate serum levels of apoB-100 levels in LDL and VLDL, factors that have been linked to the development of atherosclerosis (21). Absorbance at 214 nm is due primarily to the peptide bonds in apoB-100 and is considerably higher than at 280 nm. In the first step, the peak area at 214 nm was calibrated against the apoB-100 concentration using an unstained LDL stock solution where the [apoB-100] had been previously measured using the BCA method as described in Materials and Methods. A set of samples was prepared by serial dilution using the delipidating CE buffer. In the measurement of the peak area, a background is first subtracted and the residual total absorbance is referred to as a corrected peak area (CPA) that is used in obtaining the calibration curve. Over a range of concentrations from 3–43 mg/dL, the calibration curve is linear with a slope of 0.296 (±0.007), a y-intercept of 0.2 (±0.2), and linearity of 0.995. This concentration range was adopted as the working range of the analysis.

The method was tested using an LDL solution obtained from Calbiochem, assayed by the vendor as 950 mg/dL apoB-100 using the Lowry method (25). The sample was diluted to a concentration of 21 mg/dL us-

ing the delipidating CE buffer and analyzed by CE. A value of 24 (±1) mg/dL was obtained. The good agreement is significant because it demonstrates that when the apoB-100 concentration is within the linear range of the calibration curve where effective delipidation by the buffer is achieved, the method is accurate, even for stored LDL samples.

A more stringent test of the accuracy of the overall method for plasma/serum analysis including staining by SBB and ultracentrifuge separation was made using a plasma standard obtained from Sigma (Cardiolipid Control Level 1). The total apoB-100 concentration (VLDL + LDL) was 58 (±12) mg/dL determined by immunoturbidometric analysis. A measured aliquot (μL of UC plasma) of the plasma standard was stained with SBB and separated into VLDL, LDL, and HDL by sucrose density gradient ultracentrifugation. The VLDL and LDL layers were identified and withdrawn quantitatively (μL of VLDL or LDL fraction) from the UC tube using the distribution of the stain as a marker for the location of the band. A measured aliquot of the VLDL and LDL fraction was diluted with the delipidating CE buffer using a dilution factor (D.F.) which was typically 2 and analyzed by CE. The apoB-100 peak in the electropherograms was identified by its characteristic μ_e value and a determination of the CPA was made. The plasma concentration of apoB-100 in VLDL and LDL was calculated using the following equations:

$$[\text{ApoB} - 100]_{\text{LDL}}\ (\text{mg/dL}) = \text{D.F.} \times \frac{\text{CPA}}{0.296} \times \frac{(\mu\text{L of LDL fraction})}{(\mu\text{L of UC plasma})}$$

$$[\text{ApoB} - 100]_{\text{VLDL}}\ (\text{mg/dL}) = \text{D.F.} \times \frac{\text{CPA}}{0.296} \times \frac{(\mu\text{L of LDL fraction})}{(\mu\text{L of UC plasma})}$$

The factor 0.296 is the slope of the calibration curve obtained from the analysis of the LDL standard as described above. Following this procedure, the total apoB-100 concentration was found to be 58 (±7) mg/dL, a value in good agreement with that obtained by immunoturbidometric analysis. During the delipidation of LDL and VLDL by the CE buffer, the SBB incorporated in the LDL particle is released along with the other lipids and separated from apoB-100 by CE.

Results from clinical applications of the methodology

Using the methodology described above, the serum apoB-100 levels of several donors were measured in conjunction with the standard lipid screen for cholesterol and triglycerides levels as a preliminary test of the use of the methodology in a clinical application. The normal range of total plasma apoB-100 concentrations determined by immunoassay is 70–100 mg/dL with 90% in LDL and the remainder distributed in VLDL and intermediate density lipoproteins (30, 31). For hyperlipidemic individuals, the total [apoB-100] is typically in the range from 100 to 160 mg/dL (32). Using the CE-based analysis, the same range of values was found in a small sampling of a donor population. A young healthy male with a normal lipid profile had apoB-100_{LDL} and apoB-100_{VLDL} levels of 51 and 3 mg/dL, respectively. An individual with moderately elevated cholesterol levels had apoB-100_{LDL} and apoB-100_{VLDL} levels of 99 and 5 mg/dL, respectively. A hyperlipidemic donor exhibited apoB-100_{LDL} and apoB-100_{VLDL} levels of 159 and 6 mg/dL. This limited test of the method is an indication that the range of apoB-100 values obtained by the CE-based methodology is consistent with what has been observed from methods currently in use.

