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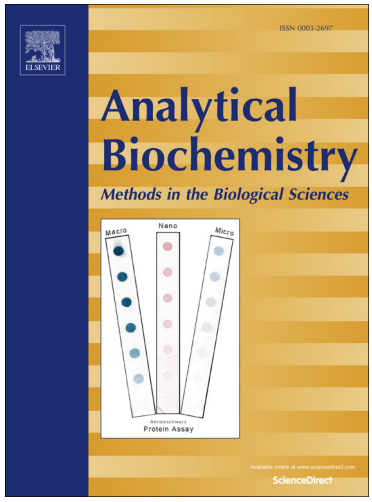

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Analytical Biochemistry

LC-MS/MS determination of human plasma 1-palmitoyl-2-hydroperoxyoctadecadienoyl-phosphatidylcholine isomers via promotion of sodium adduct formation

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A B S T R A C T

Accumulation of phosphatidylcholine hydroperoxide (PCOOH), a primary oxidation product of phosphatidylcholine, in blood plasma has been observed in various pathological conditions, including atherosclerosis. In this study, we investigated the use of LC-MS/MS to develop a method for accurate quantification of PCOOH (1-palmitoyl-2-hydroperoxyoctadecadienoyl-*sn*-glycero-3-phosphocholine, 16:0/HpODE PC), focusing on isomers such as 16:0/13-HpODE PC and 16:0/9-HpODE PC. Sodiated PCOOH ($[M+Na]^+$, *m/z* 812) provided not only a known product ion (*m/z* 147), but also characteristic product ions (*m/z* 541 for 16:0/13-HpODE PC and *m/z* 388 for 16:0/9-HpODE PC). Three multiple reaction monitorings (MRMs) could thus be performed. MRM (812/147) enabled determination of 16:0/HpODE PC, and MRM (812/541 and 812/388) allowed specific measurement of 16:0/13-HpODE PC and 16:0/9-HpODE PC, respectively. By using this method, we could determine plasma PCOOH concentrations in healthy and patients with angiographically significant stenosis. In both plasma, the concentration of 16:0/HpODE PC was close to the sum of the concentrations of 16:0/13-HpODE PC and 16:0/9-HpODE PC. This finding shows that radical- and/or enzymatic-oxidation, rather than singlet oxygen-oxidation, are recognized to cause peroxidation of PC. The newly developed LC-MS/MS method appears to be a powerful tool for developing a better understanding of in vivo lipid peroxidation and its involvement in human diseases.

Keywords: Phosphatidylcholine hydroperoxide; Hydroperoxide positional isomer; Tandem mass spectrometry; Human blood plasma; Atherosclerosis; Stenosis

Abbreviations: MxP, 2-methoxypropene; BHT, butylated hydroxytoluene; CID, collision-induced dissociation; ESI, electrospray ionization; HpODE, hydroperoxyoctadecadienoyl; ICAM-1, intracellular adhesion molecule-1; MRM, multiple reaction monitoring; PC, phosphatidylcholine; PCOOH, phosphatidylcholine hydroperoxide

Oxidative lipid modifications play important roles in various pathological conditions, including atherosclerosis [1–4]. Among oxidatively modified lipids, phosphatidylcholine hydroperoxide (PCOOH) (Fig. 1), a primary oxidation product of phosphatidylcholine (PC), is known to accumulate in arterial walls and blood plasma in atherosclerotic rabbits [5]. PCOOH was also identified in human atherosclerotic lesions [6, 7]. Furthermore, PCOOH accumulation in the plasma has been found in human subjects with pathological conditions, such as hyperlipidemia [8, 9], diabetes [10], and Alzheimer's disease [11]. Because hyperlipidemia and diabetes are strongly associated with increased incidence of atherosclerosis [12–14], higher plasma PCOOH in these patients is recognized to be involved in atherogenesis. As for the possible mechanism, our previous study showed that PCOOH induced THP-1 monocytic cell adhesion to intracellular adhesion molecule-1 (ICAM-1) via actin cytoskeletal polymerization and subsequent localization of lymphocyte function-associated antigen-1 [15, 16]. Further, we reported the involvement of Rac GTPase activation in the PCOOH-induced cell adhesion to ICAM-1 via actin polymerization. The Rho-family GTPase-dependent actin cytoskeleton organization is involved in various cellular processes such as adhesion. Via these mechanisms, phospholipid peroxidation may play a crucial role in atherogenic progress. Therefore, accurate PCOOH determination is key to understanding the pathophysiology of oxidative stress-related diseases, such as atherosclerosis.

PCOOH can be quantified by liquid chromatography (LC)-chemiluminescence detection (hydroperoxide base-specific method) [5, 8–11, 17–20], LC-mass spectrometry (LC-MS) [7, 20], and LC-tandem mass spectrometry (LC-MS/MS) [21, 22]. The concentrations of PCOOH have been shown to range from 55 to 227 pmol/ml in the plasma of healthy subjects [17, 20], though some reports have failed to detect PCOOH in human plasma [23, 24]. These conflicting results suggest the need for further investigation of analytical concerns, such as detection capability and extraction recovery, to develop

additional, more reliable methods.

In recent years, plasma PCOOH concentrations have been measured at the molecular species level using LC-MS/MS. Hui et al. reported that PCOOH (1-palmitoyl-2-hydroperoxyoctadecadienoyl-*sn*-glycero-3-phosphocholine, 16:0/HpODE PC) and (1-stearoyl-2-hydroperoxyoctadecadienoyl-*sn*-glycero-3-phosphocholine, 18:0/HpODE PC) concentrations in plasma samples were 89 and 32 pmol/ml, respectively [21], although these authors did not mention the position of the hydroperoxide group in the fatty acid. As the determination of hydroperoxide position can be helpful in the elucidation of the in vivo lipid peroxidation mechanisms (i.e. radical-, enzyme-, and/or singlet oxygen-oxidation) [25], analysis of these PCOOH isomers analysis has been hoped for the investigation of oxidative stress-related pathophysiology.

In the present study, we used LC-MS/MS to investigate the above analytical concerns (e.g., detection capability and extraction recovery), focusing on a representative PCOOH molecular species (16:0/HpODE PC) in plasma [21, 22]. Moreover, we investigated various analytical conditions in order to quantify major 16:0/HpODE PC isomers such as 1-palmitoyl-2-(13-hydroperoxyoctadecadienoyl)-*sn*-glycero-3-phosphocholine (16:0/13-HpODE PC) and 1-palmitoyl-2-(9-hydroperoxyoctadecadienoyl)-*sn*-glycero-3-phosphocholine (16:0/9-HpODE PC) in plasma from healthy subjects and patients with angiographically significant stenosis.

Materials and methods

Materials

1-Palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (16:0/18:2 PC) and lysoPC

(16:0) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Methyl linoleate was obtained from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of the highest grade available.

Preparation of 16:0/13-HpODE PC and 16:0/9-HpODE PC

16:0/13-HpODE PC (Fig. 1) was prepared from 16:0/18:2 PC using soybean lipoxygenase-1 [26, 27]. 16:0/9-HpODE PC was synthetically prepared as follows: linoleic acid methyl ester was oxidized for 24 hours at 40°C. Among the oxidation products, 9-hydroperoxyoctadecadienoic acid methyl ester was chromatographically fractionated [28] and its hydroperoxide group was protected by 2-methoxypropene (MxP) [26, 27]. The protected methyl ester was purified and saponified in base solution to yield 9-hydroperoxyoctadecadienoic acid, in which the hydroperoxide group was protected by MxP. The protected fatty acid hydroperoxide was purified and esterified with lysoPC (16:0) in chloroform containing *N, N'*-dicyclohexylcarbodiimide and dimethylaminopyridine under N_2 atmosphere for 24 hours [29]. Synthesized diacylphosphocholine was finally deprotected, and the resultant 16:0/9-HpODE PC was chromatographically purified [26, 27].

