

Characterization and Quantitation of the Apoproteins of High-Density Lipoprotein by Capillary Electrophoresis

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A method has been developed using capillary electrophoresis (CE) to quantitate plasma levels of apo-protein A-I (apoA-I) and apoprotein A-II (apoA-II) in high-density lipoprotein (HDL) samples. ApoA-I and apoA-II are resolved by CE in delipidated and non-delipidated HDL samples. Concentrations of apoA-I and apoA-II were calculated from their peak areas in the electropherogram. Results of the analysis of Sigma plasma standards (Controls 1 and 2) using CE are in good agreement with values obtained by Sigma using immunoturbidimetric assay. CE and reverse-phase high-performance liquid chromatography (RP-HPLC) were found to be complementary in the study of apoA-I and apoA-II. RP-HPLC resolves the isoforms of purified apoA-I and apoA-II, but it cannot resolve mixtures of them because the retention times of the isoforms overlap. CE separates apoA-I from apoA-II, but it does not resolve the isoforms. Matrix-assisted laser desorption/ionization mass spectrometry was used to identify the isoforms of apoA-I and apoA-II by their molecular weight (M_r) in fractions collected from RP-HPLC. © 1996

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High plasma levels of HDL² cholesterol are inversely correlated to the development of coronary artery disease (1, 2), but the mechanism by which HDL prevents this is not known. Studying the protein com-

position and molecular structure of HDL may shed some light in understanding this mechanism. The major apoproteins of HDL are apoA-I (M_r 28,078) and apoA-II (M_r 17,414), and they constitute 70 and 25% of the HDL protein content, respectively. In addition, HDL contains very small amounts of C-, D-, and E-apoproteins (3).

Human HDL consists of a heterogeneous population of lipoproteins which differ in their physicochemical and metabolic properties (4, 5). It is known that HDL consists of two major classes of apoA-I-containing particles: those that contain both apoA-I and apoA-II and those which contain only apoA-I (6, 7). Fruchart and Ailhaud (8) proposed that the HDL particles which contain only apoA-I are potentially antiatherogenic. In another study, Warden *et al.* (9) showed that mice with high levels of HDL and apoA-II developed fatty streak atherosclerotic lesions, which suggests that the ratio of apoA-I to apoA-II may be an important indicator for the development of atherosclerosis. This finding is part of the growing evidence that the apoprotein distribution in the plasma is potentially a significant marker of coronary artery disease along with the cholesterol level (10, 11).

The quantification of apoA-containing lipoproteins is generally carried out using immunological methods including sequential immunoprecipitation, enzyme immunoassay, and differential electroimmunoassay (12). However, the major drawbacks of these immunological techniques are the presence of lipids which shield antigenic sites from being recognized (13) and the effort required in the preparation and characterization of antibodies. In addition, each apolipoprotein must be assayed separately (14).

In the past 20 years, the separation, purification, and analysis of apoproteins have improved due to the development of suitable chromatographic media. Reverse-phase high pressure liquid chromatography (RP-

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² Abbreviations used: ACN, acetonitrile; apoA-I, apoprotein A-I; apoA-II, apoprotein A-II; CE, capillary electrophoresis; EOF, electroosmotic flow; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MALDI-MS, matrix-assisted laser-desorption/ionization mass spectrometry; RP-HPLC, reverse-phase high-pressure liquid chromatography; TFA, trifluoroacetic acid; VLDL, very low-density lipoprotein; μ_e , electrophoretic mobility.

HPLC) has emerged as a powerful analytical tool for the resolution of apoCs and the apoA-I isoforms (15). Hughes *et al.* (14) developed a method for analyzing the protein composition of human plasma lipoproteins by combining density gradient ultracentrifugation with HPLC. They used a C₁₈ column to separate the apoproteins of the six lipoprotein bands obtained from centrifugation with enough resolution to permit their quantification. Hancock *et al.* (16) separated apoA-I and apoA-II by HPLC using an alkyl phenyl and C₈ columns. Recently, Atwal *et al.* (17) were able to separate and quantify apoA-I, apoA-II, and apoB-100 by HPLC using a gel filtration column.

In the past decade, capillary electrophoresis (CE) has emerged as an alternate technique which can be utilized in the separation of apoproteins. Tadey and Purdy (18) studied the influence of anionic, neutral, and cationic detergents in the capillary electrophoretic separation of plasma apolipoproteins from very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and HDL. They found that the optimal separation and reproducibility of HDL and LDL apolipoproteins were obtained when using high-pH buffers containing sodium dodecyl sulfate (SDS). In another study, Liebich *et al.* (13, 19) used CE to measure apoA-I directly from human serum by utilizing a high-pH sodium borate buffer and SDS to separate apoA-I from the rest of the plasma proteins. In addition to their technique being fast and highly reproducible, they obtained a good linearity between the peak area and the concentration of apoA-I. This correlation encourages the feasibility of using CE in the quantification of apoproteins for clinical purposes.

Mass spectrometry is a highly specific technique for identifying proteins separated by CE and HPLC by their molecular mass. Weinmann *et al.* (20) developed an "off-line" combination of CE with MALDI-MS for the structural characterization of CE-separated peptides and proteins. They used this system to study a mixture of apoA-II monomer and homodimer using sample amounts of less than 1 pmol. In another study, Caprioli and DaGue (21) demonstrated the feasibility of connecting a microbore HPLC to a fast atom bombardment MS in the study of the tryptic digest products of human apoA-I.

The main objective of this work was to develop a protocol for characterizing and quantifying the major apoproteins of HDL by using a combination of MALDI-MS, RP-HPLC, and CE. This protocol was tested first on a protein mixture consisting of human serum albumin, ovalbumin, insulin, α -lactalbumin, and β -lactoglobulin B. MALDI-MS was used to identify the apoproteins separated by RP-HPLC and CE. In addition, a CE method was developed which quantifies apoA-I and apoA-II levels in delipidated and non-delipidated HDL samples.