Advantages of a CE-based method for apoB-100 quantitation

The most precise and well-founded method for apoB-100 quantitation in serum is the method of Vega and Grundy (33) where apoB-containing serum lipoproteins are precipitated, delipidated, resolubilized, and the protein content is assayed by the Lowry-Folin method (25). A second chemical method uses SDS-PAGE to separate the apoB-100 and absorbance measurements of the stained bands (34). Immunoassay has also been used for quantitation of serum apoB-100 levels (35).

A difference between the method of Vega and Grundy (33) and the CE-based method is that precipitation is used to isolate apoB-100-containing lipoproteins from serum whereas the method described here uses sucrose density gradient ultracentrifugation. Choice of method depends on the objective of the analysis. If the objective is to measure integrated apoB-100 levels, the precipitation method is faster and could be coupled with CE to measure apoB-100 levels utilizing the high resolution separation features of CE to eliminate interferences in the analysis. The ultimate goal of the ultracentrifuge/CE method is to develop a comprehensive cardiac risk profile protocol that includes the particle density profile as well as measurement of the key apolipoprotein levels in VLDL, LDL, and HDL. Consequently, the ultracentrifuge-based initial separation is the starting point in the overall scheme and, because precipitation purification is not used, is an essential step for purification from other plasma proteins, particularly albumin which co-elutes with apoB-100 in the delipidating CE buffer used in this study.

One feature that CE bring to the analysis of apoB-100 is the ability to analyze the delipidated mixture directly using on-line separation, circumventing the need to re-

move interfering lipids and, if other apolipoproteins are present in the LDL fraction, they are separated from apoB-100 during the CE analysis. A more precise method for apoB-100 quantitation using CE is through UV-absorbance by apoB-100 using an internal BSA standard in a mode that utilizes the flexibility of CE. The CE analysis can be programmed for serial injection where the delipidated apoB-100 sample is first injected followed by a second injection of the BSA concentration standard after a brief interval of electrophoresis. The electrophoresis continues to completion and the resulting electropherogram has peaks due to apoB-100 and BSA analyzed under identical conditions.

Both CE and SDS-PAGE can be used to quantitate apoB-100 in delipidated LDL solutions and both methods provide a separation from other proteins. The advantage of CE is that concentration can be linked directly to the UV-absorbance of apoB-100. In SDS-PAGE, concentrations are determined by the affinity of the apolipoprotein for a visible chromophore such as Coomassie Blue. Variability in chromogenicity within the isoforms of apoB-100 is a source of systematic error and the method of determination of optical density from a gel plate is less precise than a direct absorbance measurement.

The advantage of immunoassay over CE for the quantitation of apoB-100 is speed and ease of analysis of serum/plasma samples without separation of the apoB-100-containing lipoproteins. Immunoassay is an integrating analysis measuring total apoB-100 levels and accuracy is dependent on uniformity of immunoreactivity in the apoB-100 isoform distribution. Quantitation based on CE with on-line separation and detection is uniformly responsive to this distribution because measurement is based on UV-absorbance of apoB-100. The potential for high accuracy and precision inherent in the CE method can be utilized to develop apoB-100 standards in plasma/serum samples to monitor the accuracy of immunoassay methods.

A unique feature of CE operating at basic pH using a silica capillary is the phenomenon of electroosmotic flow which does not occur in SDS-PAGE. This marker, which is easily identified in the electropherogram, accounts for all of the instrumental variables that influence the electromigration of ions. By measuring ion mobilities relative to the EOF, highly reproducible μ_e values for apoB-100 are obtained which means that the CE analysis of apoB-100 is both quantitative and qualitative.

One of the unexplained findings in this study that was not pursued further portends the potential of CE for characterizing new features of the apoB-100 isoform distribution utilizing variables that are associated with the CE buffer. The observation was that LDL is delipidated by 99% SDS, 20% ACN giving three distinct classes of apoB-100-containing delipidation products. Different buffer systems might be sensitive to particular features of the heterogeneity of LDL.

Summary

The overall objective of the study was to investigate the use of CE to study the delipidation of LDL by surfactants in conjunction with developing a method for the on-line quantitation of apoB-100 in lipoprotein fractions isolated by density gradient ultracentrifugation. LDL was injected and transported through a silica capillary by electroosmotic flow and detected on-line by 214 nm absorbance produced an electropherogram of LDL characterized by a narrow range of μ_e values. Adding anionic surfactants to the CE buffer at low concentration increased the average μ_e due to uptake of surfactant anions. Increasing the concentration further induced delipidation giving apoB-100 and free lipids identified as two peaks in the electropherogram and verified by sucrose density gradient ultracentrifugation.

