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Short communication

An improved HPLC assay for phosphatidylcholine hydroperoxides (PCOOH) in human plasma with synthetic PCOOH as internal standard

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Abstract

Quantitative and qualitative analyses of 1-palmitoyl-2-linoleoyl-phosphatidylcholine monohydroperoxide [PC 16:0/18:2-OOH] and 1-stearoyl-2-linoleoyl-phosphatidylcholine monohydroperoxide [PC 18:0/18:2-OOH] in human plasma were improved by chemiluminescence HPLC using synthetic 1-stearoyl-2-erucoyl-phosphatidylcholine monohydroperoxide (PC 18:0/22:1-OOH) as internal standard. The calibration curves of synthetic PC 16:0/18:2-OOH and PC 18:0/18:2-OOH, obtained by their direct injections with the IS into the HPLC system, were linear throughout the calibration range (10–1000 pmol). Within-day and between-day coefficients of variation were below 8%, and the recoveries were between 84% and 101%. Plasma concentrations of PC 16:0/18:2-OOH and PC 18:0/18:2-OOH were 102 ± 59 nM (mean $\pm$ SD) and 36 ± 20 nM, respectively, in the 33 healthy volunteers. The present method might help understanding incompletely understood pathway of plasma phosphatidylcholine hydroperoxides.

Keywords: HPLC; Chemiluminescence; Luminol; Phosphatidylcholine hydroperoxide; PC; Human plasma

1. Introduction

Lipid peroxidation plays a significant role in various diseases [1]. Previously, we reported a chemiluminescence HPLC (HPLC-CL) assay for simultaneous measurement of hydroperoxides of cholesterylester (CE) and triglyceride (TG) in human plasma [2]. For phosphatidylcholine (PC) hydroperoxides (PCOOH) in human plasma discrepant results have been reported in the literature: for example, 227 nM (mean) and 73.7 nM according to two different studies by Miyazawa and coworkers [3,4], and 9.50 nM according to Adachi et al. [5]. Although the previous HPLC-CL assays for PCOOH adopted external standard calibration methods, use of an appropriate internal standard might be helpful for more precise and accurate measurements.

Here we report a reversed phase HPLC-CL method for measurement of PCOOH concentrations in human plasma, using the synthetic 1-stearoyl-2-erucoyl-PC monohydroperoxide (PC 18:0/22:1-OOH) as internal standard (IS). This IS is a member of PCOOH, but is undetectable in human plasma. To the best of our knowledge, this is the first report of plasma PCOOH measurements by internal standard calibration method.

2. Experimental

2.1. Reagents

Imidazole was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Stearic acid and cytochrome *C* (from horse heart) were obtained from Wako Pure Chemical Industry Ltd. (Osaka, Japan). 1-Palmitoyl-2-linoleoyl-PC (PC 16:0/18:2) and 1-stearoyl-2-linoleoyl-PC (PC 18:0/18:2) were obtained from Sigma (USA). Erucic acid was obtained from Alfa Aesar (Johnson Matthey Company, USA). *tert*-Butyldimethylchlorosilic

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(*tert*-BMS) was obtained from Shin-Etsu Chemical Co. Ltd. (Tokyo, Japan). Serum lipids and apolipoproteins were measured by using commercial kits (Daiichi Pure Chemicals, Tokyo, Japan).

2.2. Blood samples

Healthy volunteers ($n = 33$; 21 ± 1 years, mean $\pm$ SD, range 20–21 years; male/female = 11/22) underwent an overnight fast and their blood was drawn into tubes containing EDTA-2Na. Plasma was separated by centrifugation ($2000 \times g$, 10 min, 4 °C) and immediately stored at −80 °C in aliquots. These samples were used with the approval of the institute after obtaining informed consent from the volunteers.

