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Automatic determination of hydroperoxides of phosphatidylcholine and phosphatidylethanolamine in human plasma

Kazuaki Akasaka*, Akiko Ohata, Hiroshi Ohrui, Hiroshi Meguro

Department of Applied Biological Chemistry, Faculty of Agriculture, Tohoku University, Tsutsumidori-Amamiyamachi 1-1, Aoba, Sendai 981, Japan

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Abstract

An automatic method for the determination of hydroperoxides of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) is reported. Sample plasma was deproteinized with a fourfold volume of methanol. After centrifugation, the supernatant was injected directly into an HPLC system without further treatment. The hydroperoxides of PC and PE were concentrated and washed on an ODS column followed by introduction into two analytical columns, a silica gel and an aminopropylsilica gel column, which were connected in series, by column switching. After the separation, they were detected by postcolumn detection with diphenyl-1-pyrenylphosphine. The compounds were determined at picomole levels within 30 min with good reproducibilities. By using only a silica gel column as an analytical column, PC hydroperoxides were determined within 20 min, and samples could be injected into it at 15-min intervals. Those methods made it possible to inject a sample of up to 2 ml at one time and up to 8 ml by repeated injections and to determine phospholipid hydroperoxides in human plasma at picomole levels.

1. Introduction

Lipid hydroperoxides in living bodies have attracted much attention as one of the risk factors of some diseases and ageing [1–3]. Although there is a need for the determination of lipid hydroperoxides in biological materials, it has been difficult owing to their diversity, instability and trace concentrations. Recently, some HPLC methods have been reported for their determination in biological materials [4–10]. In these methods, the sensitivity and selectivity were improved using an iron thiocyanate method [4], an electrochemical method [5] or chemiluminescence [6–10]. A flow-injection assay was also reported, using chemiluminescence with luminol [11].

Previously, we have reported HPLC systems with postcolumn detection with diphenyl-1-pyrenylphosphine (DPPP) for the fluorimetric determination of lipid hydroperoxides [12–16] and we showed the presence of phosphatidylcholine (PC) hydroperoxides in fresh plasma and serum [12,13]. Miyazawa et al. [7] also reported its presence in fresh human plasma with a

* Corresponding author.

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chemiluminescence method, whereas Yamamoto et al. [8] reported its absence.

These previous methods involve extraction of lipids with organic solvents and the evaporation of the solvents. They are not only tedious but also have the possibility of the artificial formation or degradation of lipid hydroperoxides because polyunsaturated fatty acid moieties are easily oxidized to hydroperoxides, and they easily decompose to secondary products. Therefore, it is not absolutely clear whether PC hydroperoxide is really present in human plasma or not. To solve the problem, we designed a method in which the concentration of hydroperoxides and their determination are carried out with an on-line system using a column-switching technique. Deproteinated human plasma with methanol is injected directly into the system.

2. Experimental

2.1. Chemicals

DPPP was prepared by our previous method [17]. Dilinoleoyl- and dipalmitoylphosphatidylcholine and phosphatidylethanolamine (PE, from egg yolk) were purchased from Sigma (St. Louis, MO, USA) and 2,6-di-*tert*.-butyl-4-methylphenol (BHT) from Tokyo Kasei Kogyo (Tokyo, Japan). Other reagents and solvents were of special or super-special pure grade (Wako, Osaka, Japan). *n*-Hexane was used after distillation and other solvents were used as received.

2.2. Preparation of lipid hydroperoxides

Dilinoleoylphosphatidylcholine was autoxidized at 37°C for 6 h under an oxygen atmosphere. Hydroperoxides of PE were prepared by photooxidation with methylene blue (0.5 m*M*) in ethanol–chloroform (2:1) for 12 h at ambient temperature followed by purification by preparative silica gel thin-layer chromatography (20 × 20 cm plate, silica gel 60 GF_{254}; Merck, Darmstadt, Germany), developing with chloroform–methanol–water (65:30:5, v/v/v). They were dissolved in chloroform–methanol (1:1) and stored at −20°C. Their hydroperoxide contents were determined by fluorimetry with DPPP as described previously [18]. Dipalmitoylphosphatidylcholine was added to the standard hydroperoxide solutions so as to inject 30–100 μg of dipalmitoyl-PC on to the column.