Using 3.5 mM 70% SDS, 20% (v/v) ACN as a delipidating CE buffer, electropherograms of buoyant and dense LDL were obtained showing that the lipid content of dense LDL is lower than the buoyant form but that the μ_e values for apoB-100 for both forms are the same. Apo[a] was distinguished from apoB-100 using the delipidating CE buffer and apoB-100 was identified in VLDL based on its characteristic μ_e value. The lipid component from VLDL and LDL was found to have different μ_e distributions; the composition of these components is not known.

A CE-based quantitation method for apoB-100 in LDL and VLDL was developed using BSA as a standard. The method was tested with commercial LDL and plasma standards and measured apoB-100 levels were found to be accurate within the precision of the standard.

The overall advantage of a CE-based method for apoB-100 quantitation when linked to an ultracentrifuge-based separation is the potential for high accuracy by eliminating systematic error associated with precipitation, resolubilization, losses associated with lipid removal, and variability in chromogenicity and immunoreactivity within the apoB-100 isoform distribution. The reproducibility and control of CE of conditions for analysis makes high precision possible, and the use of electroosmotic flow as a mobility marker combined with the high resolving power of CE gives the method high specificity for apoB-100 analysis.

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REFERENCES

1. Mahley, R. W., T. L. Innerarity, S. C. Rall, Jr., and K. H. Weisgraber. 1984. Plasma lipoproteins: apolipoprotein structure and function. *J. Lipid Res.* **25:** 1277–1294.
2. Rosenfeld, L. 1989. Lipoprotein analysis. *Arch. Pathol. Lab. Med.* **113:** 1101–1110.
3. Garber, D. W. 1992. Lipoprotein classes and subclasses. *In* Plasma Lipoproteins and Coronary Artery Disease. R. A. Kreisberg, editor. Academic Press, Orlando, FL. 251–256.
4. Davis, R. A. 1991. Lipoprotein structure and secretion. *In* Biochemistry of Lipids, Lipoproteins and Membranes. D. E. Vance and J. Vance, editors. Elsevier Science Publishers, Edmonton, Alberta, Canada. 403–426.
5. Knott, T. J., R. J. Pease, L. M. Powell, S. C. Wallis, S. C. Rall, Jr., T. L. Innerarity, B. Blackhart, W. H. Taylor, Y. Marcel, R. Milne, D. Johnson, M. Fuller, A. J. Lusis, B. J. McCarthy, R. W. Mahley, B. Levy-Wilson, and J. Scott. 1986. Complete protein sequence and identification of structural domains of human apolipoprotein B. *Nature.* **323:** 734–738.
6. Yang, C-Y., S-H. Chen, S. H. Giantiurco, W. A. Bradley, J. T. Sparrow, M. Tanimura, W-H. Li, D. A. Sparrow, H. DeLoof, M. Rosseneu, F-S. Lee, Z-W. Gu, A. M. Gotto, Jr., and L. Chan. 1986. Sequence, structure, receptor-binding domains and internal repeats of human apolipoprotein B-100. *Nature.* **323:** 738–742.
7. Kane, J. P. 1983. Apolipoprotein B: structural and metabolic heterogeneity. *Annu. Rev. Physiol.* **45:** 637–650.
8. Kulkarni, K. R., D. W. Garber, M. K. Jones, and J. P. Segrest. 1995. Identification and cholesterol quantitation of low density lipoprotein subclasses in young adults by VAP-II methodology. *J. Lipid Res.* **36:** 2291–2302.
9. Musliner, T. A., and R. M. Krauss. 1988. Lipoprotein subspecies and risk of coronary disease. *Clin. Chem.* B78–83.
10. Shen, M. S., R. M. Krauss, F. T. Lindgren, and T. M. Forte. 1981. Heterogeneity of serum low density lipoproteins in normal human subjects. *J. Lipid Res.* **22:** 236–244.
11. Chapman, M. J., P. M. Laplaud, G. Luc, P. Forgez, E. Bruckert, S. Gouliniet, and D. Lagrange. 1988. Further resolution of low density lipoprotein spectrum in normal human plasma: physicochemical characteristics of discrete species separated by density gradient ultracentrifugation. *J. Lipid Res.* **29:** 442–458.
12. Chung, B. H., T. Wilkinson, J. C. Geer, and J. P. Segrest. 1980. Preparative and quantitative isolation of plasma lipoproteins: rapid, single discontinuous density gradient ultracentrifugation in a vertical rotor. *J. Lipid Res.* **21:** 284–291.
