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Analysis of Oxidized 1-Palmitoyl-2-Arachidonoyl-Sn-Glycero-3 Phosphocholine Products in Uremic Patients by LC-ESI/MS

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Abstract: A simple liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI/MS) method has been developed to analyze oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (ox-PAPC) products on the lipoproteins of uremic patients. The native PAPC standard was in vitro oxidized by the Fenton reaction, and the ox-PAPC products were analyzed by LC- ESI/MS. For LC, a C_8 column and a mobile phase (acetonitrile-isopropanol containing 0.1% formic acid (70:30, v/v)) were selected. For ESI/MS, the optimal conditions included sheath gas pressure (10 psi), capillary temperature (270 °C), and injection time (1000 ms). The identification of ox-PAPC products on human lipoproteins was based on the extracted ion chromatograms (EICs) and the ESI-MS spectra of the in vitro oxidation products of PAPC standard. The EICs and ESI-MS spectra showed good repeatability and sensitivity. A total of 21 ox-PAPC products was determined. Linear analysis has been performed for the phospholipid standard, 1, 2-Di-O-hexadecyl-sn-glycero-3-phosphocholine (PC(O-16:0/O-16:0)). The linear range was 5.0–100.0 μg/mL, and the coefficient of determination (R^2) was 0.989. The concentration limit of detection (LOD) was 1.50 μg/mL, and the concentration limit of quantitation (LOQ) was 4.54 μg/mL. The selected 21 ox-PAPC products have been identified and quantified in very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) of uremic and healthy subjects. Interestingly, the results showed that the levels of 18 products in VLDL, one product in LDL, and 19 products in HDL were significantly higher for uremic patients than healthy controls. This simple LC-ESI/MS method might accelerate the searching for biomarkers of uremia in the future.

Keywords: oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine; lipoproteins; uremia; LC-ESI/MS

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1. Introduction

Minimally modified LDL (MM-LDL) activates human aortic endothelial cells (HAECs) to bind monocytes. Monocyte recruitment is a key step in the progression of atherosclerosis. It was found that biologically active oxidized phospholipids played a key role in the activation of endothelial cells by MM-LDL. Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (ox-PAPC) showed similar biological activity to MM-LDL [1–5].

The chemical structure of PAPC is shown in Figure 1. It was found that the oxidation products of PAPC up-regulated about 600 genes and down-regulated about 400 genes in endothelial cells. Ox-PAPC products increased the expression of some important chemokines such as interleukin-6 (IL-6), interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), and the tissue factor (TF) of HAECs [1,2]. Three bioactive oxidized phospholipids, 1-palmitoyl-2-oxovaleroyl-sn-glycero-3-phosphocholine (POVPC), 1-palmitoyl-2-glutarol-sn-glycero-3-phosphocholine (PGPC) and 1-palmitoyl-2-(5, 6–epoxyisoprostane E_2)-sn-glycero-3-phosphocholine (PEIPC), were found to play critical roles on the biological activity of MM-LDL. All of them were the oxidation products of PAPC and were found in atherosclerotic lesions [1–5].

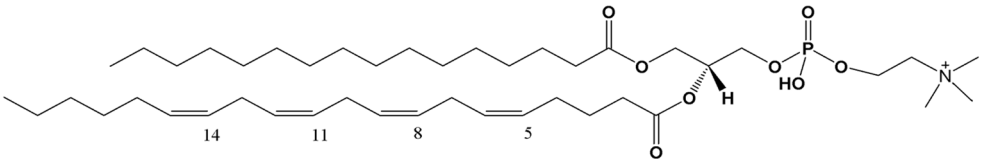


Figure 1. Structure of 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PAPC).

The polyunsaturated fatty acyl chain on the *sn*-2 position of PAPC is the target for non-enzymatic and enzymatic oxidation. Non-enzymatic oxidation is initiated by free radicals or non-radical reactive oxygen species (ROS). Free radicals include OH radical, OOH radical, NO radical, NO_2 radical, and superoxide anion radical. Sources of free radicals are air pollution, smoking, UV-light, radiation, and several enzymes (e.g., NADPH oxidase and xanthine oxidase). Examples of non-radical ROS included singlet oxygen and ozone [6–8]. Enzymes including 12/15 lipoxygenases and cytochrome c were capable of oxidizing (not via free radicals) phospholipids containing polyunsaturated fatty acids [9–11].

The determination of phospholipids and their oxidation products in tissues and biological fluids has opened a new window for research. Detailed knowledge about the structural changes of phospholipids under oxidative stress is critical for understanding their biological activities and the associated pathological processes [12–15].

ESI-MS is a soft ionization method without extensive fragmentation, and it is suitable for analyzing biological molecules. Previously, some researchers have analyzed the chemical structures of ox-PAPC products based on LC-MS and LC-MS/MS [16–21]. The oxidation of PAPC produces a large diversity of molecular species, and many of them are bioactive. In addition, oxidation products also vary with oxidative stress. Many of the oxidation products of PAPC have not been identified so far, and their biological effects have not been understood. A study of the structural modification of phospholipids under oxidative stress is critical for exploring their biological activities and understanding the associated pathological processes. However, the separation and identification of ox-PAPC molecules are extremely difficult due to the structural similarity of the oxidation products.

The aim of this study was to develop an LC ESI-MS method to analyze the in vitro oxidation products of the native PAPC standard. The developed method was then applied on the lipoprotein samples of uremic patients and healthy subjects to observe its utility. A total of 21 ox-PAPC products have been analyzed and quantified in VLDL, LDL, and HDL of uremic patients. Moreover, their concentrations have been compared between healthy and uremic subjects. To the best of our knowledge, this is the first study to determine ox-PAPC products on the lipoproteins of uremic patients by LC-ESI/MS.

2. Materials and Methods

2.1. Chemicals

The chemicals used in this study included acetonitrile (CH_3CN; CAS number: 75-05-8, HPLC grade, Echo Chemical, Miaoli, Taiwan), isopropanol (C_3H_7OH; CAS number: 67-63-0, Echo Chemical), deionized water (di water; CAS number: 77-32-18-5, Millipore Simplicity; Millipore, Billerica, MA, USA), formic acid (HCOOH; CAS number: 64-18-6, Acros Organics, Geel, Belgium), chloroform ($CHCl_3$; CAS number: 67-66-3, J. T. Baker, Phillipsburg, NJ,

USA), methanol (CH_3OH; CAS number: 67-56-1, Echo chemical), phosphate buffered saline (PBS; MDL number: MFCD00131855, Sigma Chemical Sigma, Chemical, St. Louis, MO, USA), hydrogen peroxide (H_2O_2, 34–36.5%; CAS number: 7722-84-1, Sigma Chemical), ferrous chloride ($FeCl_2 \cdot 4H_2O$; CAS number: 13478-10-9, J. T. Baker), potassium bromide (KBr; CAS number: 7758-02-3, J. T. Baker), ethylenediaminetetraacetic acid (EDTA; CAS number: 60-00-4, Sigma Chemical), 1-pammitoyl-2-arachidonoyl-3-glycerophosphocholine (PAPC; CAS number: 35418-58-7, Avanti Polar Lipids, Inc., Alabaster, AL, USA), and 2,6-di-tert-butyl-4-methylphenol ($[(CH_3)_3C]_2C_6H_2(CH_3)OH$) (BHT; CAS number: 128-37-0, Sigma Chemical).