2.3. Operation conditions of HPLC-CL

HPLC-CL with post-column detection was performed with the LC-10AD pump system equipped with a SCL-10A system controller and a CLD-10A chemiluminescence detector (Shimadzu Co., Kyoto, Japan). A Mightysil RP-18 GP analytical column (4.6 mm × 150 mm, 5 μm, Kanto Chemical Co., Japan) was eluted with a mobile phase of methanol–isopropanol (20:1, v/v) at a flow rate of 0.5 ml/min at room temperature. The column eluant was mixed with luminescent reagent solution (0.5 ml/min) in a post-column mixing joint at 40 °C. The luminescent reagent consisted of cytochrome C (10 μg/ml) and luminol (2 μg/ml) in 50 mM borate buffer (H_3BO_3/KCl–Na_2CO_3, pH 10) [2].

2.4. Preparation of PCOOH standards

PC 16:0/18:2 and PC 18:0/18:2 have been reported as the two major PC species in human plasma [6]. Correspondingly, PC 16:0/18:2-OOH and PC 18:0/18:2-OOH have been identified as the major PCOOH species in human plasma by using LC–MS [5]. Hence, PC 16:0/18:2-OOH and PC 18:0/18:2-OOH were chosen as analytes in this study.

Hydroperoxidation of PC was performed using our previous method [2,7,8], with some modification in purification steps [9,10]. 1-Palmitoyl-2-linoleoyl-PC monohydroperoxide (PC 16:0/18:2-OOH) was obtained from PC 16:0/18:2 with 73% yield. ^{1}H NMR ($CDCl_3$, δ) 0.87 (m, 6H, –C*H*$_3$), 1.24–1.57 (m, 42H, –C*H*$_2$), 2.03 (m, 4H, –C=C–C*H*$_2$–), 2.27 (m, 4H, –OCO–C*H*$_2$–), 3.30 (m, 10H, –N(C*H*$_3$)$_3$ and –OO*H*), 3.74 (m, 2H, –C*H*$_2$–N–), 3.96 (m, 2H, 3-*H*), 4.11–4.37 (m, 5H, 1-*H*, –POC*H*$_2$CH$_2$N, and –C*H*–OOH), 5.20 (m, 1H, 2-*H*), and 5.32–6.53 (m, olefinic *H*). 1-Stearoyl-2-linoleoyl-PC monohydroperoxide (PC 18:0/18:2-OOH) was obtained from PC 18:0/18:2 with 70% yield. ^{1}H NMR ($CDCl_3$, δ) 0.87 (m, 6H, –C*H*$_3$), 1.24–1.57 (m, 46H, –C*H*$_2$), 2.02 (m, 4H, –C=C–C*H*$_2$–), 2.28 (m, 4H, –OCO–C*H*$_2$–), 3.30 (m, 10H, –N (C*H*$_3$)$_3$ and –OO*H*), 3.73 (m, 2H, –C*H*$_2$–N–), 3.97 (m, 2H, 3-*H*), 4.13–4.34 (m, 5H, 1-*H*, –C*H*–OOH, and –POC*H*$_2$CH$_2$N), and 5.21 (m, 1H, 2-*H*), 5.32-6.51 (m, olefinic *H*).

2.5. Synthesis of IS

PC 18:0/22:1-OOH (IS) was synthesized with 15% overall yield, as shown in Fig. 1. Stearoylglycerol (I) was synthesized from stearic acid and glycerol (76% yield) [8]. *tert*-BMS (4 mmol) and imidazol (1 mol) were added to a solution of compound I (4 mmol) in dried pyridine at 0 °C. The mixture was stirred at 0 °C for 2 h and extracted with diethyl ether. The organic phase was washed with 2 M HCl and water. The residue was purified by SiO_2 column chromatography with hexane–ethyl acetate (20:1, v/v) to yield pure 1-stearoyl-3-*tert*-butyldimethylsilylglycerol (II, 83% yield).

