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Characterization of *in vitro* modified human high-density lipoprotein particles and phospholipids by capillary zone electrophoresis and LC ESI-MS

Chung-Yu Wu, Yu-Nong Peng, Jing-Huei Chiu, Yu-Ling Ho, Chin-Pong Chong, Ying-Ling Yang, Mine-Yine Liu*

Department of Chemistry, National Changhua University of Education, Changhua, Taiwan

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ABSTRACT

A simple capillary zone electrophoresis (CZE) method was used to characterize native, *in vitro* oxidized and glycated human high-density lipoprotein (HDL) particles. Both native and *in vitro* oxidized HDL capillary electrophoresis (CE) profiles showed a major peak, but the oxidized HDL particles had higher effective mobilities. The *in vitro* glycated HDL particles showed a major peak and one or two minor peaks. The effective mobility of the major peak of glycated HDL was similar to that of the major peak of native HDL, whereas the effective mobilities of the two minor peaks were much lower. For the analysis of HDL phospholipids, a solid phase extraction procedure was optimized and a LC ESI-MS method was developed. Several possible HDL phospholipid molecular species including phosphatidylcholine (PC 16:0/18:2, 16:0/18:1, 18:0/18:2 and 18:0/18:1), sphingomyelin (SM 16:0) and lyso-phosphatidylcholine (lysoPC 16:0 and 18:0) were found. It appeared that the ion intensity ratios of hydroperoxy-PC or epoxyhydroxy-PC (16:0/hydroperoxy-18:2 or 16:0/epoxyhydroxy-18:2, *m*/*z* 790.4) and trihydroxy-PC (16:0/trihydroxy-18:2, *m*/*z* 808.3) relative to PC (C16:0/C18:2, *m*/*z* 758.5) were higher for oxidized HDL than for native and glycated HDL. It should be helpful to use both CZE and LC ESI-MS methods for analyzing high-density lipoproteins from patients of cardiovascular disease. Their combination may be also useful for further studies concerning the role of oxidized and glycated HDLs in the development of atherosclerosis.

1. Introduction

HDL particles are known to be cardioprotective due to their promoting cholesterol efflux ability as well as antioxidant, anti-inflammatory and antithrombotic properties. Many evidences also indicated that HDL-cholesterol levels in human plasma were inversely correlated with atherosclerotic disease. Considerable therapeutic efforts have been focusing on raising HDL-cholesterol levels in patients of cardiovascular disease. However, HDL might become pro-oxidant and enhance low-density lipoprotein (LDL) oxidation during chronic inflammation such as atherosclerosis. Recent findings have proposed that structural alterations of HDL particles may contribute to the pro-inflammatory properties. But, it is not understood yet how the chemical modifications of phospholipids, cholesterols and apolipoproteins within HDL particles influence their pro-inflammatory functions [1–7].

Previously, some researchers have studied HDLs by various methods. Wang and Stocker determined specifically oxidized apolipoproteins A-I and A-II on *in vitro* oxidized HDL by a reverse phase HPLC method [8]. Wiesner et al. quantitated lipid classes of very low-density lipoprotein (VLDL), LDL and HDL by an ESI-MS–MS method. The analyzed lipids included PC, lysoPC, SM, PE, PE-based plasmalogen (PE-pI), ceramide (CER), cholesterol and cholesteryl ester [9].

CE has many advantages for analyzing biomolecules including high speed, high sensitivity, minute volumes of buffer and sample needed as well as simultaneous measuring absorbance and electrophoretic mobility. Stocks and Miller reviewed the analysis of apolipoproteins and lipoproteins by various CE methods [10]. Macfarlane et al. used CZE to profile HDL particles by non-delipidating and delipidating CE buffers. HDL appeared as intact particles under non-delipidating condition (0.5 mM 99% SDS, borate buffer, pH 9.1), while apo A-I and A-II proteins showed as major peaks under delipidating condition (3.5 mM 70% SDS, 20% v/v acetonitrile, borate buffer, pH 9.1) [11–13]. Weiller et al. used CZE to separate HDL particles with Tricine buffer and methylglutamine (pH 9.0) as the

Abbreviations: HDL, high-density lipoprotein; SPE, solid phase extraction; CZE, capillary zone electrophoresis; ESI-MS, electrospray ionization mass spectrometry; TIC, total ion chromatography; EIC, extracted ion chromatography.

* Corresponding author. Tel.: +886 4 7232105x3530; fax: +886 4 7211190.
E-mail address: myliu@cc.ncue.edu.tw (M.-Y. Liu).

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background electrolyte. HDL showed a broad peak by both UV and fluorescence detections [14]. Several researchers have also utilized capillary isotachophoresis to separate human HDL into several subfractions [15–22].

Previously, some researchers have studied human HDL phospholipids by LC-MS analysis. Hayakawa and Okabayashi quantitated eight phospholipid classes on HDL by a normal-phase LC ESI-MS method. The mobile phase consisted of chloroform, methanol, 1-propanol and ammonium hydroxide [23]. They also measured the time course change of several phospholipid classes on HDL which was treated with human secretory phospholipase A_2-X by LC/ESI-MS. The mobile phase used for the normal-phase LC separation included acetonitrile, methanol and ammonium formate [24]. Tolonen et al. analyzed a unique phospholipid (phosphatidylethanol) on HDL by reverse phase HPLC and TOF-MS. A C_8 column was used for the HPLC separation, and a solvent system composed of ammonium acetate, acetonitrile and isopropanol was used as the mobile phase [25].

The aim of this study was to characterize native, *in vitro* oxidized and *in vitro* glycated human HDL particles by a simple CZE method, and to analyze HDL phospholipid molecular species by a new LC ESI-MS method. To the best of our knowledge, this is the first report for analyzing *in vitro* oxidized and glycated human HDL particles by CZE. This is also the first report to study phospholipid molecular species from *in vitro* oxidized and glycated human HDL particles using LC ESI-MS method.

2. Experimental

2.1. Chemicals

The following chemicals were used for this study: potassium bromide (KBr; J. T. Baker, Phillipsburg, NJ, USA), sodium phosphate monobasic (NaH_2PO_4; Sigma Chemical, St. Louis, MO, USA), sodium phosphate dibasic (Na_2HPO_4; Sigma Chemical), ethylenediaminetetraacetic acid (EDTA; Sigma Chemical), D-(+)-glucose (Sigma Chemical), copper(II) sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$; Riedel-de Haën, Seelze, Germany), phosphoric acid 85% (H_3PO_4; Riedel-de Haën), sodium hydroxide (NaOH; Riedel-de Haën) and deionized water (Millipore Simplicity; Millipore, Billerica, MA, USA), methanol(MeOH; Mallinckrodt Chemicals, Phillipsburg, NJ, USA), acetic acid (AcOH; Mallinckrodt Chemicals), chloroform($CHCl_3$; Mallinckrodt Chemicals), phosphatidylcholine (PC 16:0/18:2, 16:0/18:1,18:0/18:2, 18:0/18:1; Sigma Chemical), lyso-phosphatidylcholine (lysoPC 16:0, 18:0; Sigma Chemical), phosphatidyl ethanolamine (PE 16:0/18:2; Sigma Chemical), sphingomyelin (SM 16:0; Sigma Chemical).

2.2. Healthy subjects

Taichung Blood Donation Center (Taichung, Taiwan) kindly provided the five healthy human blood plasma samples. Informed consent was obtained from each participant. This study was approved by National Changhua University of Education and Taichung Blood Center (Taichung, Taiwan). Concentrations of HDL-cholesterol and Apo-AI in the HDL fractions were measured by Changhua Christian Hospital (Changhua, Taiwan).

2.3. Separation of human high-density lipoprotein fractions by ultracentrifugation

Isolation of lipoprotein fractions was performed using a Beckman Coulter Optima™ XL-100K following a standard procedure [26]. "The plasma volume used for ultracentrifugation preparation was 85 mL. After the addition of KBr to adjust density to 1.019 g/mL, the plasma was added into 8 tubes with 10 ml in each tube. Therefore, the actual plasma volume used was 80 mL. First, the adjusted plasma was subjected to ultracentrifugation at 45,000 rpm (174,000 × *g*) and 5 °C for 18 h. VLDL fraction was collected from the floating layer (2 mL from each tube). Second, density of the remained plasma was adjusted to 1.063 g/ml and divided into 6 tubes. Subsequently, the plasma was subjected to ultracentrifugation at 45,000 rpm (174,000 × *g*) and 5 °C for 18 h. LDL fraction was collected from the floating layer (3 mL from each tube). Third, density of the remained plasma was adjusted to 1.210 g/ml and divided into 4 tubes. The adjusted plasma was then subjected to ultracentrifugation at 45,000 rpm (174,000 × g) and 5 °C for 48 h. HDL fraction was collected between densities of 1.063 and 1.210 g/ml. The collected HDL fraction (3 mL from each tube) was immediately used for reaction and separation, otherwise kept at −80 °C until used.

