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Research Article

Analysis of phospholipids using an open-tubular capillary column with a monolithic layer of molecularly imprinted polymer in capillary electrochromatography-electrospray ionization-tandem mass spectrometry

In this study, an open-tubular capillary electrochromatography (OT-CEC) column with a monolithic layer of molecularly imprinted polymer (MIP) based on methacrylic acid, ethylene glycol dimethacrylate, and 4-styrenesulfonic acid was utilized for the simultaneous separation and characterization of phospholipid (PL) molecular structures by interfacing with electrospray ionization-tandem mass spectrometry (ESI-MS-MS). Introducing an MIP-based monolith along with charged species at the OT column made it possible to separate PL molecules based on differences in head groups and acyl chain lengths in CEC. For the interface of OT-CEC with ESI-MS-MS, a simple nanospray interface utilizing a sheath flow was developed and the resulting OT-CEC-ESI-MS-MS was able to separate PL standards (phosphatidylserines, phosphatidylethanolamines, phosphatidylglycerols, phosphatidic acid, and lysophosphatidylglycerols). The developed method was applied to human urinary lipid extracts, and resulted in the separation and structural identification of 18 molecules by data-dependent collision-induced dissociation.

Keywords:
Capillary electrochromatography / Monolith / Open-tubular column / Phospholipids / Tandem mass spectrometry

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1 Introduction

Phospholipids (PLs) are the major constituents of cellular membranes and play important roles in signal transduction, energy storage, and cell proliferation and death [1–3]. The molecular structures of PLs are based on a glycerol backbone containing a polar head group and one or two acyl chains. Because of the different polar head groups and the different degrees of chain length and unsaturation in the acyl chain, the composition of PLs is very diverse. Lipidomics has recently grown alongside proteomics, and involves the study of extensive classes of lipid molecules, changes in lipid metabolism, and lipid-mediated signaling processes in relation to the development of biomarkers for adult disease. Analysis of PLs can be conducted with TLC either by densitometry [4, 5] or by MALDI-TOF-MS [6, 7], GC-MS [8, 9]. While GC requires a derivatization step to replace the polar head groups, HPLC provides an intact separation of PLs as well as simultaneous identification of the molecular structure when it is combined with ESI-tandem mass spectrometry (LC-ESI-MS-MS) [10–12]. A recent advancement in MS enables ESI-MS to provide an outstanding performance in the analysis of lipid molecules [13–16], however a separation method still requires prior to MS analysis due to the complexity of lipid molecules and the different signal intensities influenced by chain lengths and degrees of unsaturation in acyl chain as well as the different head groups.

CE is an alternative method for the analysis of PLs in terms of speed and separation efficiency with simple instrumental requirements. Compared with the efficiency of CE on a variety of biological substances, analysis of PLs with CE in the aqueous phase is rather limited due to the poor solubility of PLs as well as poor detection with UV. CE analysis of PLs has been attempted with MEKC using UV detection [17] or fluorescence detection [18, 19] with indirect UV detection using a background electrolyte [20, 21]. While

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Abbreviations: BPC, base peak chromatogram; **EDMA,** ethylene glycol dimethacrylate; **LPG,** lysophosphatidylglycerols; **MAA,** methacrylic acid; **MIP,** molecularly imprinted polymer; **OTC,** open-tubular capillary; **OT-CEC,** open-tubular capillary electrochromatography

Color Online: See the article online to view Figs. 1 & 5 in colour.

these approaches are reasonable for the assessment of PLs, the use of MEKC method has limitations in MS, and the latter two methods are indirect. To overcome these limitations, the use of organic solvents was introduced to CE to improve PL solubility and to facilitate the ESI-MS interface. NACE was utilized to separate four PL standards [22] or PL mixtures extracted from the rat peritoneal surface in combination with ESI-MS [23], and PLs from plant seeds with UV detection [24]. In most of these trials, the separation of PLs in CE was based on the polar head groups due to the polarity of the capillary surface. The separation of different acyl chain lengths has not been reported except for one case that used micelles [17], which is incompatible with ESI-MS.