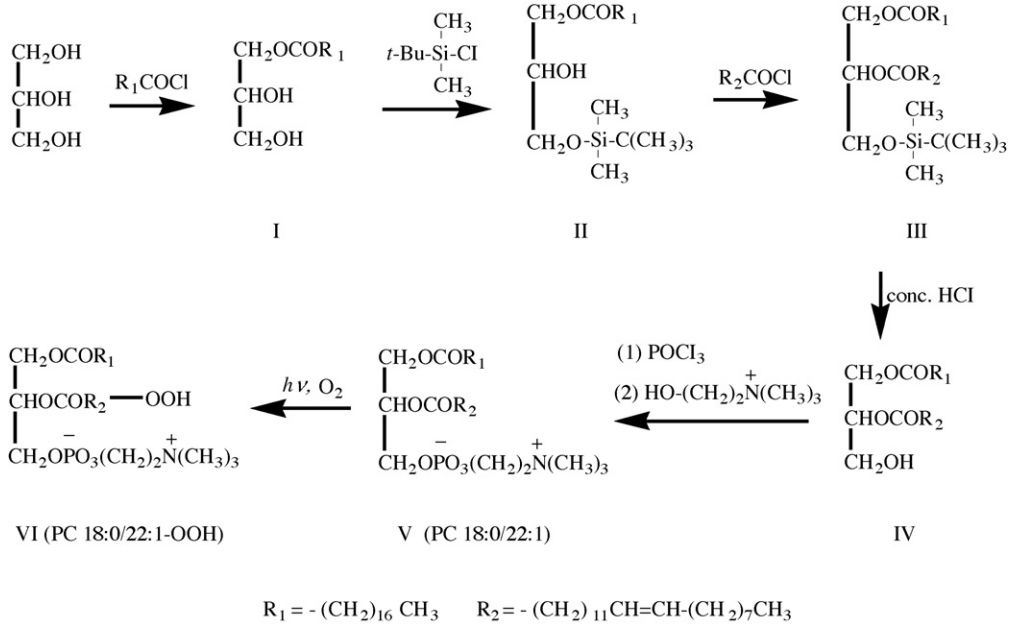

Fig. 1. Synthesis of the 1-stearoyl-2-erucoyl-phosphatidylcholine monohydroperoxide (PC 18:0/22:1-OOH) for use as internal standard.

1-Stearoyl-2-erucoyl-3-(*tert*-butyldimethylsilyl) glycerol (III) was synthesized from compound II and erucic acid (92% yield) [8]. The compound III (2 mmol) was dissolved in ethanol–benzene, and the solution was stirred in an ice bath. Concentrated HCl was added dropwise until compound III was converted to 1-stearoyl-2-erucoyl glycerol (IV) (66% yield). The reaction mixture was evaporated, extracted with ethyl acetate and washed with water. The residue was subjected to SiO_2 column chromatography eluted with *n*-hexane–ethyl acetate (20:1, v/v). The compound IV was then converted to 1-stearoyl-2-erucoyl-PC (PC 18:0/22:1, V) by using previous methods (54%) [9,10]. PC 18:0/22:1-OOH (VI) was obtained from compound V with 71% yield. ^{1}H NMR ($CDCl_3$, δ) 0.87 (m, 6H, –C*H*$_3$), 1.24–1.57 (m, 58H, –C*H*$_2$), 2.00 (m, 4H, –C=C–C*H*$_2$–), 2.28 (m, 4H, –OCO–C*H*$_2$–), 3.32 (m, 10H, –N (C*H*$_3$)$_3$ and –OO*H*), 3.76 (m, 2H, –C*H*$_2$–N–), 3.92 (m, 2H, 3-*H*), 4.09–4.39 (m, 5H, 1-*H*, –C*H*OOH, and –POC*H*$_2$CH$_2$N), 5.18 (m, 1H, 2-*H*), and 5.33–5.71 (m, 2H, olefinic *H*).

2.6. Extraction and refinement of PCOOH

Extraction was started within 2 min after plasma sample was taken out of a −80 °C freezer. Each plasma sample (0.5 ml) was mixed with 10 μL of 6.0 μM IS solution in methanol (finally 120 nM in plasma), 0.5 ml freshly prepared 0.4% *tert*-butylhydroxytoluene (BHT) (antioxidant) in methanol and 2.0 ml chloroform. The mixture was vigorously mixed for 1 min and then centrifuged at 2000 × *g* for 5 min at 4 °C. The chloroform layer was collected and evaporated in vacuo. The residue was refined using a solid-phase extraction according to the method of Adachi et al. [5], with some modification. A 3-ml capacity silica column (Sep-Pak, Waters Co., Milford, USA) packed with aminopropyl-derivatized silica was pre-washed with 5 ml distilled acetone and 10 ml *n*-hexane. The residue dissolved in a small amount of chloroform was added to the column, which was then flushed with a mixture of 2 ml chloroform and 1 ml isopropanol, giving an eluate containing the hydroperoxides of CE and TG. PCOOH and the IS were eluted with following 1 ml methanol containing 0.005% BHT. The methanol was dried under a nitrogen stream. The residue was dissolved in 50 μl methanol–isopropanol (20:1, v/v) and a 20-μl aliquot was injected into the HPLC-CL system.