MATERIALS AND METHODS

Chemical reagents. SDS (70 and 99% purity), insulin from bovine pancreas, ovalbumin, human serum albumin, α -lactalbumin, β -lactoglobulin B, and apoA-II were purchased from Sigma Chemical Co. (St. Louis, MO). ApoA-I was bought from PerImmune, Inc. (Rockville, MD). Other reagents used include sodium borate (Fisher Scientific, Fair Lawn, NJ), sodium bromide (Fisher Scientific), acetonitrile (ACN) (Baxter Healthcare Corp., Muskegon, MI), trifluoroacetic acid (TFA) (Pierce Chemical Co., Rockford, IL), methanol (Mallinckrodt, Inc., Paris, KY), and ethyl ether (EM Science, Gibbstown, NJ). Deionized water was prepared using a Milli Q water purification system (Millipore, Bedford, MA), and it was used in the preparation of all solutions.

Isolation of HDL by sequential flotation centrifugation. Venous blood was drawn from the antecubital vein from a 12-h fast individual into ethylenediaminetetraacetic acid-containing Vacutainer tubes (Becton-Dickson Vacutainer Systems, Franklin Lakes, NJ). The blood plasma was separated from the red cells by a 161g centrifugation for 30 min at 4°C. Then the plasma lipoproteins were separated into VLDL, LDL, HDL₂, and HDL₃ by sequential flotation centrifugation (22) using a Sorvall OTD 70B centrifuge (Du Pont Medical Products, Newtown, CT) and a Beckman Type 40 rotor. First, the density of a 3-ml plasma sample was adjusted to 1.019 g/ml with NaBr and centrifuged for 28 h at 110,878g and 19°C to yield VLDL as the supernatant. Next, LDL was isolated after adjusting the density to 1.063 g/ml and centrifuging under the same conditions. HDL₂ was obtained after adjusting the density to 1.125 g/ml and centrifuging for 36 h. Finally, HDL₃ was isolated after adjusting the density to 1.210 g/ml and centrifuging for 36 h. All the lipoprotein samples were stored at 4°C.

Isolation of HDL by density gradient ultracentrifugation. Plasma lipoproteins from the Cardiolipid Control 1 and 2 samples from Sigma were separated by density gradient ultracentrifugation (23). Each plasma sample (150 and 550 μ l) was stained with 1% (w/w) Sudan black B in ethylene glycol (20 and 50 μ l) and diluted with water to a final volume of 0.6 ml. Then each stained plasma sample was layered on top of 0.6 ml of 25% (w/w) sucrose (Fisher Scientific) inside a 1.5-ml polycarbonate open-mouth centrifuge tube (Beckman Instruments, Fullerton, CA). Then the samples were centrifuged in a Beckman TL-100 ultracentrifuge equipped with a TLA100.2 rotor for 4 h at 436,000g and 20°C. After the centrifugation, the HDL was removed with a syringe and its volume was measured.

Delipidation of HDL. HDL was delipidated using the procedure described in Ref. (24). HDL (125 μ l) was delipidated with methanol (1.5 ml) and ethyl ether (3.5 ml). The isolated proteins were dried under vacuum at

room temperature and then dissolved in 0.1% TFA in water. The protein solution was filtered through a nylon 0.2- μm -pore-size membrane filter (Millipore).

RP-HPLC separation. The HPLC system was a Model 130A from Applied Biosystems (Foster City, CA) equipped with an Aquapore RP-300 (C_8) column (50×1.00 mm). HPLC separation of the proteins was carried out with an ACN–water gradient at low pH (0.1% TFA) and at a constant temperature of 30°C. All the reagents used in the preparation of the mobile phases were HPLC grade. The mobile phases were filtered through a 0.22- μm -pore-size membrane filter (Millipore) and degassed extensively under vacuum and by sonication before use. The uv absorbance detector was set to 220 nm. The eluted proteins were collected in 1-min intervals using a Cygnet fraction collector (Isco, Inc., Lincoln, NE). The HPLC data were recorded and analyzed by a Waters 745B data module integrator (Millipore Corp., San Francisco, CA).

Capillary electrophoresis. The CE instrument used was Beckman P/ACE Model 5510 equipped with a diode-array detector. The uv absorbance was monitored at 214 nm. A voltage of 17.5 kV, applied under normal polarity, was used in the separation of the proteins. The electrophoretic separations were carried out in untreated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ) of 75 μm i.d., 354 μm o.d., 56.8 cm total length, and 50.1 cm from injector to detector. The capillary was thermostated at 20°C. The buffer consisted of 50 mM sodium borate, 3.5 mM SDS (70% purity), and 20% (v/v) ACN, pH 9.7 (25). The electroosmotic flow (EOF) was strong enough to carry the negatively charged proteins to the cathode. The samples were degassed by a 5-min 161g centrifugation at room temperature before being injected into the capillary by a 4-s pressure pulse. Water was used as the EOF maker, which was injected by 1 s pressure injection following the sample plug. The sample tray of the instrument was thermostated at 10°C.

Molecular mass determinations. The M_r of the apo-proteins was determined with a HP G2025A MALDI–time-of-flight instrument (Hewlett–Packard Co., Palo Alto, CA) equipped with a N_2 laser (337 nm) and using a +28-kV acceleration voltage. The MALDI matrix was α -cyano-3-hydroxy-trans-cinnamic acid (30 mg/ml in methanol, Aldrich Chemical Co., Milwaukee, WI). An aliquot of 1 μl matrix solution was placed on a gold probe forming a fine crystal layer within a few seconds. Then 0.5 μl of sample–matrix solution (1:1 ratio) was added to the surface of the crystalline deposit. The protein α -chymotrypsinogen A (Sigma), which has a M_r of 25,656, was used as the external standard. Each mass spectrum was the sum of 40 laser shots. Multiple charged species (MH_n^{n+} , where $n = 1, 2 \dots$) were pro-

duced during the desorption/ionization process due to the low laser power utilized.

RESULTS AND DISCUSSION

Protein separation by RP-HPLC and CE. RP-HPLC and CE are techniques that employ different mechanisms to separate proteins. RP-HPLC uses a nonpolar stationary phase and a polar mobile phase, where the retention and separation of proteins depend upon the magnitude of the hydrophobic contact area established between the molecule and the stationary phase. As the polarity of the proteins increases, their interaction with the stationary phase at a given eluent composition becomes weaker (15). Therefore, the proteins elute in order of increasing hydrophobicity. The retention factor, k' , is directly proportional to the hydrophobicity of the protein and is defined as

$$k' = \frac{t_R - t_0}{t_0}, \quad [1]$$

where t_R is the retention time of the protein and t_0 is the elution time of the components not retained by the column. In addition, the k' value depends on the type of column used, the organic modifier, pH, the gradient, and the temperature of the separation.