CEC has the potential to be an alternative to CE analysis of PLs since it offers both electrophoretic and chromatographic features [25–27]. While CEC has rapidly progressed by utilizing polar stationary phases in packed or open-tubular capillary (OTC) columns, an OTC column with a porous, monolithic polymer layer provides flexibility to control the strong polar nature of the capillary wall [27]. However, due to the strong hydrophobicity of polymeric monolith layers, copolymerization with hydrophilic monomers is generally utilized and applied for the separation of oligosaccharides [28] and peptides or drugs [29] with polyacrylamide-based monoliths; polymethacrylate-based monoliths are used for the separation of alkaloids [30] or phenols [31].

In this study, we applied open-tubular CEC (OT-CEC) using a silica capillary column with a monolithic layer of molecularly imprinted polymer (MIP) for the separation of PLs and the simultaneous characterization of molecular structures by interfacing with ESI-MS-MS. The OT-CEC MIP column used in this study was prepared with a specific diluted monomer mixture composed of *S*-ketoprofen, methacrylic acid (MAA, functional monomer), ethylene glycol dimethacrylate (EDMA, cross-linker), and 4-styrenesulfonic acid (4-SSA, for electro-osmotic flow) [32]. The current OT-CEC monolithic column has recently been developed for the chiral separation of ketoprofen [32], but in this study it was directly utilized for the separation of PLs by chain length and polar head groups since the developed column exhibits both hydrophobic and hydrophilic properties. For the ESI interface, a simple nanospray interface between the OT-CEC and ESI-MS-MS was developed. The developed OT-CEC-ESI-MS was evaluated with PL standards and was applied to PL extracts from a human urine sample.

2 Materials and methods

2.1 Reagents

The phospholipid standards examined in this study included four phosphatidylserines (6:0/6:0 PS, 12:0/12:0 PS, 14:0/14:0 PS, and 18:0/18:0 PS), three phosphatidylglycerols (12:0/12:0 PG, 14:0/14:0 PG, and 18:0/18:0 PG), one phosphatidylethanolamine (14:0/14:0 PE), one phosphatidic acid (14:0/14:0 PA), and two lysophosphatidylglycerols (14:0-lyso-PE, 18:0-lyso-PE) purchased from Avanti Polar Lipids (Alabaster, AL, USA).

2.2 Preparation of samples and standard solutions

The urinary phospholipid sample was from a healthy human donor with a written consensus and PL extraction was conducted by lyophilization of 10 mL urine followed by the Folch method [33] with slight modification. Details of the urinary lipid extraction can be found in a previous report [12]. The extracted lipid mixture was dissolved in CH_3OH/CH_3CN (1:1) in a final volume of 0.1 mL and then diluted to a concentration of 46 μg/μL with CH_3OH/CH_3CN (9:1) for CEC-ESI-MS-MS analysis. To test the new CE-ESI-MS interface, the following standard peptides were used: bradykinin (1060.2 Da), luteinizing hormone-releasing hormone (LHRH, 1182.3 Da), angiotensin II (1046.2 Da), and oxytosin (1007.2 Da) obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade solvents (CH_3CN, CH_3OH, $CHCl_3$, isopropanol, and water) were used. Ammonium acetate and ammonium hydroxide were obtained from Sigma-Aldrich.

The OTC column with a monolithic layer of MIP was the same type utilized in a previous study [32]. The column was prepared by thermally initiated polymerization of the mixture composed of *S*-ketoprofen, MAA, EDMA, and 4-SSA in 9:1 CH_3CN/isopropanol solvent. The details for OT-CEC column fabrication can be found in a previous report [32]. The OT-CEC column used in this study was prepared from the silica capillary with a length of 30 cm (50 μm id, 365 μm od) purchased from Alltech (Deerfield, IL, USA). For nanospray ionization, the CEC column was inserted into a 3-cm GlassTip™ (0.94 mm id, 1.2 mm od, and 4 μm for tip id) obtained from New Objective, Inc. (Woburn, MA, USA).