2.3. High-performance liquid chromatography

Fig. 1 shows the HPLC system. The HPLC pumps were CCPM multi-pumps (P1 and P2) and two CCPE pumps (P3 and P4) (Tosoh, Tokyo, Japan). Their flow-rates were 0.8 ml/min (P1 and P3), 0.3 ml/min (P2) and 1.2 ml/min (P4), respectively. The six-port switching-valve devices (V1 and V2) used were PT-8000 (Tosoh). A Rheodyne Model 7125 injector was used as a sample injector (I) with a 2.0-ml sample loop. The reaction oven was an RE-8000 reactor (Ro) (Tosoh). The detector used was an FS-8000 spectrofluorimeter (F) (Tosoh). The data processor (D) used was an SC-8010 (Tosoh) or a Chromatocorder 12 (System Instrument, Tokyo, Japan). The HPLC columns used were Develosil ODS-HG-5 (C1; 50 mm × 4.6 mm I.D.; 5 μm), Develosil 60-3 (C2; 75 mm × 4.6 mm I.D.; 3 μm) and Develosil NH_2-5 (C3; 75 mm × 4.6 mm I.D.; 5 μm) (Nomura Chemical, Aichi, Japan). The mobile phase solvents were methanol–water (4:1, v/v) (S1) and *n*-hexane–1-

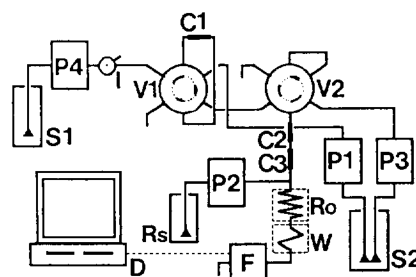

Fig. 1. HPLC system (system A) P1–P4 = pumps; S1 = solvent 1; S2 = solvent 2; I = injector; V1 and V2 = six-port valve device; C1 = ODS column; C2 = silica gel column; C3 = aminopropyl column; Rs = reagent solution; Ro = reactor; W = water-jacket; F = fluorescence detector; D = data processor.

butanol–methanol–water (30:100:400:30, v/v) (S2).

2.4. Column-switching procedure

Fig. 2 shows the column-switching time programmes. The initial positions of the column-switching devices are shown by bold lines in Fig. 1. First, the hydroperoxides were trapped on an ODS column (C1) and washed with methanol–water (4:1) at a flow-rate of 1.2 ml/min for 8.8 min. Then the mobile phase was changed to solvent S2 by switching V1 to elute the hydroperoxides from C1. The hydroperoxides eluted from C1 were introduced into the analytical columns (C2 + C3) by switching V2 at 10 min. Both valve devices were returned to the initial positions at 27 min. Pump P3 pumped solvent S2 at 0.8 ml/min (system A).

For the determination of only PC hydroperoxides, the aminopropyl column (C3) was eliminated from system A. With this system (System B), the valve devices were switched at the same timings as described above without returning to their initial positions at 10.5 min (V2) and 13.0 min (V1), as shown in Fig. 2.

2.5. Postcolumn detection of hydroperoxides

The DPPP solution [Rs; 3 mg in 400 ml of 1-butanol–methanol (1:1, v/v), containing of 1 mg of BHT in 2 ml] was mixed with the eluate

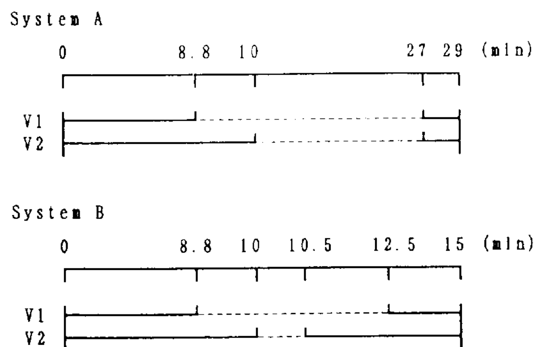

Fig. 2. Valve-switching time programme. The bold and broken lines correspond to the valve positions shown by those lines in Fig. 1.

from the analytical columns at a rate of 0.3 ml/min by pump P2. It was reacted at 80°C by passing it through a reaction coil (stainless steel, 20 m × 0.5 mm I.D.) followed by a cooling coil (stainless steel, 0.5 m × 0.5 mm I.D.) in a water-jacket (W), and the fluorescence intensity was monitored at 380 nm (excitation at 352 nm). The reagent solution was degassed by ultrasonic treatment under reduced pressure and kept for more than 5 h below 4°C before use; it was kept in an ice-bath during the system operation.