CE uses a capillary filled with a buffer in which the charged proteins separate during the application of voltage. The ionic components move with different mobilities depending on their charge-to-volume ratio. As the charge-to-volume ratio increases, the electrophoretic mobility (μ_e) increases. When SDS is present in the buffer, the proteins adsorb SDS through hydrophobic interactions, increasing the amount of negative charges and thus their μ_e values. If ACN is added to buffer, the partition of SDS within the proteins is affected and the resolution can be improved (25). The μ_e of a negatively charged protein that moves against the EOF can be calculated with the equation

$$\mu_e = \frac{L_c L_d}{U} \left[\frac{1}{t} - \frac{1}{t_{e0}} \right], \quad [2]$$

where L_c is the total length of the capillary in centimeters, L_d is the length of the capillary from injector to detector in centimeters, U is the applied voltage in volts, t_{e0} is the migration time of the EOF marker in seconds, and t is the migration time of the protein in seconds. The electrophoretic mobility of a protein is affected by the type and concentration of buffer used, the quantity and the nature of the organic modifier, the temperature, pH, and the amount of SDS absorbed by the protein, which is directly proportional to its hydrophobicity.

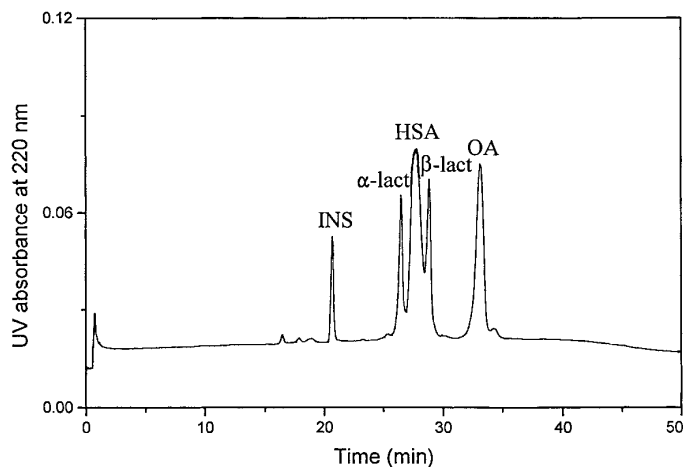


FIG. 1. RP-HPLC chromatogram of a protein mixture consisting of insulin (INS), α -lactalbumin (α -lact), human serum albumin (HSA), β -lactoglobulin B (β -lact), and ovalbumin (OA). The concentration of each protein was $15 \mu\text{M}$. The HPLC conditions used were ACN-water (with 0.1% TFA) linear gradient of 0 to 70% at 1.55%/min, a flow rate of $200 \mu\text{l}/\text{min}$, the column was heated to 30°C , and the sample loop was $5 \mu\text{l}$.

These two techniques separate proteins on the basis of their hydrophobicity, but their separation mechanisms are different. In RP-HPLC, only a small part of the protein interacts with the stationary phase in one interaction, whereas in CE the whole surface of the protein interacts with SDS at the same time. Another difference is that increasing the volume of the proteins at constant charge decreases their μ_e values resulting in a loss of resolution, whereas protein volume is not a significant factor in the RP-HPLC separation. This is what we found when we studied the separation of a protein test mixture.

A protein test mixture consisting of human serum albumin, ovalbumin, insulin, α -lactalbumin, and β -lactoglobulin B was separated by RP-HPLC (Fig. 1) and CE (Fig. 2). Table 1 summarizes the M_r of the proteins as well as the measured k' and μ_e values. The order of elution (determined by injection of each component separately) and the resolution of the mixture are different for each technique. In both techniques, insulin migrates first, meaning that it is the least hydrophobic of the proteins. The proteins α -lactalbumin and β -lactoglobulin B are well separated in RP-HPLC, but they comigrate in CE, indicating that they have similar charge-to-volume ratios. Albumin and ovalbumin are very well separated in HPLC and in CE. The fact that they have a large volume (large M_r) results in low μ_e values. Thus, the elution of the proteins is different for each separation technique because RP-HPLC is surface sensitive while CE is sensitive to charge and surface.

In the electropherogram shown in Fig. 2, the SDS used had a purity of 70%. The SDS consisted of a mix-

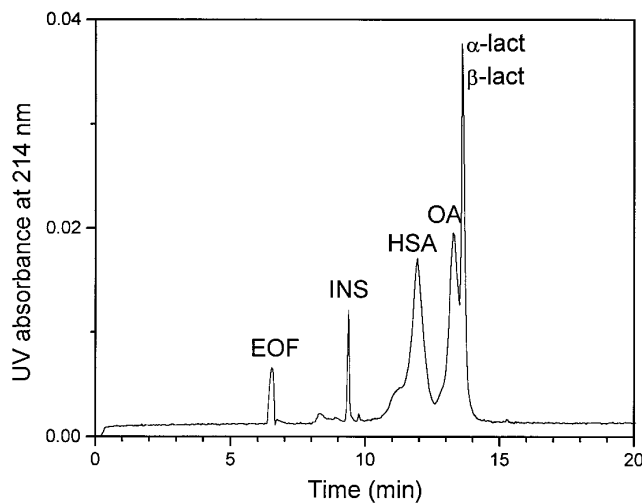


FIG. 2. Electropherogram of a protein mixture consisting of insulin (INS), human serum albumin (HSA), ovalbumin (OA), α -lactalbumin (α -lact), and β -lactoglobulin B (β -lact). The concentration of each protein was $30 \mu\text{M}$. CE separation conditions were buffer composed of 20% ACN, 3.5 mM SDS (70% purity), and 50 mM sodium borate (pH 9.7), the temperature was 20°C , the applied voltage was 17.5 kV, and the sample was injected by applying pressure for 4 s.

ture of 70% SDS (C_{12} chain), 25% myristyl sulfate (C_{14} chain), and 5% cetyl sulfate (C_{16} chain). Figure 3 shows the electropherogram of the same protein test mixture, except that the SDS used was 99% pure. The separation is poor because the 99% SDS is not as hydrophobic as the 70% SDS, which means that the overall interaction with the proteins is weaker. This observation suggests that the resolution and selectivity of a protein separation in CE can be improved by using a combination of different surfactants.

