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Fast C_{18} solid-phase desalting/delipidation of the human serum apolipoproteins for matrix-assisted laser desorption ionization and electrospray ionization mass spectrometric analysis☆

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Abstract

A new method for the delipidation of human serum lipoproteins involving the use of a reversed-phase C_{18} solid-phase extraction (SPE) cartridge is introduced for use with matrix-assisted laser desorption ionization and electrospray ionization mass spectrometry. This method is compared with two other methods of lipoprotein delipidation. The SPE method of delipidation produces a higher and more reproducible protein yield than the conventional liquid–liquid methanol–diethyl ether delipidation technique. Furthermore, the SPE method implements a fast, sequential, desalting and delipidation of the lipoproteins for subsequent mass spectrometric analysis providing high quality spectra.

Keywords: Desalting method; Delipidation methods; Apolipoproteins; Lipoproteins; Proteins

1. Introduction

Human serum lipoproteins are pseudo-micellar structures comprised of proteins known as apolipoproteins (apos) and non-covalently bound lipids. The lipid components are cholesterol, cholesterol esters, phospholipids, and triglycerides. The triglycerides and cholesterol esters form a hydrophobic lipid core which is stabilized by the apolipoproteins. The apolipoproteins, phospholipids, and cholesterol form the amphipathic surface of the lipoprotein particles. The major functions of the apolipoproteins are lipid transport and metabolic control through the activation or inhibition of enzymes [1]. Apos structural and quantitative variations and their possible correlation with coronary heart disease (CHD) is an active field of research using different analytical techniques [2–8]. Recently, we applied two modern mass spectrometry methods, matrix-assisted laser desorption

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ionization (MALDI) and electrospray ionization (ESI), to the fast screening of apo polymorphism. Accurately measured M_r values of the intact apos provided information about their structural variations (related to genetic polymorphism and post-translational modification) which previously could not be assayed [9].

Lipoproteins from human serum are typically separated into fractions by ultracentrifugation (UC) according to their flotation density. The mixture of apos in each lipoprotein fraction contains several impurities of different origins including salts from human serum, salt (NaBr) or sucrose from the UC density gradient media, and the non-covalently bound lipids. For a high resolution, high accuracy mass spectrometric analysis, complete separation of the apos from the lipids and salts is important. Traditional delipidation methods employ liquid–liquid, precipitation–extraction techniques to remove the lipids from the apos. While this is effective at removing the lipids, the removal of the salts requires a second step of dialysis which can be very time consuming. The need for a quick and efficient desalting/delipidation technique for apolipoprotein analysis is of importance for the application of mass spectrometry towards the clinical assessment of CHD. This apo purification procedure is the focus of this paper.

2. Materials and methods

2.1. Chemical reagents

Reagent grade acetonitrile (ACN), methanol, and deionized water were obtained from EM Science (Gibbstown, NJ, USA). Other chemicals used include acetic acid (Fisher Scientific, Fair Lawn, NJ, USA), apolipoproteins A-I, A-II, C-I, C-II, C-III, and E (Calbiochem, San Diego, CA, USA), bicinchoninic acid assay reagents A and B (Pierce, Rockford, IL, USA), bovine serum albumin (BSA; Sigma, St. Louis, MO, USA), dimethyl sulfoxide (DMSO; Burdick and Jackson Labs., Muskegon, MI, USA) ferulic acid (Aldrich, Milwaukee, WI, USA), formic acid (88% solution; EM Science) ethylenediaminetetraacetic acid (EDTA; J. T. Baker, Phillipsburg, NJ, USA), high density lipoprotein (HDL; Sigma), sodium azide (Aldrich), sodium bromide (NaBr; Fisher Scientific), sudan black b (SBB; Sigma), sucrose (Fisher Scientific), and trifluoroacetic acid (TFA; Pierce). Deionized water was obtained from a Milli-Q water purification system and was used in the preparation of all solutions except the solid-phase extraction (SPE) solutions (Millipore, Bedford, MA, USA).

2.2. Isolation of high density lipoproteins by single spin preparative ultracentrifugation

The serum used for this study was from normolipidemic subjects collected after a 12 h fast. Serum samples were obtained by a blood draw into an untreated 7 ml Vacutainer tube (Becton Dickinson Systems, Franklin Lakes, NJ, USA). The blood was allowed to clot for 45 min at room temperature followed by centrifugation at 161 *g* for 20 min at 4°C. The supernatant (serum) was aspirated from the red blood cells. A 200 μl volume of the serum was stained (for visualization) with 7 μl of SBB in DMSO (1%, w/v) and incubated at room temperature for 30 min. A 250 μl aliquot of 1.26 g/ml NaBr was transferred to a 34×11 mm polycarbonate open-top centrifuge tube (Beckman Instruments, Fullerton, CA, USA). The tube was then layered with 200 μl of 1.10 g/ml NaBr, 207 μl of the stained serum sample, and 600 μl of 1.00 g/ml NaBr. All of the NaBr solutions contained 0.01% EDTA and 0.01% sodium azide [10]. The tube was then sealed and centrifuged in a Beckman Optima TLX-120 ultracentrifuge equipped with a 30° fixed angle TLA 120.2 rotor at 627 379 *g* at 10° for 2 h 26 min. After centrifugation, a 200 μl volume of the HDL (1.05 <density<1.15) fraction was collected by aspiration.

