Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 806 (2004) 41–46
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of phosphatidylcholine monohydroperoxides using quadrupole time-of-flight mass spectrometry

Junko Adachi*, Naoki Yoshioka, Rika Funae, Hideyuki Nushida, Migiwa Asano, Yasuhiro Ueno

Department of Legal Medicine, Kobe University Graduate School of Medicine, 7 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan

Available online 18 March 2004

Abstract

An improved technique for the analysis of phosphatidylcholine (PC) monohydroperoxides was developed using quadrupole time-of-flight (Q-TOF) mass spectrometry with electrospray ionization. Separation was obtained using an HPLC C8 column with a gradient of methanol and 10 mM aqueous ammonium acetate. Monohydroperoxides of palmitoyl-linoleoyl (C16:0/C18:2) PC, stearoyl-linoleoyl (C18:0/C18:2) PC, and oleoyl-linoleoyl (C18:1/C18:2) PC were detected mainly as MH^+ and $[M+Na]^+$ ions in the heart of the intact rat. Using standard synthetic PCOOH (C16:0/C18:2-OOH), the lipid extract component was identified as (C16:0/C18:2-OOH) PC based on the product ions of ESI-MS-MS and, the PCOOH concentration was quantitated using HPLC with chemiluminescence detection. Two epoxyhydroxy derivatives of the three PCs mentioned above were also detected. This is the first report to show the presence of monohydroperoxides and epoxyhydroxy-derivatives of (C16:0/C18:2)PC, (C18:0/C18:2)PC, and (C18:1/C18:2) PC in the rat heart.

Keyword: Phosphatidylcholine monohydroperoxides

1. Introduction

Using gas chromatography-mass spectrometry (GC-MS), we previously identified cholesta-3,5-dien-7-one, a metabolite of 7-hydroperoxycholesterol, in the erythrocyte membrane of alcoholic patients [1]. Recently, we identified and confirmed by LC-MS using an atmospheric chemical ionization interface the presence of 7-hydroperoxycholesterol,7-ketocholesterol, cholesterol-α- and β-epoxide in alcoholic fatty liver tissue [2].

The reaction of linoleic acid hydroperoxide is as follows [3]. 13-Hydroperoxy-9-*cis*,11-*trans*-octadecadienoic acid formed by the peroxidation of linoleic acid in vivo reacts with hematin to produce *erythro*-11-hydroxy-12,13-epoxy-9-octadenoic acid, *threo*-11-hydroxy-12,13-epoxy-9-octadecenoic acid, 9,12,13-trihydroxy-10-octadecenoic acid, 13-oxo-9,11-octadecadienoic acid and 13-hydroxy-9,11-octadecadienoic acid [3]. Similar reactions are believed to occur with phosphatidylcholine (PC) hydroperoxide. The aim of this study is to elucidate the molecular structure of oxidation products of PC in control rat heart by quadrupole time-of-flight (Q-TOF) mass spectrometry with electrospray ionization.

The possible products of phosphatidylcholine oxidation are shown in Fig. 1. (A) 13-Hydroperoxy-PC and 9-hydroperoxy-PC; (B) 12,13-epoxy-11-hydroxy-9-en-PC, 12,13-epoxy-9-hydroxy-10-en-PC, and 9,10-epoxy-11-hydroxy-12-en-PC.

The proposed molecular species of oxidatively modified PC and protonated molecular ions are shown in Table 1.