The results with the protein test mixture established the basis for understanding the separation of HDL apo-proteins in RP-HPLC and CE. Since these techniques probe proteins differently, they can be used as complementary methods in the study.

TABLE 1

Molecular Weight (MW_r), Retention Factor (k'), and Electrophoretic Mobility (μ_e) Values of the Proteins Separated by RP-HPLC^a and CE^b

Protein	MW_r	k'	μ_e ($10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$)
Insulin	5,737	61.8 (± 0.3)	-12.6 (± 0.1)
α -Lactalbumin	14,178	80.2 (± 0.3)	-21.6 (± 0.1)
β -Lactoglobulin B	18,277	88.2 (± 0.7)	-21.5 (± 0.1)
Ovalbumin	44,400	101.6 (± 0.3)	-20.8 (± 0.2)
Human serum albumin	66,438	83.9 (± 0.8)	-18.6 (± 0.1)

^a RP-HPLC conditions as described in the legend to Fig. 1.

^b CE separation conditions as described in the legend to Fig. 2.

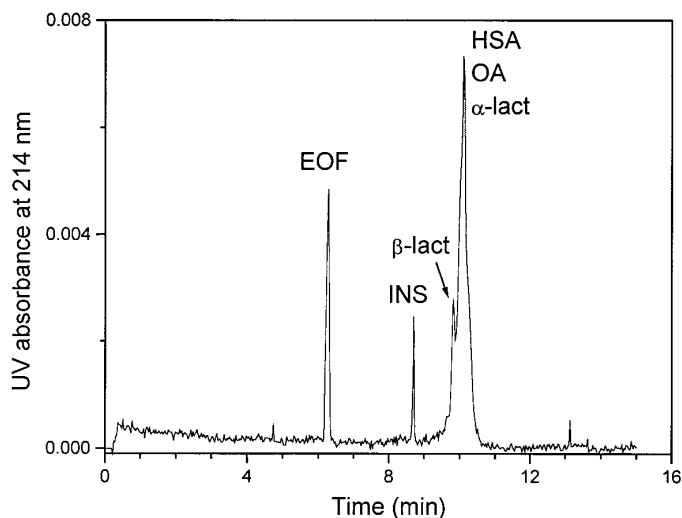


FIG. 3. Electropherogram of a protein mixture consisting of insulin (INS), β -lactoglobulin B (β -lact), human serum albumin (HSA), ovalbumin (OA), and α -lactalbumin (α -lact). The concentration of each protein was $15 \mu\text{M}$. CE separation conditions were the same as described in the legend to Fig. 2, except that the buffer consisted of 20% ACN, 3.5 mM SDS (99% purity), and 50 mM sodium borate (pH 9.6).

Characterization of purified apoA-I and apoA-II by RP-HPLC, CE, and MALDI-MS. Commercial samples of human apoA-I and apoA-II were analyzed by RP-HPLC and CE using the protocol developed for the protein test mixture. The RP-HPLC chromatogram of the commercial apoA-I sample shows three major peaks (Fig. 4). The same apoA-I solution studied by CE contains a single peak with a μ_e value of $-24.1 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ (Fig. 5). Fractions corresponding to the three RP-HPLC peaks of apoA-I were collected and analyzed by MALDI-MS and CE. Multiple protonated species of apoA-I were detected in the MALDI-MS spectra. MALDI-MS results confirmed that the three peaks are indeed apoA-I because they have the same M_r of 28,140 (Fig. 4). When each RP-HPLC fraction was studied by CE, they all gave a single peak with an identical μ_e value ($-24.1 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$). We conclude that each peak observed for apoA-I in RP-HPLC corresponds to an apoA-I isoform. However, these apoA-I isoforms cannot be resolved by CE.

The RP-HPLC chromatogram of the commercial apoA-II sample contains two major peaks (Fig. 6), but only a single peak was observed in its CE electropherogram (Fig. 7). The μ_e value obtained for apoA-II was $-22.2 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$. Fractions from the apoA-II peaks separated by RP-HPLC were collected and analyzed by MALDI-MS and CE. It was found that both peaks have the same M_r of 17,370 and an identical μ_e value ($-22.2 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$). This means that apoA-II isoforms can also be separated by RP-HPLC

but, as it was observed for apoA-I, the isoforms of apoA-II migrate as a single peak in CE.

Separation of apoA-I and apoA-II isoforms by RP-HPLC has been reported earlier (14, 16). Weinberg *et al.* (26) observed two peaks for apoA-I and three peaks for apoA-II by HPLC when using a C_{18} column. They also concluded that the multiple peaks were due to different isoforms of apoA-I and apoA-II because their molecular masses were identical when determined by SDS-polyacrylamide gel electrophoresis.

In conclusion, RP-HPLC is superior to CE in the separation of apoA-I and apoA-II isoforms. However, these isoforms have overlapping k' values (Table 2), which suggests that a mixture of apoA-I and apoA-II will be poorly resolved by RP-HPLC. On the other hand, the μ_e values of apoA-I and apoA-II are different and they are very well separated by CE. This difference is important because, in the analysis of HDL samples, CE can be used to measure apoA-I and apoA-II levels while HPLC cannot due to the presence of the isoforms.

Characterization of HDL₂ and HDL₃ apoproteins by RP-HPLC, CE, and MALDI-MS. There are two major classes of HDL on the basis of density: HDL₂ (1.063–1.125 g/ml) and HDL₃ (1.125–1.210 g/ml). Another difference is their apoprotein composition: the apoA-I/apoA-II weight ratio is higher in HDL₂ than in HDL₃. Because of this feature, low levels of HDL₂ are associated with premature development of coronary artery disease (27). Therefore, the development of a method for obtaining an accurate concentration ratio of apoA-I/apoA-II in HDL is one of the goals of this study.

A RP-HPLC chromatogram of a delipidated HDL₂ sample shows that the apoproteins are contained in one major peak (Fig. 8). Fractions from different segments of this peak were analyzed by MALDI-MS and CE. The MALDI-MS and CE results of three of these fractions (a, b, and c) are shown in Figs. 9 and 10, respectively. Both MALDI-MS and CE gave the same result with regard to the composition of the broad peak in the RP-HPLC chromatogram. Fraction a contained a small amount of apoA-I. Fraction b was predominantly apoA-I (based on M_r and μ_e values), but both MALDI-MS and CE detected a trace level of apoA-II. Fraction c had a lower apoA-I/apoA-II ratio than fraction b. The molar ratios of apoA-I/apoA-II can be calculated from the peak areas of the electropherograms, but it is impossible to quantify the MALDI-MS peak intensities due to differences in ionization probability.