2.2. In Vitro Oxidation of PAPC Standard

The procedures for oxidation, extraction, and analysis of PAPC standard are described in this section. First, a 2.5 mg of PAPC standard was mixed with freshly prepared 1.0 mL of $FeCl_2 \cdot 4H_2O$ and H_2O_2 (in di water). The ratios of $FeCl_2$:H_2O_2 = 2:1 (0.4 mM:0.2 mM). The solution mixture was incubated at 37 °C in a water bath for 15 min. Ox-PAPC products were extracted by a 0.5 mL of ice-cold methanol/chloroform (1:2 *v*/*v*) and briefly vortexed. The solution mixture was centrifuged at 13,000 rpm and room temperature for 3 min. Then, it was kept in ice-cold water for 3 min. The above two steps were repeated for another two times. The lower organic phase containing ox-PAPC products was collected and kept in ice-cold water. The above liquid-phase extraction procedure was repeated another two times, and the ice-cold organic phases were combined and dried under a stream of nitrogen gas. Then, a 1.0 mL of ice-cold chloroform was added to reconstitute the dried ox-PAPC products. The reconstituted ox-PAPC products were kept at −30 °C until analyzed by LC-ESI/MS.

In all of the procedures for the oxidation, extraction and analysis of PAPC standard, glassware is used as the equipment. The glassware has also been carefully cleaned and dried to avoid contamination. The reagents, including native PAPC standard, $FeCl_2 \cdot 4H_2O$, H_2O_2, di water, methanol, and chloroform, have been checked for impurities before reaction and analysis. Extra care has been performed during all of the experimental procedures to avoid contamination from the equipment and reagents. In addition, extra care has been taken to avoid re-oxidation of the ox-PAPC products. Ice-cold solvents were used for extraction. Between centrifugations, the solution mixtures were kept in ice-cold water. The final ox-PAPC products were kept at −30 °C before analysis.

2.3. Collection of Human Blood and Separation of Lipoproteins by Ultracentrifugation

Healthy human blood was collected from healthy subjects who had no known medical history. Uremic patients' blood was collected before the dialysis session at the time of routine laboratory investigations. Written informed consent was obtained from 10 healthy subjects and 10 uremic patients before blood sampling. The study protocol was approved by the China Medical University and Hospital (Taichung, Taiwan) institutional review board (reference number CMUH 110-REC2-047).

Lipoprotein fractions, including VLDL, LDL, and HDL, were isolated from each subject's plasma. The ox-PAPC products were analyzed for the VLDL, LDL, and HDL fractions of the individual subject. Therefore, a total of 10 uremic VLDL samples and 10 healthy VLDL samples were analyzed. LDL and HDL samples were analyzed in a similar way.

The separation of lipoprotein fractions was performed using a Beckman Coulter Optima™ XL-100K ultracentrifuge (Brea, CA, USA). First, KBr was added into human plasma to adjust the density to 1.019g/mL, and then the plasma was divided into 8 tubes with 10 mL in each tube. For VLDL, the plasma was subjected to ultracentrifugation at 45,000 rpm (174,000 g) and 5 °C for 18 h. The floating layer (2 mL for each tube) was collected as a VLDL fraction. For LDL, the plasma density was adjusted to 1.063 g/mL, and it was divided into 6 tubes. The plasma was then subjected to ultracentrifugation at 45,000 rpm (174,000 g) and 5 °C for 18 h. The floating layer (3 mL for each tube) was

collected as a LDL fraction. For HDL, the plasma density was adjusted to 1.210 g/mL, and it was divided into 4 tubes. The plasma was then subjected to ultracentrifugation at 45,000 rpm (174,000 g) and 5 °C for 48 h. The floating layer (3 mL for each tube) was collected as a HDL fraction. The collected lipoprotein fractions were immediately used for in vitro oxidation; otherwise they were kept at −80 °C until they were used.

2.4. Extraction of Phospholipids from Human Lipoproteins

A 400 µL of isolated lipoprotein fraction (VLDL, LDL, or HDL) was mixed with a 500 µL of cold methanol/chloroform (1:2 *v*/*v*) solution and briefly vortexed. The methanol portion contained 15 µg/mL of BHT to prevent phospholipid oxidation during the extraction procedures. The solution mixture was incubated in a cold water bath for 20 min. It was then centrifuged at 13,000 rpm and room temperature for 3 min. The whole solution was again incubated in a cold water bath for 2 min. It was then again centrifuged at 13,000 rpm and room temperature for 2 min. The lower organic phase containing phospholipids was collected. The above liquid-phase extraction procedure was repeated for a total of three times, and the lower organic phases were combined and dried under a stream of nitrogen gas. Then, a 200 µL of methanol was added to reconstitute the dried phospholipids. Glass containers and syringes were used for the above procedures to prevent the dissolution of polymers by organic solvents. Finally, LC-ESI/MS analysis was performed to analyze ox-PAPC products in human lipoproteins.

2.5. LC-ESI/MS Analysis of Ox-PAPC Products

First, we have developed an LC ESI-MS method to analyze the in vitro oxidation products of native PAPC standard. The developed method was then applied on the lipoproteins of uremic patients and healthy subjects to observe its utility.

For the analysis of in vitro oxidation products of a PAPC standard, a native PAPC standard was oxidized and extracted as described in Section 2.2. A 20 µL sample was introduced into a Waters HPLC system (Waters Corporation, Milford, MA, USA). The HPLC instrument was equipped with a photodiode array detector (Waters 966).

In order to optimize the LC ESI-MS method, a huge number of experiments have been performed. The ion at *m*/*z* 846.5 is one of the abundant in vitro oxidation products of the PAPC standard, and it has been selected to test the effects of various experimental parameters of HPLC and ESI-MS.

For HPLC, two reverse-phase columns, including C_{18} and C_8 columns, were examined. Both columns had particle sizes of 3.5 µm, internal diameters of 4.6 mm, and lengths of 15 cm. At the beginning, various mobile phases were tested for the C_{18} and C_8 columns. Several organic solvents, including methanol, acetonitrile, isopropanol, and di water, have been tested as mobile phases. In summary, the solvent containing 70% acetonitrile + 30% isopropanol + 0.1% formic acid tested for the C_8 column showed the best column efficiency for the ox-PAPC ion at *m*/*z* 846.5. As a result, this separation system was selected as being the best for HPLC conditions.