2. Experimental

2.1. Materials

3,5-Di-*tert*-butyl-4-hydroperoxytoluene (BHT), luminol and cytochrome C (from horse heart, type IV) were purchased from Wako Pure Chemical Co. (Osaka, Japan). 1-Palmitoyl-2-linoleoyl-phosphatidylcholine hydroperoxide (C16:0/C18:2-OOH) was synthesized as follows. Methylene blue·$4H_2O$ (2 mg) was dissolved in a 1:1 mixture (50 ml) of $CHCl_3$ and methanol. A solution of 1-palmitoyl-2-linoleoyl-L-phosphatidylcholine (160 mg) in

* Corresponding author. Tel.: +81-78-382-5582; fax: +81-78-382-5599.
E-mail address: adachi@med.kobe-u.ac.jp (J. Adachi).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.02.025

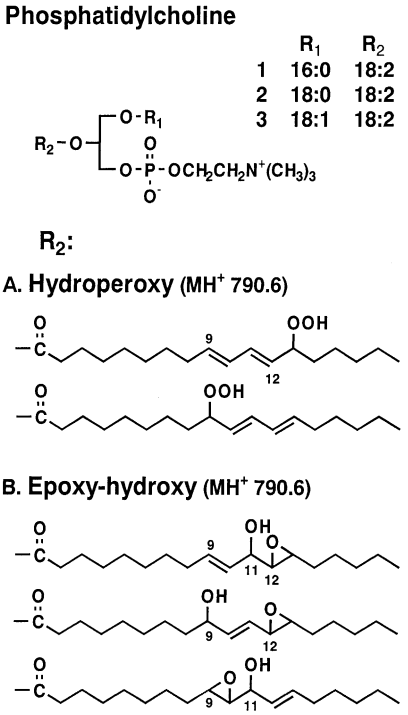

Fig. 1. Possible products of phosphatidylcholine oxidation.

methylene blue solution (20 ml) prepared as mentioned above was irradiated using a tungsten lamp (30 W) at 15 °C for 8 h monitoring by HPLC and TLC. The reaction mixture was then subjected to HPLC (Daiso-gel, SP-120-40/60-ODS-B, $CHCl_3$-methanol, UV 235 nm, 50 ml/min). The product (67.3 mg) obtained was checked by MS, TLC, and HPLC. In addition, the amount of phospholipid was quantitatively analyzed as 1.95 mg/ml. MS: 790.6 (MH^+, monoperoxide) and 812.5 ($[M + Na]^+$, monoperoxide). H-NMR (δ) 6.8–5.2 (m, olefinic H and CH(OOH)), 5.20 (1H, m, 2‴-H), 4.4–4.0 (m, 1‴, 1″, and CH(OOH), 3.95 (2H, m, 3‴-H_2), 3.75 (2H, m, 2″-H_2), 3.3 (10H, m, $N(CH_3)_3$ and OOH), 2.3 (2H, m, 2-H_2), 2.05 (2H, m, CH_2CH–CH=CH=CH–), 1.6 (4H, m, 3-H_2 and 3′-H_2), and 0.95 (6H, m, 16-CH_3 and 18′-CH_3).

Table 1
Molecular species of oxidatively modified phosphatidyicholine (PC) and protonated molecular ions

Compounds	—	Molecular species of PC		
		R_1	R_2	MH^+
Standard PCOOH	1	16:0	18:2	790.6
Hydroperoxy PC	2	16:0	18:2	790.6
	3	18:1	18:2	816.6
	4	18:0	18:2	818.6
Epoxyhydroxy PC	5	16:0	18:2	790.6
	6	18:1	18:2	816.6
	7	18:0	18:2	818.6

2.2. Tissue extraction

Six control rats were killed under deep anesthesia induced by Nembutal. The hearts were dissected on ice and the total lipid content was extracted by adding 4 ml of ice-cold chloroform:methanol (3:1 v/v), containing 0.005% BHT to approximately 0.1 g of heart followed by homogenization under ice-cold conditions. The homogenate was mixed with another 4 ml of chloroform: methanol (3:1 v/v) and 1 ml distilled water, spun vigorously for 1 min, and then centrifuged at 800 × *g* for 20 min. The chloroform layer was aspirated away, concentrated in a rotary evaporator and then dried under a nitrogen stream. The phospholipid fraction then was isolated from the total lipid sample by solid phase extraction. A silica column (Sep-Pak, Waters. Milford, MA) of 3 ml capacity packed with aminopropyl-derivatized silica (–NH_2) was used. The total lipid sample was dissolved in a small amount of chloroform and layered on the column, which then was flushed with a mixture of 2 ml chloroform and 1 ml of *iso*-propanol. The column was next flushed with methanol containing 0.005% BHT, giving an eluate consisting mainly of phospholipid. This was concentrated using a rotary evaporator, dried under a nitrogen stream and then dissolved in 150 μl of methanol. A 10 μl portion was injected into the HPLC column with chemiluminescence detection (HPLC-CL). Additionally, a 1 μl portion was injected into the LC-MS.