2.4. Preparation of native HDL samples for CE analysis

After the ultracentrifugation procedure, the buffer of a 200 μl HDL fraction was exchanged 4 times from KBr solution to 2.5 mM sodium phosphate solution using a 30 kDa ultrafiltration filter (Amicon, Micron Centrifugal Filter Devices, Ultracel YM-30). Finally, the HDL solution was reconstituted to 200 μl in 2.5 mM sodium phosphate buffer, pH 7.40.

2.5. In vitro oxidation of HDL by Cu^{2+}

After the ultracentrifugation procedure, the buffer of a 200 μl HDL fraction was exchanged from KBr solution to PBS buffer using a 30 kDa ultrafiltration filter. The final volume of HDL was reconstituted to 200 μl. Then, a 200 μl of 10 μM $CuSO_4$ solution was added and the microcentrifuge tube was gently inverted several times. The final concentration of Cu^{2+} was 5 μM. The HDL solution mixture was incubated in 37 °C water bath and shaken at 80 rpm for 18 h. Finally, 0.0009 g of EDTA was added to quench the oxidation reaction. The buffer of the HDL sample was exchanged 4 times from the oxidation solution mixture to 2.5 mM sodium phosphate buffer using a 30 kDa ultrafiltration filter. CE analysis was then carried out.

2.6. In vitro glycation of HDL by glucose

As in the above section, a 200 μl HDL fraction in PBS buffer was prepared. Then, a 200 μl of 120 mM glucose solution (in PBS buffer) and 0.0009 g EDTA were added into the HDL solution. The microcentrifuge tube was gently inverted several times. The final glucose concentration was 60 mM. The solution mixture was then incubated in 37 °C oven for 7 days. At the end, the buffer of HDL was exchanged to 2.5 mM sodium phosphate as described in the above section. CE analysis was then performed to characterize HDL particles.CE analysis of HDL particles

All of the CE analysis in this study was performed using a Beckman P/ACE MDQ capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA). The instrument was equipped with a diode array detector and capillary cartridge coolant tubing. An IBM Pentium 4 computer was connected to the CE instrument for data acquisition and analysis. Electropherograms were analyzed by a 32 Karat software (version 7.0, Beckman). Capillary used was uncoated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 75 μm i.d. and 375 μm o.d. with a total length of 60.2 cm and an effective length of 50.0 cm. Normally, a new capillary was sequentially rinsed with 1 N NaOH for 5 min, 0.1 N NaOH for 10 min, and deionized water for 10 min before use. Every day before sample analysis, a capillary was also rinsed with 1 N NaOH for 1 min, 0.1 N NaOH for 5 min, deionized water for 5 min, and CE buffer for 5 min. CE separation was run from anode to cathode. A voltage of 16 kV was applied and the capillary was kept at 25 °C. For HDL sample

Table 1
The average effective mobility, peak area and peak area ratios of native HDL particles analyzed by CZE (data are means ± S. D. of six replicates).

N-HDL						
	μ_{eff} ($\times10^{-5}$ cm^2 V^{-1} s^{-1})	CV(%)	Area(214) ($\times10^5$)	CV(%)	CPA ratio (A_{234}/A_{214}) ($\times10^{-2}$)	CPA ratio (A_{280}/A_{214}) ($\times10^{-2}$)
Donor A	−27.51 ± 0.35	0.73	36.50 ± 0.30	4.35	30.08 ± 3.20	7.37 ± 0.33
Donor B	−25.52 ± 0.39	1.09	13.54 ± 0.97	4.69	31.55 ± 0.56	8.30 ± 0.45
Donor C	−25.25 ± 0.20	0.53	14.21 ± 0.74	2.83	30.19 ± 0.72	7.48 ± 0.82
Donor D	−26.09 ± 0.25	0.55	15.52 ± 1.44	4.02	31.17 ± 0.57	7.52 ± 0.83

Table 2
The average effective mobility, peak area and peak area ratios of *in vitro* oxidized HDL particles analyzed by CZE (data are means ± S. D. of six replicates).

ox-HDL						
	μ_{eff} ($\times10^{-5}$ cm^{2}V^{-1} s^{-1})	CV(%)	Area(214) ($\times10^5$)	CV(%)	CPA ratio (A_{234}/A_{214}) ($\times10^{-2}$)	CPA ratio (A_{280}/A_{214}) ($\times10^{-2}$)
Donor A	−29.25 ± 0.15	0.39	36.92 ± 1.25	1.94	50.77 ± 0.11	14.41 ± 0.26
Donor B	−28.15 ± 0.24	0.59	13.51 ± 1.01	5.28	48.39 ± 0.14	11.99 ± 0.11
Donor C	−26.44 ± 0.13	0.45	14.51 ± 0.47	3.08	45.47 ± 2.17	12.21 ± 0.46
Donor D	−27.67 ± 0.24	0.40	15.69 ± 0.36	2.05	46.77 ± 0.70	12.12 ± 0.36

introduction, the injection sequence was: (1) a 4-s pressure injection of deionized water, (2) a 4-s pressure injection of HDL sample and (3) a 4-s pressure injection of CE buffer. A pressure of 0.5 psi was applied for sample injection. Between runs, the capillary was always conditioned with 0.1 N NaOH for 2 min and deionized water for 2 min.

2.8. Solid phase extraction of HDL phospholipids

The procedure for solid phase extraction (SPE) of HDL was modified from a previous procedure [27]. Briefly, in a microcentrifuge tube, an 800 μl HDL fraction from the ultracentrifugation procedure, 450 μl chloroform, 300 μl methanol and 150 μl deionized water were mixed together. Then, the tube was gently inverted several times, and centrifuged at 5000 rpm for 10 min to extract lipids. After the centrifugation procedure, the lower layer was separated from the upper layer. In another microcentrifuge tube, the lower layer was mixed with 1 ml of a solvent mixture (chloroform:methanol:water = 3:48:47). Subsequently, the tube was gently inverted several times, and centrifuged at 5000 rpm for 10 min. Again, the lower layer was separated from the upper layer and added into another microcentrifuge tube. Then, it was dried with nitrogen gas, and 1 ml of chloroform was added to dissolve the lipid sample. In a Sep-Pak cartridge column (Waters, Sep-Pak light silica cartridge, particle size: 55–105 μm, pore size: 125 Å, silica mass: 120 mg, column volume: 0.4 ml), first, 2 ml chloroform was added onto the column to rinse it. Second, 1 ml of lipid sample in chloroform from the above extraction procedure was slowly loaded onto the column. Third, 4 ml chloroform was added onto the column to elute neutral lipids. Fourth, 4 ml methanol was added onto the column to elute polar lipids. The methanol solution was then dried under nitrogen gas to reduce the solution volume to 0.5 ml, and LC ESI-MS analysis was carried out to analyze phospholipids.

2.9. LC ESI-MS analysis of phospholipid standards

Phospholipid standards including phosphatidylcholine (PC 16:0/18:2, 16:0/18:1, 18:0/18:2, 18:0/18:1), lyso-phosphatidylcholine (lysoPC 16:0, 18:0), phosphatidyl ethanolamine (PE 16:0/18:2), sphingomyelin (SM 16:0) were chosen to study phospholipids on HDL particles. A 3 mg of each of the above phospholipid standards was mixed together and dissolved in 1 ml of methanol. A 30 μl of the lipid mixture was introduced into HPLC to obtain the total ion chromatogram (TIC). The mobile phase was composed of 95% methanol and 5% acetic acid with a flow rate of 0.5 ml/min.