2.3 Construction of the electrospray ionization interface for CEC-ESI-MS

A simple interface device for CEC-ESI-MS was constructed in our laboratory as shown in Fig. 1. The OT-CEC column was inserted into a Teflon tube (1/16″ od, 0.03″ id) using a Tefzel® Cross from Upchurch Scientific (Oak Harbor, WA, USA), through which a sheath flow was introduced and connected with a Pt wire (Fig. 1). The CEC column plumbing (the left side port of the Cross), Pt wire (the upper port), and a PEEK™ tubing sleeve (1/16″ od and 405 μm id) with a ferrule (1/16″) were utilized. For the Teflon (the right side port) and the sheath flow (the bottom side port) tubes, the same ferrule (1.16″) was used with each male nut. With this simple plumbing, electric voltage for nanoflow ESI was provided along with sheath flow to enable sufficient flow for ESI. The Teflon tube was connected with

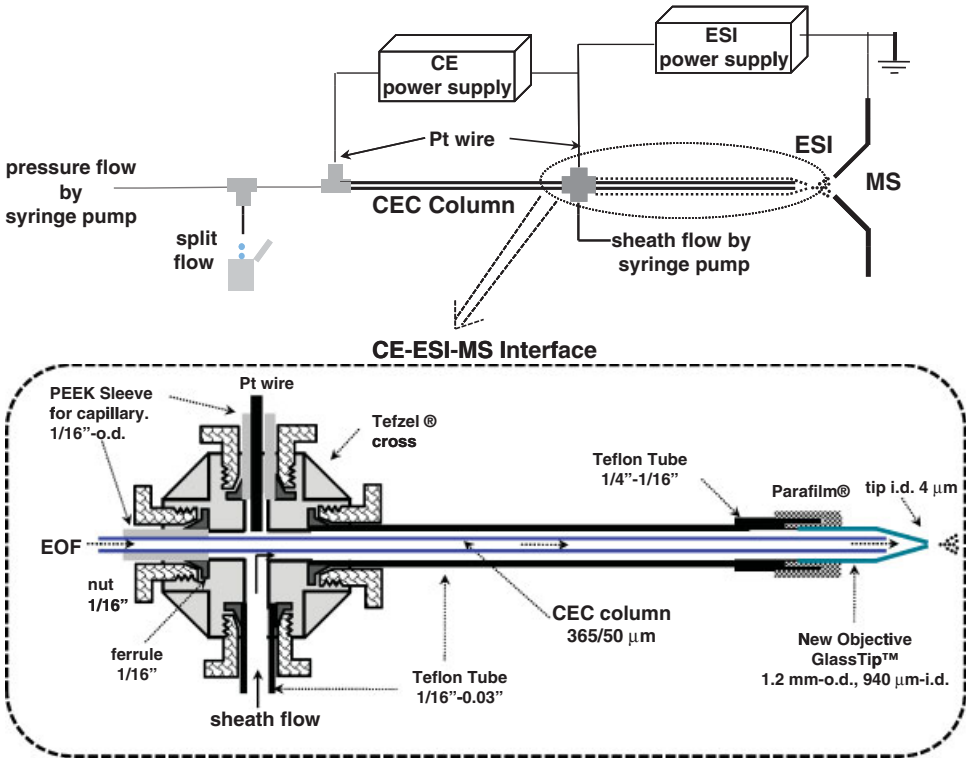

Figure 1. Schematic of the CE-ESI-MS; the interface developed herein is shown at the bottom.

a 3-cm long GlassTip^{TM} emitter with a 4 μm-id tip diameter using a short piece of Teflon tube (1/4″ od and 1/16″ id) wrapped with Parafilm® (right side of Fig. 1). The OT-CEC column was inserted into the GlassTip until the emitter tip so that dead volume between the column and the tip can be minimized. Sheath flow was delivered at a rate of 300 nL/min by a syringe pump equipped in the mass spectrometer. The solutions for sheath flow were 50:50 CH_3OH/water with 0.02% formic acid for peptide analysis and 80:20 CH_3OH/water with 0.05% NH_4OH for PL analysis.