MS/MS and LC-MS/MS

A 4000 QTRAP quadrupole/linear ion-trap tandem mass spectrometer (AB SCIEX, Tokyo, Japan) was used for MS/MS analysis. Initially, we analyzed the prepared reference PCOOH (16:0/13-HpODE PC or 16:0/9-HpODE PC). To evaluate MS/MS fragmentation, product ion scanning was performed via direct infusion of the PCOOH solution (1 nmol/ml methanol) into the MS/MS system (10 μl/min). Electrospray ionization (ESI) was used as an ion source with a collision energy of 61 eV, a turbo gas temperature of 700°C, and a spray

voltage of 5500 V. Nitrogen pressure values for turbo, nebulizer, and curtain gases were set at 80, 70, and 20 psi, respectively. Positive ion spectra were collected in the *m/z* range of 100–1000.

By using the observed fragments, MRM of PCOOH (16:0/13-HpODE PC or 16:0/9-HpODE PC) was performed on an LC-MS/MS system. For LC-MS/MS analysis, a Shimadzu liquid chromatography system, including a vacuum degasser, a quaternary pump, and an autosampler (Shimadzu, Kyoto, Japan), was equipped with a 4000 QTRAP mass spectrometer. PCOOH was analyzed using an ODS column (Atlantis T3 column, 3.5 μm, 2.1 × 100 mm; Waters, Tokyo, Japan) with a binary gradient consisting of solvent A (methanol containing 5 mM ammonium acetate or 0.1 mM sodium acetate) and solvent B (water containing 5 mM ammonium acetate or 0.1 mM sodium acetate). The gradient profile was as follows: 0–4 min, 70%–90% B linear; 4–10 min, 90% B; 10–17 min, 90%–100% B linear; 17–30 min, 100% B; 30–30.1 min, 100%–70% B linear; 30.1–35.0 min, 70% B. The flow rate was 0.2 ml/min, and the column temperature was 40°C. PCOOH isomers were detected using MRM for the transition of parent ions to product ions (Table 1), and external standard curves were individually constructed.

Extraction of PCOOH from plasma

Blood (10 ml) was collected from 3 healthy subjects (mean age, 24 ± 2 years) into a tube containing heparin as an anticoagulant. Plasma was prepared from the blood by centrifugation at 1,000 × *g* for 10 min at 4°C. Plasma samples from 3 subjects were pooled and used in the following studies.

To effectively extract PCOOH from plasma samples, different extraction conditions were examined (Scheme 1) as follows:

Step 1: plasma (0.3 ml) was diluted with 0.3 ml of 0%–5.0% KCl aqueous solution.

Step 2: total lipids were extracted from the diluted plasma using 2.4 ml chloroform:methanol (1:1 or 2:1, v/v) with 0.002% butylated hydroxytoluene (BHT). The extract was partitioned by centrifugation at 1,000 × *g* for 20 min at 25, 4, or -20°C into 2 layers: the chloroform layer (lower organic layer) and the methanol-water layer (upper layer). The lower chloroform layer (lipid fraction) was collected. The remaining aqueous layer containing a semisolid interface was re-extracted with Folch's theoretical lower phase [30] and subjected to centrifugation at 1,000 × *g* for 20 min at 25, 4, or -20°C. The combined lipid fraction was either rinsed with Folch's theoretical upper phase or left without rinsing. Similarly, lipids were extracted by mixing the diluted plasma with 2.4 ml chloroform:methanol (1:2, v/v) or 100% methanol containing 0.002% BHT. The mixture was centrifuged at 1,000 × *g* for 20 min and the supernatant was collected. The extraction was repeated, and the supernatants containing lipid moieties were combined.

Step 3: the lipid fraction was evaporated and dried under nitrogen gas.

Step 4: the dried extract was redissolved in 0.24 ml chloroform:isopropanol (2:1, v/v), and 0.04 ml of this mixture was loaded onto an aminopropyl Sep-Pak cartridge (Waters) equilibrated in chloroform:isopropanol (2:1, v/v). The cartridge was rinsed with 0.75 ml chloroform:isopropanol (2:1, v/v). PCOOH was eluted with 1.5 ml methanol. The eluent was evaporated, and the residue was dissolved in 0.2 ml methanol.

Step 5: a 10-µl final aliquot was injected into the LC-MS/MS system.

To evaluate the extraction efficiency, we spiked standard PCOOH (120 pmol 16:0/13-HpODE PC/10 µl methanol) into the plasma (Step 1), lipid fraction (Step 2), total lipids (Step 3), dried extract (Step 4), or final aliquot (Step 5; see Scheme 1). PCOOH was then extracted and quantified by LC-MS/MS, and extraction recoveries were calculated using equation corresponding to the external standard curves.

Matrix effect

The ion suppression effect was evaluated by injecting either the plasma extract or mobile phase (10 μl) into the LC-MS/MS system in parallel with continuous post-column infusion of the PCOOH standard solution (0.1 pmol 16:0/13-HpODE PC or 16:0/9-HpODE PC/ml methanol) at 10 μl/min.

Plasma PCOOH in healthy subjects and patients with stenosis

After optimization of the method, we measured the concentration of plasma PCOOH in healthy subjects and patients with angiographically significant stenosis (≥ 50% in at least one major coronary vessel). Blood samples were collected from 8 healthy subjects (2 women and 6 men, mean age 25 ± 2 years) and 12 patients (3 women and 9 men, mean age 69 ± 11 years). Plasma samples were prepared from heparinized blood via centrifugation at 1,000 × *g* for 10 min at 4°C, and plasma PCOOH was extracted and quantified under optimized conditions. Plasma PCOOH concentrations were calculated using equation corresponding to the external standard curves. This study was approved by the institutional review board of the Cardiovascular Institute (Tokyo, Japan). Informed consent was obtained from all participants.

Statistics

Data are expressed as means ± SDs. For comparisons between 2 groups, statistically significant differences were determined by Student's *t*-tests. To compare an overall difference among 3 or more groups, one-way ANOVA was used. If a statistically significant difference was detected, the Turkey-Kramer test was performed for comparison between individual groups. Differences were considered significant at $P < 0.05$.

Results

Preparation of authentic 16:0/13-HpODE PC and 16:0/9-HpODE PC

PCOOH (16:0/13-HpODE PC) was enzymatically synthesized from PC (16:0/18:2 PC) using soybean lipoxygenase-1 and chromatographically purified to yield essentially pure PCOOH (Fig. 1) [26, 27]. The structure of PCOOH was confirmed by NMR (data not shown). Another PCOOH isomer (16:0/9-HpODE PC) was prepared by esterifying the 9-hydroperoxyoctadecadienoyl group into lysoPC (16:0).