2.10. LC ESI-MS analysis of HDL phospholipids

After the SPE procedure, a 50 μl of the methanol solution containing polar lipids was introduced into a Waters HPLC system (Waters Corporation, Milford, MA, USA) for separation. The HPLC instrument is equipped with a controller (Waters 600), a pump (Waters 600) and a photodiode array detector (Waters 966). The HPLC column used was a C_{18} column which had a

Table 3
The average effective mobility, peak area and peak area ratios of *in vitro* glycated HDL particles analyzed by CZE (data are means ± S. D. of six replicates).

	μ_{eff} ($\times10^{-5}$ cm^{2}V^{-1} s^{-1})	CV(%)	Area(214) ($\times10^5$)	CV(%)	CPA ratio (A_{234}/A_{214}) ($\times10^{-2}$)	CPA ratio (A_{280}/A_{214}) ($\times10^{-2}$)
Gly-HDL I						
Donor A	Not measurable		Not measurable			
Donor B	Not measurable		Not measurable			
donor C	−13.41 ± 0.31	1.12	7.26 ± 0.53	13.61	31.46 ± 2.76	9.05 ± 1.23
Donor D	−13.98 ± 0.07	0.65	0.48 ± 0.05	5.07	31.71 ± 2.85	9.19 ± 1.15
Gly-HDL-II						
Donor A	−17.82 ± 0.18	1.24	11.10 ± 0.38	4.66	30.84 ± 1.17	7.76 ± 0.19
Donor B	−17.74 ± 0.52	1.83	2.82 ± 0.73	8.48	30.34 ± 0.52	8.63 ± 0.24
Donor C	−16.54 ± 0.46	1.55	3.16 ± 0.34	6.56	33.92 ± 0.74	8.47 ± 1.10
Donor D	−16.90 ± 0.38	1.30	1.52 ± 0.09	4.23	33.40 ± 1.11	9.07 ± 2.32
Gly-HDL-III						
Donor A	−27.49 ± 0.32	0.78	24.76 ± 0.92	3.17	30.38 ± 0.29	7.58 ± 0.20
Donor B	−25.64 ± 0.31	0.56	10.87 ± 0.87	5.48	32.23 ± 1.18	8.67 ± 0.25
Donor C	−25.31 ± 0.11	0.44	9.82 ± 0.30	1.56	32.07 ± 0.64	7.80 ± 0.48
Donor D	−26.09 ± 0.05	0.22	13.07 ± 0.1	2.37	32.44 ± 0.39	8.08 ± 0.40

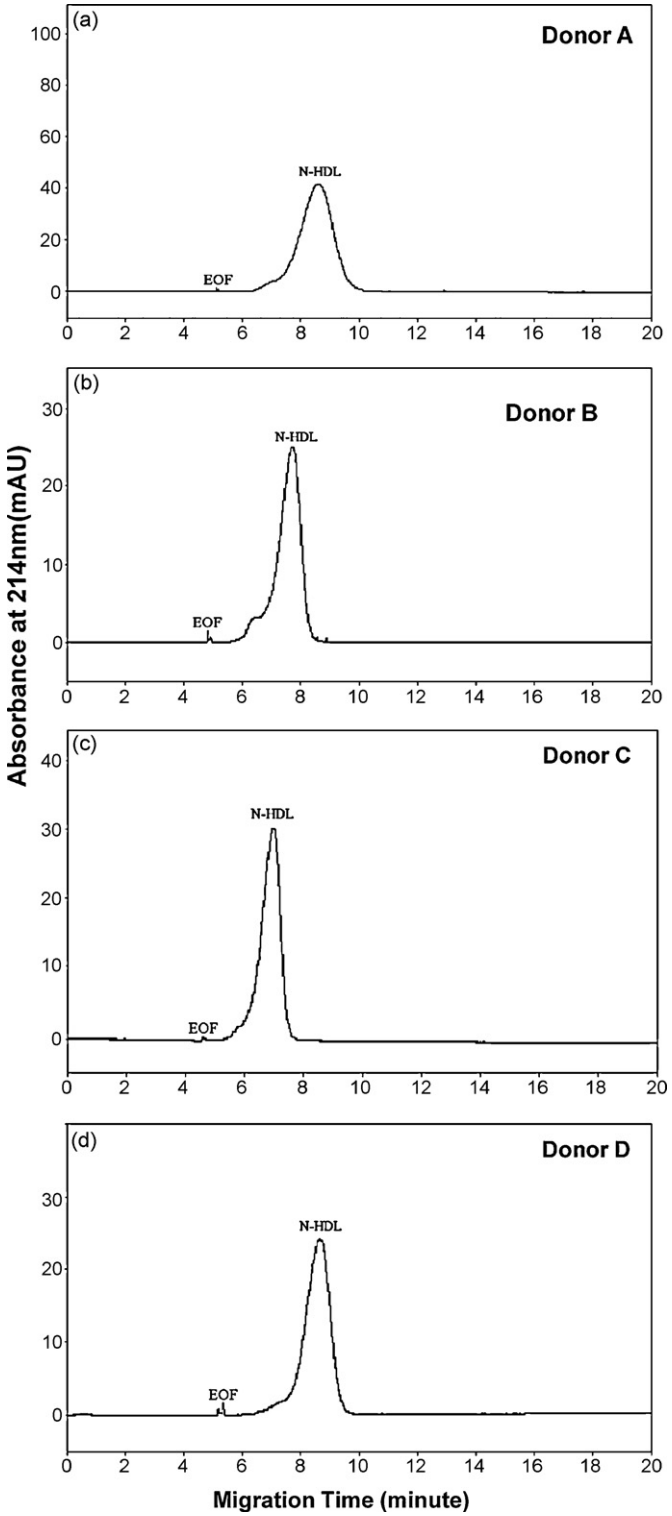

Fig. 1. Electropherograms of native HDL particles of four healthy donors. The buffer of a 200 μl HDL fraction isolated by ultracentrifugation was exchanged from KBr solution to 2.5 mM sodium phosphate using a UF filter. For HDL sample introduction, a pressure of 0.5 psi and a 4-s injection were used. The CE background electrolyte was 5 mM sodium phosphate, pH 7.40, and the CE separation voltage was 16 kV.

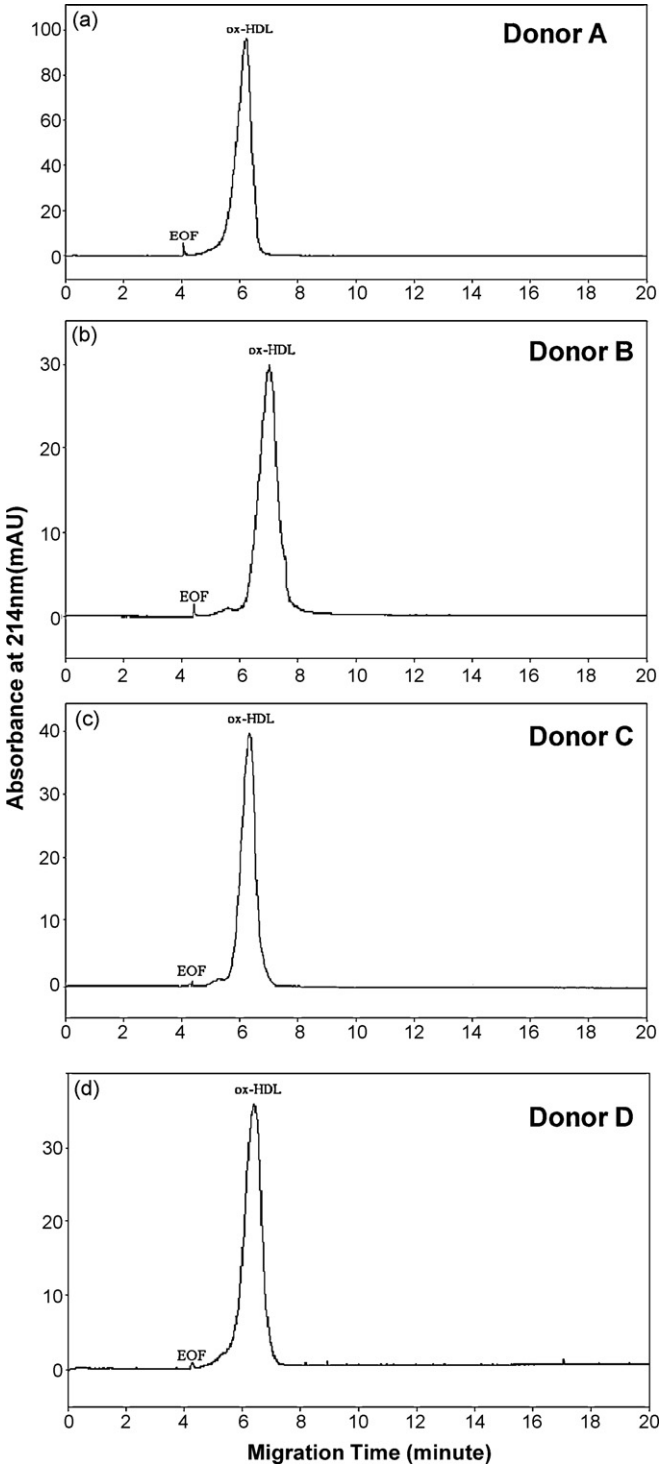

Fig. 2. Electropherograms of oxidized HDL particles of four healthy donors. HDL sample was oxidized by 5 μM Cu^{2+} in PBS buffer at 37 °C for 18 h, and the buffer was then exchanged to 2.5 mM sodium phosphate using a UF filter. CE conditions are the same as Fig. 1.