2.4 CEC-ESI-MS-MS

Samples were loaded at the end of the capillary via siphoning for 15 s at a height of 10 cm and 18 kV was applied for EOF. For the new ESI interface performance test, an empty capillary (40 cm long, 50 μm id) for CZE was utilized by conditioning the capillary with 0.1 M NaOH for 1 min, water for 15 min, and CZE buffer for 15 min. For the CZE-ESI-MS test, peptide mixtures were utilized as test samples with a 60/40 CH_3CN/10 mM ammonium formate buffer solution (pH 4.0). For OT-CEC-ESI-MS analysis of PLs, a 60:30:10 2-propanol/ACN/50 mM ammonium acetate solution (pH 9.0 adjusted by adding ammonium hydroxide [34]) was utilized.

An LCQ Deca XP MAX ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) was employed in this study. For the ESI of CEC effluent with sheath flow, 1.70 kV (for peptides) was applied via the Pt wire at the positive ion mode of MS with a capillary temperature of 200°C, and 2.2 kV (for PLs) at the negative ion mode at the same temperature. The mass range of MS detection was 600–1000 amu for the first MS scan. For CID of eluted PLs, the data-dependent analysis mode was used for the three prominent ions from each MS scan under 35% normalized collision energy. The structural identification of PLs from the human urine sample was made by manual examination of characteristic fragment ions along with the m/z value of corresponding precursor ions.

3 Results and discussion

3.1 Evaluation of CEC-ESI-MS interface

For evaluating the performance of the developed CEC-ESI-MS interface (Fig. 1), an empty uncoated capillary column for CZE, in lieu of an OT-CEC column, was utilized to assess peptide mixtures. Using an uncoated capillary for peptide separation is to check the performance of the interface without the influence of the interaction of PLs with the MIP-based monolithic column. To detect peptides at the positive ion mode of ESI-MS, the CZE capillary cathode was inserted at the end of the emitter tip for ESI such that electrophoretic separation could be accomplished. Samples were loaded into the capillary anode via a height difference of 10 cm for 15 s. The sheath flow (50:50 CH_3OH/water with 0.2% formic acid) was applied via the Tefzel Cross at a flow rate of 300 nL/min to provide better ESI and a stable electric

current. With this configuration, ESI voltage was applied via the Tefzel Cross, without a metal emitter for direct contact with the electric power, and without an electrode wire inserted at the end of the emitter tube. Figure 2 shows the CZE-ESI-MS result of the standard peptide mixtures (1, bradykinin; 2, LHRH; 3, angiotensin II; 4, oxytocin; each concentration is 10 μM) separated at 18 kV and the eluted peptides are ionized at 1.7 kV. The half height widths of the eluted peptide peaks were 8–12 s, and the calculated plate number (*N*) values were 12 400, 9500, 6900, and 10 400 for peaks 1–4, respectively. Since the CE-ESI-MS interface was successful in providing electrospray ionization with 300 nL/min of sheath flow, an additional pressure flow was not considered for the CE-ESI-MS test of peptides.

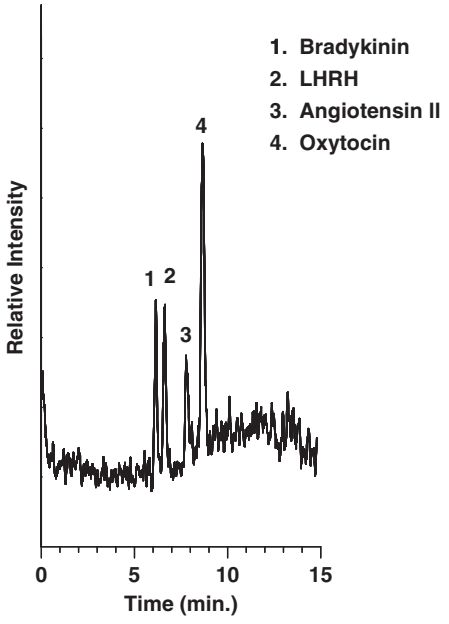

Figure 2. CZE-ESI-MS electropherogram of peptide standards (10 μM each) obtained at 18 kV. For separation, an uncoated 50 μm id × 30 cm fused-silica capillary column was used in 60:40 ACN/10 mM ammonium formate (pH 4) with a sheath flow (300 nL/min) of 50:50 methanol/water added with 0.2% formic acid.