New MRM programs for PCOOH detection

MS analysis was carried out using the synthesized PCOOH (16:0/13-HpODE PC or 16:0/9-HpODE PC) as a reference compound. $[M+H]^+$ and $[M+Na]^+$ ions were detected at *m/z* 790 and 812, respectively, by positive ESI (Figs. 2A, B). Collision-induced dissociation (CID) of $[M+H]^+$ produced fragment ions including $[M+H-H_2O]^+$ (*m/z* 772), $[M+H-H_2O-O]^+$ (*m/z* 756), and choline phosphate ion (*m/z* 184) (Figs. 2C, D). It was found that CID of $[M+Na]^+$ yielded unique neutral losses (i.e., 88 Da for 16:0/13-HpODE PC and 169 Da for 16:0/9-HpODE PC). For example, product ion (*m/z* 541) was generated from 16:0/13-HpODE PC through the neutral loss of 88 Da and phosphatidylcholine (Fig. 2E). Whereas CID of 16:0/9-HpODE PC produced a product ion (*m/z* 388) through the neutral loss of 169 Da and 255 Da (corresponding to the palmitic acid) (Fig. 2F). Sodiated cyclophosphane (*m/z* 147) was observed in both spectra [31-33].

Based on these results, the authentic PCOOH was analyzed with LC-MS/MS under various MRM programs (e.g., 790/184, 812/541, 812/388, and 812/147). While MRM enabled detection of PCOOH, the peak intensity was found to be affected by Na^+ in the

mobile phase (Figs. 3A, B). Thus, we added extra Na^+ (i.e., 0.1 mM sodium acetate) to the mobile phase, and achieved stable and quantitative detection of sodiated PCOOH by MRM (812/541, 812/388, and 812/147) (Figs. 3C, D). MRM (812/541 and 812/388) could detect PCOOH only if there was a hydroperoxide group at the 13- or 9-position, respectively, of linoleic acid in the PCOOH molecule. MRM (812/147) enabled detection of PCOOH, regardless of the position of the hydroperoxide group in linoleic acid. Under optimized conditions, calibration curves showed good linearity within the range of 0.01 to 10 pmol/injection (Fig. 4).

Extraction of PCOOH from plasma

Next, we investigated PCOOH extraction from plasma samples. PCOOH (16:0/13-HpODE PC) spiked into plasma (Step 1 of Scheme 1) was extracted under different extraction conditions to check PCOOH recovery. Higher PCOOH recovery was obtained when chloroform:methanol (1:1 and 2:1, v/v) was used as an extraction solvent (Fig. 5A). When plasma was diluted with 0-1% KCl followed by extraction with chloroform:methanol (2:1, v/v), higher PCOOH recovery was observed (Fig. 5B). A somewhat lower PCOOH recovery was found when the combined lipid fraction was rinsed with Folch's theoretical upper phase (Fig. 5C). PCOOH recovery was not affected by extraction temperature (Fig. 5D).

In this study, the optimal extraction conditions were as follows: plasma (300 µl) was diluted with water (0% KCl, 300 µl) and mixed with 2.4 ml chloroform:methanol (2:1, v/v) at 4°C. The lipid fraction was collected, and the remaining layer was re-extracted with Folch's theoretical lower phase. The combined lipid fraction was evaporated and applied to solid-phase extraction. As shown by the recovery of PCOOH spiked into each step of the extraction (Scheme 1, Fig. 5E), our method enabled efficient and quantitative extraction of

PCOOH from plasma. Higher recovery was also observed when 16:0/9-HpODE PC spiked in plasma was extracted by the optimal extraction conditions (data not shown). Consistent with the quantitative results, the matrix effect was not observed following injection of plasma extracts into the LC-MS/MS system in parallel with continuous post-column infusion of reference PCOOH (16:0/13-HpODE PC or 16:0/9-HpODE PC) (Fig. 6).

Quantification of PCOOH in plasma samples from healthy subjects and patients

Under optimized quantification procedures (e.g., detection and extraction conditions), we measured PCOOH in plasma samples of healthy subjects. A very clear PCOOH peak was detected at 17 min, especially for MRM 812/541 and 812/388 (Fig. 7). Plasma PCOOH concentrations in healthy subjects were calculated to be 36.1 ± 11.5 pmol/ml (means ± SDs, n = 8) for 16:0/13-HpODE PC (MRM 812/541) and 33.1 ± 10.2 pmol/ml for 16:0/9-HpODE PC (MRM 812/388), while a higher concentration (72.3 ± 23.5 pmol/ml) was observed for MRM (812/147) (Table 2 and supplementary Table I). In patients with angiographically significant stenosis, the PCOOH concentrations were 52.4 ± 24.6 (812/541), 45.2 ± 18.1 (812/388), and 97.3 ± 39.5 (812/147) pmol/ml (means ± SDs, n = 12); these concentrations tended to be higher than those of healthy subjects (P = 0.06-0.13). In healthy and patient plasma, the concentration of 16:0/HpODE PC was found to be close to the sum of the concentrations of 16:0/13-HpODE PC and 16:0/9-HpODE PC.

Discussion

As mentioned in the Introduction, several studies have investigated the physiological concentrations of PCOOH, particularly in human plasma, due to its possible contribution to pathological conditions. Using synthesized authentic 16:0/13-HpODE PC and 16:0/9-HpODE PC, we developed an LC-MS/MS method to analyze PCOOH to obtain quantitative data and

information on the position of the hydroperoxide group.

Analysis of authentic 16:0/13-HpODE PC and 16:0/9-HpODE PC by QTRAP MS/MS yielded the same product ion mass spectra, and generated a well-known product ion *m/z* 184 (phosphocholine) (Figs. 2C, D). This fragment ion (*m/z* 184) is commonly used for LC-MS/MS MRM (790/184) analysis of PCOOH [21, 22]; however, MRM (790/184) does not give the structural information of the fatty acid moiety. On the other hand, sodiated PCOOH (*m/z* 812) generated unique product ions (i.e., *m/z* 541 for 16:0/13-HpODE PC and *m/z* 388 for 16:0/9-HpODE PC). Therefore, three MRM of sodiated PCOOH could be performed via LC-MS/MS analysis (Figs. 3A, B). MRM (812/541 and 812/388) allowed specific measurement of 16:0/13-HpODE PC and 16:0/9-HpODE PC only if there was a hydroperoxide group at the 13- and 9-position of the fatty acid, respectively. MRM (812/147) enabled detection of 16:0/HpODE PC, regardless of the position of the hydroperoxide group in the fatty acid of the PCOOH molecule. Nevertheless, PCOOH peak intensities were easily affected by instrumental conditions in LC-MS/MS MRM (812/541, 812/388, 812/147), especially metals (Na^+) in the flow path (and/or ion source). In the present study, stable and quantitative detection of PCOOH was achieved by addition of Na^+ to the mobile phase (Figs. 3C, D). Generally, nonvolatile salts containing Na^+ put a burden on the MS instrument [34]. In the present study, PCOOH and other matrix compounds were separated as much as possible, and the amount of Na^+ could be reduced to only 0.1 mM in the mobile phase. Despite the use of a nonvolatile salt (sodium acetate), our method could provide hundreds of hours of trouble-free operation and allow stable continuous analysis over 400 samples. Under optimized conditions, the external standard curves showed good linearity. It is worthy to say that when 16:0/13-HpODE PC and 16:0/9-HpODE PC were analyzed with MRM (812/147), there was no difference in their standard curves (Figs. 4A, B). This means that MRM (812/147) actually enables determination of all 16:0/HpODE PC isomers. With optimized methods, the detection limit of PCOOH was either superior to, or in the same range of,

previous LC-MS/MS methods [21, 22]. Based on these results (Figs. 2–4), we expect that this newly developed LC-MS/MS MRM could become a powerful tool for providing accurate quantitative data, as well as structural information of PCOOH.