2.6. Deproteination procedure of human plasma

To fresh human plasma, a fourfold volume of methanol was added and vortex mixed for more than 30 s. It was then centrifuged for 5 min at 6000 *g* below 5°C, and the supernatant was separated and stored below 0°C until analysis. Up to 2 ml of the supernatant were directly injected into the system at a time. More than 2 ml of sample were introduced into the system in stages at intervals of more than 1.7 min. The time programming for valve switching was started at the last injection.

Fresh human plasma was sampled, with heparin sodium as an anticoagulant, from healthy volunteers who had fasted for more than 12 h.

3. Results and discussion

3.1. Concentration and clean-up of hydroperoxides

First, hydroperoxides of PC and PE were trapped on an ODS column and washed with methanol–water solution. On the ODS column used (C1), these hydroperoxides were never eluted within 15 min by eluting with methanol–water solutions with methanol contents less than 80%. When methanol–water (85:15, v/v) was used as the mobile phase, the hydroperoxides gave broad peaks within 15 min. Therefore, methanol–water (4:1, v/v) (S1) was used as the mobile phase both to concentrate the hydroperoxides and to wash the column.

The trapped hydroperoxides were eluted with a less polar solvent and introduced into the analytical columns. Normal-phase columns were used as analytical columns. The retentions of hydroperoxides on the ODS column were effectively controlled by the *n*-hexane content in the mobile phase without a significant influence to the separations on both analytical columns (C2 and C3). By eluting with *n*-hexane–1-butanol–methanol–water (400:100:30:30, v/v) (S2) on the ODS column, hydroperoxides of PC and PE gave a single, sharp peak near the solvent front.

There were no significant differences in the recoveries of hydroperoxides of PC and PE from the ODS column after washing for 8, 11.5 and 15 min. Their recoveries were 91% (PC-HPO; 138.5 pmol on-column) and 98–101% (PE-HPO; 146.5 pmol on-column). This meant that these hydroperoxides were effectively trapped on the ODS column for at least 15 min and recovered almost quantitatively by elution with solvent S2. This might allow us to inject larger volumes of a sample as a methanol–water (4:1) solution.

When hydroperoxides of PC (139 pmol on-column) and PE (147 pmol on-column) were loaded on to the system by various volume injections (10–2000 μl), their peak areas were almost constant without significant differences in their peak widths and shapes. There was good agreement between injection times (2 ml $\times$ 1–4 times) and peak areas of PC hydroperoxides (83.1 pmol per 2 ml; correlation coefficient $r = 0.999$) and PE hydroperoxides (87.9 pmol per 2 ml; $r = 0.999$) without influence on their peak widths and shapes. These results allowed us to inject a sample of 2 ml in one injection and up to 8 ml in four injections with the proposed systems. It should be noted that more than 1.7-min intervals should be allowed between each injection, because it was necessary to pass more than 2 ml of the solvent to introduce a whole sample into the loop into the ODS column.

When deproteinated human plasma was injected into the system, it was necessary to wash the ODS column with solvent S1 for more than 8.8 min to remove unfavourable substances for the determination. Therefore, valve V1 was switched at 8.8 min after the last sample injection.

3.2. Separation of hydroperoxides

The effects of the timing of V2 valve switching on peak areas after switching V1 were studied. The peak areas were almost the same with elution volumes between 0.95 and 0.97 ml before switching V2, and gradually decreased with larger elution volumes. This meant that PC and PE hydroperoxides did not elute from the ODS column within 1.2 min (0.96 ml elution with S2) after switching V1. Therefore, V2 was switched at 1.2 min after switching V1 so as not to introduce solvent S1 but the whole hydroperoxide fraction into the analytical columns.

Two columns, aminopropyl and silica gel, were connected in series for the simultaneous determination of PC and PE hydroperoxides. Fig. 3a shows the chromatogram of PC and PE hydroperoxides obtained with this system (system A).