particle size of 3.5 μm, an i.d. of 4.6 mm, and a length of 15 cm (Waters Sunfire™ C_{18} column). The mobile phase contained 95% methanol and 5% acetic acid with a flow rate of 0.5 ml/min. Column temperature was maintained at 25 °C, and pressure was kept between 850 and 900 psi. Chromatograms were analyzed by the Millennium^{32} Chromatography Manager software (version 3.2, Waters).

Phospholipids separated by HPLC were then analyzed by ESI-MS. The HPLC system was coupled to a LCQ ion-trap mass spectrometer (Finnigan Corporation, San Jose, CA, USA). Samples were analyzed in positive ion mode with an ion spray voltage of 5 kV. The capillary voltage and temperature were maintained at 14 V and 200 °C, respectively. Nitrogen was used as sheath gas. Mass spectrum range

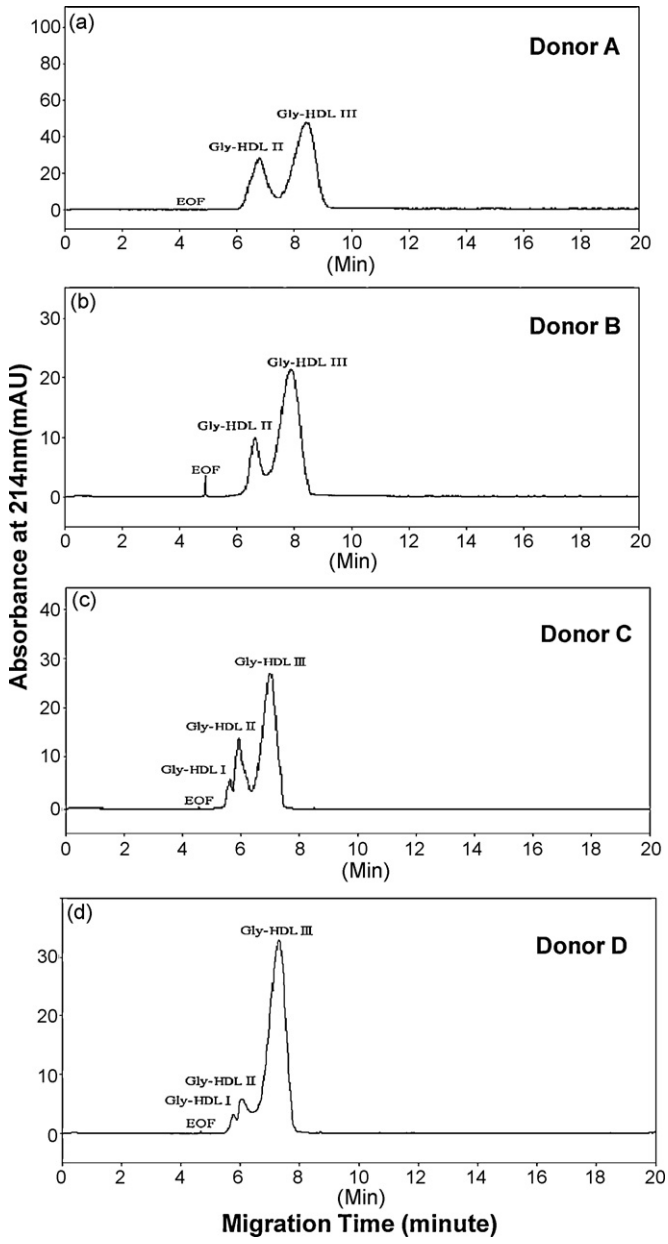

Fig. 3. Electropherograms of glucose modified HDL particles of four healthy donors. HDL was incubated with 60 mM glucose in PBS buffer at 37 °C for 7 days, and the buffer was then exchanged to 2.5 mM sodium phosphate using a UF filter. CE conditions are the same as Fig. 1.

between m/z 450 and 900 was recorded. Data were analyzed by the Xcalibur software (Version 1.2; Finnigan Corporation).

3. Results and discussion

3.1. HDL CE analysis for four healthy subjects

KBr was added to plasmas to adjust densities for the ultracentrifugation procedure, and anti-coagulants were added while healthy human plasmas were prepared by Taichung Blood Center. Therefore, it was important to purify HDL fractions before *in vitro* modification and CE analysis. In this study, extensive ultrafiltration procedures were applied for HDL sample purification. Ultrafiltration procedures were tested for repeating a total of 1, 2, 3, 4 and 5 times using a 30 kDa ultrafiltration filter. After the ultrafiltration procedure, the HDL sample was analyzed by CE to know if the

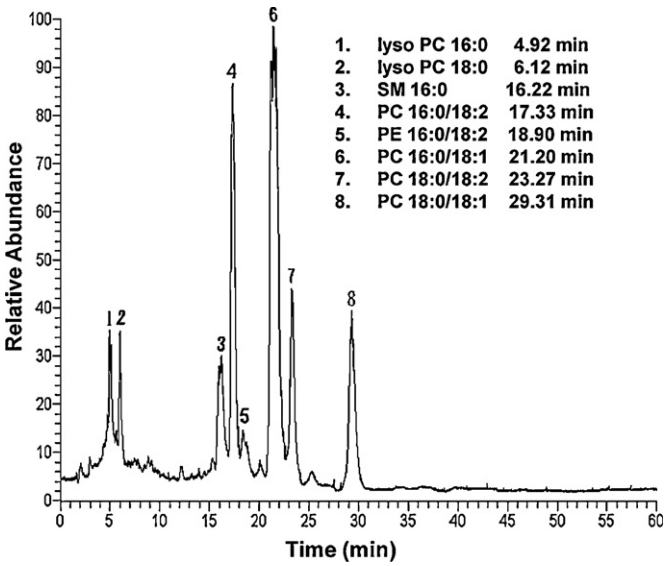

Fig. 4. Total ion chromatogram of 8 phospholipid standards analyzed in positive ion mode. Mass range scanned was m/z 450–900.

impurities were cleaned. The peak of HDL showed irregular shape and peak area when 1, 2 and 3 times of ultrafiltration was used. But, the peak shape and area of HDL became reproducible using 4 and 5 times of ultrafiltration. Therefore, repetition of ultrafiltration for 4 times was chosen because it was enough for cleaning the chemicals and impurities accompanying HDL sample. Voltages of 10, 12, 14, 16, 18 and 20 kV were tested for CE analysis. The HDL peaks appeared wider using 10, 12 and 14 kV, while separation efficiency was not good using 18 and 20 kV because of joule heating. Therefore, 16 kV was chosen as the optimal voltage. To select the optimal CE separation buffer, several buffers including 2.5, 5, 10, 30, 50 and 100 mM PB, pH 7.4 were tested (data not shown). Finally, 5 mM PB, pH 7.4 was chosen as the optimal buffer for CE separation, which was similar to our previous study for LDL particles [28,29].

3.2. Native HDL CE profiles

To observe the difference between native and *in vitro* modified HDL particles, good reproducibility of HDL electropherograms is essential. The results of native HDL profiles for four healthy subjects are shown in Fig. 1. The feature of HDL profiles was similar for each donor. Each donor showed a major HDL peak with a small shoulder on the lower mobility end of the peak.