Centrifugation was also performed using a 20% (w/w) sucrose solution as a density gradient medium. The solution contained ~0.1% sodium azide to prevent bacterial and fungal growth. An 800 μl aliquot of this solution was pipetted into a polycarbonate open-top centrifuge tube. A 200 μl volume of serum was stained with 7 μl of SBB in DMSO (1%, w/v) for visualization, diluted with deionized water to a volume of 400 μl, and applied on top of the sucrose solution. The UC separation was carried out as described in the NaBr procedure except that the UC conditions were 20° C for 4 h and 10 min. After

centrifugation, a 200 μl volume of the HDL fraction was collected by aspiration.

2.3. Bicinchoninic acid assay protein quantitation

Protein quantitation was determined using this bicinchoninic acid (BCA) assay based on the procedure described by Smith et al. [11]. Briefly, the BCA standard working reagent (SWR) was prepared by mixing reagent A with reagent B in a ratio of 50:1 (v/v). A set of BSA standards was prepared with known concentrations. A 1 ml aliquot of the SWR was mixed with 10 μl of each BSA solution, each sample, and a blank (deionized water). The solutions were vortexed and incubated at 60°C for 30 min followed by a 15 min equilibration period at room temperature. The absorbance of each solution was measured at 562 nm using a 1 ml quartz cuvette with a 1cm path length (Fisher Scientific) and a Perkin–Elmer Lambda 4B UV–Vis spectrometer (Norwalk, CT, USA). A linear calibration curve was established between the absorbance of the BSA standards and the concentrations (0–3.2 mg/ml) with a slope of 0.54 (±0.01), an intercept of 0.05 (±0.01), and a correlation coefficient of 0.9997. Protein concentrations in the samples were determined from the calibration curve.

2.4. Delipidation of lipoprotein particles

Three delipidation methods were investigated in order to find the best purification procedure for subsequent mass spectrometric analysis of the apos: (1) the conventional delipidation method using methanol–ether liquid–liquid extraction, (2) a two-step method, dialysis of the HDL fraction followed by methanol delipidation, and (3) C_{18} SPE, desalting and delipidation.

2.5. Methanol–diethyl ether liquid–liquid extraction

An HDL fraction (200 μl) separated by the UC technique from a 200 μl volume of serum was delipidated with methanol (1.5 ml) and diethyl ether (3.5 ml). HDL isolated using either NaBr or sucrose as the UC density gradient was examined in separate experiments. The methanol–diethyl ether delipidation protocol was used according to the procedure described by Osborne [12]. The precipitated proteins retained in the methanol were dried under vacuum at room temperature and then dissolved in 200 μl of 2.5% aqueous formic acid (pH 2.1). The protein solution was filtered through a nylon 0.2-μm-pore-size membrane filter (Millipore, San Francisco, CA, USA) and used for ESI-MS and MALDI-MS analysis.

2.6. Dialysis of the HDL fraction followed by methanol delipidation

An HDL fraction (200 μl) from a 200 μl volume of serum separated by the UC using the NaBr density gradient medium was placed inside a Slide-A-Lyzer (Pierce) and dialyzed against 10 m*M* ammonium acetate for 4 h. The desalted HDL fraction was slightly acidified by acetic acid to pH 4 and analyzed by ESI using several different sample-to-methanol ratios.

2.7. C_{18} solid-phase extraction, desalting and delipidation

A 100 μl aliquot of an HDL fraction collected from a UC separation was acidified with 2.5% (v/v) formic acid (88% solution) to pH 2.1 and applied to a tC_{18} light cartridge (Sep-Pak, No. 51910, Waters, Milford, MA, USA), previously conditioned with 5 ml of 2.5% (v/v) formic acid in acetonitrile and 5 ml of 2.5% (v/v) aqueous formic acid. The total protein concentration in the HDL fraction was determined by the BCA protein assay to be ~1.5 mg/ml. After applying the sample, the SPE cartridge was washed with 5 ml of 2.5% (v/v) aqueous formic acid solution to remove the density gradient solute (sucrose or NaBr) and water soluble components of the plasma or serum sample. The cartridge was then purged with 5 ml of air to remove as much excess water as possible. Then, isocratic elution was performed with four 50 μl aliquots of 2.5% formic acid in acetonitrile, purging with 1 ml of air between each aliquot, collecting fractions 2–4. The same procedure was done using 0.1% TFA as the acid. The BCA

protein assay was used to identify the fractions eluted from the C_{18} cartridge that contained protein.

2.8. Efficiency of the SPE delipidation method

Numerous studies were carried out in the development of the C_{18} method to evaluate the efficiency of the method on the recovery of the apolipoproteins both in the presence and absence of lipids. The application of a mixture of the commercial purified apos (A-I, A-II, C-I, C-II, C-III, and E) to the cartridge showed that all of the apo standards were eluted in the third 50 μl fraction and that the protein recovery was between 70 and 90% in the absence of the lipids. The mixture of the commercial apos prior to application to the SPE cartridge and the recovered C_{18} fractions were analyzed by MALDI-MS and capillary electrophoresis [13] (data not shown) to determine if any fractionation of the apos had occurred. It was determined that the relative concentrations of the apos remained the same after elution from the SPE cartridge. Similar experiments were carried out using commercial HDL. It was determined that 150 μg of protein was the optimum amount applied to the cartridge when the HDL lipids were present. Several of the 50 μl fractions were collected and analyzed for protein by the BCA assay. Only the third 50 μl fraction was found to contain protein and the yield was determined to be ~80%. CE analysis of fractions 2–4 determined that fractions 2 and 4 contained no apos and verified that the apos eluted in the third 50 μl fraction. When the relative concentrations of the apos were compared to the CE analysis of the HDL prior to its application to the SPE cartridge it was found that there was no fractionation of the apo mixture.