As it took less than 1.5 min to initialize the ODS column with solvent S1, the sample was injected more than 2 min after returning valve V1 to its initial position. As no PE hydroperoxide was detected in plasma samples, the aminopropyl column was omitted from the system to shorten the time of analysis of PC hydroperoxide. With this modified system (system B), the valve switching programmes were also modified as described under Experimental. As more than 93% of PC hydroperoxide was

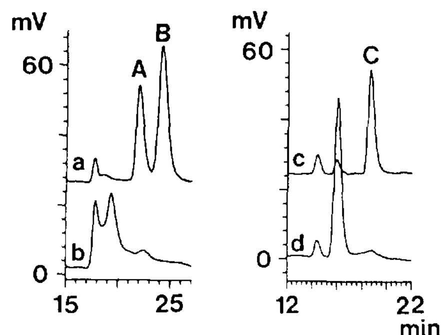

Fig. 3. Typical chromatograms of phospholipid hydroperoxides obtained with (a, b) system A and (c, d) system B. Peaks A and C = standard PC–OOH (111 pmol on-column) and peak B = standard PE–OOH (117 pmol on-column). Deproteinated plasma samples (2 × 2 ml) were injected for chromatograms b and d.

eluted from the ODS column within 0.5 min after switching V2 (at 10 min), V2 was returned to the initial position at 10.5 min, whereas V1 was switched at 13 min to wash the ODS column for another 2.5 min. This meant that consecutive samples could be injected into the system at 15-min intervals, although it took 20 min to measure PC hydroperoxide with this system (Fig. 3c).

The standard PC hydroperoxides used should contain dihydroperoxide (about 10%) whereas most was monohydroperoxide because 17.5% of the linoleoyl moiety in PC was oxidized to hydroperoxide. With the proposed systems, the mono- and dihydroperoxides were eluted at the same time because the sample gave a single peak even when it was loaded directly on to the separation columns.

The standard PE hydroperoxide used, which was prepared from egg yolk PE, was a mixture of hydroperoxides of unsaturated fatty acid esters such as linoleate, arachidonate, eicosapentaenoate and docosahexaenoate. Although the ratio of mono- and dihydroperoxides was not estimated, no peak was observed other than peak B in Fig. 3a.

As the detected substance was only DPPP oxide, which was produced by the reaction of DPPP with hydroperoxy groups, the fluorescence intensity should be proportional to the amount of the hydroperoxy group. At least with PC hydroperoxides, there was no significant difference in the reactivities of DPPP with mono- and dihydroperoxides.

3.3. Detection of hydroperoxides

The eluate from analytical columns was mixed with DPPP solution followed by reaction in the stainless-steel coil and the fluorimetric detection as in our previous methods. This method was highly sensitive and selective with respect to lipid hydroperoxides and was not influenced by radical trapping agents such as BHT and tocopherols in the sample.

Table 1 shows the determination ranges, detection limits and reproducibilities of the standard hydroperoxides with the present methods. It is possible with these systems to determine hydroperoxides with high selectivity and reproducibility and without the need for a procedure for extraction of lipids by organic solvents and their concentration. As there were some variations in the calibration graphs on different days, it is recommended to calibrate each day by injection of standard hydroperoxide solutions (two or three points).

3.4. Determination of PC and PE hydroperoxides in human plasma

Human plasma was deproteinated by addition of a fourfold volume of methanol and centrifugation. As the supernatant could be regarded as methanol–water (4:1, v/v) solution, it was injected directly into the systems. Fig. 3b shows a typical chromatogram for the hydroperoxides in human plasma. The peak at 22 min was assigned as PC hydroperoxides from the retention time. Whereas a 2-ml injection gave only a faintly detectable peak, the peak increased proportionally to the sample volume and gave an apparent peak with 4- and 6-ml injections (Table 2). However, the tailing of an unknown peak at 20 min made it difficult to elucidate the peak of PC hydroperoxides and increased its detection limit 2–3-fold, and this might be one reason why only a faint peak was detected with a 2-ml injection.

As PE hydroperoxide was not detected with system A, we modified the system to determine only PC hydroperoxides (system B). With system B, almost the same level of PC hydroperoxides was detected at 19 min (Fig. 3d).

The concentration of hydroperoxides, especially PE hydroperoxides, gradually decreased in the supernatant during storage, as shown in Fig. 4. The samples were treated below 5°C during the deproteination procedure and injected into the system within 10 min after preparation. As 90–99% of the PC and PE hydroperoxides spiked were recovered from fresh human plasma, as shown in Table 1, the decrease in the hydroperoxides was negligible during the procedure. Fig. 4 also shows that the decrease in PC hydroperoxides was lowered to less than 2% at 3 h and 9% at 6-h by keeping it below 0°C. This allowed us to keep it for 6-h at 0°C to determine PC hydroperoxides. It should be noted that the

Table 1
Determination ranges, detection limits and reproducibilities for standard phospholipid hydroperoxides and their recoveries from fresh human plasma