Table 1 shows the average effective mobility $\mu_{eff,}$ peak area (A_{214}), peak area ratios (A_{234}/A_{214} and A_{280}/A_{214}) and CV(%) for HDL particles. For each donor, the experiments including ultracentrifugation, ultrafiltration and CE analysis were repeated for 6 times to obtain the measurements. The purpose of simultaneous analysis of absorbances at 214, 234 and 280 nm was for estimating lipids, conjugated dienes and protein contents in HDL particles, respectively. Table 1 suggests that the native HDL electropherograms are highly reproducible with good precision of effective mobility and peak area.

Concentrations of HDL-cholesterol and Apo-AI in the HDL fraction for each donor are listed as follows:

HDL-cholesterol (mg/dl): 177 (donor A), 93 (donor B), 93 (donor C), 174 (donor D).
Apo-AI (mg/dl): >260 (donor A), 176 (donor B), 241 (donor C), >260 (donor D).

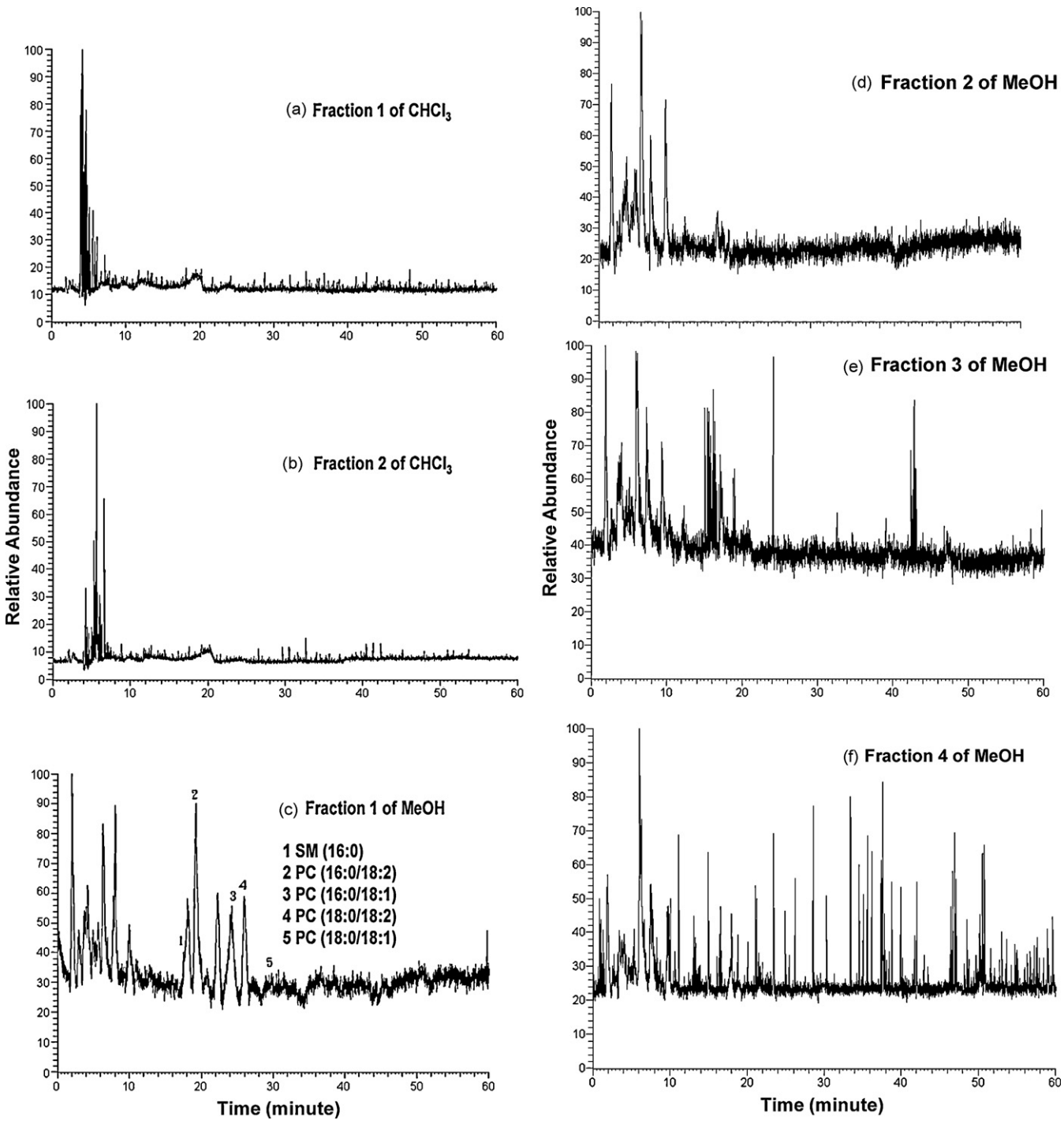

Fig. 5. Total ion chromatograms of HDL phospholipids from donor E. Phospholipids were purified by a solid phase extraction procedure. A 2 ml volume was collected for each chloroform or methanol fraction. (a) the first chloroform fraction, (b) the second chloroform fraction, (c) the first methanol fraction, (d) the second methanol fraction, (e) the third methanol fraction and (f) the fourth methanol fraction. ESI-MS conditions are the same as Fig. 4.

For donor A, both concentrations of HDL-cholesterol and Apo-AI are the highest among the four donors. It is possible the reason that donor A has the highest peak areas of native and modified HDL particles by CE analysis.

3.3. Cu^{2+} in vitro oxidized HDL CE profiles

To understand *in vivo* oxidized HDL particles associated with cardiovascular diseases, HDL particles of the four healthy donors were *in vitro* oxidized by 5 μM Cu^{2+}, and then CE analysis was performed to characterize HDL particles. Fig. 2 shows the HDL electropherograms of Cu^{2+} oxidation for 18 h. Table 2 shows the average effective mobility $\mu_{eff,}$ peak area (A_{214}), peak area ratios (A_{234}/A_{214} and A_{280}/A_{214}) and CV(%) for oxidized HDL particles. The whole procedures including ultracentrifugation, ultrafiltration, oxidation reaction and CE analysis were repeated for at least 6 times to obtain the measurements for each donor. One major peak appeared with mobility higher than that of the major peak of native HDL, indicating it was more negatively charged. For each donor, the average peak area ratios

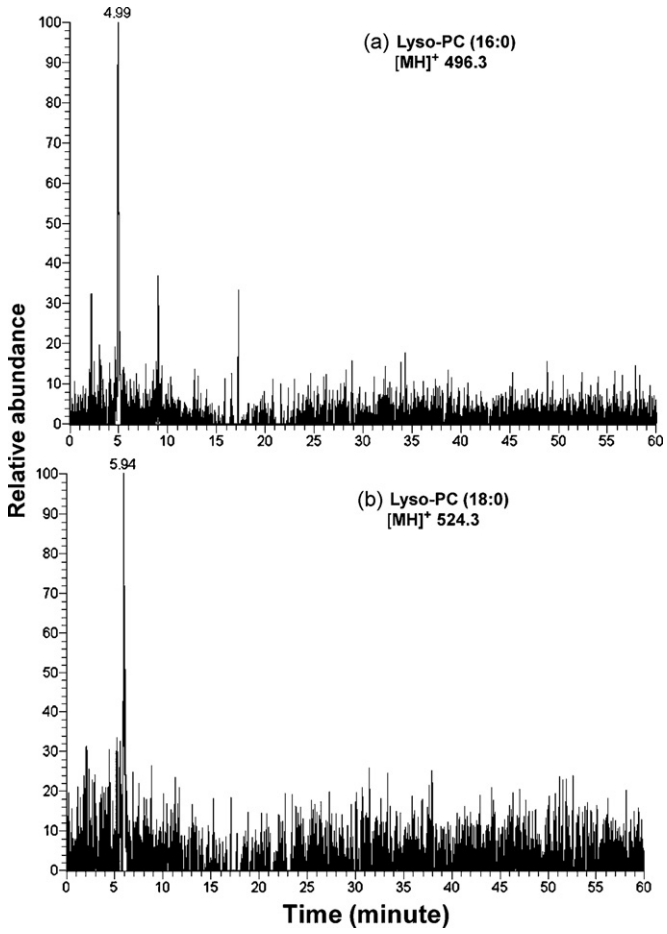

Fig. 6. Extracted ion chromatograms of phospholipids isolated from oxidized HDL particles of donor E. (a) ion at *m*/*z* 496.3 for lyso-PC (16:0) and (b) ion at *m*/*z* 524.3 for lyso-PC (18:0). ESI-MS conditions are the same as Fig. 4.