The protein fraction of the commercial HDL was analyzed to determine if any of the lipids had eluted using commercial cholesterol and triglyceride kits. No cholesterol/cholesterol esters or triglycerides were found in this fraction. Further application of acidified ACN (twenty 50 μl fractions) failed to elute the lipids. The solvent was changed to acidified isopropanol and the lipids were eluted after the application of 2 ml. From these studies, it was determined that the proteins were recovered from the C_{18} cartridge in the third 50 μl fraction when using acidified ACN and that this fraction is free of lipid contamination.

2.9. ESI-MS and MALDI-MS analysis

The ESI-MS analysis was performed using an ESI time-of-flight (TOF) instrument constructed in our laboratory [14]. The apo mixtures delipidated by each of the methods described above were injected individually into the ESI-MS instrument at flow rate of ~1 ml/min. Each mass spectrum was acquired for 10 min. The MALDI-MS analysis was performed using a commercial MALDI-TOF mass spectrometer (Voyager Elite XL, PerSeptive Biosystems, Framingham, MA, USA) [15] in the linear mode. The sample preparation was carried out similar to the procedure described by Edmundson and Russell [16] using sinapic or ferulic acid as a MALDI matrix.

3. Results and discussion

3.1. Methanol–diethyl ether liquid–liquid extraction

The conventional delipidation method using methanol–diethyl ether liquid–liquid extraction allowed for the complete separation of the apos from the lipids, however, this method was not efficient at removing the salts, leading to significant peak broadening in the ESI and MALDI mass spectra. The ESI mass spectrum was weak and un-interpretable while the MALDI spectrum contained Na^+ and K^+ adduct peaks as well as the protonated apolipoprotein ions (Fig. 1).

3.2. Dialysis of the HDL fraction followed by methanol delipidation

The dialyzed, acidified HDL fraction was directly analyzed by the ESI-MS technique (Fig. 2a). A large bump was observed in the mass spectrum, which is believed to be due to multiply protonated intact HDL particles (M_r between 100 000 and 300 000). Figs. 2b and 2c show how the addition of methanol to the electrospray solution aids in the delipidation of the lipoprotein particles. The methanol disrupts the non-covalent bonds between the apos and the lipids

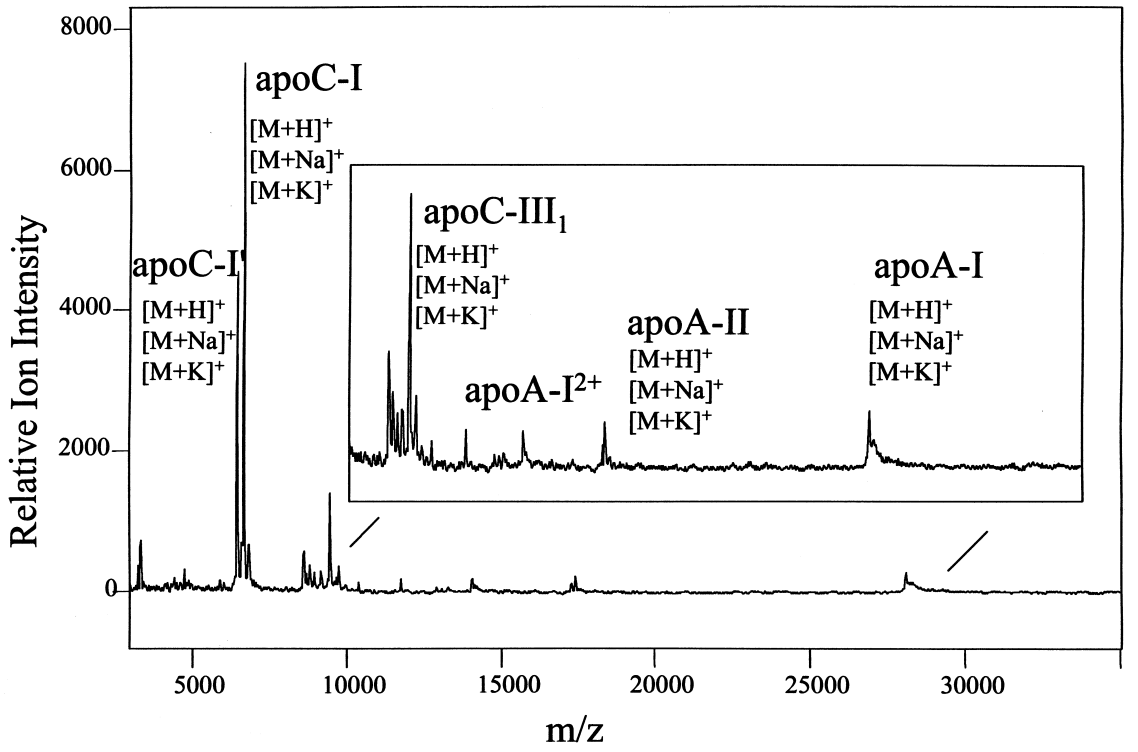

Fig. 1. The positive ion MALDI mass spectrum of methanol–diethyl ether delipidated HDL.

releasing the apos and making them available for the MS analysis. Sharp peaks were obtained using this technique, however, the presence of the lipids in the sample created a large background in the spectra. The combination of the dialysis with the conventional method of delipidation removes the background created by the lipids and increases the intensity of the protonated apos (data not shown). High quality spectra are obtained using this combination. However, the long dialysis time and the large loss of apos associated with the methanol diethyl ether liquid–liquid extraction method of delipidation [17] make this method unsuitable for fast quantitative analysis. Protein recovery using the conventional method of delipidation varies as much as 40% for the same sample within a single delipidation procedure with yields as low as 30%.