3.2 OT-CEC-ESI-MS of PL standards

For PL separation, the OT-CEC column with the polymeric monolithic layer was inserted into the interface (Fig. 1). The three OT-CEC-ESI-MS electrochromatograms in Fig. 3 show the separation of (A) PLs by different head groups, and (B) PSs, and (C) PGs by different chain lengths. Separation of PLs with different polar heads (PE, PS, PA, and PG) having the two identical acyl chains (14:0/14:0) was attempted in Fig. 3 and the elution order was similar to a previously published study using NACE [21], but PA was not successfully resolved from PS and PG molecules. Table 1 lists the calculated values of LOD and LOQ for the four different PLs having identical acyl chains. Depending on polar head, LOD varies up to 10 fold. The CEC running solution was (60:30:10) 2-propanol/ACN/(50 mM ammonium acetate) (pH 9.0) and the same solution was driven at the anodic end of the CEC column via a syringe pump at a flow rate of 56 nL/min (split from the pump flow rate of 1.4 μL/min). Without this additional flow, no separation was achieved due to low EOF. Separation was also attempted with the pressure flow alone (without EOF), however it was not successful. Since the OT-CEC column utilized in this study had 4-SSA and MAA moieties contributing to the EOF by forming negative charge, the EOF at higher pH was faster. When the pH of the running solution was lower than 9.0, the EOF was too slow to drive the sample components and separation was not successful. With the current run conditions, PLs with the same polar heads but different chain lengths were examined (Fig. 3B and C). The concentrations of PL standards in Fig. 3 were 100 mM for each PS and PE, and 75 mM for each PG. Siphoned injection was conducted for 45 s at a 10 cm height. As shown with PSs and PGs, a relatively poor but acyl chain-dependent separation was achieved in decreasing order of chain length, which was opposite to the separation order obtained with reversed-phase nLC-ESI-MS [11]. As the chain length increased, hydrophobicity of the PL

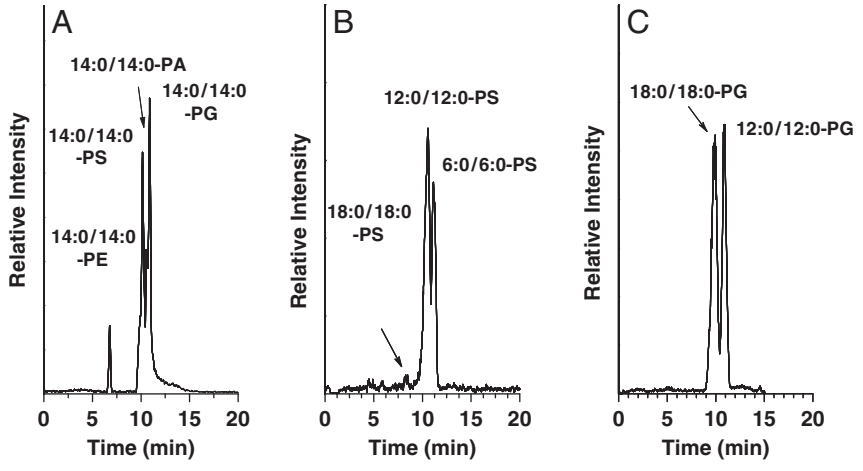

Figure 3. OT-CEC-ESI-MS electrochromatograms of (A) four PLs with different head groups having identical acyl chains (14:0 for both); (B) PSs; and (C) PGs with different chain lengths. An OT-CEC column with a polymeric monolithic layer was utilized in a solution of 60:30:10 isopropanol/ACN/50 mM ammonium acetate at 18 kV. ESI voltage at the negative ion mode was 2.2 kV with a sheath flow (500 nL/min) of 80:20 methanol/water added with 0.05% ammonium hydroxide.

molecule increased, and thus retention on the C18 column increased. However, due to the polar nature of the OT-CEC column with the monolithic polymeric layer, PS molecules with longer acyl chains eluted earlier. Likewise, if the PL molecule contained unsaturated acyl chains, it is expected to retain longer than that those with saturated acyl chains.