(A_{234}/A_{214} and A_{280}/A_{214}) of oxidized HDL were much higher than that of native HDL, suggesting it had a higher level of conjugated dienes and protein to lipid ratio due to oxidation. Meanwhile, the higher A_{280}/A_{214} ratio may also indicate higher contents of some oxidized lipids such as dihydroxy-eicosatetraenoic acid which has 3 conjugated double bonds with absorbance at 280 nm.

3.4. Glucose in vitro modified HDL CE profiles

To understand *in vivo* glycation reaction of HDL particles for diabetic patients who often show high levels of blood glucose, HDL particles of the four healthy donors were *in vitro* modified by 60 mM glucose and then CE analysis was performed. Since EDTA was included in the reaction to eliminate possible metal ions present, the modification of HDL particles was glycation rather than oxidation. The CE profiles of glucose modified HDL are shown in Fig. 3. It appeared that two peaks were found for donors A and B, and three peaks were found for donors C and D.

Table 3 shows the average effective mobility $\mu_{eff,}$ peak area (A_{214}), peak area ratios (A_{234}/A_{214} and A_{280}/A_{214}) and CV(%) for glucose modified HDL particles. For each donor, all experiments including ultracentrifugation, ultrafiltration, glycation reaction and CE analysis were repeated for 6 times to obtain the measurements.

The average effective mobilities of peaks Gly-I and Gly-II were much lower than that of native HDL. The peak area ratios (A_{234}/A_{214} and A_{280}/A_{214}) for these two peaks were similar to that of native HDL. The lower mobility was probably because of Schiff base products formed between carbonyl groups of glucose and amine groups of apolipoproteins on HDL. Schiff base products increased the molecular weight without changing the charges of HDL, leading to lower mobility of the particle. The average effective mobility and peak area ratios (A_{234}/A_{214} and A_{280}/A_{214}) of peak Gly-III were similar to that of native HDL, suggesting it represented unmodified HDL particles.

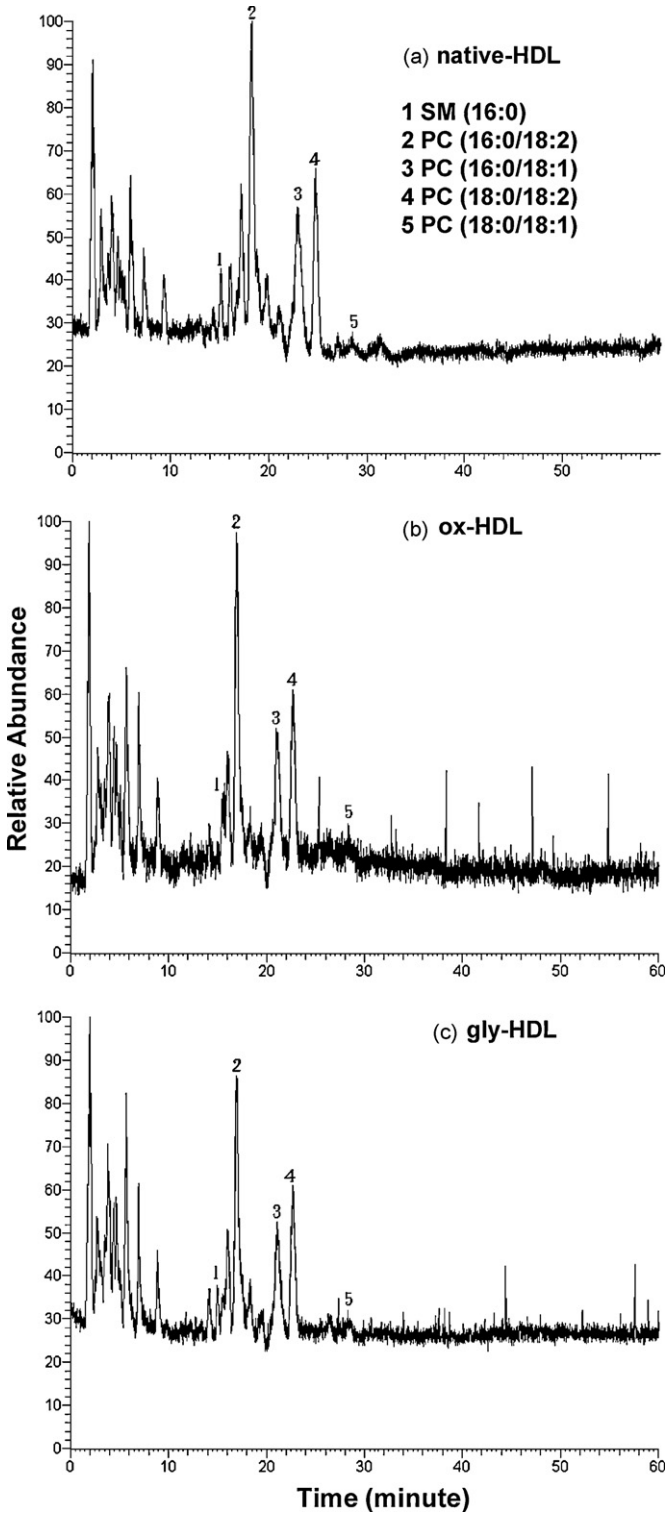

Fig. 7. Total ion chromatograms of phospholipids isolated from (a) native HDL, (b) *in vitro* oxidized HDL and (c) *in vitro* glycated HDL particles of donor E. A 4 ml chloroform followed by a 4 ml methanol elution was used for the solid phase extraction procedure for phospholipids. ESI-MS conditions are the same as Fig. 4.

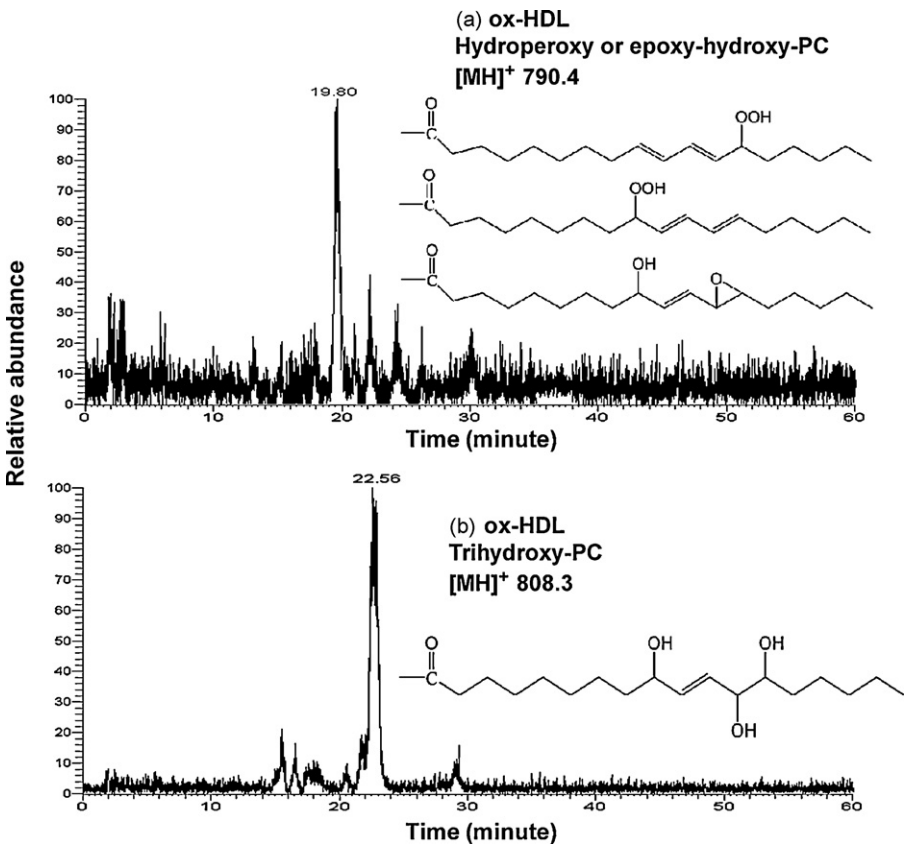

Fig. 8. Extracted ion chromatograms of phospholipids isolated from oxidized HDL particles of donor E. (a) ion at *m*/*z* 790.4 for hydroperoxy-PC (16:0/hydroperoxy-18:2) or epoxyhydroxy-PC (16:0/epoxyhydroxy-18:2) and (b) ion at *m*/*z* 808.3 for trihydroxy-PC (16:0/trihydroxy-18:2). ESI-MS conditions are the same as Fig. 4.