An LCQ ion-trap mass spectrometer (Finnigan Corporation, San Jose, CA, USA) was connected to the HPLC instrument. Ox-PAPC products were analyzed in positive ion mode. The ion spray voltage chosen was 4.5 kV. The capillary voltage was maintained at 46 kV. The tube lens offset was set at −5 V. Mass spectrometry data were analyzed by Xcalibur software (version 1.2; Finnigan Corporation). For the ESI/MS analysis of ox-PAPC products, the selected optimal capillary temperature was 270 °C, the sheath gas pressure was 10 psi, and the injection time was 1000 ms.

3. Results and Discussion

3.1. Optimization of LC ESI-MS Conditions for Analyzing In Vitro Oxidized PAPC Products

In this study, an LC ESI-MS method has been developed to analyze the in vitro oxidation products of native PAPC standard. The optimal method was applied to analyze the lipoproteins of uremic patients and healthy subjects to observe its utility. The ion at

m/z 846.5 is one of the abundant in vitro oxidation products of PAPC, and it has been selected for method development. As described in Section 2.5, a tremendous number of experiments have been carried out to develop the LC ESI-MS method. The best HPLC conditions included a solvent mixture of 70% acetonitrile + 30% isopropanol + 0.1% formic acid, and a C_8 column. Figure 2 shows the EIC of ox-PAPC product m/z 846.5 analyzed by the best conditions. A major peak shows near 10 min. The peak is narrow and has a width about 1.0 min. This selected system has much better separation efficiency than the other systems, as mentioned in Section 2.5. The high S/N ratio is due to the very low concentration of ox-PAPC. Although the original concentration of the native PAPC standard used for in vitro oxidation is 100 µg/mL, the produced ox-PAPC products actually have much lower concentrations.

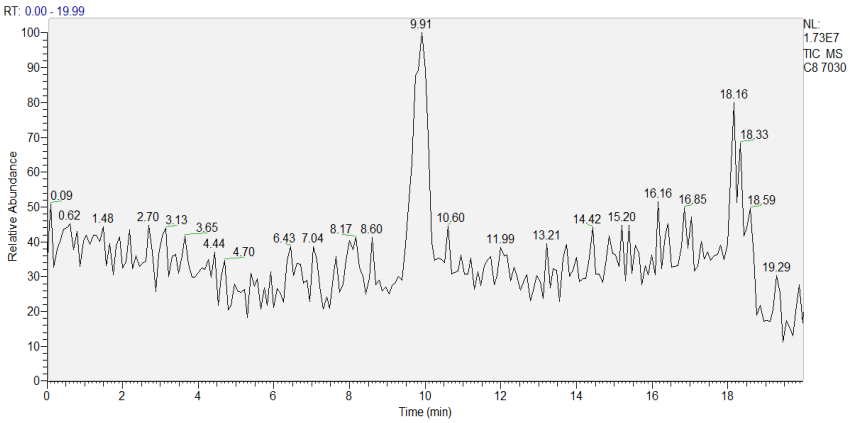
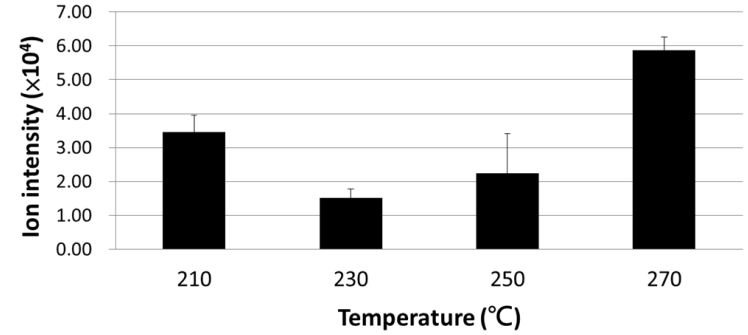


Figure 2. The EIC of ox-PAPC product m/z = 846.5. Ox-PAPC was prepared by in vitro oxidation ($FeCl_2$:H_2O_2 = 2:1) of 100 µg/mL native PAPC standard at 37 °C for 15 min. The selected stationary phase was a C_8 column. The selected mobile phase contained 70% acetonitrile, 30% isopropanol, and 0.1% formic acid.

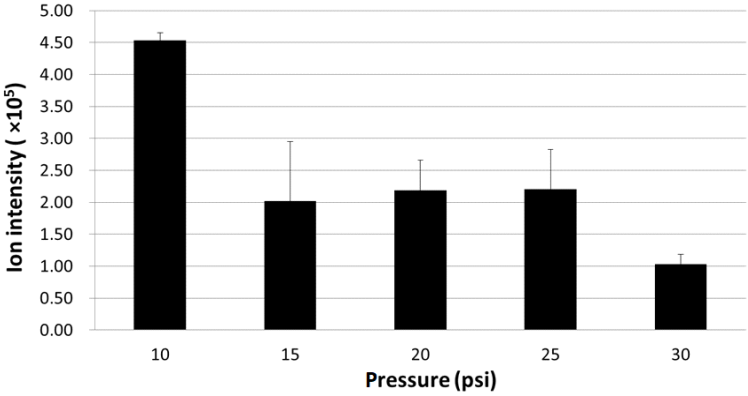
To find the best ESI/MS conditions for analyzing ox-PAPC products, three important experimental parameters, including capillary temperature, sheath gas pressure, and injection time have been examined. The ion intensity of the abundant in vitro ox-PAPC product m/z 846.5 has been chosen to test the effects. To optimize the capillary temperature, various temperatures including 210, 230, 250 and 270 °C were examined. It was found that 270 °C showed the highest relative ion intensity (Figure 3A). To optimize the sheath gas pressure, various pressures including 10, 15, 20, 25 and 30 psi were examined. It was found that 10 psi showed the highest relative ion intensity (Figure 3B). To optimize the injection time, various injection times including 20, 200 and 1000 ms were examined. It was found that 1000 ms showed the highest relative ion intensity (Figure 3C). As a result, the best conditions for the ESI/MS analysis included capillary temperature (270 °C), sheath gas pressure (10 psi) and injection time (1000 ms).