13. Tadey, T., and W. C. Purdy. 1993. Effect of detergents on the electrophoretic behavior of plasma apolipoproteins in capillary electrophoresis. *J. Chromatogr.* **652:** 131–138.
14. Tadey, T., and W. C. Purdy. 1992. Characterization of plasma apolipoproteins by capillary electrophoresis. *J. Chromatogr.* **583:** 111–115.
15. Edelstein, C. 1991. Conventional and high performance liquid chromatography as applied to human plasma apolipoproteins. *In* Analysis of Fats, Oils and Lipoproteins. E. G. Perkins, Editor. AOCS, Champaign, IL. 589–598.
16. Simons, K., and A. Helenius. 1970. Effects of sodium dodecyl sulphate on human plasma low density lipoproteins. *FEBS Lett.* **7:** 59–63.
17. Helenius, A., and K. Simons. 1971. Removal of lipids from human plasma low-density lipoprotein by detergents. *Biochemistry.* **10:** 2542–2547.
18. Gotto, A. M., R. I. Levy, F. T. Lindgren and D. S. Fredrickson. 1969. Partial delipidation of human serum β-lipoprotein by sodium decyl sulfate. *Biochim. Biophys. Acta.* **176:** 667–669.
19. Fruchart, J. C., and J. M. Bard. 1991. Lipoprotein particle measurement: an alternative approach to classification of lipid disorders. *Curr. Opin. Lipidol.* **2:** 362–366.
20. Naito, H. K. 1988. Reliability of lipid, lipoprotein and apolipoprotein measurements. *Clin. Chem.* **34:** 84–94.
21. Rifai, N., J. F. Chapman, L. M. Silverman, and J. T. Gwynnes. 1988. Review of serum lipids and apolipoproteins in risk assessment of coronary heart disease. *Ann Clin. Lab. Sci.* **18:** 429–439.
22. Hu, A. Z., I. D. Cruzado, J. W. Hill, C. J. McNeal, and R. D. Macfarlane. 1995. Characterization of lipoprotein [a] by capillary zone electrophoresis. *J. Chromatogr.* **717:** 33–39.
23. Cruzado, I. D., A. Z. Hu, and Ronald D. Macfarlane. 1996. Influence of dodecyl sulfate ions on the electrophoretic mobilities of lipoprotein particles measured by HPCE. *J. Cap. Elect.* **3:** 25–29.
24. Cruzado, I. D., S. Song, S. F. Crouse, B. C. O'Brien, and R. D. Macfarlane. 1996. Characterization and quantitation of the apoproteins of high-density lipoprotein by capillary electrophoresis. *Anal. Biochem.* **243:** 100–109.
25. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
26. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150:** 76–85.
27. Janzen, R., K. K. Unger, H. Giesche, J. N. Kinkel, and M. T. W. Hearn. 1987. Evaluation of advanced silica packings for the separation of biopolymers by high-performance liquid chromatography. IV. Mobile phase and surface-mediated effects on recovery of non-porous, monodisperse 1.5-μm reverse-phase silicas. *J. Chromatogr.* **397:** 81–89.
28. Khoo, J. C., E. Miller, P. McLoughlin, and D. Steinberg. 1990. Prevention of low density lipoprotein aggregation by high density lipoprotein or apolipoprotein A-I. *J. Lipid Res.* **31:** 645–652.
29. Gaubatz, J. W., C. Heideman, A. M. Gotto Jr., J. D. Morrisett, and G. H. Dahlen. 1983. Human plasma lipoprotein [a]. *J. Biol. chem.* **258:** 4582–4589.
30. Spady, D. K. 1991. Lipoproteins in biological fluids and compartments: synthesis, interconversions and catabolism. *In* Lipoproteins as Carriers of Pharmacological Agents. J. M. Shaw, editor. Marcel Dekker, Inc., New York, NY. 1–44.

31. Gotto, A. M., Jr., H. J. Pownall, and R. J. Havel. 1986. Introduction to plasma lipoproteins. *Methods Enzymol.* **128:** 3–41.
32. Marcovina, S. M., and J. J. Albers. 1989. Standardization of the immunochemical determination of apolipoprotein A-I and B: a report of the IFCC meeting on standardization of apolipoprotein A-I and B measurements. *Clin. Chem.* **35:** 2009–2015.
33. Vega, G. L., and S. M. Grundy. 1996. Quantitation of apolipoprotein B by chemical methods. *Methods Enzymol.* **263:** 63–82.
34. Bergeron, N., L. Kotite, and R. J. Havel. 1996. Simultaneous quantification of apolipoproteins B-100, B-48, and E separated by SDS-PAGE. *Methods Enzymol.* **263:** 82–94.
35. Krul, E. S. and G. Schonfeld. 1986. Immunochemical methods for studying lipoprotein structure. *Methods Enzymol.* **128:** 527–553.