Our group previously developed an LC-chemiluminescence method and reported a mean plasma PCOOH concentration of 227 pmol/ml [17], while Yamamoto et al. detected no PCOOH in human plasma [23, 24]. The reason for the differences observed in the reported PCOOH concentrations may be rooted in the extraction conditions, particularly considering the fact that we failed to detect PCOOH in plasma extracts prepared using 100% MeOH as an extraction solvent in previous experiments (Fig. 5) [23, 24]. PCOOH has been reported to be easily decomposed by several factors, such as acids [35], metal ions [36], and temperature [37]. Thus, we spiked standard PCOOH (16:0/13-HpODE PC) into plasma and checked PCOOH recovery under different extraction conditions (Scheme 1). We found that PCOOH recovery was notably affected by the extraction solvent (Fig. 5A). In addition, recovery was low when 5% KCl was used for plasma dilution before extraction with chloroform:methanol, although such concentrations of KCl have been frequently used for total lipid extraction from biological samples [30]. Unexpectedly, recovery was not affected by extraction temperature. These results suggest that PCOOH can be stably extracted by avoiding contact of PCOOH with water-soluble materials (e.g., metals) in the plasma during extraction procedures. Moreover, these results suggest that the present extraction procedures and LC-MS/MS MRM assays not only allowed accurate quantification, but also provided useful structural information of the analyte, even in the presence of background contaminants from plasma.

Our method exhibited the ability to detect 16:0/13-HpODE PC and 16:0/9-HpODE PC in human plasma (Fig. 7). MRM (812/147) detected some peaks close to the retention time of PCOOH; these peaks may be attributed to other PCOOH molecular species, other PC oxidation products (e.g., phosphatidylcholine hydroxide), and nonoxidized PC, because this product ion (147) originated from a PC backbone, not from a hydroperoxide group on a fatty

acid chain. Nevertheless, PCOOH detection using PC backbone-derived product ions has generally been performed [21, 22]. In contrast, very clear chromatograms were obtained for MRM (812/541 and 812/388). It should be noted that the plasma PCOOH (16:0/13-HpODE PC PC) peak (17.6 min) would be consisted of two *cis-trans* isomers (13-hydroperxyoctadeca-9*Z*, 11*E*-dienoyl PC and 13-hydroperxyoctadeca-9*E*, 11*E*-dienoyl PC), whereas two peaks of PCOOH (16:0/9-HpODE PC) would represent 9-hydroperxyoctadeca-10*E*, 12*Z*-dienoyl PC (17.4 min) and 9-hydroperxyoctadeca-10*E*, 12*E*-dienoyl PC (17.6 min). Quantification of PCOOH (16:0/9-HpODE PC) was carried out based on the total peak area of both peaks.

In patients with angiographically significant stenosis, the PCOOH concentration tended to be higher than those of healthy subjects (Table 2 and supplementary Table I), suggesting the involvement of phospholipid peroxidation in the development of atherosclerosis. Accumulation of PCOOH such as 16:0/13-HpODE PC in plasma may cause the development of atherosclerosis, as our in vitro cell culture studies showed that 16:0/13-HpODE PC induced atherogenic responses (i.e., monocyte adhesion and angiogenesis induction). Future trials are needed to further investigate these possibilities (e.g., age-matched control studies), as the patients participating in this study were 69 ± 11 years of age, and age is thought to correlate with increased phospholipid peroxidation [8]. While these data are still preliminary, the concentration of 16:0/HpODE PC was found to be close to the sum of the concentrations of 16:0/13-HpODE PC and 16:0/9-HpODE PC in healthy and patient plasma (Table 2 and supplementary Table I). Considering these concentrations, other isomers (e.g., 16:0/10- and 12-HpODE PC) would scarcely exist in human plasma. Lack of 16:0/10- and 12-HpODE PC might provide insight into biochemical processes that initiate phospholipid peroxidation in the plasma lipoprotein membrane. It is possible that radical- and/or enzymatic-oxidation, rather than singlet oxygen-oxidation, are apt to cause peroxidation of unsaturated fatty acid residues of PC. Thus, new method is required to

distinguish radical- and enzymatic-oxidation in future.

As mentioned above, there are published reports about LC-MS/MS determination method of PCOOH [21, 22]. But, by using the published method, it is impossible to analyze the position of hydroperoxide group in the fatty acid of PCOOH molecule. In the present study, we could differentially determine 16:0/13-HpODE PC and 16:0/9-HpODE PC, and found that there was no difference in these levels of healthy and patient plasma. Meanwhile, it is likely that different LOXs (15-LOX and 5-LOX) generate different 16:0/HpODE PC species (13-HpODE PC and 9-HpODE PC) in inflammatory state [38, 39]. Hence, difference of 16:0/13-HpODE PC and 16:0/9-HpODE PC may appear in inflammatory atherosclerotic lesions. This will provide better understanding of the pathophysiology of atherosclerosis. These possibilities need to be addressed in future studies.

In summary, we developed a method for accurate quantification of PCOOH, specifically isomers such as 16:0/13-HpODE PC and 16:0/9-HpODE PC in human plasma by LC-MS/MS. MRM (812/147) enabled determination of plasma PCOOH (16:0/HpODE PC), and MRM (812/541 and 812/388) allowed specific measurement of 16:0/13-HpODE PC and 16:0/9-HpODE PC, respectively. Using this method, we determined plasma PCOOH concentrations in healthy subjects and in patients with angiographically significant stenosis. The LC-MS/MS MRM system is useful for studying biological samples, including human plasma, and will be useful for developing a better understanding of the pathophysiology of oxidative stress-related diseases, such as atherosclerosis.

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Figure legends

Fig. 1. Chemical structures of PC (1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine, 16:0/18:2 PC) (A), (1-palmitoyl-2-(13-hydroperoxyoctadecadienoyl)-*sn*-glycero-3-phosphocholine, 16:0/13-HpODE PC) (B), and (1-palmitoyl-2-(9-hydroperoxyoctadecadienoyl)-*sn*-glycero-3-phosphocholine, 16:0/9-HpODE PC) (C).

Fig. 2. Q1 mass spectra of 16:0/13-HpODE PC and 16:0/9-HpODE PC in positive ESI mode (A and B, respectively). The product ion mass spectra of protonated PCOOH (*m/z* 790 $[M+H]^+$) for 16:0/13-HpODE PC (C) and 16:0/9-HpODE PC (D), and sodiated PCOOH (*m/z* 812 $[M+Na]^+$) for 16:0/13-HpODE PC (E) and 16:0/9-HpODE PC (F). Standard PCOOH (1 nmol16:0/13-HpODE PC or 16:0/9-HpODE PC/ml methanol) was infused directly into the MS/MS system at a flow rate of 10 μl/min. Detailed analytical conditions are described in Materials and Methods.

Fig. 3. MRM chromatograms of standard PCOOH (16:0/13-HpODE PC or 16:0/9-HpODE PC). 16:0/13-HpODE PC (1 pmol) was analyzed with MRM (*m/z* 790/184, 812/541, and 812/147) (A, C), whereas 16:0/9-HpODE PC (1 pmol) was analyzed with MRM (*m/z* 790/184, 812/388, and 812/147) (B, D). Mobile phases included either 5 mM ammonium acetate (A, B) or 0.1 mM sodium acetate (C, D). Insets show Q1 mass spectra of PCOOH detected at 17.0 (A), 16.9 (B), 17.5 (C), and 17.4 (D) min. Detailed analytical conditions are described in Materials and Methods.