The chemical structure of PAPC (Figure 1) contains four double bonds at C-5, C-8, C-11, and C-14 of the *sn*-2 fatty acyl chain. The ESI-MS spectra of native PAPC and in vitro ox-PAPC are shown in Figure 4A,B. In Figure 4A, one major ion appeared in the ESI-MS spectrum. The major ion at m/z 782.8 represented the $[M+H]^+$ ion, and the ion at m/z 804.5 represented the $[M+Na]^+$ ion of PAPC. In Figure 4B, one major ion and numerous minor ions appeared in the ESI-MS spectrum of ox-PAPC. The two ions at m/z 782.8 and 804.8 represented the $[M+H]^+$ and $[M+Na]^+$ ions of PAPC, respectively. The large numbers of minor ions represented the oxidation products of PAPC. The *sn*-2 acyl chain of PAPC is susceptible to oxidation since it contains four double bonds, and thus the oxidation of PAPC produces a large diversity of molecular species. The chemical structures of the oxidation products are very similar; therefore, the separation and identification of ox-PAPC molecules is significantly difficult. Several researchers have analyzed ox-PAPC products based on

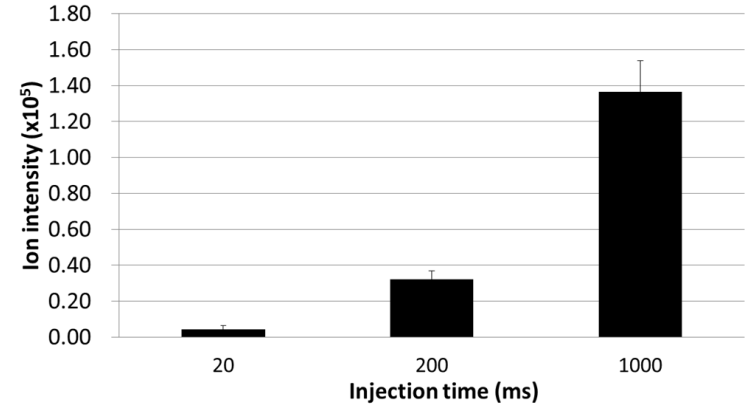
LC-MS and LC-MS/MS previously [16–21]. However, many the oxidation products have not been identified, and their biological effects in human disease are not understood.



(**A**)



(**B**)



(**C**)

Figure 3. Effects of (**A**) capillary temperature, (**B**) sheath gas pressure and (**C**) injection time for in vitro ox-PAPC product m/z 846.5. Ox-PAPC was prepared by in vitro oxidation ($FeCl_2$:H_2O_2 = 2:1) of 100 μg/mL native PAPC standard at 37 °C for 15 min. The ion intensities were measured from the selected ion monitoring (SIM) spectra of the ion m/z 846.5.

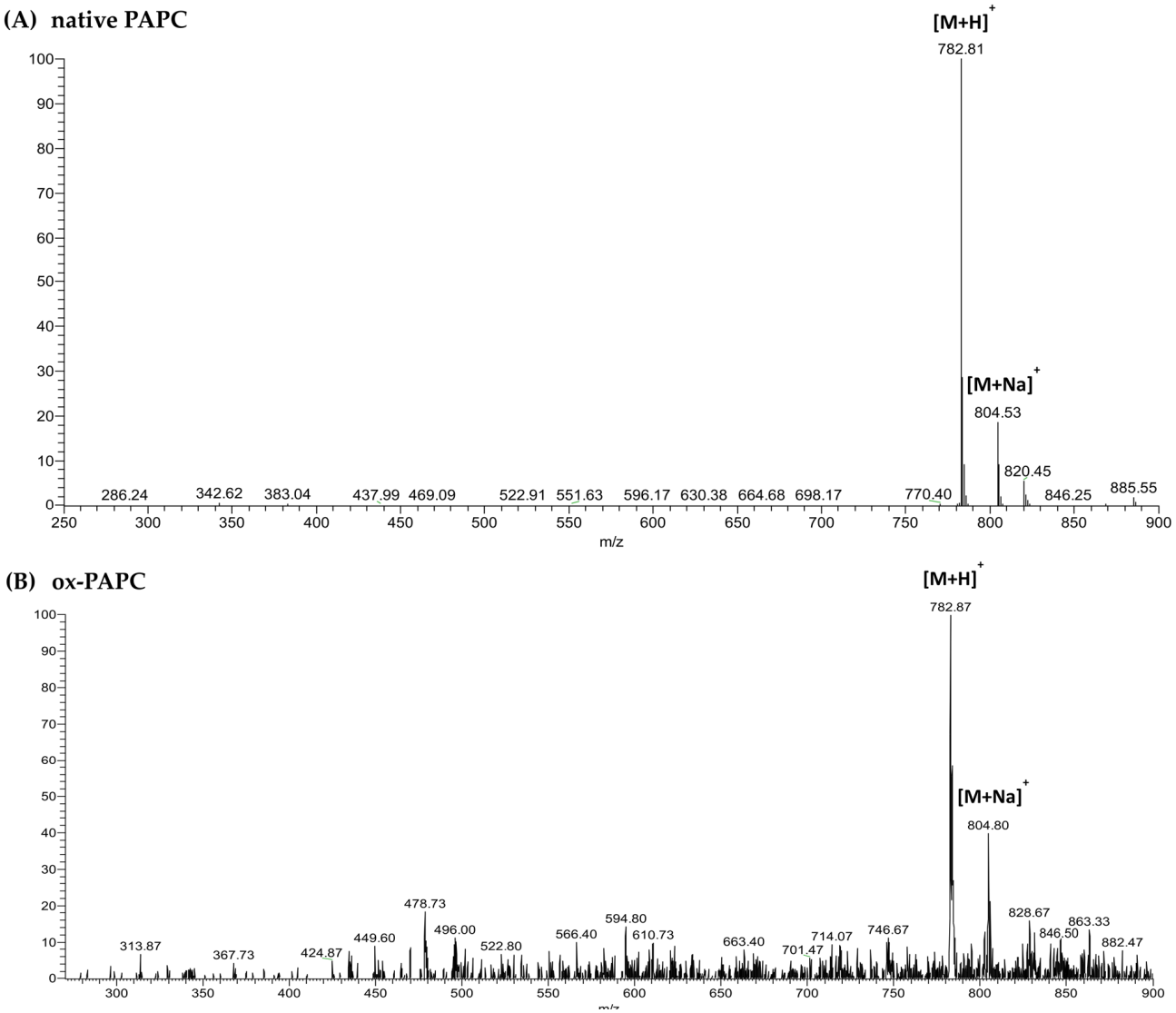


Figure 4. The ESI-MS spectra of (**A**) native PAPC and (**B**) ox-PAPC. Native PAPC standard was prepared at a concentration of 100 μg/mL. Ox-PAPC was obtained by in vitro oxidation ($FeCl_2$:H_2O_2 = 2:1) of native PAPC standard at 37 °C for 15 min. The conditions used for ESI-MS analysis were sheath gas pressure, 10 psi; capillary temperature, 270 °C; and injection time, 1000 ms.