2.3. LC-MS conditions

An HPLC model 1100 system (Agilent Technologies, Waldbronn, Germany) with a Luna C_8 column (1.0 mm × 150 mm, 5.0 μm; Phenomenex, Torrance, CA) was used. Injections of rat sample (1 μl (MS) and 2 μl (MS/MS) were made. The column was maintained at 40 °C and eluted at 100 μl/min. The mobile phase consisted of 5% methanol with 10 mM aqueous ammonium acetate (solvent A) and 95% methanol with 10 mM aqueous ammonium acetate (solvent B). Separation was carried out with a liner gradient starting with 90% solvent B followed by ramping up to 100% solvent B at 20 min and then maintaining for 17 min. The total run time was 37 min.

Mass spectrometric analysis was performed on a quadrupole orthogonal acceleration time-of-flight, Micromass Q-TOF Micro (Waters Corporation, Milford, MA) equipped with an electrospray interface. The instrument was operated in positive ion mode with a capillary voltage of 3200 V and a cone voltage of 40 V. The desolvation gas was set at 600 l/h with a desolvation temperature of 150 °C and source temperature of 80 °C. Full scan spectra were recorded in profile mode. The range between *m/z* 100–1000 was recorded at a resolution of 5000 (FWHM) and the accumulation time was 1 s/spectrum. Accurate masses were measured by comparison to a reference compound, leucine enkephalin ($[M + H]^+ = 556.2771$ ml mol mass units) infused into the lock spray reference channel.

2.4. HPLC-CL analysis

Phosphatidylcholine hydroperoxide (PCOOH, C16:0/C18:2-OOH) was quantitated by HPLC-CL. An LC-18-DB column (SUPELCO, 250 mm × 4.6 mm i.d.) was isocratically run using a mobile phase consisting of 0.01% triethylamine methanol. The mobile phase and chemiluminescent reagent (cytochrome C and luminol) were delivered at 0.7 ml/min. After the column eluant passed through a UV detector, it was mixed with luminescent reagent in the post-column mixing joint of the chemiluminescence detector. Individual peak areas were calculated using an integrator (Chromatopac C-R8A, Shimadzu).

3. Results

Accurate masses of 10 components of PC oxidation products in rat were measured (a single run) using the Q-TOF system. The results of the measurements are shown in Table 2.

Fig. 2 shows mass chromatograms (MCs) of phospholipids extracted from a control rat heart by LC-MS at specific mass-to charge ratios (*m/z*). In positive-ion mode the strongest species observed were PCs due to the positive charge on the choline headgroup. For a given chain length, lipids containing more unsaturated fatty acid eluted earlier from the column: for example C34:2 PC (e.g. palmitoyl-linoleoyl PC) at *m/z* 758.6 eluted earlier (22.12 min) than C34:1 PC (e.g palmitoyl-oleoyl PC) at *m/z* 760.6 (24.38 min). Increased fatty acid chain length resulted in longer lipid retention times, as seen by comparing the elution of C34:2 PC (e.g. palmitoyl-linoleoyl PC) with a mass of 758.6 at 22.12 min and C36:2 PC (e.g. stearoyl-linoleoyl PC) with a mass of 786.6 at 25.62 min. Fig. 3 shows mass chromatograms from 0 to 16 min by LC-MS. Mass chromatograms of standard PCOOH (column 1) and rat extracts (columns 2–4) are shown in Fig. 3. Coinciding peaks are seen originating from the MH^+ of standard PCOOH (C16:0/C18:2-OOH) at *m/z* 790.6 at a retention time of 10.6 min, from the MH^+ of epoxyhydroxy derivatives (estimated) of PC C16:0/C18:2 at *m/z* 790.6 with retention times of 6.5 and 7.3 min (column 2), from the MH^+ of epoxyhydroxy derivatives and mono-hydroperoxides of PC C18:1/C18:2 at *m/z* 816.6 (column 3), and from the MH^+ of PC C18:0/C18:2 at *m/z* 818.6 (column 4), respectively. The ESI-mass spectra of peaks 2a and 2b show a MH^+ (*m/z* 790.6) and $[M + Na]^+$ (*m/z* 812.5) ions as shown in