3. Results and discussion

3.1. HPLC analyses of synthetic PCOOH and IS

A mixture of methanol–isopropanol (20:1, v/v) was used for the preparation of standard solutions. As shown in Fig. 2A, synthetic PC 16:0/18:2-OOH, PC 18:0/18:2-OOH, and the IS were separated with 7.3 min, 9.0 min, and 13.5 min of retention times, respectively. By photosensitized oxidation, PC 16:0/18:2-OOH and PC 18:0/18:2-OOH were expected to be consisted of respective 9-, 10-, 12-, and 13-regioisomers [11,12]. In the chromatogram (Fig. 2A), however, a single peak was obtained for each of PC 16:0/18:2-OOH and PC 18:0/18:2-OOH, showing that these regioisomers were not separated in our HPLC system. The response factor of the IS was 2.47 for PC 16:0/18:2-OOH and 2.64 for PC 18:0/18:2-OOH.

3.2. Precision and accuracy

PC 16:0/18:2-OOH and PC 18:0/18:2-OOH were added to plasma at the concentrations of 10 nM, 100 nM, and 400 nM in

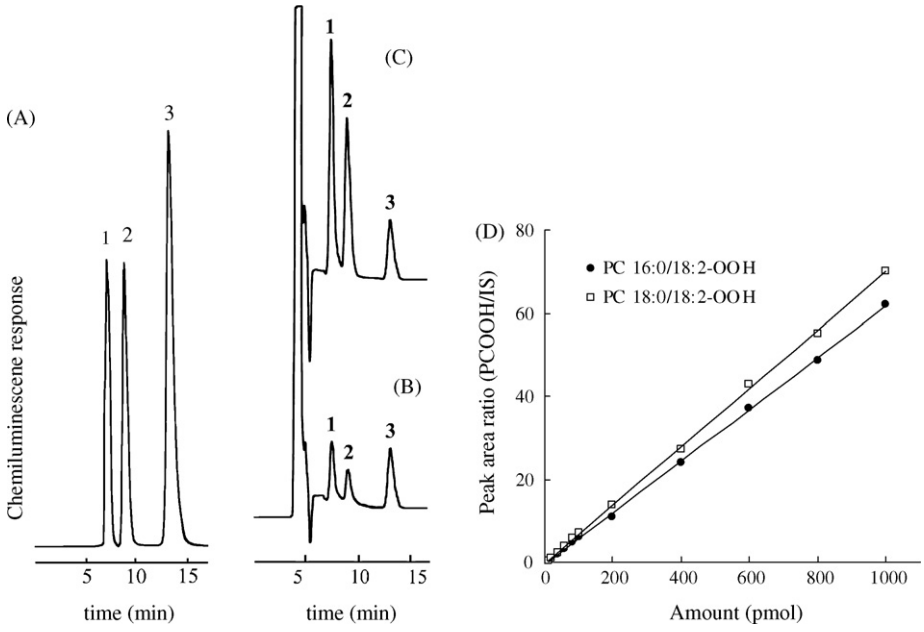

Fig. 2. (A) Chromatogram of a mixture of synthetic PC 16:0/18:2-OOH (10 pmol), PC 18:0/18:2-OOH (10 pmol), and PC 18:0/22:1-OOH (IS, 60 pmol) with HPLC-CL detection. Chromatograms of human plasma extracts with HPLC-CL detection from a healthy subject (B) before and (C) after addition of synthetic PC 16:0/18:2-OOH and PC 18:0/18:2-OOH. Peaks 1, 2, and 3 correspond to PC 16:0/18:2-OOH, PC 18:0/18:2-OOH and PC 18:0/22:1-OOH (IS), respectively. (D) The calibration curves of synthetic PC 16:0/18:2-OOH and PC 18:0/18:2-OOH. For abbreviation and HPLC-CL conditions see text.