3.3. C_{18} solid-phase extraction, desalting and delipidation

A new procedure described in Section 2 was developed, making it possible to quickly prepare purified apolipoproteins from lipoprotein fractions in a form suitable for high-resolution MALDI-MS and ESI-MS analysis using a reversed-phase SPE cartridge. Figs. 3 and 4 show MALDI and ESI mass spectra, respectively, of an HDL fraction desalted and delipidated by this method.

The elution volume used in this technique was chosen equal to the cartridge hold-up volume (V_c) of 50 μl. Each eluent fraction was tested for protein using the BCA assay method [11]. The first fraction tested negative for protein content. The second and third fraction tested positive while subsequent fractions contained only a small amount of protein. The mass spectrometric analysis, which will be described further, identified albumin as the major constituent of the second fraction and the apolipoprotein mixture in the third fraction. The use of the low pH eluent with a high percentage of organic modifier enabled the elution of the albumin in the second hold-up volume of the cartridge ($2V_c$) and the apolipoprotein mixture in the third hold-up volume ($3V_c$) corresponding to

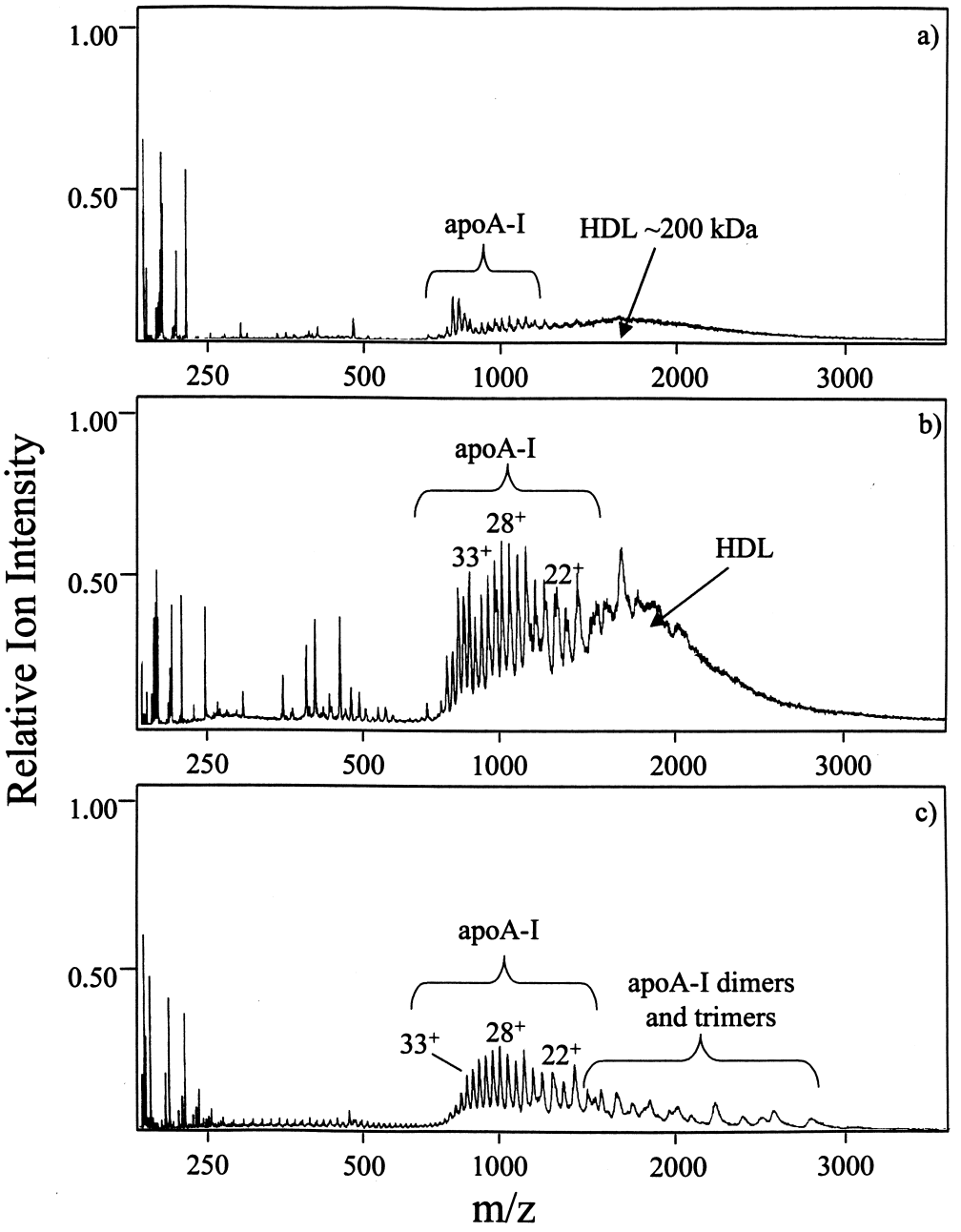

Fig. 2. The positive ion ESI mass spectra of dialyzed HDL. Electrospray matrix conditions are as follows: (a) 80% deionized water, 15% methanol, 5% acetic acid; (b) 50% deionized water, 45% methanol, 5% acetic acid; (c) 95% methanol, 5% acetic acid.