Figure 5 shows the relative ion intensities of some important ox-PAPC products from in vitro oxidation of native PAPC standard. They included 10 short-chain and 9 long-chain ox-PAPC ions and were analyzed by the optimal ESI-MS conditions. Among them, the ions at m/z 594, 610, 820.5, 828.5, and 846.5 are more abundant than others. In this study, these 19 ox-PAPC ions and two other ions m/z 478, 496 were analyzed for healthy and uremic subjects, and their relative levels were compared. The proposed structures of the 9 long-chain and 12 short-chain ox-PAPC products are shown in Supplementary Tables S1 and S2 [16–21].

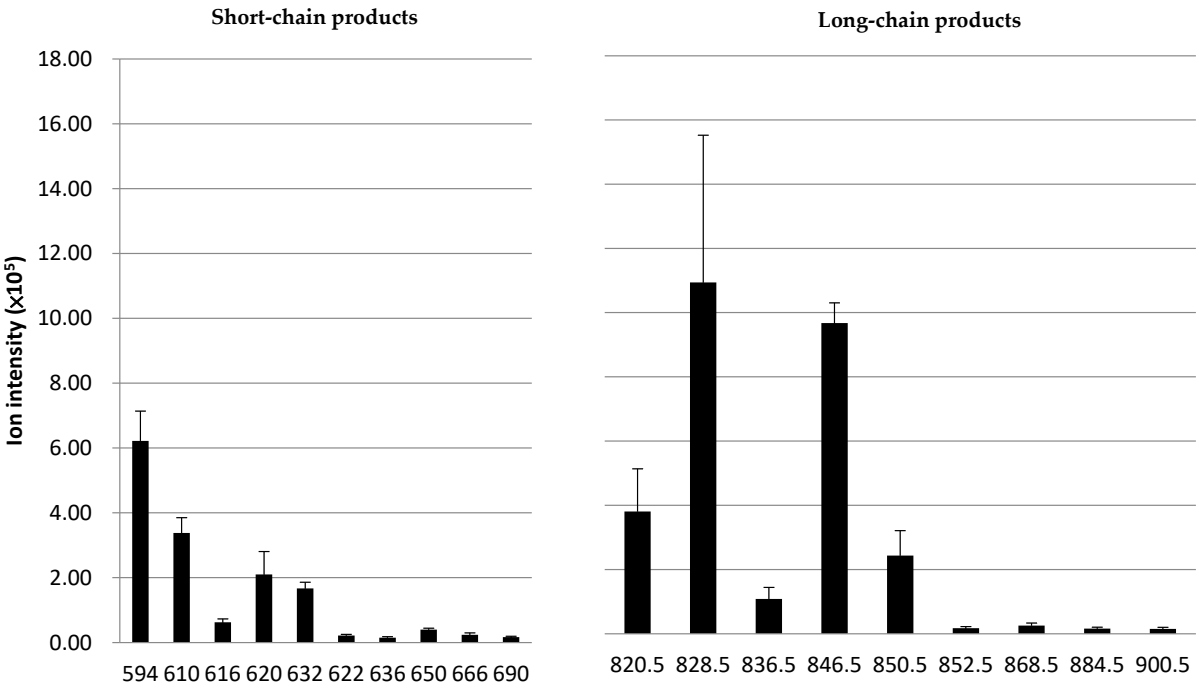
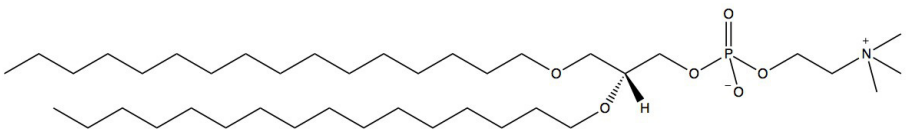


Figure 5. Comparison for the short-chain and long-chain ox-PAPC products. Ox-PAPC was obtained by in vitro oxidation ($FeCl_2$:H_2O_2 = 2:1) of 100 μg/mL native PAPC standard at 37 °C for 15 min. The ion intensities of ten short-chain (*m*/*z* 594.0, 610.0, 616.0, 620.0, 622.0, 632.0, 636.0, 650.0, 666.0, and 690.0) and nine long-chain (*m*/*z* 820.5, 828.5, 836.5, 846.5, 850.5, 852.5, 868.5, 884.5, and 900.5) ox-PAPC products were analyzed by ESI-MS. The ESI/MS conditions were as in Figure 4.

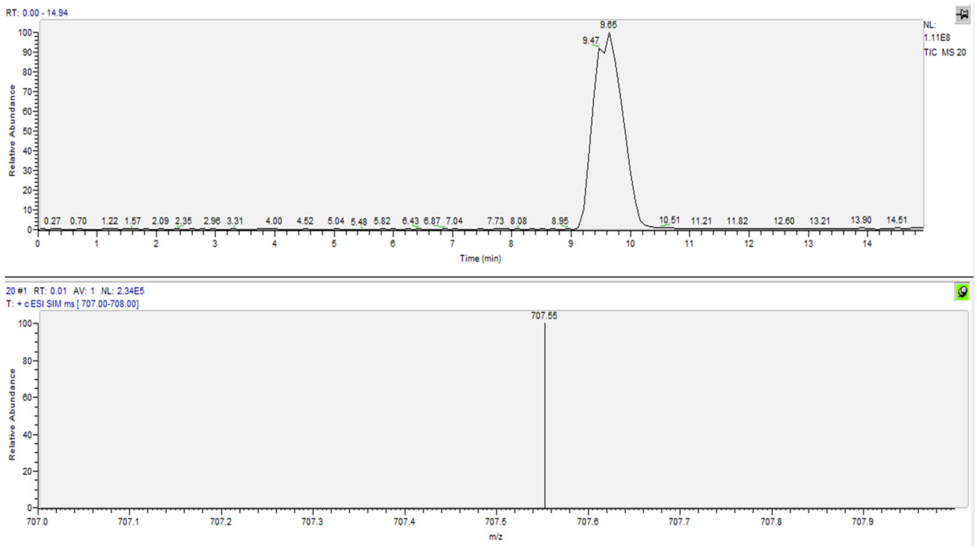
3.2. Linear Analysis of the Phospholipid Standard, PC(O-16:0/O-16:0) by LC ESI-MS

Using the optimal LC ESI-MS conditions, a phospholipid standard, 1,2-Di-O-hexadecyl-sn-glycero-3-phosphocholine (PC(O-16:0/O-16:0)), was chosen to determine the linear range of the optimized method. Seven different concentrations of the phospholipid standard (5, 10, 20, 40, 60, 80, and 100 μg/mL) were measured to determine the linear range. The linear range was 5.0–00.0 μg/mL, and the coefficient of determination (R^2) was 0.989. The concentration limit of detection (LOD) of the phospholipid standard was 1.50 μg/mL. It was calculated using 3σ/S, where σ was the standard deviation of the Y-intercept of the regression line and S was the slope of the regression line. The concentration limit of quantitation (LOQ) of the phospholipid standard was 4.54 μg/mL. It was calculated by using three times of LOD (3 × LOD). Figure 6A shows the chemical structure, and Figure 6B shows the EIC and ESI-MS spectrum for the phospholipid standard, PC(O-16:0/O-16:0).