Table 2
Accurate mass measurement results for components found in the rat heart

Peak	RT (min)	Measured *m/z*	Calculated *m/z*	Composition	Differences[a] (ppm)
1[b]	10.6	790.5564	790.5598	C42H81NO10P	−4.3
2a	6.5	790.5588	790.5598	C42H81NO10P	−1.2
2b	7.3	790.5621	790.5598	C42H81NO10P	2.9
2c	10.7	790.5590	790.5598	C42H81NO10P	−1.1
3a	8.0	816.5759	816.5755	C44H83NO10P	0.6
3b	10.7	816.5774	816.5755	C44H83NO10P	2.4
3c	11.5	816.5729	816.5755	C44H83NO10P	−3.1
4a	9.4	818.5891	818.5911	C44H8SNO10P	−2.4
4b	11.0	818.5931	818.5911	C44H85NO10P	2.4
4c	15.2	818.5917	818.5911	C44H8SNO10P	0.7

[a] Differences in values between measured and calculated.
[b] Standard PC (Cl 6:0/Cl 8:2-OOH).

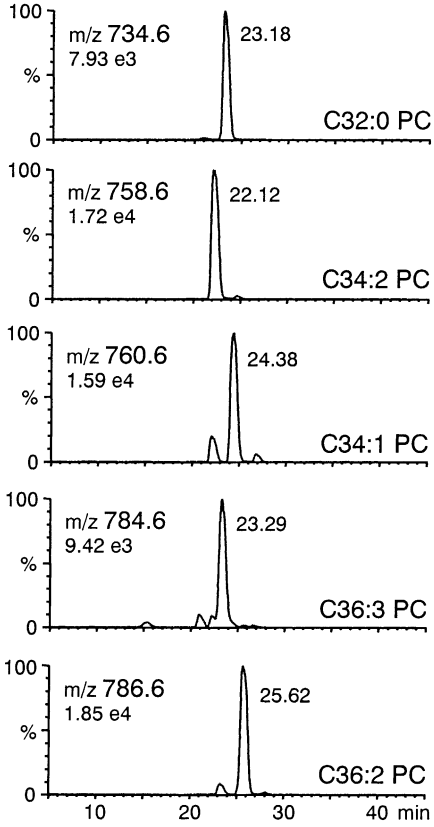

Fig. 2. Mass chromatograms of phospholipids from control rat heart using LC-MS from 20 to 40 min.

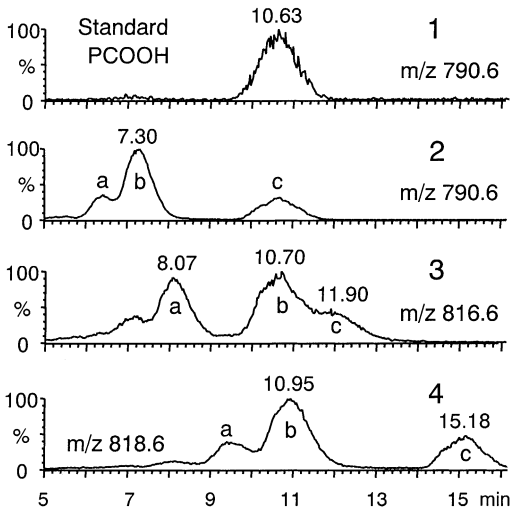

Fig. 3. Mass chromatograms of lipid extract from 0 to 16 min using LC-MS.