Table 1
Concentrations of phosphatidylcholine hydroperoxides, other lipids and apolipoproteins in plasma in the studied population

Variable	Unit	Mean ± SD (n = 33)	Range	vs. PC 16:0/18:2-OOH+PC 18:0/18:2-OOH	
				r	p
Age	Year	21 ± 1	20–21	–	–
PC 16:0/18:2-OOH[a]+PC 18:0/18:2-OOH[b]	nM	138 ± 77	50–424	–	–
PC 16:0/18:2-OOH	nM	102 ± 59	33–311	0.993	<0.0001
PC 18:0/18:2-OOH	nM	36 ± 20	11–113	0.933	<0.0001
PC 16:0/18:2-OOH/PC 18:0/18:2-OOH ratio	–	2.9 ± 0.8	1.8–5.3	0.201	0.264
Total cholesterol	mM	4.74 ± 0.68	3.60–6.27	0.396	0.022
Triglyceride	mM	0.94 ± 0.42	0.51–2.46	0.249	0.163
Phospholipid	mM	2.66 ± 0.32	2.14–3.24	0.276	0.121
HDL-cholesterol	mM	1.79 ± 0.39	1.09–2.51	−0.045	0.805
LDL-cholesterol	mM	2.71 ± 0.60	1.40–4.07	0.395	0.022
ApoA-I	mg/dl	149 ± 17	112–188	−0.129	0.477
ApoA-II	mg/dl	30 ± 3	22–36	−0.022	0.906
ApoB	mg/dl	78 ± 17	50–131	0.366	0.036
ApoC-II	mg/dl	3.4 ± 1.3	1.4–6.9	0.356	0.041
ApoC-III	mg/dl	8.6 ± 2.0	5.1–14.1	0.209	0.245
ApoE	mg/dl	4.5 ± 1.3	2.4–7.4	0.243	0.175

[a] 1-Palmitoyl-2-linoleoyl-phosphatidylcholine monohydroperoxide.
[b] 1-Stearoyl-2-linoleoyl-phosphatidylcholine monohydroperoxide.

the presence of the IS (120 nM in plasma), and analyzed in five replicates within the same assay and in five assays on 5 different days. The mean recoveries (within-day and between-day) ranged between 87% and 101% for PC 16:0/18:2-OOH and 84% and 95% for PC 18:0/18:2-OOH. All within-day and between-day coefficients of variation were less than 8%. The limit of detection (LOD) and the limit of quantification (LOQ) were determined using synthetic standards. The LOD (signal-to-noise ratio, 3:1) was at 1.0 pmol, and the LOQ (signal-to-noise ratio, 10:1) was at 10 pmol for both of PC 16:0/18:2-OOH and PC 18:0/18:2-OOH.

3.3. Stability during storage

The synthesized monohydroperoxides (IS, PC16:0/18:2-OOH, PC18:0/18:2-OOH) and their solutions (1 μM, 2 μM, 4 μM, 6 μM, 8 μM, 10 μM, 20 μM, 40 μM, 60 μM, 80 μM, and 100 μM for PC16:0/18:2-OOH and PC18:0/18:2-OOH, 6 μM for IS in methanol) were stored at −80 °C. Their stabilities were checked under the above storage conditions by reversed-phase TLC analyses (ODS silica gel, 100–200 mesh, Fuji Silysia Chemical Ltd., Kasugai, Japan, developed by chloroform–methanol–water, 1:2:0.1, v/v/v, colorized by phosphomolybdic acid reagent) and also by HPLC-CL analyses as above. The results indicated that any impurities and decomposition products were not observed, and the concentrations of the standard stock solutions did not change significantly for at least 3 months. The plasma samples spiked with PC16:0/18:2-OOH and PC18:0/18:2-OOH (10 nM, 100 nM, and 400 nM) were also stored at −80 °C, and then studied by the HPLC-CL. No significant change was observed in their concentrations during 3 months.