Fig. 4. Calibration curves of reference PCOOH (16:0/13-HpODE PC (A) or 16:0/9-HpODE

PC (B)). Different amounts of PCOOH (0.01–10 pmol) were analyzed by optimized LC-MS/MS MRM (*m/z* 812/541 812/388, and 812/147) with mobile phase containing sodium acetate. Detailed analytical procedures are described in Materials and Methods.

Fig. 5. PCOOH recovery rates from plasma spiked with standard PCOOH (16:0/13-HpODE PC). PCOOH-spiked plasma (300 µl) was diluted with 300 µl of water and mixed with 2.4 ml of chloroform:methanol (1:2 or 2:1, v/v). Lipid fractions were collected, and the remaining phase was re-extracted with Folch's theoretical lower phase at 4°C. Combined lipid fractions were evaporated and applied to solid phase extraction followed by LC-MS/MS determination of PCOOH. PCOOH-spiked plasma (300 µl) was diluted with 300 µl of water and mixed with 2.4 ml of chloroform:methanol (1:2, v/v) or 100% methanol. The supernatants were collected and applied to solid phase extraction followed by LC-MS/MS. (A) Recovery of PCOOH extracted with different solvent ratios (chloroform:methanol (1:2, 1:1, or 2:1, v/v) or 100% methanol). (B) Recovery of PCOOH extracted using 300 µl of different concentrations of KCl (instead of water) and 2.4 ml of solvent ($CHCl_3$-MeOH, 2:1). (C) Recovery of PCOOH in the case of washing (rinsing) the combined lipid fraction (obtained from extraction conditions using $CHCl_3$-MeOH 2:1 and 0% KCl) with Folch's theoretical upper phase. (D) Recovery of PCOOH extracted with $CHCl_3$-MeOH 2:1, 0% KCl, and no rinsing at different temperatures (25, 4, or -20°C). (E) Recovery of PCOOH after addition of PCOOH standards to different extraction steps (Scheme 1). Extracts were analyzed by optimized LC-MS/MS condition with mobile phase containing sodium acetate. Detailed analytical conditions and procedures are described in Materials and Methods. Means without a common letter differ significantly at $P < 0.05$.

Fig. 6. Experimental setup for evaluating LC-MS/MS ion suppression (A) and MRM chromatograms of PCOOH when the plasma extract (10 µl) was injected into the LC-MS/MS

system with mobile phase containing sodium acetate in parallel with continuous post-column infusion of a PCOOH standard solution (0.1 pmol 16:0/13-HpODE PC (B, D) or 16:0/9-HpODE PC (C, E)/ml methanol) at 10 μl/min. MRM conditions were 812/541 (B), 812/388 (C), and 812/147 (D, E). Detailed analytical conditions are described in Materials and Methods.

Fig. 7. MRM chromatograms of PCOOH in the plasma of healthy subjects. Plasma extracts (10 μl) were analyzed by optimized LC-MS/MS MRM (812/541 (A), 812/388 (B), and 812/147 (C)) with mobile phase containing sodium acetate. Detailed analytical conditions are described in Materials and Methods.

TABLE 1. Optimal MS/MS parameters for PCOOH detection

Precursor ion (*m/z*)	790.4 $[M+H]^+$	812.4 $[M+Na]^+$		
Product ion (*m/z*)	184.3	541.4	388.3	147.0
Declustering potential (V)	126	126	126	126
Entrance potential (V)	10	10	10	10
Collision energy (V)	47	59	41	69
Collision cell exit potential (V)	10	30	16	6
Curtain gas (psi)		20		
Collision gas (psi)		7		
Ion spray voltage (V)		5000		
Temperature (°C)		500		
Ion source gas 1 (psi)		40		
Ion source gas 2 (psi)		40		

TABLE 2. PCOOH (16:0/HpODE PC) concentrations in the plasma of healthy subjects and patients with angiographically significant stenosis

MRM	812/541	812/388	812/147*
		pmol/ml	
Healthy subjects n = 8	36.1 ± 11.5	33.1 ± 10.2	72.3 ± 23.5 (69.3 ± 23.5)
Patients n = 12	52.4 ± 24.6	45.2 ± 18.1	97.3 ± 39.5 (97.6 ± 42.2)
P value	0.063	0.105	0.127

*Contents in parentheses ( ) represent the sum of 16:0/13-HpODE PC (812/541) and 16:0/9-HpODE PC (812/388). Values are means ± SD.

Fig. 1.

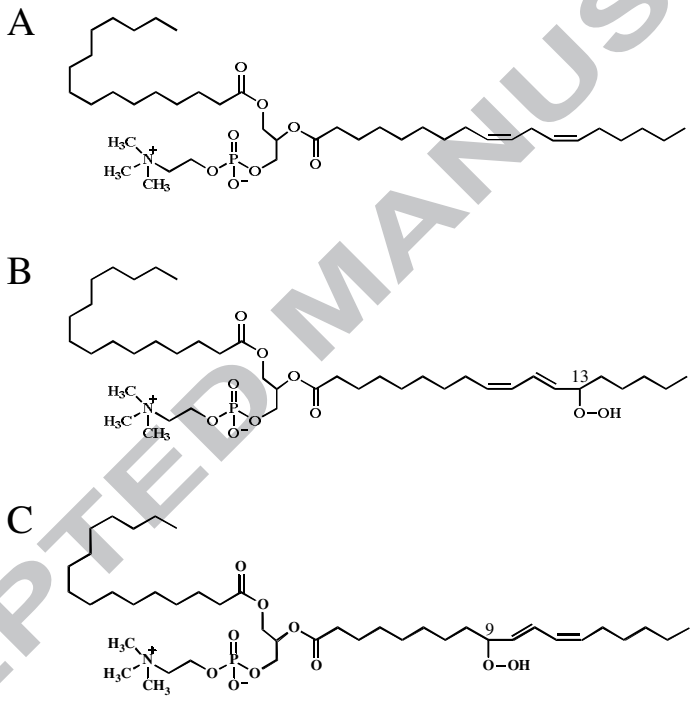


Fig. 2.

