papers and notes on methodology

Chemiluminescent simultaneous determination of phosphatidylcholine hydroperoxide and phosphatidylethanolamine hydroperoxide in the liver and brain of the rat

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Abstract The quantification of phospholipid hydroperoxides in biological tissues is important in order to know the degree of peroxidative damage of membrane lipids. For this purpose, optimal conditions for the chemiluminescent simultaneous assay of phosphatidylcholine hydroperoxide (PCOOH) and phosphatidylethanolamine hydroperoxide (PEOOH) in rat liver and brain were determined. A chemiluminescence detection-high performance liquid chromatography (CL-HPLC) method that incorporates cytochrome *c* and luminol as a post-column hydroperoxide-specific luminescent reagent was used (Miyazawa et al. 1987. *Anal. Lett.* **20**: 915–925; Miyazawa. 1989. *Free Radical Biol. Med.* **7**: 209–217). An n-propylamine-bound silica column with hexane-2-propanol-methanol-water 5:7:2:1 (v/v/v/v) (flow rate 1.0 ml/min) as eluant was used to determine both PCOOH and PEOOH, which were separated from each other and from other lipids and lipid-soluble antioxidants. High reproducibility and sensitivity as low as 10 pmol hydroperoxide-O_2 were observed with a mixture of 10 μg/ml cytochrome *c* and 2 μg/ml luminol in 50 mM borate buffer (pH 10.0, flow rate 1.1 ml/min) as luminescent reagent and a post-column mixing joint temperature of 40°C. Using the established analytical conditions, it was confirmed that both PCOOH (1324 ± 122 pmol/g liver, 114 ± 18 pmol/g brain, mean ± SD) and PEOOH (728 ± 89 pmol/g liver, 349 ± 60 pmol/g brain, mean ± SD) are present in the liver and brain of Sprague-Dawley rats bred on a slightly modified AIN-76A semisynthetic diet for 3 months. The phospholipid hydroperoxide content in the rat liver was shown to be affected by dietary oils, but not significantly affected in the brain.—**Miyazawa, T., T. Suzuki, K. Fujimoto, and K. Yasuda.** Chemiluminescent simultaneous determination of phosphatidylcholine hydroperoxide and phosphatidylethanolamine hydroperoxide in the liver and brain of the rat. *J. Lipid Res.* 1992. **33:** 1051–1058.

Supplementary key words lipid hydroperoxide • chemiluminescence-HPLC • luminol • cytochrome *c* • dietary oils

Lipid peroxidation in biological tissues attracts much attention because of its possible contribution to the functional modulation of biomembranes due to free radical-mediated damages (1–5) and in carcinogenesis (6–8) and the aging process (9–12). Research requires specific, sensitive, and reproducible procedures to quantify the lipid hydroperoxide in each lipid class as a primary product of the peroxidation reaction. Phospholipids such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are very important functional components of biological membranes and are also major structural constituents in tissue lipids. Therefore, simultaneous determination of phosphatidylcholine hydroperoxide (PCOOH) and phosphatidylethanolamine hydroperoxide (PEOOH) is needed to determine the degree of peroxidation of biomembranes and tissue lipids. Miyazawa et al. (13, 14) have developed a chemiluminescence detection-high performance liquid chromatography (CL-HPLC) technique for the specific assay of lipid hydroperoxides, and they expanded this technique to measure PCOOH in human blood plasma and other biological materials (7, 15–17). Other methods have been reported to measure phospholipid hydroperoxides using HPLC-UV (234 nm, con-

Abbreviations: CL-HPLC, chemiluminescence-high performance liquid chromatography; PC, phosphatidylcholine; PCOOH, phosphatidylcholine hydroperoxide; PE, phosphatidylethanolamine; PEOOH, phosphatidylethanolamine hydroperoxide; BHT, butylated hydroxytoluene.

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jugated diene) (18), HPLC-EC (19), and HPLC-iron-thiocyanate assay (20). However, these methods were insufficient to determine biological hydroperoxides because of their lower selectivity and sensitivity for the hydroperoxide group. Only a few methods specific for lipid hydroperoxide have been used to measure lipid hydroperoxide in human plasma using cyclooxygenase activity (21) and iodometric measurement (22). In the cyclooxygenase assay, enzyme preparation is complicated, and the iodometric assay has lower sensitivity for the hydroperoxide group. Both of these methods do not distinguish lipid classes.

The purpose of the present work was to expand our previous CL-HPLC technique for the simultaneous determination of PCOOH and PEOOH in biological tissues such as rat liver and brain. CL-HPLC optimum conditions described here have provided definitive evidence for hydroperoxidation of phospholipids in many biological systems.