3.4. Identification of PCOOH in human plasma

Fig. 2B shows a typical chromatogram for plasma from a healthy volunteer. For identification of Peaks 1 and 2, synthetic PC 16:0/18:2-OOH and PC 18:0/18:2-OOH were added to the same subject's plasma and found to overlap with Peaks 1 and 2, respectively (Fig. 2C).

In order to rule out possible interference with other phospholipid hydroperoxides, we synthesized monohydroperoxides of phosphatidylethnolamine (PEOOH), lysophosphatidylcholine (lysoPCOOH), and sphingomyelin (SMOOH), and analyzed by the HPLC-CL. The retention times of PEOOH, lysoPCOOH, and SMOOH were, respectively, 2.5 min, 3.8 min, and 11.2 min, that were distinct from those of PC 16:0/18:2-OOH (7.3 min), PC 18:0/18:2-OOH (9.0 min), and the IS (13.5 min). Additionally, we never detected PEOOH, lysoP-

Table 2
Comparison between two methods of quantitation of phosphatidylcholine hydroperoxides in two human plasma samples

	Replicate	PC 16:0/18:2-OOH[a] (nM)		p[b]	PC 18:0/18:2-OOH[c] (nM)		p[b]
		External standard (Mean ± SD)	Internal standard (Mean ± SD)		External standard (Mean ± SD)	Internal standard (Mean ± SD)	
Plasma A	5	12 ± 7.9	33 ± 3.7	0.036	5 ± 4.1	15 ± 1.9	0.009
Plasma B	5	31 ± 9.6	99 ± 4.7	0.009	4 ± 3.7	29 ± 3.0	0.009

[a] 1-Palmitoyl-2-linoleoyl-phosphatidylcholine monohydroperoxide.
[b] vs. external standard method.
[c] 1-Stearoyl-2-linoleoyl-phosphatidylcholine monohydroperoxide.

COOH, and SMOOH in human plasma in our HPLC-CL system. As described in Section 2.6, monohydroperoxides of CE and TG were removed from PCOOH during extraction.

3.5. Calibration curves

The calibration curves of synthetic PC 16:0/18:2-OOH and PC 18:0/18:2-OOH were obtained by direct injections of them and the IS in methanol into the HPLC-CL system (Fig. 2D). They were plotted as the peak area ratios of PC 16:0/18:2-OOH and PC 18:0/18:2-OOH to that of the IS versus the amounts of added PC 16:0/18:2-OOH or PC 18:0/18:2-OOH. The calibration curves showed good linearity throughout the range of 10–1000 pmol (PC 16:0/18:2-OOH, $y = 0.0624x - 0.5041$, $r = 0.9997$; PC 18:0/18:2-OOH, $y = 0.0707x - 0.3213$, $r = 0.9998$; x = amount of hydroperoxides; y = peak area ratio).

3.6. Concentrations of PCOOH in plasma

Results are summarized in Table 1. The plasma concentrations of PC 16:0/18:2-OOH was 2.9-fold higher in average than those of PC 18:0/18:2-OOH. In previous studies on plasma PC concentrations, the mean ratio of PC 16:0/18:2 to PC18:0/18:2 were reported as 1.91 for Caucasians [6] and 1.66 for Japanese [13], showing a relationship similar to that between PC 16:0/18:2-OOH and PC 18:0/18:2-OOH. As shown in Fig. 3, plasma PC 18:0/18:2-OOH concentrations were correlated with those of PC 16:0/18:2-OOH. The sum of PC 16:0/18:2-OOH and PC 18:0/18:2-OOH was significantly correlated with total cholesterol, LDL-cholesterol, and apoB (the major apolipoprotein of LDL), but was not correlated with HDL-cholesterol and the apolipoproteins in HDL, namely apoA-I and apoA-II. Based on these results, LDL might serve as the major carrier of PCOOH in plasma. We have no explanation of the significant correlation between apoC-II and the sum of PC 16:0/18:2-OOH and PC 18:0/18:2-OOH.