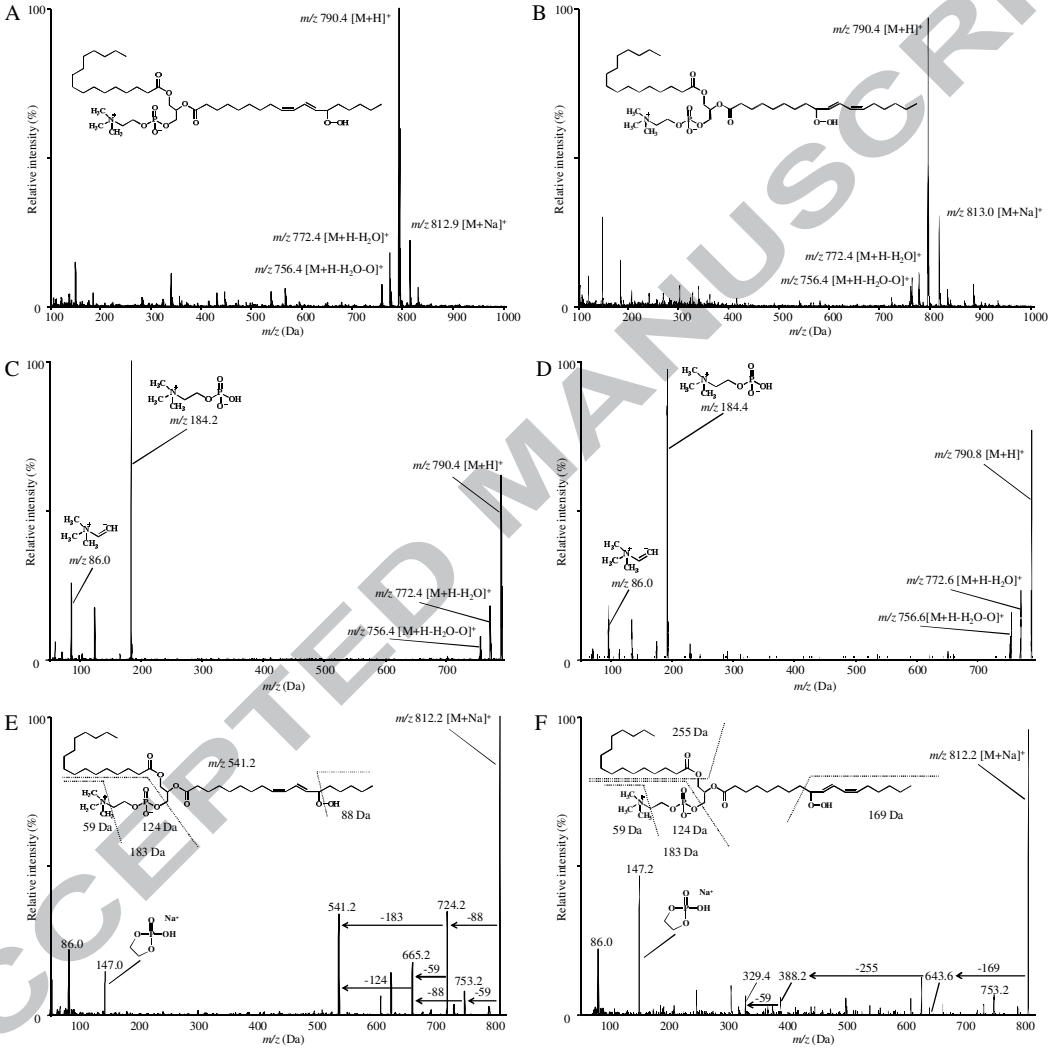


Fig. 3.

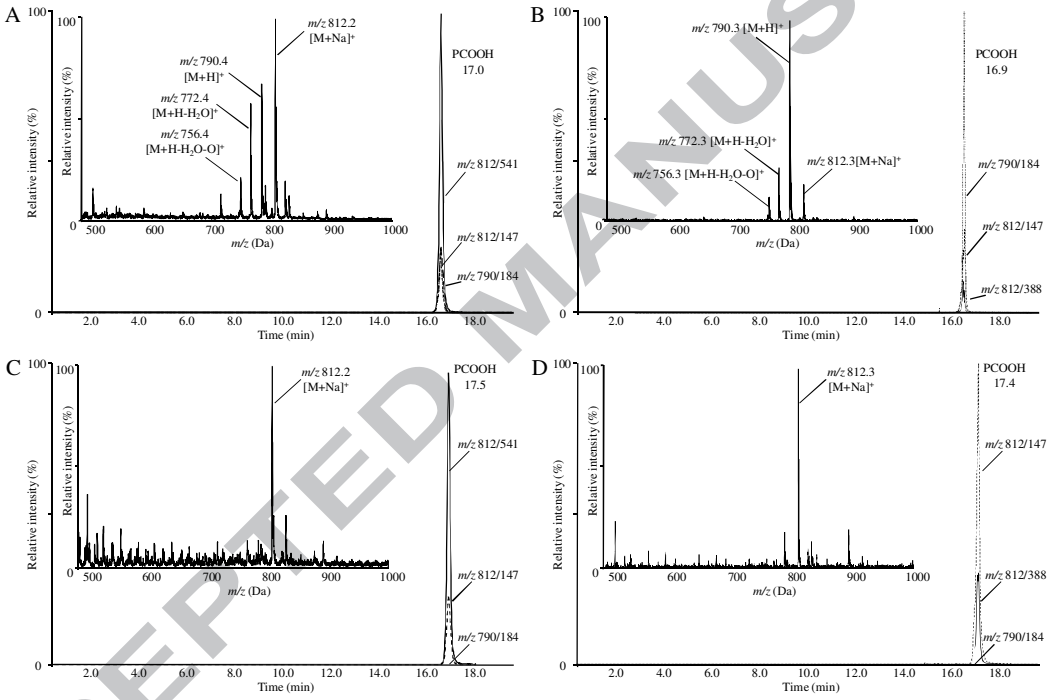
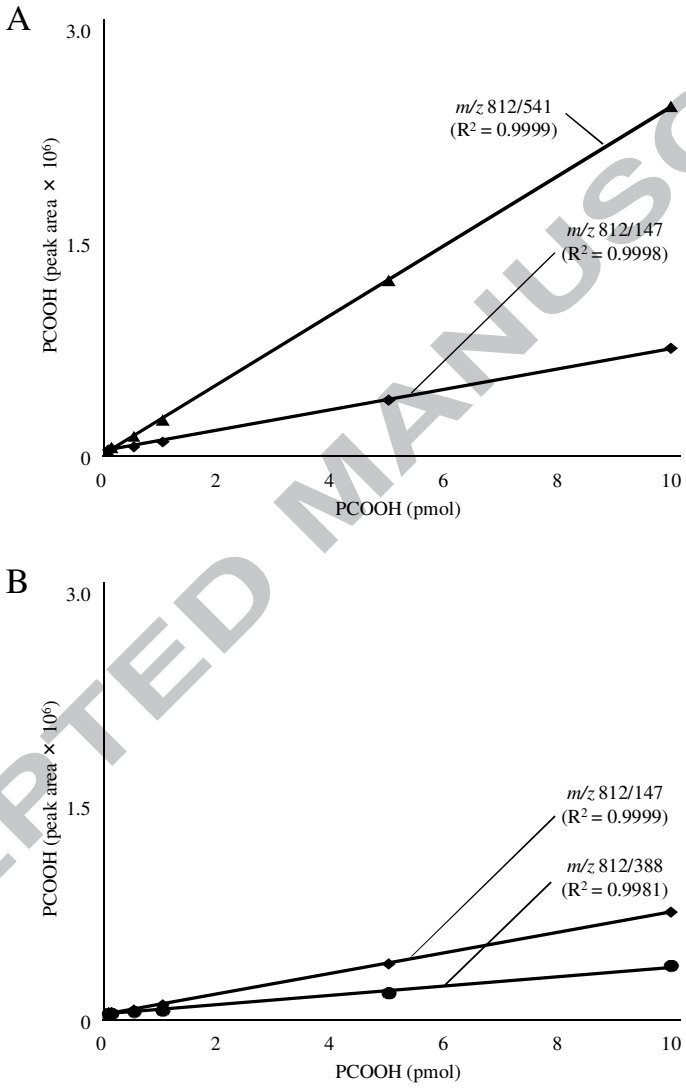


Fig. 4.