capacity factors of 1 and 2, respectively. Human serum albumin (HSA) is not a structural part of the HDL particles. However, it is frequently detected in this lipoprotein fraction after separation by ultracentrifugation. HSA has the capacity to bind cholesterol as well as free fatty acids in the blood stream [1]. The association of the lipids, combined with the natural density of albumin approaches the density of

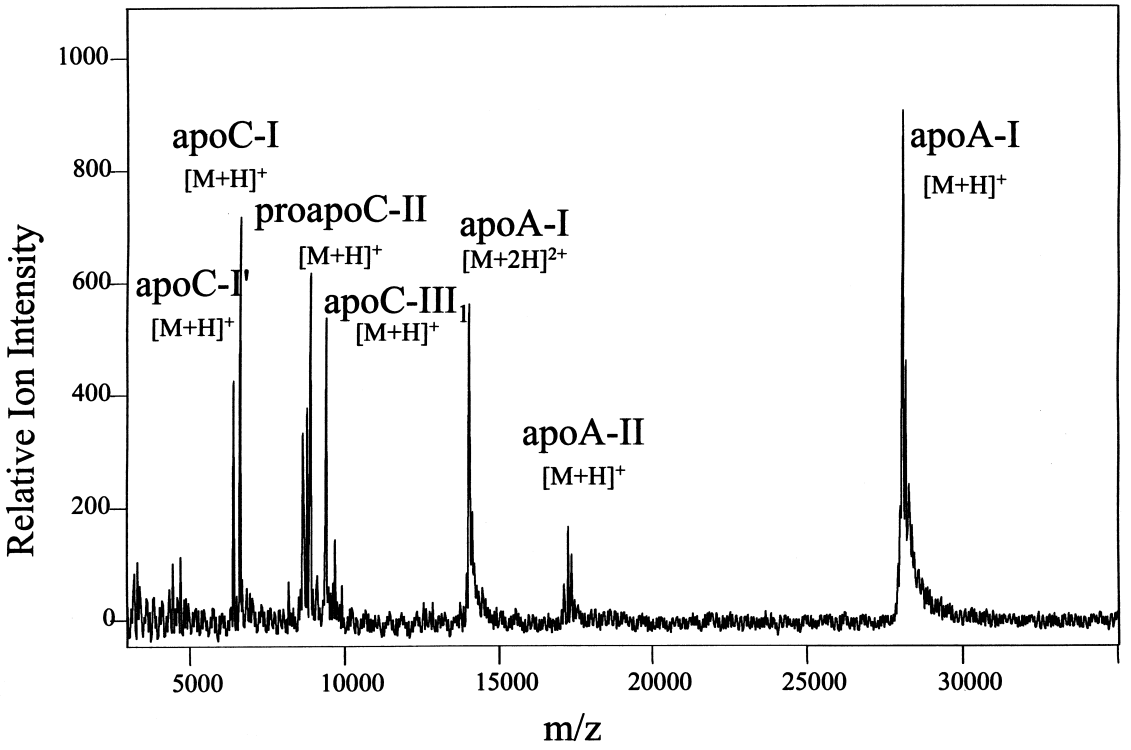

Fig. 3. The positive ion MALDI mass spectrum of the C_{18}-SPE delipidated HDL.

the HDL particles making it difficult to separate the albumin from the HDL particles by gradient ultracentrifugation. The differences in the capacity factors of the albumin and the apos enabled the separation of the albumin from the apos under the employed experimental conditions. This difference in capacity factors corresponds well with typical reversed-phase chromatography [13]. In addition, the low capacity factor of the apos prevented an undesired dilution of the purified apo solution. Quantitation of the apolipoprotein mixture eluted in the third aliquot was carried out according to the procedure described in Section 2 and found to be 70±5%. The concentration of the apos in the third 50 μl aliquot was determined to be ~1 mg/ml.

Figs. 5 and 6 are the ESI and the MALDI mass spectra of the consecutive elutions from the C_{18} cartridge. In both figures albumin is present in (a), the apos in (b), and (c) contains no protein. The mass spectra are of high quality with essentially no evidence of adduct peaks due to alkali metal ion attachment. While the presence of albumin in the MALDI mass spectra would not be a great concern, its removal from the apos in the ESI-MS sample greatly simplifies the spectrum.