In Fig. 4A, separation of a mixture of six PLs under the same run conditions is demonstrated. Eluted PL molecules were examined via a data-dependent CID for structural identification. Figure 4B shows the MS-MS spectra of a 14:0/14:0-PE molecule showing the parent ion $[M\text{-}H]^-$ at m/z 634.6 with characteristic fragment ions at m/z 590.0 $[M\text{-}H\text{-}43]^-$ for the loss of ethanolamine (CH_2CH_2NH), and m/z 424.2 and m/z 406.6 for the loss of fatty acids in the form of ketene and carboxylic acids, respectively. The spectra also showed a peak corresponding to the acyl chain fragment as a form of carboxylate ion at m/z 227.9 $[RCOO^-]$. It was noted that the two lysophosphatidylglycerols (18:0-LPG and 14:0-LPG) were separated from each other at the end of the run. Lysophospholipid (LPL) has only one acyl chain attached either to terminal or at the center hydroxide of glycerol backbone, making it more polar than PL. In Fig. 4, 18:0-LPG eluted with 12:0/12:0-PG, however it showed a clear separation from 14:0-LPG. This is supported by the CID spectra of 14:0-LPG (m/z 455.9 at precursor scan) in Fig. 4C with the fragment ion peaks at m/z 153.7 and 227.4 of which the former ions were generated from the dissociation of carboxylic acid from the parent ion without the glycerol head group (–$CHCHOHCH_2OH$, 74 Da) as $[M\text{-}H\text{-}74\text{-}RCOOH]^-$ and the latter ions from the carboxylate anion $[C_{13}H_{27}COO]^-$. It is also noted that a $[M\text{-}H\text{-}74\text{-}OH]^-$ peak appeared with relatively weak intensity at m/z 361.7.

Table 1. LOD and LOQ for four different PLs with an identical acyl chain length

Phospholipids	Migration time	Area		
	RSD (%)	RSD (%)	LOD (μM)	LOQ (μM)
14:0/14:0-PA	2.80	17.56	14.38	19.60
14:0/14:0-PE	3.55	12.44	9.05	24.13
14:0/14:0-PG	3.98	8.53	1.53	2.93
14:0/14:0-PS	4.44	18.11	8.44	9.74

The acyl chain structure of all four PLs ($n = 5$) was identical (14:0/14:0), as shown in Fig. 3A.

3.3 OT-CEC-ESI-MS-MS for human urinary PLs

The developed run condition was applied for the separation and characterization of PL extracts from a human urine sample. Since human urine is a noninvasive sample that can be readily obtained and the amount of urinary PLs is much smaller than that of blood sample, it can be more informative to develop an OT-CEC-ESI-MS-MS method for urinary PLs. Figure 5 shows the base peak chromatogram (BPC) of the urinary PL extract obtained by CEC-ESI-MS-MS at the negative ion mode. Due to the complexity of the urinary extract, it was presumed to contain a variety of lipids, and the observed MS chromatogram appeared to be broad in distribution. By CID experimentation, the molecular structure of PLs can be identified from their fragmentation spectra. For instance, parent ions of m/z 810.0 observed by the MS scan at $t_r = 11.73$ min in Fig. 5A were subjected to data-dependent MS-MS analysis resulting in the corresponding CID spectra shown in Fig. 5B. The base peak ion in the CID spectra represents $[M\text{-}H\text{-}87]^-$, indicating the loss of serine ($C_3H_4NO_2$, 87 Da), and the fragment ions of m/z 438.7