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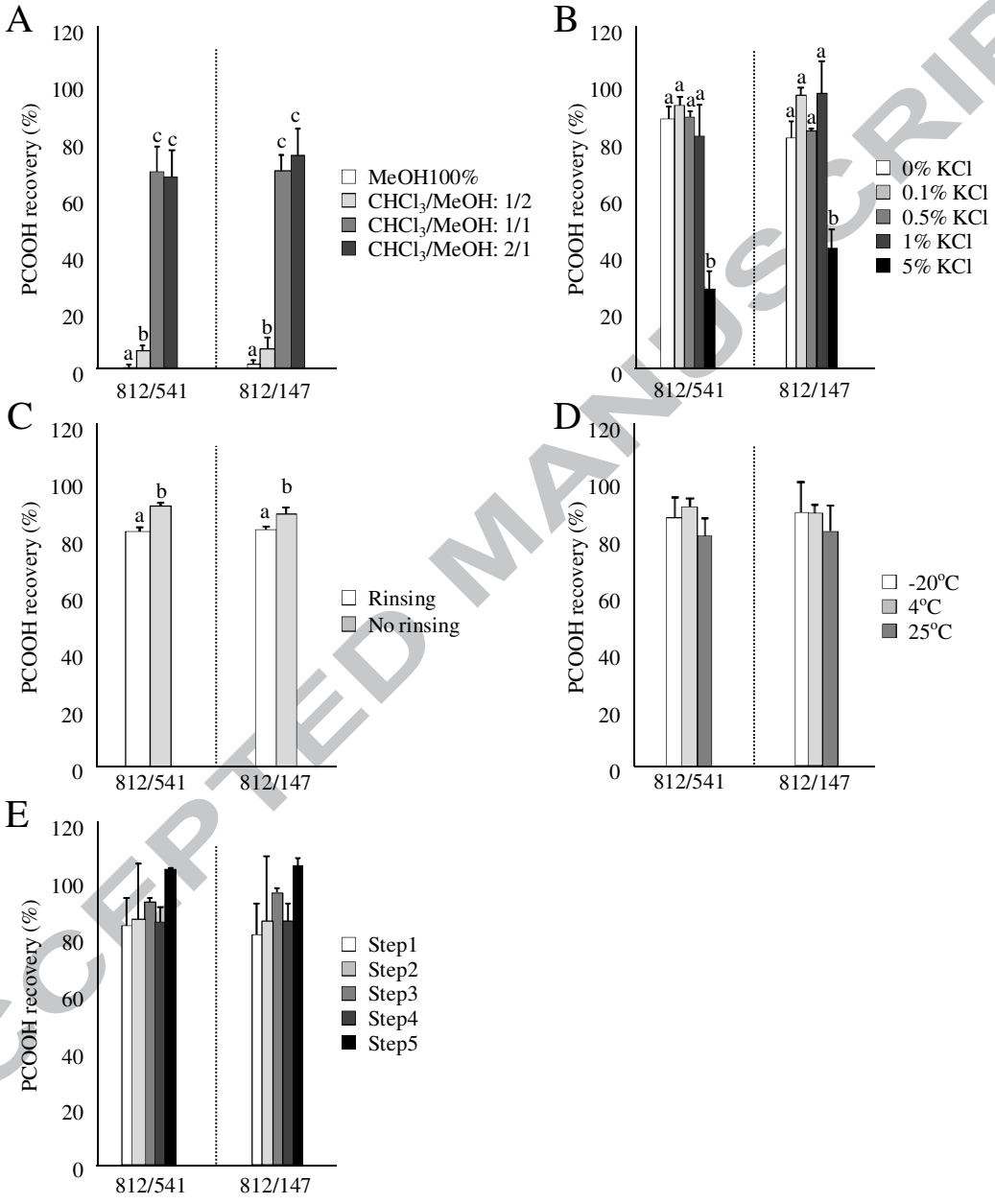
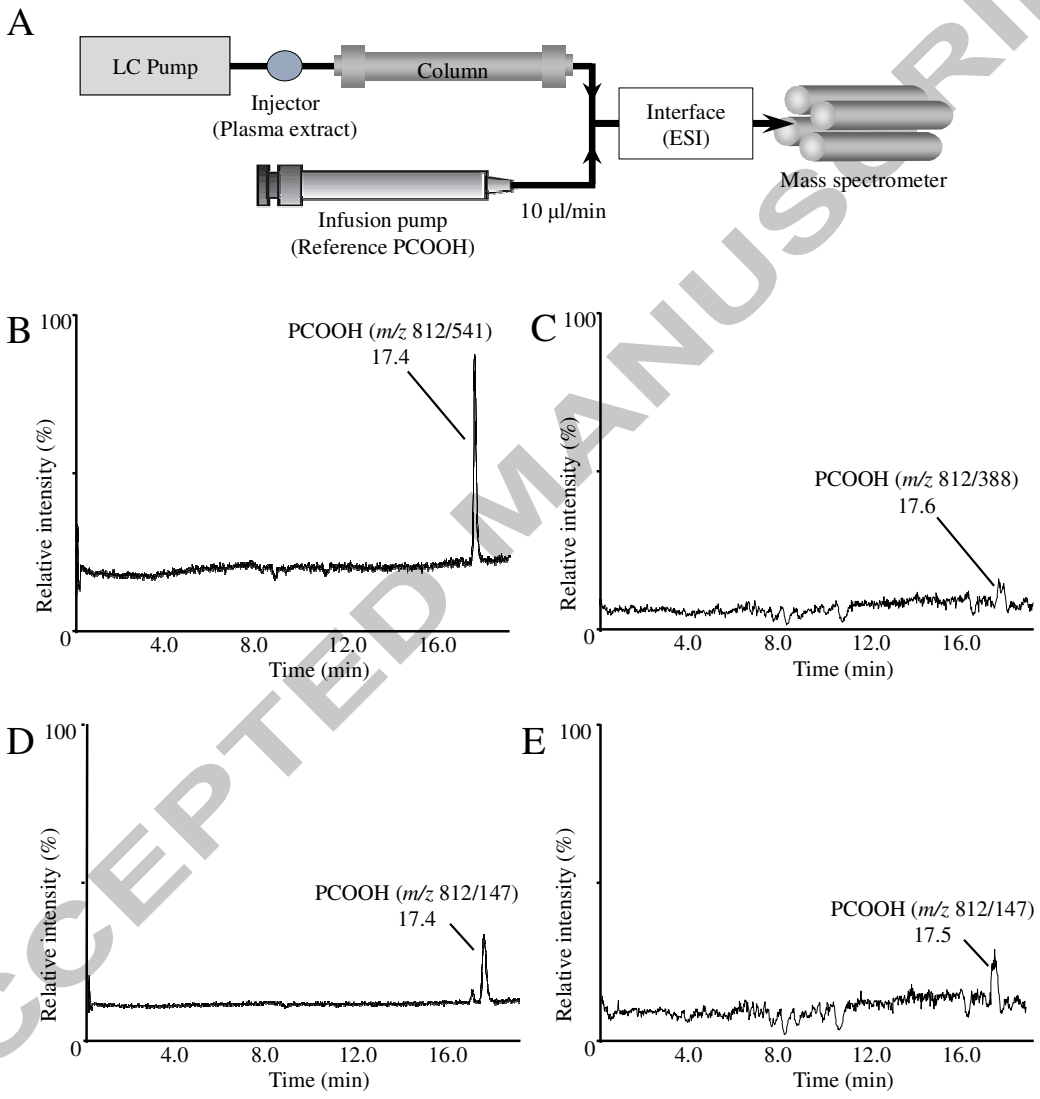


Fig. 6.