3.4. Clinical application

The C_{18} method of delipidation with subsequent MALDI-MS analysis was implemented as part of a comprehensive apo analysis regime using healthy individuals and individuals with known coronary heart disease. Through this study, an acute phase protein normally not found in HDL, serum amyloid A (SAA) previously known as apolipoprotein L, was discovered in the HDL fraction of a cardiac patient after experiencing myocardial infarction (MI). The patient's blood was analyzed for three consecutive days by MALDI-MS and CE (Fig. 7). SAA was not present in the blood drawn immediately following the MI; however, it was present on the second and third day with increasing intensity. The CE electropherograms obtained from the C_{18} delipidation were compared to those delipidated according to the

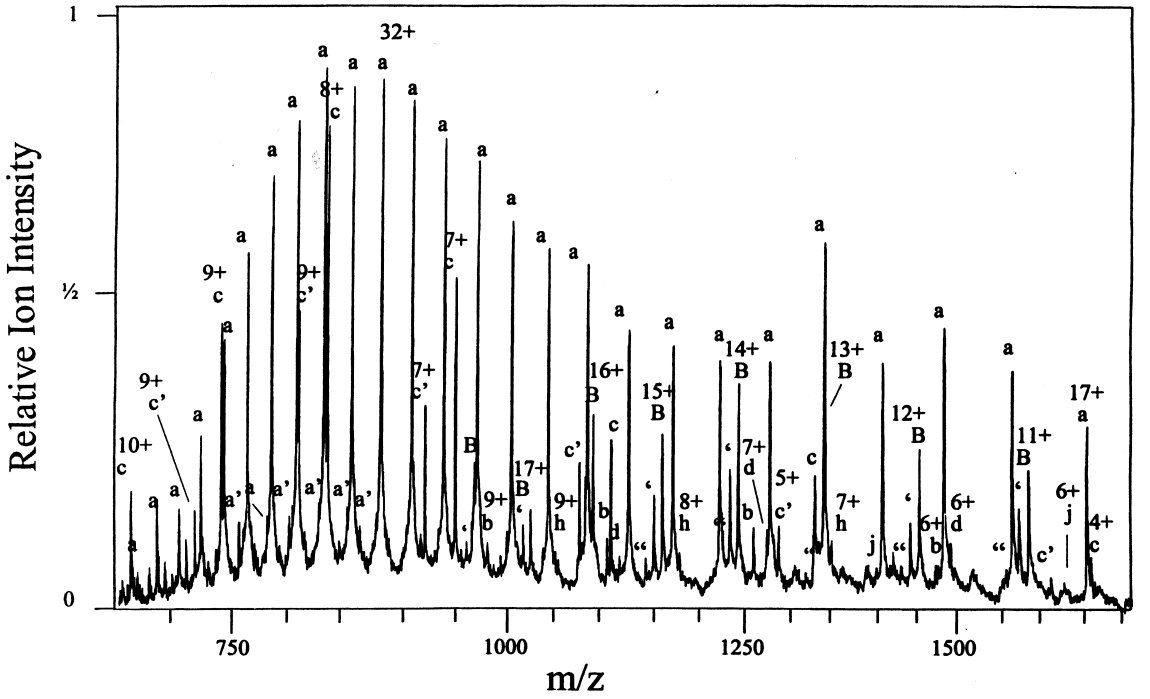

Fig. 4. The positive ion ESI mass spectrum of the C_{18}-SPE delipidated HDL. The positive numbers identify the number of attached protons and, respectively, the charge of the molecular ions. The one letter code system for the apo isoforms is: **c**′=truncated apoC-I, **c**=apoC-I, **b**=single chain apoA-II, **d**=proapoC-II, **h**=apoC-III_1, **j**=apoC-III_2, **B**″=truncated apoA-II, **B**′=truncated apoA-II, **B**=apoA-II, **a**′=truncated apoA-I, **a**=apoA-I.

method of Cruzado et al. [13] and the relative intensities of the apos and the SAA were determined to be consistent. The CE was helpful in the identification of an unknown component in the HDL fraction while the MALDI-MS analysis provided the information required for identification.

4. Conclusions

A new SPE method has been developed for the sequential desalting and delipidation of the lipoprotein fractions of human serum obtained from fast ultracentrifugation. Using the SPE technique, the speed and efficiency of apolipoprotein purification was greatly reduced. Furthermore, this new technique provides higher and more reproducible protein yields when compared to the solvent extraction method of delipidation. The SPE method of delipidation produces a lipid-free mixture of apos with no evidence of fractionation. The SPE delipidation technique gives samples that produce high quality MALDI and ESI mass spectra. This purification protocol has been applied to the clinical analysis of the apolipoprotein population of the major lipoprotein for donors in routine screening as well as for patients with coronary heart disease where the acute phase response protein, serum amyloid A, was detected in the SPE purified HDL by CE and MALDI-MS.