The *in vitro* glycation reaction of HDL sample was compared with the control experiment (same incubation conditions but without glucose). From the CE analysis, it was found that the control HDL sample showed similar profiles to the native HDL sample. Therefore, more peaks found in glycated HDL was the result of glycation and not incubation alone.

3.5. LC ESI-MS analysis of phospholipid standards

In order to characterize phospholipid molecular species of HDL, several phospholipid standards were analyzed by LC ESI-MS. The search of optimal conditions is crucial for the separation and identification of phospholipids. For the HPLC study, both normal-phase and reverse-phase columns were tested, and finally reverse-phase column was selected because of better separation efficiency. The reverse-phase column utilized was a new C_{18} column developed by Waters Corporation which had a particle size of 3.5 μm, an i.d. of 4.6 mm, and a length of 15 cm. In this study, both organic solvents (methanol, acetonitrile and chloroform) and aqueous phases (acetic acid and water) were tested for the use of mobile phase. Several solvent systems including 95% methanol + 5% water, 99% methanol + 1% water, 100% methanol, 95% methanol + 5% acetic acid, 80% methanol + 20% acetonitrile were tested for the separation of phospholipid standards. Finally, the solvent system consisted of 95% methanol and 5% acetic acid was selected as the optimal condition because of its best separation and ionization efficiency. Fig. 4 shows the total ion chromatogram (TIC) of several phospholipid standards including phosphatidylcholine (PC 16:0/18:2, 16:0/18:1,18:0/18:2, 18:0/18:1), lyso-phosphatidylcholine (lysoPC 16:0, 18:0), phosphatidyl ethanolamine (PE 16:0/18:2) and sphingomyelin (SM 16:0).

Each phospholipid standard was characterized by its own total ion chromatogram, extracted ion chromatogram and ESI-MS spectrum. After scanning the mass range *m*/*z* 150–1000, it was found that each phospholipid standard molecule showed only protonated molecular ion without fragmentations since ESI-MS was a soft ionization method. Therefore, we have recorded the mass range *m*/*z* 450–900 since this is the mass range for the above phospholipid standards. Subsequently, the standards were combined in equal quantity to obtain the total ion chromatogram as shown in Fig. 4.

Although all phospholipid standards were mixed in equal quantities, the relative abundances were different due to various ionization efficiency of each phospholipid. Ionization was easy for some phospholipids, while it was difficult for others. The irregular shape of peak 6 was due to different ion intensities obtained between mass scans. There were a total of 32 sans for peak 6 (i.e. between 20.54 and 22.78 min), and the irregular shape represented various ion intensities at each time point.

3.6. Solid phase extraction of HDL phospholipids

In order to obtain phospholipids, first, HDL fraction of healthy donor E was extracted by chloroform, methanol and deionized water twice to separate lipids from apolipoproteins. Second, solid phase extraction (SPE) was carried out to separate polar lipids from neutral lipids. The SPE procedure was a modification from a previous method which was used to separate phospholipids in rat and human brain tissues [27]. In our study, to optimize SPE procedure, the total ion chromatograms were obtained. The results are shown in Fig. 5. First, the SPE cartridge loaded with HDL lipids was eluted by chloroform, and two fractions were collected. Each fraction had a volume of 2 ml. The chloroform fractions contained neutral lipids.

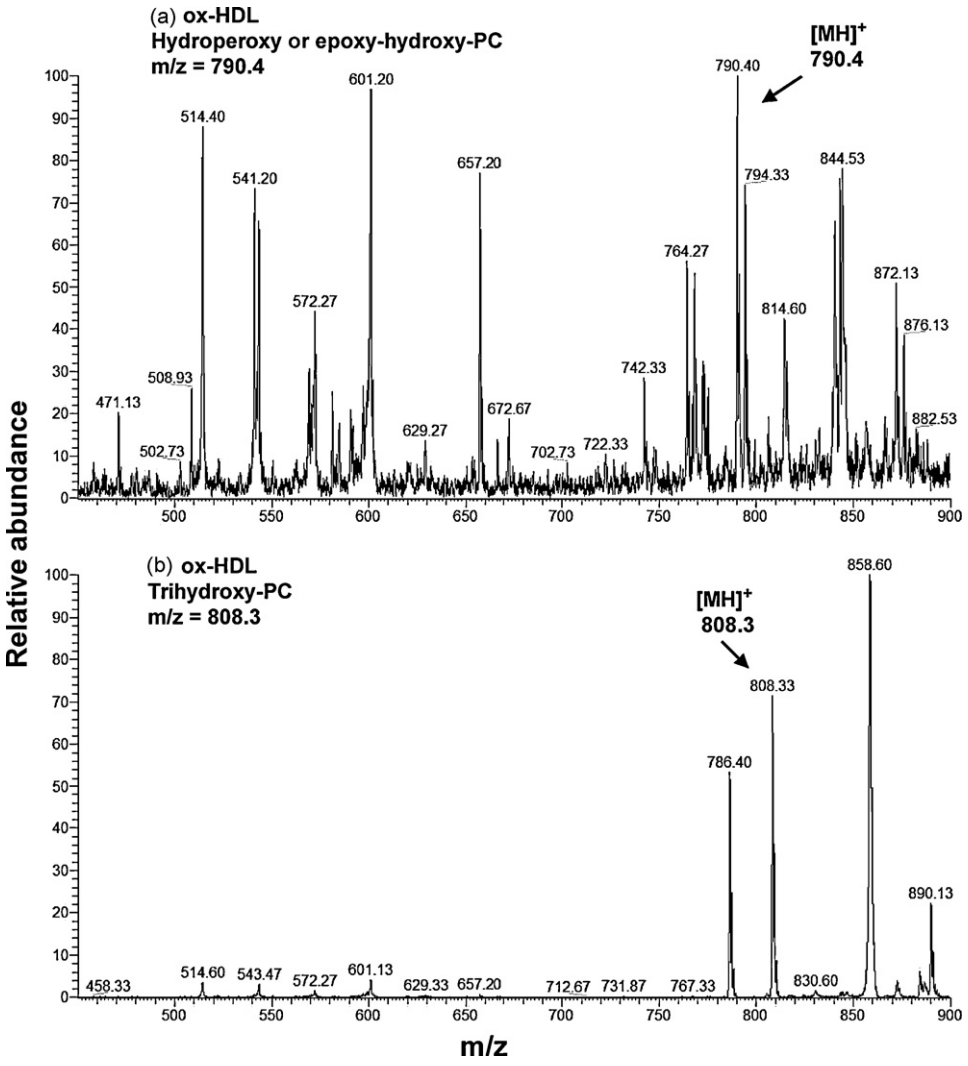

Fig. 9. Mass spectra of phospholipids isolated from oxidized HDL particles of donor E. (a) ion at *m/z* 790.4 for hydroperoxy-PC (16:0/hydroperoxy-18:2) or epoxyhydroxy-PC (16:0/epoxyhydroxy-18:2) and (b) ion at *m/z* 808.3 for trihydroxy-PC (16:0/trihydroxy-18:2). ESI-MS conditions are the same as Fig. 4.

The fractions were dried under nitrogen gas, re-dissolved in 200 μl methanol and analyzed by LC ESI-MS. The procedure was repeated for 4 more times by methanol elution. Fig. 5a and b shows the results of two chloroform fractions, and Fig. 5c and d shows the results of four methanol fractions. It appeared that phospholipids were not eluted by chloroform, and they were mostly eluted in the first two fractions of methanol. Therefore, 4 ml chloroform followed by 4 ml methanol elution was chosen as the optimal condition for SPE.

3.7. LC ESI-MS analysis of HDL phospholipids

Study of phospholipid molecular species in HDL samples was based on the analysis of phospholipid standards. Several possible phospholipid molecular species were found in HDLs by comparing the total ion chromatograms of the extracted HDL phospholipids, and the extracted ion chromatograms as well as the ESI mass spectra of phospholipid molecular species to the phospholipid standards.