Figure 11 shows the RP-HPLC separation of the apoproteins of delipidated HDL₃ from the same subject from which HDL₂ was studied before. The shape of the HDL₃ chromatogram is essentially the same as that of HDL₂. The large peak at low retention time is due to albumin (verified by MALDI-MS and CE). MALDI-MS and CE analysis of the RP-HPLC fractions from

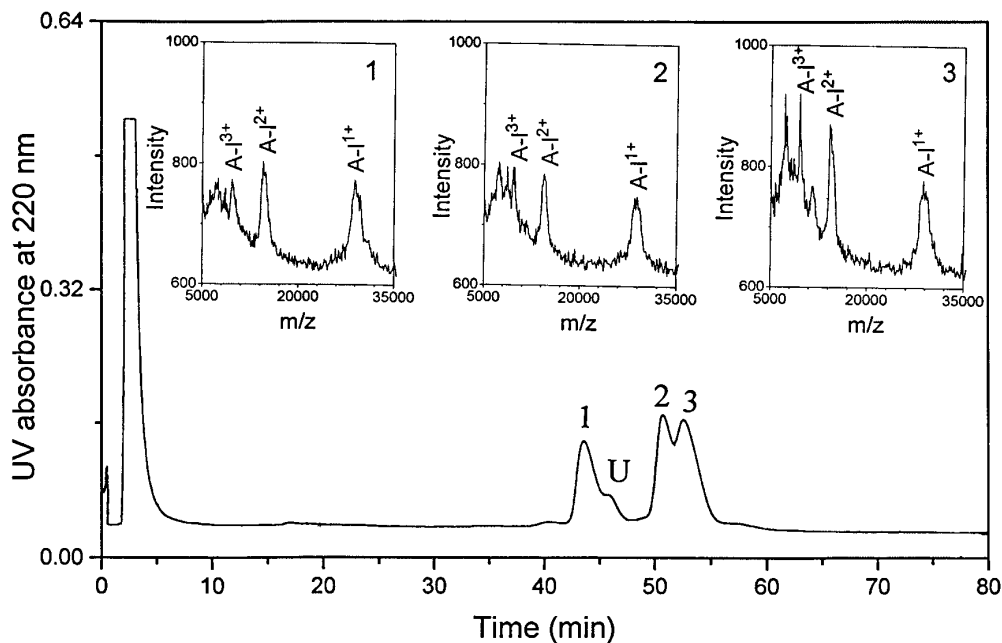


FIG. 4. RP-HPLC chromatogram of apoA-I (20 μM) and MALDI-MS spectra of the separated components. Peaks: 1, 2, and 3, apoA-I isoforms; U, unknown. HPLC conditions as described in the legend to Fig. 1, except the flow rate was 40 $\mu\text{l}/\text{min}$, and the linear gradient was 35–40% at 1%/min the first 5 min, and then it went from 40 to 55% at 0.2%/min.

the apoprotein peak gave the same results as obtained for HDL₂: apoA-I and apoA-II were poorly resolved.

The delipidated HDL₂ and HDL₃ solutions from the same individual were analyzed by CE. The results are shown in Fig. 12. Both A-I and A-II apoproteins were detected in both HDL samples, as well as albumin in HDL₃. The main advantages of CE over RP-HPLC are that it can separate apoA-I from apoA-II and that peak

areas can be directly correlated to the concentration of each apoprotein.

Quantitation of apoA-I and apoA-II by CE. One of the main objectives of this research was to develop a method for the quantitation of apoA-I and apoA-II in HDL samples. This objective can be achieved by CE because it is capable of resolving the apoA-I isoforms from the apoA-II isoforms as two distinct classes. Thus, the two peak areas can be directly correlated to their concentrations. To test the validity of this analysis protocol, the corrected peak areas from the electropherograms of the commercial apoA-I and apoA-II samples were measured as a function of their concentrations (Fig. 13). A good linear relationship was obtained for both apoproteins. Using this calibration curve, it is possible to measure the concentrations of apoA-I and apoA-II in a delipidated HDL fraction.

Previous studies using RP-HPLC showed that complete delipidation of HDL is necessary in order to obtain reproducible chromatograms of the apoproteins (15). However, delipidation is not necessary in our CE method. Figure 14 shows the electropherograms of a HDL sample before and after delipidation. The two electropherograms have common features. An extra peak is present in the non-delipidated HDL, which is absent after delipidation. Liebich *et al.* (13) observed both a major and a minor apoA-I peak when utilizing CE to resolve apoA-I from serum proteins of a plasma sample. They suggested that the two peaks were from

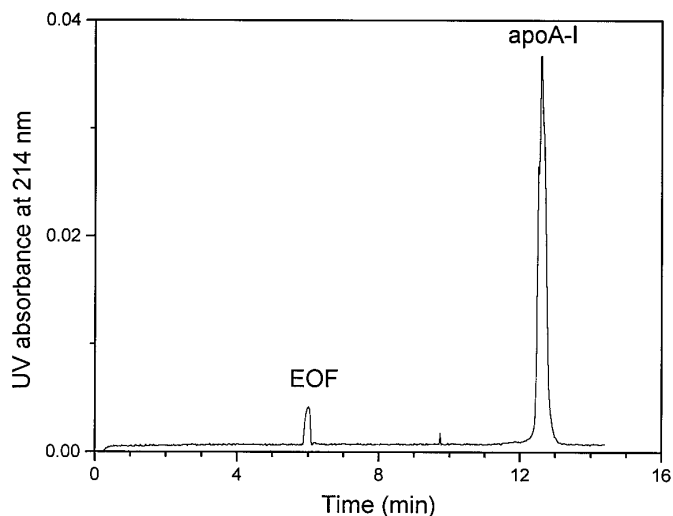


FIG. 5. Electropherogram of apoA-I (54 μM). CE conditions were the same as described in the legend to Fig. 2.