(**A**)

Figure 6. *Cont.*



(**B**)

Figure 6. (**A**) Structure and (**B**) EIC and ESI/MS spectrum of the phospholipid standard 1,2-Di-O-hexadecyl-sn-glycero-3-phosphocholine, PC(O-16:0/O-16:0). For HPLC, the stationary phase selected was a C_8 column, and the mobile phase selected was 70% acetonitrile, 30% isopropanol, and 0.1% formic acid. The ESI/MS conditions were as in Figure 4.

3.3. Analysis of ox-PAPC Products for Healthy and Uremic Subjects by LC ESI-MS

Figure 7A,B show the EIC and ESI-MS spectrum of one representative long-chain ox-PAPC product (m/z 820.5) obtained from the *in vitro* oxidation of native PAPC standard. The ESI-MS spectrum shows several long-chain ox-PAPC ions including m/z 782.5, 820.5, 828.5, 836.5, 846.5, 850.5, 852.5, 868.5, 884.5, and 900.5. It is because these 10 ox-PAPC ions were detected together by the selected ion monitoring (SIM) of ESI-MS. Although the EIC peak is broad, extending from 8.0 to 10.4 min, quantitation based on the EIC peak areas shows very promising results. Figure 7C,D show the EIC and ESI-MS spectrum of one representative short-chain ox-PAPC product (m/z 666) obtained from the in vitro oxidation of native PAPC standard. The ESI-MS spectrum shows several short-chain ox-PAPC ions including m/z 632.5, 636.5, 650.5, 666.5, 690.5 and 782.5. It is because these six ox-PAPC ions were detected together by the SIM mode of ESI-MS. Although the EIC peak is broad, extending from 7.0 to 11.0 min, quantitation based on the EIC peak areas shows very promising results. The long-chain ox-PAPC products have very similar structures, and they are extremely difficult to separate and identify. Likewise, the short-chain ox-PAPC products are very difficult to separate and identify due to their similar structures. The SIM mode of ESI-MS is a powerful and convenient method to detect several ions simultaneously. In addition, the EIC peak area of individual ion allows for quantitation based on the calibration curve of the phospholipid standard. The results demonstrate that EIC and ESI-MS are very powerful and simple methods for detecting complex phospholipid molecules in one biological sample.

A total of 21 ox-PAPC products were analyzed for the VLDL, LDL, and HDL fractions of healthy and uremic subjects. Two important data were investigated for the individual human ox-PAPC product: (1) the migration time and peak shape of EIC and (2) the ESI-MS spectrum. The determination of the in vivo ox-PAPC molecular species was based on comparing their EICs and ESI-MS spectra with the in vitro oxidation products of the native PAPC standard. The concentration of each ox-PAPC product was measured based on its EIC peak area and the calibration curve of the phospholipid standard.

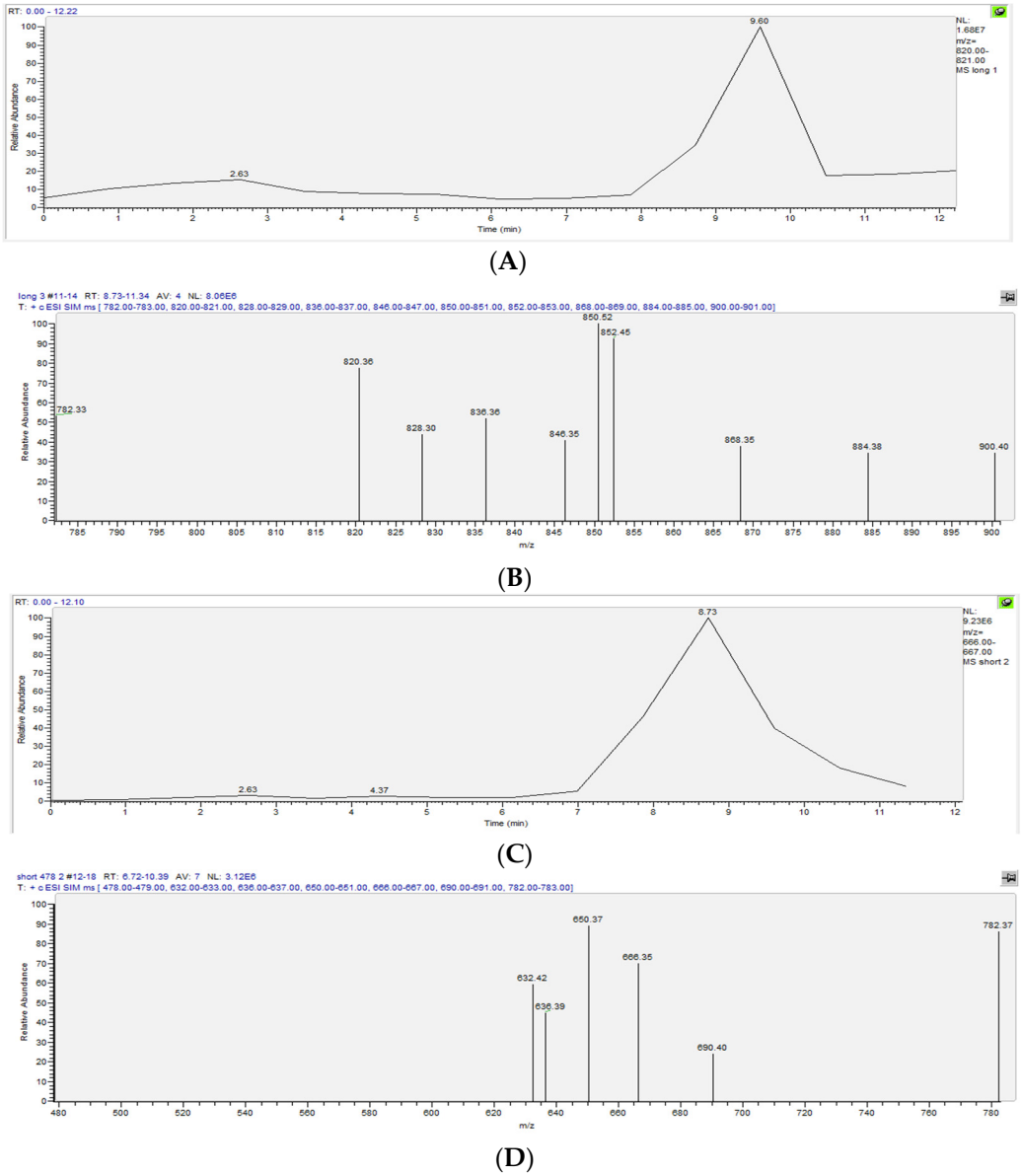


Figure 7. (**A**) EIC and (**B**) ESI-MS spectrum of the in vitro ox-PAPC product m/z 820.5. (**C**) EIC and (**D**) ESI-MS spectrum of the in vitro ox-PAPC product m/z 666.0. Ox-PAPC was obtained by in vitro oxidation ($FeCl_2$:H_2O_2 = 2:1) of 100 µg/mL native PAPC standard at 37 °C for 15 min. The LC-ESI/MS conditions were as in Figure 6.