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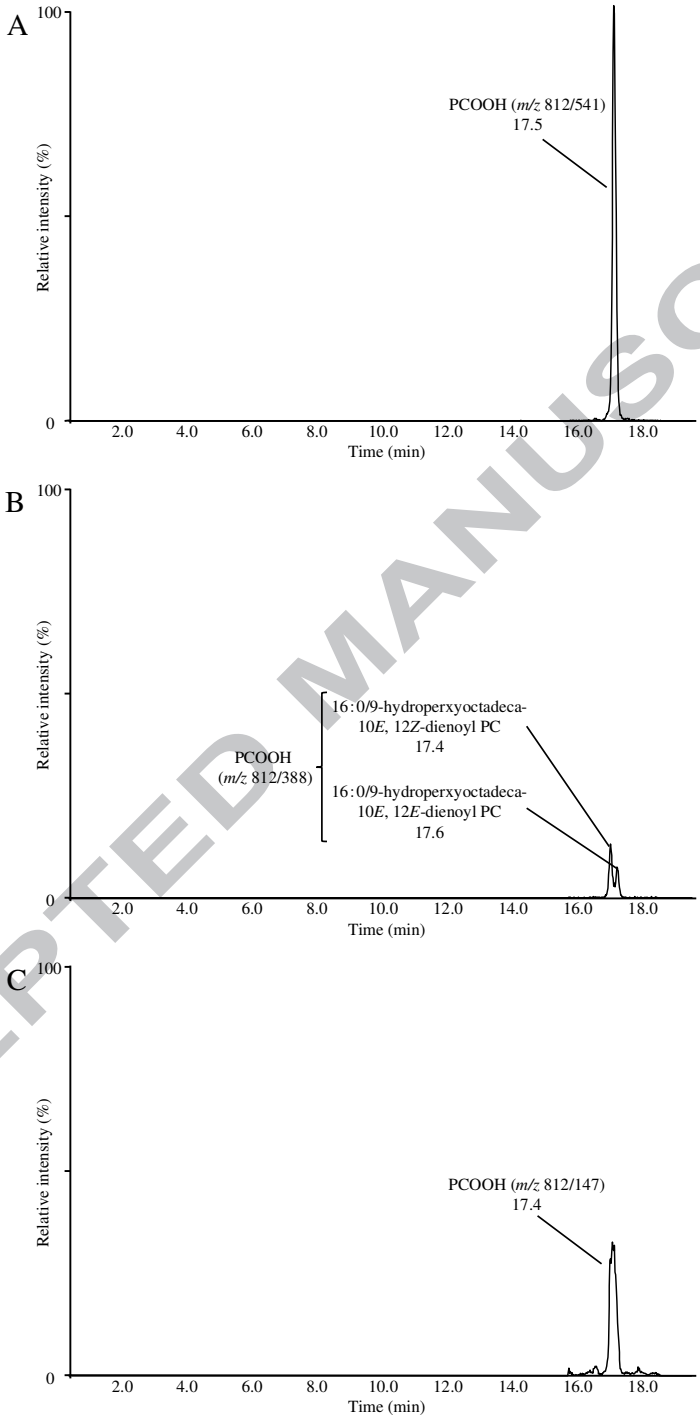
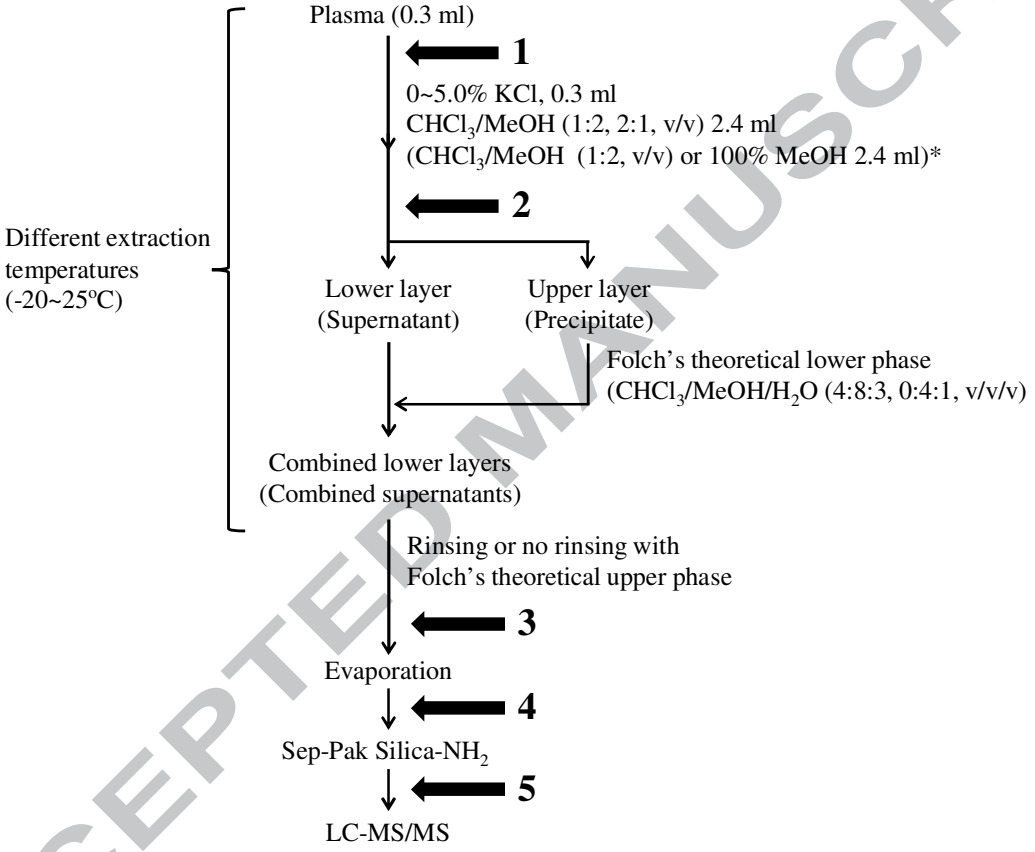


Legend of scheme

Scheme 1. Procedure for the extraction of PCOOH from plasma. Reference PCOOH (120 pmol 16:0/13-HpODE PC/10 µl methanol) was spiked into the plasma (Step 1), lipid fraction (Step 2), total lipids (Step 3), dried extract (Step 4), or final aliquot (Step 5), and PCOOH recovery rates were calculated and evaluated in order to develop efficient and quantitative extraction procedures.

Scheme 1.



*Contents in parentheses ( ) represent the case of extraction with $CHCl_3$/MeOH (1:2, v/v) or 100% MeOH

Supplementary Table I. Individual PCOOH (16:0/HpODE PC) concentrations in plasma of healthy subjects and patients with angiographically significant stenosis

MRM	812/541	812/388	812/147*
		pmol/mL	
Healthy subjects n = 8	25.4	22.6	49.8 (48.0)
	46.3	41.0	86.4 (87.3)
	50.5	46.0	103.4 (96.5)
	34.7	34.3	67.3 (69.0)
	31.9	30.9	66.1 (62.7)
	49.3	43.5	102.7 (92.8)
	19.0	16.6	38.2 (35.6)
	31.9	30.3	64.8 (62.2)
Patients n = 12	86.9	66.0	138.6 (152.8)
	32.9	32.2	64.4 (65.1)
	45.5	35.0	90.3 (80.5)
	39.0	36.0	77.8 (75.1)
	42.8	38.9	87.2 (81.8)
	112.5	84.3	194.6 (196.8)
	49.8	50.1	99.5 (99.9)
	67.4	69.4	132.5 (136.8)
	33.3	30.2	61.9 (63.6)
	43.6	34.9	80.6 (78.6)
	32.3	32.9	61.3 (65.2)
	42.9	32.8	79.1 (75.6)

*Contents in parentheses ( ) represents the sum of 16:0/13-HpODE PC (812/541) and 16:0/9-HpODE PC (812/388).