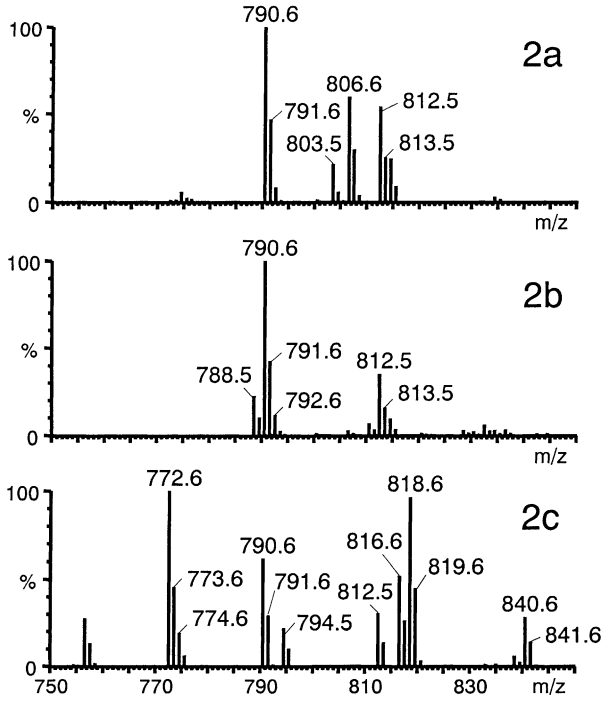

Fig. 4. ESI-mass spectra of peaks 2a–c.

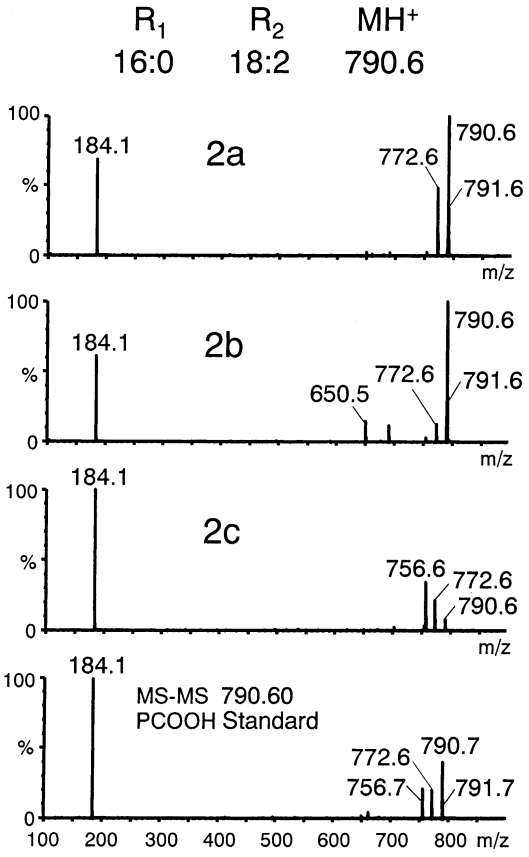

Fig. 5. Product ion spectra on LC-MS-MS of the $[M+H]^+$ ion of standard PCOOH and peaks 2a–c.

Fig. 4. The ESI-mass spectrum of peak 2c contained four pairs (A–D) of the MH^+ and $[M + Na]^+$ ions. (A) 772.6 and 794.5; (B) 790.6 and 812.5; (C) 816.6 and 838.6; (D) 818.6 and 840.6.