Student's *t*-test was used to evaluate the data for healthy and uremic subjects. Table 1 shows the comparison of the concentrations of the nine long-chain ox-PAPC products and native PAPC (m/z 782.5) between healthy and uremic VLDL. The concentrations of several ox-PAPC products show significant difference between the two groups. They include m/z 820.5 with p-value < 0.001; m/z 868.5 and 884.5 with p-value < 0.01; and m/z 828.5, 836.5, 846.5, 850.5, and 900.5 with p-value < 0.05. Table 2 shows the comparison of the concentrations of the 12 short-chain ox-PAPC products between healthy and uremic VLDL. The significantly different ox-PAPC products include m/z 666 with p-value < 0.001; m/z 496, 594, 616, 620, 632, 636, and 650 with p-value < 0.01; and m/z 610, 622, and 690 with p-value < 0.05.

Table 1. Long-chain ox-PAPC product concentrations in VLDL of healthy controls and uremic patients.

ox-PAPC	Healthy Controls (n = 10) µg/mL Average ± SD	Uremic Patients (n = 10) µg/mL Average ± SD
m/z 820.5	11.71 ± 2.00	16.36 ± 3.13 ***
m/z 828.5	10.14 ± 1.52	12.88 ± 3.24 *
m/z 836.5	10.25 ± 1.63	13.15 ± 3.34 *
m/z 846.5	10.19 ± 1.41	12.00 ± 1.86 *
m/z 850.5	10.59 ± 1.80	13.78 ± 3.55 *
m/z 852.5	11.10 ± 2.56	13.41 ± 3.59
m/z 868.5	10.08 ± 1.24	12.28 ± 1.89 **
m/z 884.5	9.82 ± 1.17	11.83 ± 1.73 **
m/z 900.5	9.78 ± 1.15	12.01 ± 2.01 *
m/z 782.5	11.65 ± 3.45	12.23 ± 2.91

* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ controls vs. patients.

Table 2. Short-chain ox-PAPC product concentrations in VLDL of healthy controls and uremic patients.

ox-PAPC	Healthy Controls (n = 10) µg/mL Average ± SD	Uremic Patients (n = 10) µg/mL Average ± SD
m/z 478	16.05 ± 4.25	35.56 ± 36.70
m/z 496	9.56 ± 0.53	10.89 ± 2.21 **
m/z 594	10.67 ± 1.39	12.48 ± 1.58 **
m/z 610	10.85 ± 1.48	12.71 ± 1.80 *
m/z 616	11.80 ± 2.10	14.91 ± 2.80 **
m/z 620	11.27 ± 1.93	14.50 ± 3.02 **
m/z 622	11.50 ± 2.38	18.58 ± 9.08 *
m/z 632	12.23 ± 2.23	15.55 ± 3.08 **
m/z 636	11.82 ± 2.16	14.94 ± 2.34 **
m/z 650	10.39 ± 1.22	12.08 ± 1.38 **
m/z 666	10.42 ± 1.17	12.47 ± 1.34 ***
m/z 690	10.29 ± 1.12	12.39 ± 1.81 *

* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ controls vs. patients.

Table 3 compares the concentrations of the nine long-chain ox-PAPC products and native PAPC (m/z 782.5) between healthy and uremic LDL. There is no significant difference between the two groups. Table 4 shows the comparison of the concentrations of the 12 short-chain ox-PAPC products between healthy and uremic LDL. Only one ox-PAPC product m/z 478 is significantly different with p-value < 0.001.

Table 3. Long-chain ox-PAPC product concentrations in LDL of healthy controls and uremic patients.

ox-PAPC	Healthy Controls (n = 10) µg/mL Average ± SD	Uremic Patients (n = 10) µg/mL Average ± SD
m/z 820.5	4.92 ± 0.89	4.49 ± 0.21
m/z 828.5	4.53 ± 0.16	4.48 ± 0.32
m/z 836.5	4.66 ± 0.28	4.53 ± 0.38
m/z 846.5	4.58 ± 0.34	4.40 ± 0.09
m/z 850.5	4.64 ± 0.38	4.44 ± 0.10
m/z 852.5	4.62 ± 0.23	4.66 ± 0.70
m/z 868.5	4.57 ± 0.22	4.50 ± 0.32
m/z 884.5	4.49 ± 0.11	4.43 ± 0.22
m/z 900.5	4.56 ± 0.34	4.44 ± 0.27
m/z 782.5	4.45 ± 0.14	4.42 ± 0.04

Table 4. Short-chain ox-PAPC product concentrations in LDL of healthy controls and uremic patients.

ox-PAPC	Healthy Controls (n = 10) µg/mL Average ± SD	Uremic Patients (n = 10) µg/mL Average ± SD
m/z 478	6.22 ± 0.61	5.06 ± 0.39 ***
m/z 496	4.41 ± 0.04	4.38 ± 0.06
m/z 594	4.69 ± 0.43	4.57 ± 0.39
m/z 610	4.62 ± 0.19	4.50 ± 0.23
m/z 616	4.91 ± 0.73	4.66 ± 0.54
m/z 620	4.75 ± 0.58	4.54 ± 0.36
m/z 622	4.66 ± 0.43	4.50 ± 0.28
m/z 632	5.41 ± 1.32	4.97 ± 0.85
m/z 636	5.52 ± 1.77	4.87 ± 0.66
m/z 650	4.60 ± 0.34	4.52 ± 0.34
m/z 666	4.58 ± 0.31	4.47 ± 0.31
m/z 690	4.60 ± 0.41	4.50 ± 0.33

*** $p < 0.001$ controls vs. patients.

Table 5 compares the concentrations of the nine long-chain ox-PAPC products and native PAPC (m/z 782.5) between healthy and uremic HDL. The significantly different ox-PAPC products include m/z 820.5 with p-value < 0.001; m/z 782.5, 828.5, 836.5, 846.5, 850.5, 852.5, and 868.5 with p-value < 0.01; and m/z 884.5 and 900.5 with p-value < 0.05. Interestingly, even the native PAPC m/z 782.5 shows a significant difference between the two groups for HDL. Table 6 compares the concentrations of the 12 short-chain ox-PAPC products between healthy and uremic HDL. The significantly different ox-PAPC products include m/z 594, 632, and 636 with p-value < 0.001; m/z 478, 610, 616, 620, 622, and 650 with p-value < 0.01; and m/z 666 with p-value < 0.05.