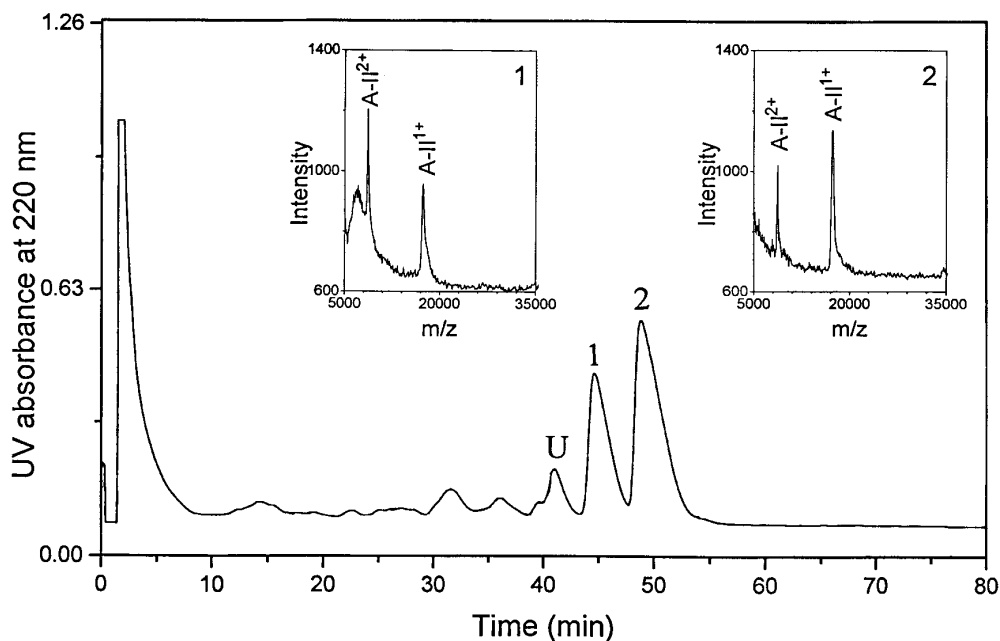


FIG. 6. RP-HPLC chromatogram of apoA-II ($125 \mu\text{M}$) and MALDI-MS spectra of the separated components. Peaks: 1 and 2, apoA-II isoforms; U, unknown. HPLC conditions were as described in the legend to Fig. 4.

different isoforms of apoA-I and concluded that the areas of both peaks were needed to quantify apoA-I. Using our CE conditions, apoA-I isoforms cannot be resolved. We postulate that the extra peak observed for the apoA-I peak is from a partially delipidated particle.

To test the use of CE in the quantitation of apoA-I and apoA-II, two plasma samples from Sigma (Controls 1 and 2), whose apoA-I concentration had been determined by an immunoturbidimetric assay, were used.

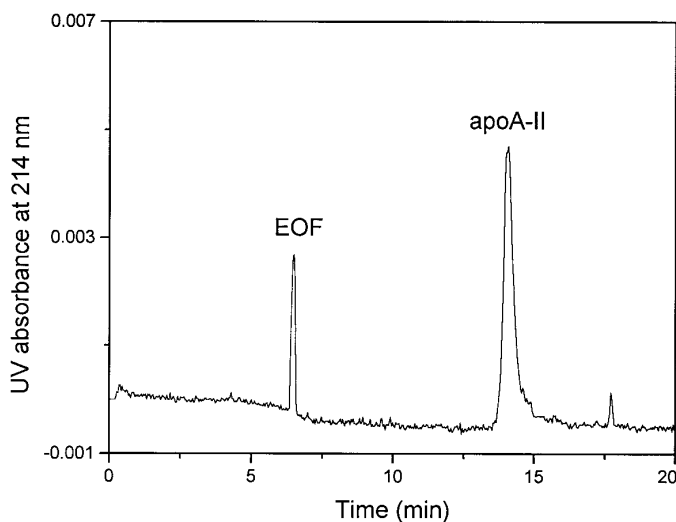


FIG. 7. Electropherogram of apoA-II ($31 \mu\text{M}$). CE conditions were as described in the legend to Fig. 2.

Their HDL bands were isolated by density gradient centrifugation and analyzed directly without prior delipidation by CE (Fig. 15). The peak areas of the apoA-I and apoA-II peaks were measured and their concentrations were determined with the calibration curves shown in Fig. 13. Table 3 compares the immuno-based assay and the CE values for the apoA-I and apoA-II concentrations from these two plasma samples. The agreement between the two methods is in the quoted uncertainty. The concentration of apoA-II was not known for the Sigma plasma samples, and therefore we were unable to test the quantitation of the apoA-II calibration curve.

Using our CE methodology, A-I and A-II apoprotein concentrations can be measured in delipidated and

TABLE 2

Retention Factor (k') and Electrophoretic Mobility (μ_e) Values Obtained for the Isoforms of ApoA-I and ApoA-II Studied by RP-HPLC^a and CE^b

Apoprotein	Isoform	k'	μ_e ($10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$)
ApoA-I	1	22.7	-24.2
	2	26.5	-24.1
	3	27.5	-24.1
ApoA-II	1	25.0	-22.2
	2	27.4	-22.3

^a RP-HPLC conditions as described in the legend to Fig. 4.

^b CE separation conditions as described in the legend to Fig. 2.

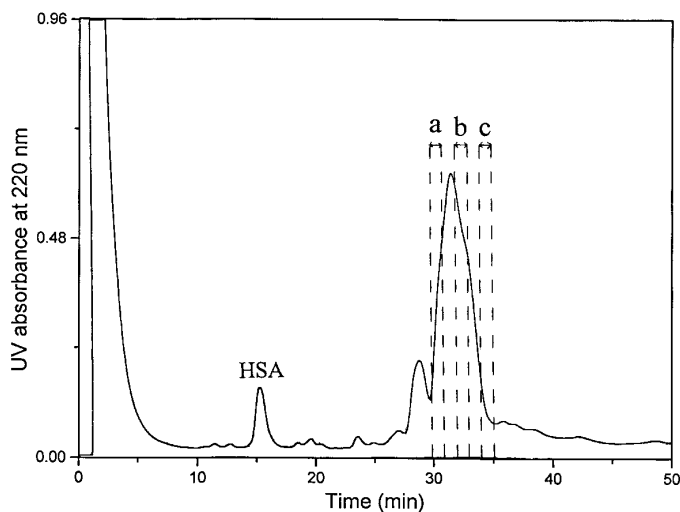


FIG. 8. RP-HPLC chromatogram of delipidated HDL₂. Peaks: HSA, human serum albumin; a, apoA-I; b and c, mixture of apoA-I and apoA-II. HPLC conditions were as described in the legend to Fig. 1, except the flow rate was 40 μ l/min, and linear gradient was from 35 to 40% at 1%/min, and then it went from 40 to 55% at 0.43%/min.