Parameter	System A		System B
	PC-OOH	PE-OOH	PC-OOH
Range (pmol)[a]	2.8–1108	2.9–1172	5.5–1108
Equation[b] ($n = 3$)	$y = 12.4(\pm0.29)x + 57.6(\pm12.1)$	$y = 19.7(\pm0.59)x + 9.5(\pm18.1)$	$y = 9.86(\pm0.22)x - 44.6(\pm31.3)$
r[c]	0.9996	0.9993	0.9992
Detection limit (pmol)[d]	1	1	1
R.S.D. (%)[e]	1.8 ($n = 4$) (55.4 pmol)	1.3 ($n = 4$) (58.6 pmol)	1.8 ($n = 5$) (111 pmol)
Recovery test:			
Found (pmol/ml)[f]	24.4 ± 3.9[g] ($n = 4$)	N.D.[h]	16.0 ± 0.5 ($n = 3$)
Added (pmol/ml)	129	137	55.4
Total (pmol/ml)	152 ± 3.7 ($n = 3$)	130 ± 6.4 ($n = 3$)	67.0 ± 0.3 ($n = 3$)
Recovery (%)	98.9 ± 2.9 ($n = 3$)	94.9 ± 4.7 ($n = 3$)	92.1 ± 0.5 ($n = 3$)

[a] pmol = Amounts on-column.
[b] y = Peak area; x = concentration (pmol on-column); values ± standard deviations of the slope and the intercept.
[c] r = Correlation coefficient.
[d] Detection limit with signal-to-noise ratio = 3.
[e] R.S.D. = relative standard deviation.
[f] pmol/ml = concentrations in plasma.
[g] Mean ± S.D.
[h] N.D. = not detected.

supernatant should be separated from the precipitate because the hydroperoxides decomposed much more rapidly in the presence of precipitate.

Table 2
Effect of amounts of deproteinated plasma injected on the peak of PC hydroperoxides

Injection volume	PC hydroperoxide		R.S.D.[c] ($n = 4$) (%)
	pmol[a]	pmol/ml[b]	
1 × 2 ml	Trace[d]	–	–
2 × 2 ml	6.1	7.6	7.2
3 × 2 ml	8.3	6.9	4.2

[a] pmol = Amounts of PC hydroperoxides on-column.
[b] pmol/ml = Concentration of PC hydroperoxides in human plasma.
[c] R.S.D. = relative standard deviation.
[d] Trace = peak was detected but amount could not be determined.

With these systems, 4–24 pmol/ml of PC hydroperoxides were detected in the fresh human plasma tested ($n = 8$); PE hydroperoxides were not detected. As PE comprises only about 3% of phospholipids in human serum compared

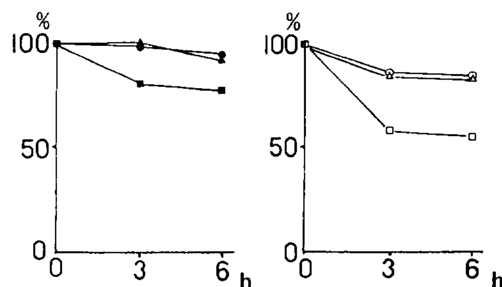

Fig. 4. Decreases in spiked (●, ▲, ■) PC–OOH and (○, △, □) PE–OOH in deproteinated plasma solution. Hydroperoxides of PC (92.3 pmol/ml in plasma) and PE (97.7 pmol/ml in plasma) were added to fresh human plasma followed by deproteination with methanol. The supernatant was stored at (■, □) room temperature, (▲, △) 0°C or (●, ○) −30°C.

with 67% for PC [19,20], the apparent absence of PE hydroperoxides in human plasma is mainly due to its lower concentration. These values were slightly lower than those obtained in previous work [12,13] and much lower than those reported by Miyazawa et al. [7]. Although the levels of PC hydroperoxides in plasma (or serum) were altered by some factors such as the season, these differences might be partly attributed to the artificial formation of hydroperoxides during the extraction and concentration procedures, because the proposed systems minimized it with the on-line sample preparation system. Therefore, it is necessary to take precautions, such as avoiding light irradiation, adding a sufficient amount of antioxidation agent, handling under a nitrogen atmosphere and using freshly purified solvents, during the sample preparation procedures if samples are prepared without the on-line system.

As the proposed methods give reliable results with a very simple procedure, they should be powerful tools in clinical analysis and for the study of the formation and metabolism of lipid hydroperoxides in living bodies.

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