Fig. 5 shows the product ion profiles for standard PCOOH and peaks 2a, 2b, and 2c. For standard PCOOH, the base peak was *m/z* 184.1 corresponding to choline phosphate; small peaks associated with fragment ions appeared at *m/z* 772.6 and 756.7. These breakdown products lost 18 and 34 mass units, corresponding to dehydration and the loss of hydrogen peroxide; this supported the assignment as hydroperoxide. For peak 2c, the base peak was *m/z* 184.1; small peaks attributed to fragment ions appeared at *m/z* 772.6, and 756.6. Thus, peak 2c was identified as PC C16:0/C18:2-OOH. For peaks 2a and 2b, the base peak was *m/z* 790.6; small peaks associated with fragment ions appeared at *m/z* 772.6 as $[M + H–H_2O]^+$ and 184.1.

Fig. 6 shows the product ion profiles for peaks 3a–c (upper column) and peaks 4a–c (lower column). For peaks 3c, and 4c the base peak was *m/z* 184.1 corresponding to choline phosphate. Two small peaks attributed to fragment ions appeared at *m/z* 798.6 and 782.6 for peak 3c and at *m/z* 800.6 and 784.6 for peak 4c. These breakdown products lost 18 and 34 mass units. Thus, peaks 3c and 4c were confirmed to be the monohydroperoxide of PC C18:1/C18:2 and PC C18:0/C18:2, respectively. For peaks 3a and 3b, the base peak was *m/z* 816.6 with small peaks associated with fragment ions appearing at *m/z* 798.6 as $[M + H–H_2O]^+$ and *m/z* 184.1. For peaks 4a and 4b, the base peak was *m/z* 818.6 with small peaks attributed to fragment ions appearing at *m/z* 800.6 as $[M + H–H_2O]^+$ and *m/z* 184.1. Peaks 2a, 2b, 3a, 3b, 4a, and 4b were assumed to be the epoxyhydroxy derivatives of PC C16:0/C18:2, PC C18:1/C18:2, and PC C18:0/C18:2, respectively.

The mean concentration of PC C16:0/C18:2-OOH in the rat heart determined by HPLC-CL was 25.8 ± 18.6 nmol/g wet wt. ($n = 6$).

4. Discussion

Q-TOF micro combines the simplicity of a quadrupole [MS 1], the high ion conductance of a hexapole collision cell and the high efficiency of an oa-TOF mass analyzer [MS 2]. Q-TOF micro exploits oa-TOF MS to achieve the simultaneous detection of ions across the full mass range. This is in contrast to conventional instruments that must scan over one mass at a time. Q-TOF micro offers up to 100 times more sensitivity than tandem quadrupole instruments when acquiring full product ion (MS-MS) mass spectra essential to metabolism studies and peptide characterization.

In the present study, we applied this LC-MS-MS system to analyze oxidized molecular species of PC. The mass accuracy obtained using the Q-TOF system was good, with differences within 4.3 ppm between the calculated values of known molecular masses of sample compounds and their