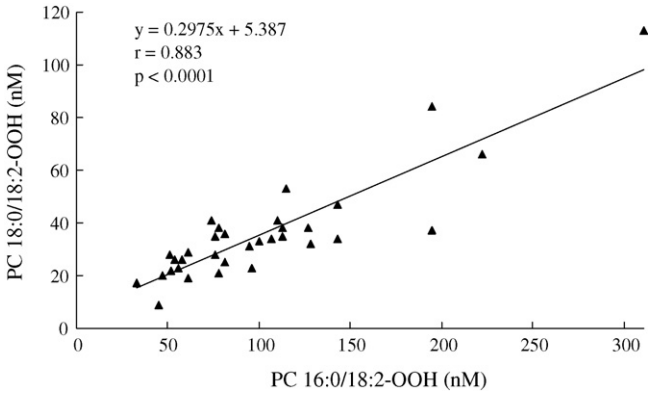

Fig. 3. Correlation between the concentrations of PC 16:0/18:2-OOH and PC 18:0/18:2-OOH in plasma samples of 33 healthy young volunteers. For abbreviation see text.

3.7. Comparison between two methods of quantitation

Two plasma samples were measured in five replicates by HPLC-CL method in comparison between the two calibration methods: one is the present internal standard method and the other is the external standard calibration method using synthetic PC 16:0/18:2-OOH and PC 18:0/18:2-OOH as standards. As shown in Table 2, the external standard calibration method gave significantly lower concentrations than the present method: 31–36% for PC 16:0/18:2-OOH and 14–33% for PC 18:0/18:2-

Table 3
Reported concentrations of phosphatidylcholine hydroperoxides (PCOOH) in human plasma by chemiluminescence HPLC methods

Authors (reference)	Column Column length	Calibration method Calibrator	*n*	Age (year)	PCOOH (nM)	PCOOH species measured
Miyazawa et al. [3]	Silica (TSK-Gel SIL 60) 4.6 mm × 250 mm	External standard Egg yolk PCOOH	14	27 ± 6.8	227 ± 119	Not specified
Miyazawa and coworkers [4,14]	Aminopropyl (Finepak SIL NH_2-5)	External standard	18	23 ± 41	73.7 ± 32.4	Not specified
	4.6 mm × 250 mm	Egg yolk PCOOH	47	49 ± 4	160 ± 65	Not specified
Adachi et al. [5]	Aminopropyl (Finepak SIL NH_2-5)	External standard	12	24 ± 1.5	32.3 ± 17.6	Not specified
	4.6 mm × 250 mm	Synthetic PC 16:0/18:2-OOH	10	34 ± 2.8	62.6 ± 28.7	Not specified
	ODS (Supelcosil LC-18 DB)	External standard	12	24 ± 1.5	9.5 ± 6.4[a]	PC 16:0/18:2-OOH
	4.6 mm × 250 mm	Synthetic PC 16:0/18:2-OOH	10	34 ± 2.8	19.0 ± 9.9[a]	PC 18:0/18:2-OOH
Hui et al. (present study)	ODS (Mightysil RP-18 GP)	Internal standard	33	21 ± 0.5	138 ± 76.9[a]	PC 16:0/18:2-OOH
	4.6 mm × 150 mm	Synthetic PC 18:0/22:1-OOH				PC 18:0/18:2-OOH

[a] Sum of PC 16:0/18:2-OOH and PC 18:0/18:2-OOH

OOH. This inconsistency might partly explain the discrepancy in the reported plasma PCOOH concentrations (Table 3). The coefficients of variation ($n = 5$) of the external standard method for samples A and B were, respectively, 66% and 31% for PC 16:0/18:2-OOH, and 82% and 93% for PC 18:0/18:2-OOH, showing poor reproducibility of the external standard method.

Additionally, Adachi et al. compared two column systems and suggested that the main peak corresponding to PC 16:0/18:2-OOH was contaminated in the aminopropyl column system, yielding higher concentrations than in the ODS column system (Table 3) [5]. Further, a remarkable age-related increase in plasma PCOOH levels was reported in this literature (Table 3) [4,14], which also might contribute to the discrepancy.

The formation and metabolism of PCOOH remains to be elucidated, and our proposed method might serve as a good tool for further investigation.

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