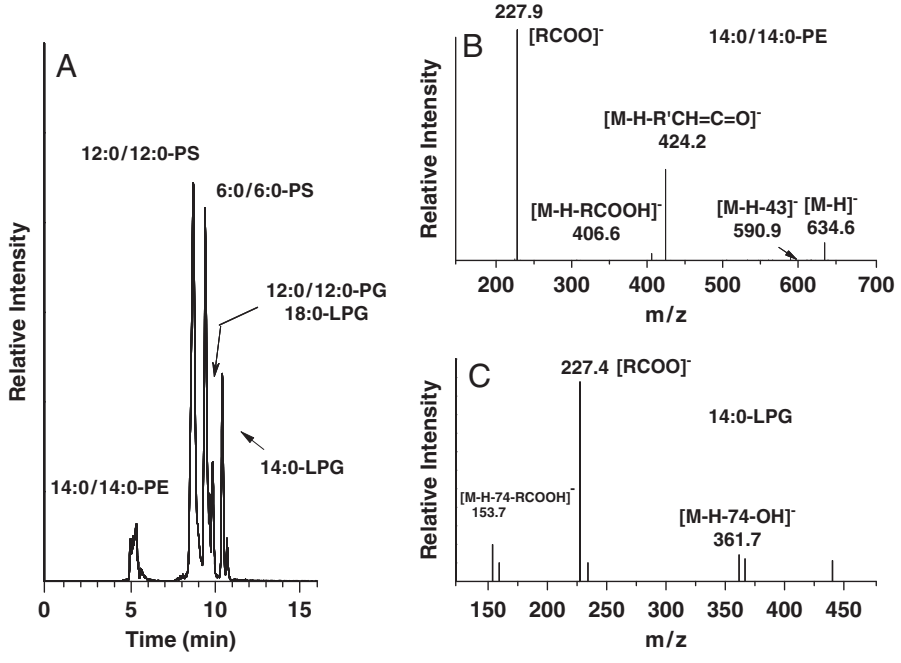

Figure 4. (A) BPC of OT-CEC-ESI-MS of six phospholipids at the negative ion mode; data-dependent MS-MS spectra of (B) 14:0/14:0-PE and (C) 14:0-LPG representing the characteristic fragment ions. Experimental conditions in run (A) were the same as those described in Fig. 3.

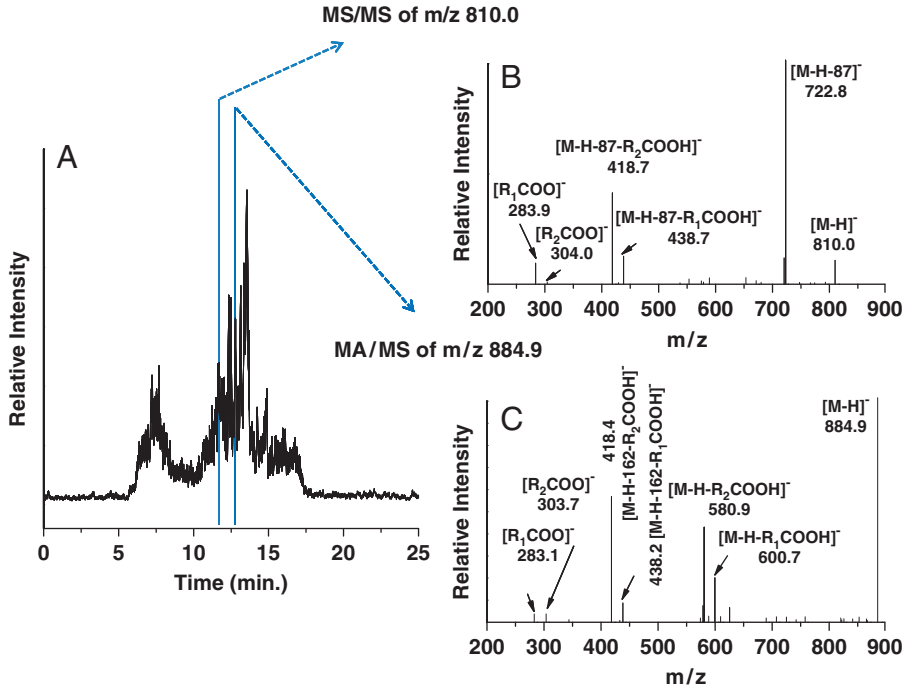

Figure 5. (A) BPC of urinary PL extracts from a healthy adult obtained by OT-CEC-ESI-MS at the negative ion mode along with MS-MS spectra of ions (B) *m/z* 811.0 (t_r = 11.74 min) and (B) *m/z* 885.8 (t_r = 12.69 min) during OT-CEC-ESI-MS-MS. Separation conditions were the same as those described in Fig. 3.