The possible phospholipids found in native HDL were phosphatidylcholine (PC 16:0/18:2, 16:0/18:1, 18:0/18:2 and 18:0/18:1) and sphingomyelin (SM 16:0). In addition, two lysophosphatidylcholine molecular species (lysoPC 16:0 and 18:0) were also possibly present, and their extracted ion chromatograms are shown in Fig. 6. Phosphatidyl ethanolamine (PE) was not found probably because of its low concentration on HDL particle.

Previously, some researchers have identified numerous phospholipid molecular species in human serum, plasma or blood. All of the above phospholipid molecular species except PC (18:0/18:1) have been reported. Takatera et al. determined and quantitated PC and lysoPC molecular species in neonatal serum by LC MS–MS analysis [30]. Using normal-phase LC ESI-MS analysis, Uran et al. identified many phospholipid molecular species including 17 new disaturated phospholipid species in human blood by MS2 or MS3 methods [31]. Wang et al. identified many phospholipid molecular species in PC, lysoPC, PE, SM and phosphatidylserine (PS) classes in human blood using normal-phase LC-MS analysis. The authors used collisionally activated dissociation (CAD) and product ion spectra of molecular ions to identify more than 100 including two new phospholipid molecular species [32]. Pang et al. determined and quantitated seven phospholipid classes including PC, lysoPC, PE, SM, PS, phosphatidylinositol (PI) and phosphatidylglycerol (PG) in human blood using normal-phase LC ESI-MS analysis. More than 100 phospholipid molecular species were identified by the ion trap MS2 and MS3 analysis [33]. Ishida et al. determined several molecular species of lysoPEs, lysophosphatidic acids and their precursors in human plasma by LC ESI-MS analysis, product ion scanning and precursor ionscanning [34]. Hidaka et al. determined all lipid classes

Table 4
ESI-MS ion intensity ratios for oxidized PC molecular species at *m/z* 790.4 and 808.3 relative to 758.5.

	m/z = 758.5 PC (16:0/18:2)	*m/z* = 790.4 Hydroperoxy, epoxyhydroxy-PC	*m/z* = 808.3 Trihydroxy-PC	$I_{790.4}/I_{758.5}$	$I_{808.3}/I_{758.5}$
N-HDL	6.69×10^8	1.33×10^6	3.55×10^6	0.19×10^{-2}	0.53×10^{-2}
Ox-HDL	3.35×10^8	5.44×10^6	3.37×10^6	1.62×10^{-2}	1.00×10^{-2}
Gly-HDL	5.31×10^8	1.10×10^6	3.21×10^6	0.20×10^{-2}	0.60×10^{-2}

in human serum lipoproteins by matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis [35].

Allthough the total ion chromatograms, extracted ion chromatograms and ESI-MS spectra of our present study as well as other researchers' studies suggested the presence of the above phospholipid molecular species in HDLs, we would like to have more definitive direct evidence. The ESI-MS–MS method is the most definitive analysis to prove the existence of phospholipid molecular species if an instrument capable of performing this method is available in the future.

In order to compare the phospholipids of native, oxidized and glycated HDLs, LC ESI-MS analysis of the three different HDL particles were performed. Fig. 7a–c shows the TICs of native, oxidized and glycated HDL phospholipids. It appeared that the three TICs were similar. Meanwhile, their EICs of various phospholipid molecular species were also similar (data not shown).

Since PC (16:0/18:2) was the most abundant phospholipid on HDL (the base peak) as shown in Fig. 7, its oxidized molecular species were studied for the three different HDL particles. Previously, Adachi et al. have done extensive studies to prove the existence of several oxidized PC molecular species including hydroperoxy-PC (*m/z* 790.6), epoxyhydroxy-PC (*m/z* 790.6), trihydroxy-PC (*m/z* 808.6) and other molecular species in human plasma. They have analyzed the oxidation products of PC (16:0/18:2 and 18:0/18:2) in human plasma and rat heart using quadrupole time of flight mass spectrometry with electrospray ionization. The authors used standard synthetic PC oxidation products, mass chromatogram, ESI-MS and ESI-MS–MS spectra to elucidate the structures of these oxidized PC molecular species [36,37].

In our study, four possible oxidized PC molecular species including two hydroperoxy-PC (16:0/hydroperoxy-18:2, *m/z* 790.4), one epoxyhydroxy-PC (16:0/epoxyhydroxy-18:2, *m/z* 790.4) and one trihydroxy-PC (16:0/trihydroxy-18:2, *m/z* 808.3) were found. The proposed structures and EICs of these oxidized PC molecular species are shown in Fig. 8a and b. The ESI-MS spectra of these oxidized molecules show MH^+ (*m/z* 790.4) and MH^+ (*m/z* 808.3) without fragmentations, as illustrated in Fig. 9a and b. Since for a given *m/z* value, there is limited number of possibilities, we have used EICs and ESI-MS spectra as well as referred to the studies of Adachi et al. to propose the possible oxidized PC molecular species at *m/z* values 790.4 and 808.3. Hopefully, in the future, we will have access to an instrument which could carry out the ESI-MS–MS studies. Then, we could determine oxidized standard PC (16:0/18:2) molecular species by the MS–MS method, and characterize the structures of these oxidized molecules on human HDL particles.

The ion intensities and ratios of *m/z* 790.4 and 808.3 relative to 758.5 are shown in Table 4. The peak at *m/z* 758.5 represented $[M+H]^+$ ion of non-oxidized PC (C16:0/C18:2). The ion intensity ratio of *m/z* 790.4 relative to 758.5 ($I_{790.4}/I_{758.5}$) suggested that the concentrations of hydroperoxy-PC and epoxyhydroxy-PC were higher in oxidized HDL than in native HDL, while their concentrations were close in glycated and native HDL. The ion intensity ratio of *m/z* 808.3 relative to 758.5 ($I_{808.3}/I_{758.5}$) indicated that the level of trihydroxy-PC was also higher in oxidized HDL than in native and glycated HDL. Since the glycation reaction in this study was carried out in the presence of EDTA to prevent oxidation reactions, the results of ion intensity ratios were consistent with our experiments.

4. Conclusions

A simple and highly reproducible CZE method was applied to analyze native, *in vitro* oxidized and glycated HDL particles for four healthy subjects. The native HDL CE profile showed a major peak with good precision of effective mobility and peak area. The *in vitro* oxidized HDL showed a major peak with higher effective mobility and peak area ratios (A_{234}/A_{214} and A_{280}/A_{214}) due to oxidation. The *in vitro* glycated HDL showed a major peak and one or two minor peaks. The effective mobility of the major peak was similar to that of native HDL, while the effective mobilities of the two minor peaks were much lower. The lower mobility was possibly because of Schiff base products formed between glucose and apolipoproteins of HDL.

A SPE procedure was optimized to isolate phospholipids from HDL particles, and a new LC ESI-MS method was developed to analyze HDL phospholipids. The possible phospholipid molecular species present in HDLs were phosphatidylcholine (PC 16:0/18:2, 16:0/18:1, 18:0/18:2 and 18:0/18:1), sphingomyelin (SM 16:0) and lyso-phosphatidylcholine (lysoPC 16:0 and 18:0). The total and extracted ion chromatograms for the above phospholipids isolated from native, oxidized and glycated HDL were similar. However, concentrations of proposed oxidized molecular species including hydroperoxy-PC (*m/z* 790.4), epoxyhydroxy-PC (*m/z* 790.4) and trihydroxy-PC (*m/z* 808.3) were higher in oxidized HDL than in native and glycated HDL based on their ion intensity ratios.

In summary, we have utilized our recently developed CZE method to analyze HDL particles. Native, *in vitro* Cu^{2+} oxidized and glucose modified HDL particles showed distinctively different electropherograms. We have also optimized a SPE procedure and developed a new LC ESI-MS method to study HDL phospholipid molecular species. It appeared that oxidized HDL contained higher levels of oxidized PC molecular species than native and glycated HDL. It should be helpful to combine the CZE and LC ESI-MS methods for the analysis of high-density lipoproteins from patients. The combination of the two methods may also help for exploring the role of oxidized and glycated HDLs in the development of atherosclerosis.

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