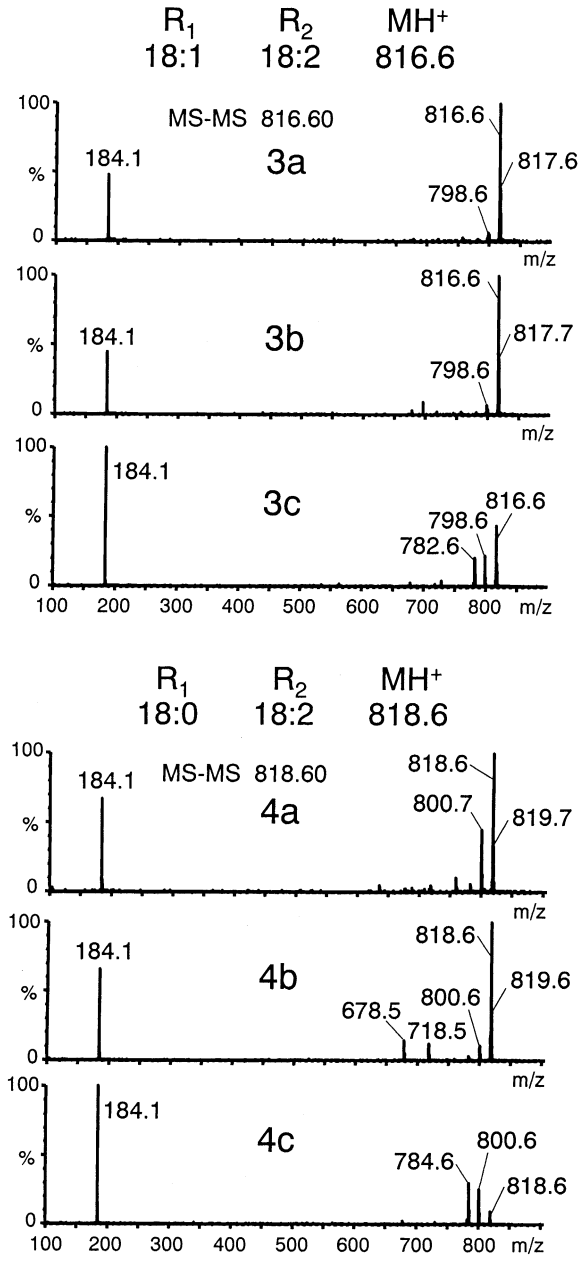

Fig. 6. Product ion spectra on LC-MS-MS of the $[M+H]^+$ ion of peaks 3a–c, 4a–c.

measured values. These results are nearly the same as those obtained using multiple sprayer nano-ESI-MS combined with nano-HPLC [4].

We detected monohydroperoxides of palmitoyl-linoleoyl-PC, and stearoyl-linoleoyl-PC in the heart of a control rat. Using a standard synthetic compound we confirmed its identification as PC C16:0/C18:2-OOH based on the product ions of MS-MS. Studies on the peroxidation of 1-palmitoyl-2-linoleoyl PC revealed that the oxidation products are 9- and 13-hydroperoxyoctadecadienoate (HPODE), 9- and 13-hydroxyoctadecadienoate (HODE) [5], 13-hydroxy [HODE] and 13-hydroperoxy [HPODE] PC [6]. Monohydroperoxides of palmitoyl-linoleoyl and stearoyl-linoleoyl PC were detected in cultured cells treated with *t*-butylhydroperoxide plus Fe^{3+} using LC-ESI-MS [7].

Moreover, we detected monohydroperoxides of oleoyl-linoleoyl PC in the rat, since C36:3 is an abundant unsaturated lipid present in the heart. Thus, this is the first report showing the presence of monohydroperoxides of (C16:0/C18:2) PC, (C18:0/C18:2) PC, and (C18:1/C18:2) PC in the heart of the intact rat.

Several studies have used LC-ESI-MS-MS techniques to examine the structure of oxidized products of arachidonic acid in glycerophospholipids from red blood cells [5,6], 1-palmitoyl-2-arachidonyl PC of macrophage scavenger receptor CD 36 [8], and 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphoryl-choline in human aortic endothelial cells [9]. These products were 5-, 8-, 9-, 12-, and 15-hydroxyeicosatetraenoate (HETE) [5,6] and 5-, 12- and 15-hydroperoxyeicosatetraenoate (HPETE) [5] and 15-hydroperoxy (15-HPETE) derivatives of plasmalogen [6], 15-hydroxy (15-HETE) and 15-hydroperoxy (15-HPETE) derivatives of PC [8].

Finally, we detected epoxyhydroxy derivatives of PC in the rat. They are believed to be epoxyhydroxy derivatives of palmitoyl-linoleoyl and stearoyl-linoleoyl as well as oleoyl-linoleoyl PC based on a recent report which stated that linoleic acid hydroperoxide reacts with hematin to produce 11-hydroxy-12,13-epoxy-octadecenoic acid [3].