Table 2. PLs identified from a healthy human urine extract by CEC-ESI-MS-MS at the negative ion mode

Class	Molecular species	*m/z*	t_r (min)
PE	16:0/18:1	717.8	7.33
	16:0/18:2	716.0	7.67
PG	18:1/14:2	716.2	11.63
PS	18:1/22:3	839.1	11.63
	16:0/18:1	761.4	11.66
	18:0/20:4	811.0	11.74
	18:1/18:0	788.6	11.76
	18:0/20:3	812.5	11.81
	16:0/20:4	782.4	11.81
	20:0/18:0	819.0	11.84
PI	18:0/18:1	863.0	11.71
	18:0/20:4	885.8	12.69
	18:0/20:3	887.2	12.82
	20:2/20:1	916.7	14.55
PA	20:4/20:0	750.7	12.12
	20:4/18:0	722.6	12.15
	22:2/20:4	775.5	12.23
	18:1/18:0	702.3	13.41

and 418.7 reflect the loss of fatty acids from the base peak as $[M\text{-}H\text{-}87\text{-}R_1COOH]^-$ and $[M\text{-}H\text{-}87\text{-}R_2COOH]^-$, respectively. Moreover, ions of *m/z* 283.7 and *m/z* 304.0 were the two cleaved carboxylate anions, $[R_1COO]^-$ and $[R_2COO]^-$, respectively. This PL was identified as 18:0/20:4-PS based on the peaks from the CID spectra along with the parent ion peak $[M\text{-}H]^-$ at *m/z* 810.0. Likewise, parent ions *m/z* 884.9 detected at t_r = 12.67 min were identified as 18:0/20:4-PI with the corresponding CID spectra shown in Fig. 5C. In the latter case, fragment ions that resulted from the loss of fatty acids from both parent ions and parent ions without inositol head groups (= 162 Da) were observed as $[M\text{-}H\text{-}R_1COOH]^-$ and $[M\text{-}H\text{-}162\text{-}R_1COOH]^-$, respectively. A total of 18 PL molecules (2 PEs, 1 PG, 7 PSs, 4 PIs, and 4 PAs) were identified in the urine extract (Table 2). Since the current column operates at a negative ion mode of MS, phosphatidylcholine (PC) molecules were not detected due to the difficulty in ionization of PC as a negative ion. Though the total number of identified PL molecules was lower than what was found from similar healthy control samples by nLC-ESI-MS-MS analysis in the literature (64 PLs) [35], this method shows potential for the utilization of OT-CEC-ESI-MS-MS for the characterization of PLs from urine at high speed. The use of reversed-phase nanoflow LC-ESI-MS-MS requires at least more than an hour of separation at each ion mode of MS detection.

4 Concluding remarks

This work demonstrates that the current OT-CEC-ESI-MS-MS shows a potential to separate PLs by acyl chain length and polar head groups at high speed, and the use of a proper interface to ESI-MS enables one to characterize 18 PL molecules by MS-MS. Separation of PLs by acyl chain length in CE with or without the use of organic solvents has not been reported due to the limitation in the polar nature of capillary surface. To the best of our knowledge, this is the first report showing that PLs can be separated using OT-CEC by their chain length and polar head group characteristics. The current study shows the potential of

OT-CEC-ESI-MS-MS for the analysis of PLs, and further improvement of column materials with optimization in separation could result in the further development of a high-speed separation and characterization method for PLs.

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The authors have declared no conflict of interest.

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