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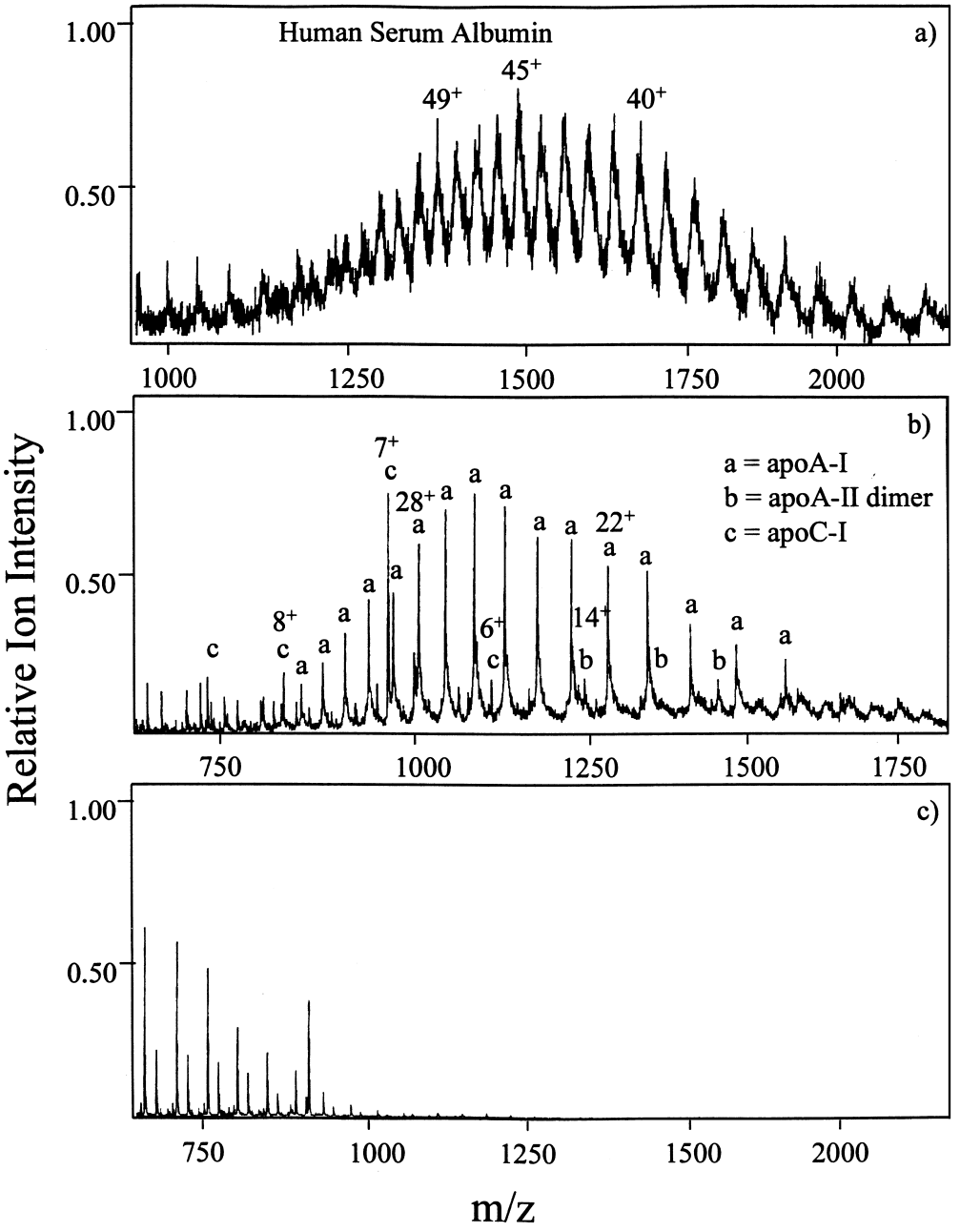

Fig. 5. The positive ion ESI mass spectra of the C_{18}-SPE delipidated HDL: (a) second 50 μl aliquot of ACN; (b) third 50 μl aliquot of ACN; (c) fourth 50 μl aliquot of ACN.