Regarding the epoxyhydroxy derivatives of PC, 1-palmitoyl-2-linoleoyl-PC-13-hydroperoxide reacts with α-tocopherol in the presence of a lipid-soluble iron chelate to form 1-palmitoyl-2-[9-(8a-dioxy-α-tocophenone)-12,13-epoxy-10-octadecenoyl] PC and 1-palmitoyl-2-[11-(8a-dioxy-α-tocophenone)-12,13-epoxy-9-octadecenoyl] PC [10]. After the incubation of submitochondrial particles, phospholipids were hydrolyzed by phospholipase A_2. In the fatty acid constituents analyzed by GC-MS and LC-MS, derivatives of hydroperoxides of linoleic acid such as keto, hydroxy, trihydroxy, and hydroxyepoxy compounds were observed [11]. Thus, 12,13-epoxy-11-hydroxy and 12,13-epoxy-9-hydroxy derivatives of PC are likely to be present. When LDL phospholipids were treated with chorohydrin, a highly toxic oxidant produced by myeloperoxidase in phagocyte, or myeloperoxidase, the major products were lipid chlorohydrins rather than lipid peroxides [12]. In cells treated with HOCl, chlorohydrins of palmitoyl-oleoyl PC were observed using LC-MS [7].

5. Conclusion

We are able to measure the accurate masses of PC oxidation products in rat extract using Q-TOF-ESI-mass spectrometry with a lock spray interface. Furthermore, the presence of monohydroperoxides of PC with three molecular species was confirmed.

Acknowledgements

We are grateful to Ms Sanae Furusho, JASCO International Ltd., Tokyo for her technical assistance. We also thank Prof. Kazuo Igarashi, Faculty of Pharmaceutical Sciences, Kobe Gakuin University for his helpful advice. This work was supported in part by a Grant-in-Aid for Scientific Research from the Japanese Society for the Promotion of Science.

References

[1] J. Adachi, K. Hojo, Y. Ueno, T. Naito, I. Ninomiya, H. Imamichi, Y. Tatsuno, Alcohol. Clin. Exp. Res. 20 (1996) 51A.
[2] J. Adachi, M. Asano, Y. Ueno, T. Naito, Alcohol. Clin. Exp. Res. 24 (2000) 21S.
[3] T.A. Dix, L.J. Marnett, J. Biol. Chem. 260 (1985) 5351.
[4] Y. Takahashi, T. Morita, Y. Ueda, J. Chromatogr. B 776 (2002) 31.
[5] L.M. Hall, R.C. Murphy, Anal. Biochem. 258 (1998) 184.
[6] R.C. Murphy, N. Khaselev, T. Nakamura, L.M. Hall, J. Chromatogr. B. 731 (1999) 59.
[7] C.M. Spickett, N. Rennie, H. Winter, L. Zambonin, L. Landi, A. Jerlich, R.J. Schaur, A.R. Pitt, Biochem. J. 355 (2001) 449.
[8] E.A. Podrez, E. Poliakov, Z. Shen, R. Zhang, Y. Deng, M. Sun, P.J. Finton, L. Shan, B. Gugiu, P.L. Fox, H.F. Hoff, R.G. Salomon, S.L. Hazen, J. Biol. Chem. 277 (2002) 38503.
[9] N. Leitinger, A.D. Watson, K.F. Faul, A.M. Fogelman, J.A. Berliner, Adv. Exp. Med. Biol. 433 (1997) 379.
[10] R. Yamauchi, K. Ozaki, M. Shimoyamada, K. Kato, Chem. Phys. Lipids 114 (2002) 193.
[11] H. Iwase, T. Takatori, M. Nagao, H. Niijima, K. Iwadate, Y. Matsuda, M. Kobayashi, Free Radic. Biol. Med. 24 (1998) 1492.
[12] A. Jerlich, A.R. Pitt, R.J. Shaur, C.M. Spickett, Free Radic. Biol. Med. 28 (2000) 673.