non-delipidated HDL samples. These concentrations can be translated to plasma concentrations by taking into account the volume of the HDL fraction and the amount of plasma used in the centrifugation. There

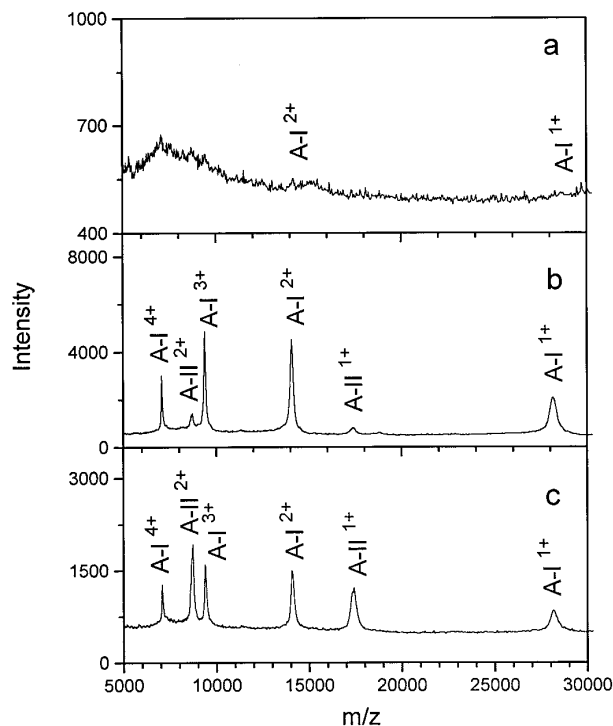


FIG. 9. MALDI-MS spectra of the RP-HPLC fractions a, b, and c of HDL₂.

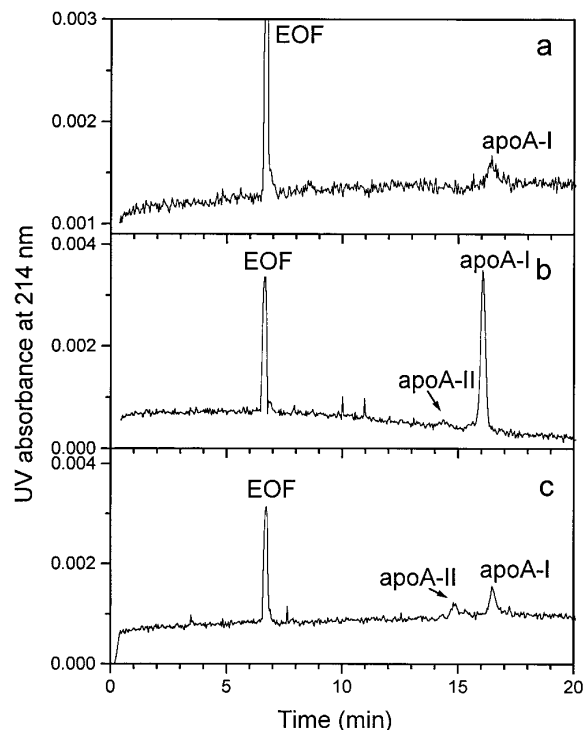


FIG. 10. Electropherograms of RP-HPLC fractions a, b, and c of HDL₂. CE conditions were as described in the legend to Fig. 2.

is an advantage in determining the apoprotein concentration in non-delipidated lipoprotein samples because it minimizes the apoprotein loss in the delipidation procedure.

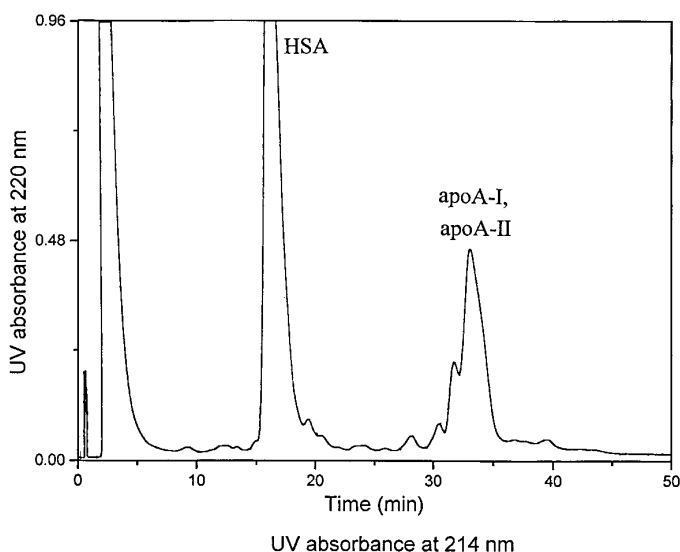


FIG. 11. RP-HPLC chromatogram of delipidated HDL₃. HPLC conditions were as described in the legend to Fig. 8.

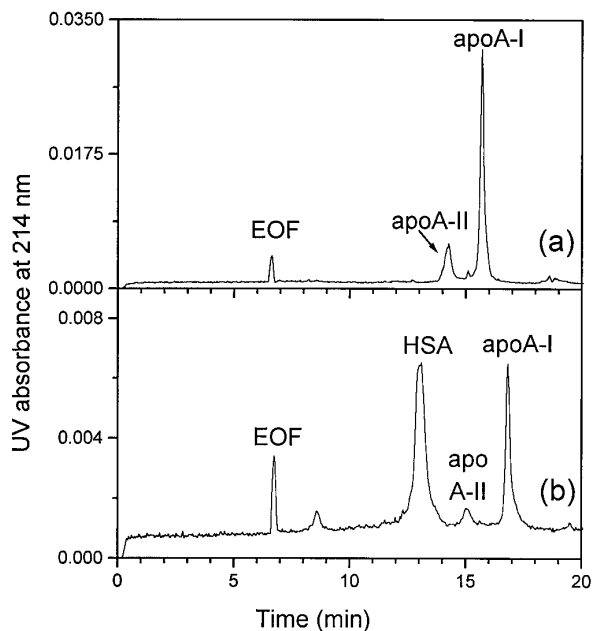


FIG. 12. Electropherograms of delipidated (a) HDL₂ and (b) HDL₃. CE conditions were as described in the legend to Fig. 2.