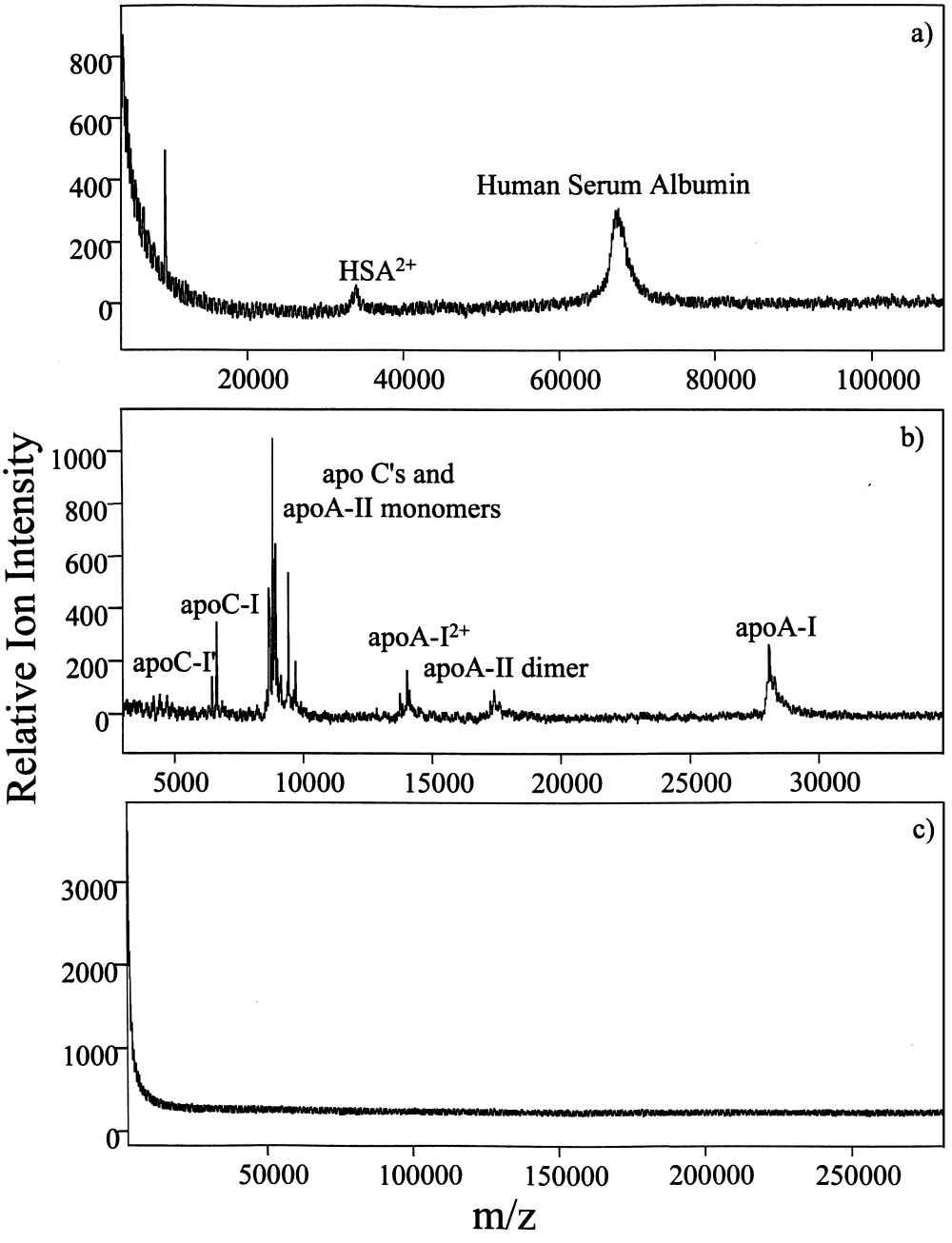

Fig. 6. The positive ion MALDI mass spectra of the SPE delipidated HDL. Conditions in (a)–(c) as in Fig. 5.

some aspects of the MALDI analysis. This work was supported by the NIH, Heart & Lung Institute (HL 54566), the United States Department of Energy, division of Chemical Sciences, Office of Basic Energy Science in support of Time of Flight Developmental Research, and Texas A & M University Research Infrastructure Program for Support of the Laboratory of Biological Mass Spectrometry.

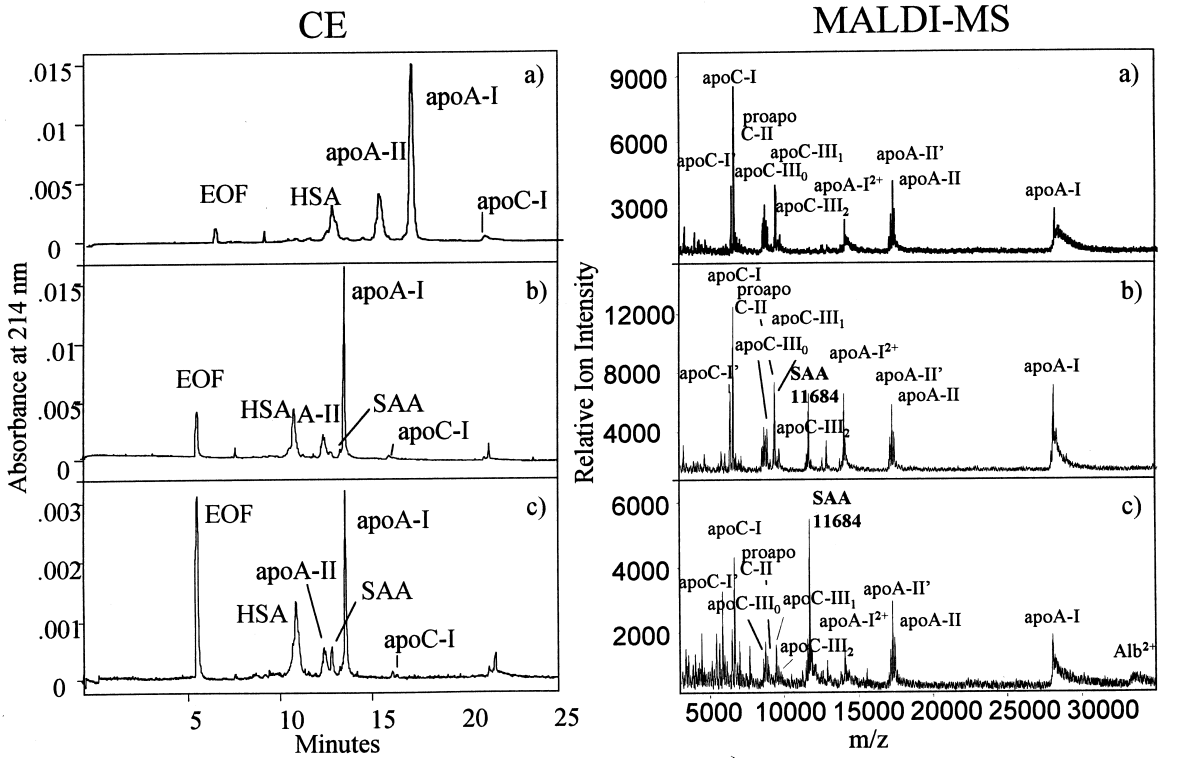

Fig. 7. The positive ion MALDI mass spectra and CE electropherograms of the SPE delipidated HDL fraction of a cardiac patient admitted following myocardial infarction: (a) immediately following admission; (b) 24 h after admission; (c) 48 h after admission. CE conditions are as described in ref. [13].

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