Table 5. Long-chain ox-PAPC product concentrations in HDL of healthy controls and uremic patients.

ox-PAPC	Healthy Controls (n = 10) µg/mL Average ± SD	Uremic Patients (n = 10) µg/mL Average ± SD
m/z 820.5	2.67 ± 0.07	2.56 ± 0.03 ***
m/z 828.5	2.58 ± 0.04	2.54 ± 0.02 **
m/z 836.5	2.64 ± 0.06	2.55 ± 0.03 **
m/z 846.5	2.59 ± 0.04	2.54 ± 0.02 **
m/z 850.5	2.61 ± 0.06	2.54 ± 0.02 **
m/z 852.5	2.61 ± 0.05	2.54 ± 0.02 **
m/z 868.5	2.63 ± 0.07	2.56 ± 0.03 **
m/z 884.5	2.58 ± 0.06	2.54 ± 0.02 *
m/z 900.5	2.57 ± 0.05	2.53 ± 0.01 *
m/z 782.5	2.55 ± 0.02	2.60 ± 0.04 **

* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ controls vs. patients.

Bagdade and Hans-Erik Norbeck et al. compared plasma lipids for uremic and healthy subjects. They found that both triglyceride and cholesterol were increased in VLDL, while triglyceride was significantly increased in LDL for uremic patients [22,23]. Meanwhile, Hans-Erik Norbeck et al. found that HDL-cholesterol was decreased in uremic patients. Janicki et al. reported significantly increased serum triglyceride and decreased serum HDL-cholesterol in HD patients [24]. Kes et al. showed increased LDL-cholesterol and decreased HDL-cholesterol in uremic patients [25]. Rao et al. indicated significantly high levels of VLDL-cholesterol in chronic kidney disease (CKD) patients, and it was one of the major lipid disorders leading to cardiovascular complications [26]. Vecino et al. investigated lysophosphatidylcholine (LPC) and PC in 7 uremic patients. They showed that plasma LPC concentrations were similar between patients and controls, while plasma PC concentrations were higher in uremic patients ($p < 0.01$) [27]. Stübiger et al. determined oxidized phospholipids in the plasma of hyperlipidemic patients by LC-ESI/MS. The authors found

higher levels of several ox-PAPC products, including m/z 594, 610, 650 and 666 in the plasma of patients, especially the ion m/z 650 [28]. Interestingly, Pawlak et al. have shown lower oxidized LDL (ox-LDL) levels in dialyzed patients without cardiovascular disease (CVD) than in healthy controls and their counterparts with CVD [29]. It was suggested that ox-LDL was probably not an important biomarker for HD patients. Coincidently, our study also showed that for the 21 ox-PAPC products analyzed in LDL, only the ion m/z 478 was significantly different between uremic patients and healthy controls.

Table 6. Short-chain ox-PAPC product concentrations in HDL of healthy controls and uremic patients.

ox-PAPC	Healthy Controls (n = 10) μg/mL Average ± SD	Uremic Patients (n = 10) μg/mL Average ± SD
m/z 478	3.98 ± 0.96	2.88 ± 0.21 **
m/z 496	2.56 ± 0.02	2.54 ± 0.05
m/z 594	2.62 ± 0.05	2.55 ± 0.03 ***
m/z 610	2.68 ± 0.09	2.57 ± 0.05 **
m/z 616	2.66 ± 0.06	2.58 ± 0.03 **
m/z 620	2.59 ± 0.03	2.55 ± 0.02 **
m/z 622	2.57 ± 0.02	2.54 ± 0.03 **
m/z 632	3.43 ± 0.26	2.88 ± 0.26 ***
m/z 636	3.40 ± 0.25	2.80 ± 0.24 ***
m/z 650	2.72 ± 0.12	2.56 ± 0.03 **
m/z 666	2.57 ± 0.04	2.54 ± 0.02 *
m/z 690	2.60 ± 0.05	2.56 ± 0.05

* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ controls vs. patients.

In summary, the results of our study showed that the levels of 18 ox-PAPC products for VLDL, one ox-PAPC product for LDL and 19 ox-PAPC products for HDL, were significantly higher for uremic patients than healthy controls. Interestingly, even the native PAPC m/z 782.5 also showed a significant difference for HDL between the two groups. Although the sample size of our study was not large, the preliminary results were promising and suggested that ox-PAPC products might be important uremic biomarkers. To the best of our knowledge, no previous studies have analyzed ox-PAPC products on lipoproteins for uremic patients. In order to understand if ox-PAPC products are important biomarkers, more uremic patient groups need to be analyzed by the optimal LC-ESI/MS method in the future. Furthermore, LC-ESI/MS/MS analysis needs to be carried out to characterize the ox-PAPC molecular species that could serve as biomarkers of uremia.

4. Conclusions

In the present study, we have developed a simple and fast LC-ESI/MS method to investigate in vivo ox-PAPC products on human lipoproteins. Several important experimental parameters including the stationary phase and the mobile phase for LC, as well as the sheath gas pressure, capillary temperature and injection time for ESI-MS, have been carefully studied. The optimal LC-ESI/MS method showed good repeatability, linearity and sensitivity. At the beginning, the in vitro oxidation products of the native PAPC standard were used for method development. A total of 9 long-chain and 12 short-chain ox-PAPC products were identified. Both the EICs and ESI-MS spectra of the in vitro oxidation products were then used for the determination of the in vivo ox-PAPC molecules of uremic patients.

A phospholipid standard, PC (O-16:0/O-16:0) has been used for the quantification of ox-PAPC products on human lipoproteins. The quantitation of each ox-PAPC product was based on its EIC peak area and the calibration curve of the phospholipid standard. A total of 21 ox-PAPC products have been quantified and compared for the lipoproteins of healthy and uremic subjects. Very interestingly, the uremic group showed significantly higher levels than healthy group for many ox-PAPC products. They included 18 products in VLDL, one product in LDL and 19 products in HDL. This simple LC-ESI/MS method might

possibly accelerate the determination of ox-PAPC biomarkers of uremia and cardiovascular disease. For future study, more uremic patient groups need to be analyzed. Furthermore, LC-ESI/MS/MS analysis needs to be performed to characterize the chemical structures of ox-PAPC products which are potential uremic biomarkers.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/separations9080192/s1. Table S1: The proposed structures of the short-chain ox-PAPC products; Table S2: The proposed structures of the long-chain ox-PAPC products.

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