SUMMARY

Purified samples of apoA-I and apoA-II were studied by CE. Separation was achieved on the basis of affinity for hydrophobic anions in a buffer containing

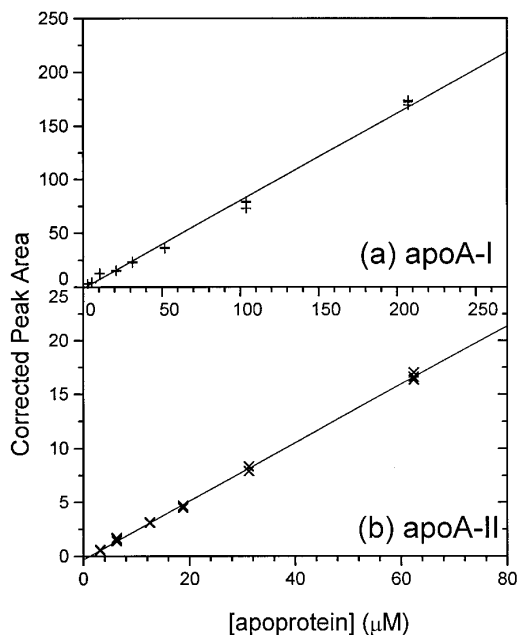


FIG. 13. Corrected peak areas from CE as a function of the concentration for standards of (a) apoA-I and (b) apoA-II. CE separation conditions were as described in the legend to Fig. 2. Linearity (r) values were (a) $r = 0.997$ and (b) $r = 0.999$.

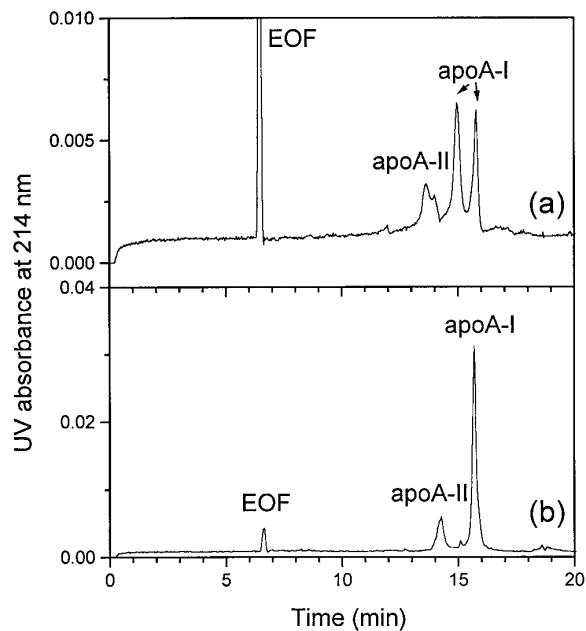


FIG. 14. Electropherograms of HDL sample (a) before delipidation and (b) after delipidation. CE conditions were as described in the legend to Fig. 2.

an organic modifier, which gives the apoproteins different charge-to-volume ratios. The basis for the separation methods was established first by studying a mixture of proteins with different hydrophobicities.

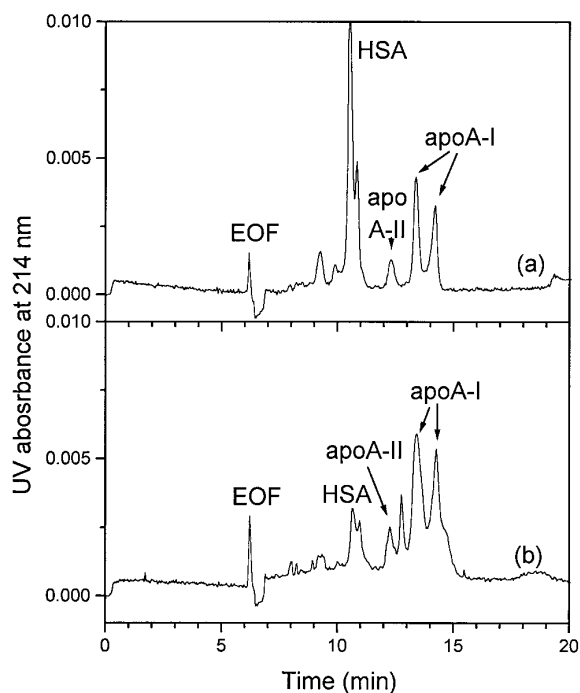


FIG. 15. Electropherogram of non-delipidated HDL of (a) control 1 and (b) control 2 plasma sample from Sigma. CE separation conditions were as described in the legend to Fig. 2.

TABLE 3
ApoA-I and ApoA-II Concentrations in Cardioliplid Plasma Samples from Sigma

Plasma sample	[apoA-I]/(mg/dl)		[apoA-II]/(mg/dl)	
	Immunoassay ^a	From CE	Immunoassay	From CE
Control 1	72 (±15)	80 (±10)	—	22 (±5)
Control 2	175 (±30)	170 (±20)	—	25 (±2)

Note. CE Values were Determined by Measuring the Peak Areas of the ApoA-I and ApoA-II (CE Separation Conditions as Described in the Legend to Fig. 2).

^a Provided by Sigma Chemical Co.

The order of elution was compared with that obtained from RP-HPLC.

The isoforms of apoA-I exhibit a range of k' values in RP-HPLC, but each isoform has essentially the same μ_e value. The same was observed for the isoforms of apoA-II. The k' values for the apoA-I and apoA-II isoforms overlap, which means that they cannot be resolved in a mixture. In CE, apoA-I and apoA-II have different μ_e values and therefore they are well separated. MALDI-MS was used to verify the M_r of the isoforms separated by RP-HPLC.

Delipidated and un-delipidated samples of HDL were analyzed by CE. The electropherograms of un-delipidated HDL contain peaks for the apoA-I and apoA-II components, which means that prior delipidation is not required for analysis of the apoproteins. A quantitative analysis for apoA-I and apoA-II was developed and its accuracy verified by immunoturbidimetric assay.

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