MATERIALS AND METHODS

CL-HPLC

The analytical system consisting of HPLC and post-column chemiluminescence detection was basically the same as described in previous reports (13, 14). The HPLC column was JASCO Finepak SIL NH_2-5 (5 μm, 250 × 4.6 mm, n-propylamine column; Japan Spectroscopic Co., Tokyo, Japan). The column mobile phase was hexane-2-propanol-methanol-water 5:7:2:1 (v/v/v/v) and the flow rate was 1.0 ml/min using a JASCO 880-PU pump. After the column eluant passed through a JASCO 875-UV detector set at 234 nm (to monitor conjugated dienes) it was mixed with a luminescent reagent at a post-column mixing joint (Y type; Kyowa Seimitsu Co., Tokyo, Japan) with temperature controlled in a JASCO 860-column oven. The luminescent reagent was prepared by dissolving cytochrome *c* (from horse heart, type VI; Sigma Chemical Co., St. Louis) and luminol (3-aminophthaloyl hydrazine; Wako Pure Chemical Co., Osaka, Japan) in alkaline borate buffer and was pumped with a JASCO 880-PU pump. The chemiluminescence generated by reacting hydroperoxide with the luminescent reagent was measured by a CLD-100 chemiluminescence detector (Tohoku Electronic Industries Co., Sendai, Japan) or a JASCO 825-CL detector.

A calibration curve was made on PCOOH and PEOOH prepared by photooxidation of egg yolk PC and PE that have been purified by silica column chromatography (23). Purified PC or PE (each 200 mg) was dissolved, respectively, in 25 ml of methanol containing 0.1 mM of methylene blue as a photosensitizer. The reaction mixture was placed in a beaker (200 ml vol) cooled with ice-cold water and photoirradiated for 20 min at 10°C. The light source, a 500-W photoreflector lamp (Toshiba Electronics Co., Tokyo, Japan), was held at a 50-cm distance above the surface of the reaction mixture. After the photooxidation, the reaction mixture was passed through a silica column (40 × 500 mm; Wako gel C-100, Wako Pure Chemical Co., Osaka, Japan) with methanol as an eluant to remove the methylene blue. Phospholipid and its oxidized products, including the hydroperoxides, were recovered in the methanol extract. The methanol extract was then dried under reduced pressure by rotary evaporation, and the hydroperoxide content was determined by KI reduction (24). In this reduction, one mol hydroperoxide quantitatively reacts with two mol KI. The hydroperoxide concentrations of the photoirradiated PC and PE were 38.4 μmol hydroperoxide-O_2/g of PC (29 mmol hydroperoxide-O_2/mol of PC) and 71.8 μmol hydroperoxide-O_2/g of PE (51 mmol hydroperoxide-O_2/mol of PE), respectively. The photooxidized PC and PE were used as standard hydroperoxides, after dilution, in the following experiments.

Temperature of the post-column mixing joint and the concentrations of cytochrome *c*, luminol, flow rate, and pH of the luminescent reagent were also examined in terms of the hydroperoxide-dependent chemiluminescence intensities. A mixing joint set in a column oven was controlled between 30 and 60°C at 5°C intervals. The borate buffer (50 mM, H_3BO_3 • KCl-NaOH) used to dissolve cytochrome *c* and luminol for the luminescent reagent was prepared between pH 7 and pH 11 at pH intervals of 0.5. The effect of flow rate of luminescent reagent was tested between 0.6 ml/min and 1.6 ml/min at 0.2 ml/min intervals. Concentrations of cytochrome *c* were prepared as 2, 5, 10, and 50 μg/ml and concentrations of luminol were 1, 2, and 5 μg/ml of borate buffer.

Preparation of rat tissue lipids

Male Sprague-Dawley rats (4 months of age, 470 g body wt, n = 12) were divided into two groups and fed a slightly modified AIN-76A semisynthetic diet (casein 20%, sucrose 20%, α-cornstarch 40%, cellulose 5%, AIN mineral mix 3.5%, AIN vitamin mix 1%, D,L-methionine 0.3%, choline bitartrate 0.2%, oil 10%) for 3 months. The diet for one group contained 10% safflower oil and the other diet contained 10% fish oil (sardine oil, from Nippon Oil and Fats Co., Tsukuba, Japan). At the end of the feeding period, blood was withdrawn by heart puncture, the liver was perfused in situ with ice-cold 0.15 M saline, and the liver and brain were removed. Total lipid was extracted with a mixture of chloroform-methanol 2:1 (v/v) (16, 25) from the liver and brain as follows. Two ml of 0.15 M NaCl containing 0.002% butylated hydroxytoluene (BHT) as antioxidant was added to 400 mg of liver or brain, and the mixture was homogenized in a Teflon-glass homogenizer under ice-cold conditions. The homogenate was added to 5 ml of chloroform-methanol 2:1 (v/v) and

mixed vigorously for 1 min with a vortex mixer. The mixture was centrifuged at 3,000 rpm for 10 min. The lower chloroform layer was collected and concentrated in a rotary evaporator and dried under a nitrogen stream. The total lipid obtained was weighed and diluted with an appropriate amount of chloroform–methanol 2:1 (v/v); a portion was subjected to hydroperoxide assay by CL-HPLC. Previously, we had confirmed that artificial lipid hydroperoxides are not formed during the extraction procedure as described above (15).

RESULTS

HPLC analysis

Fig. 1 shows a typical chromatogram of a mixture of 50 pmol each of PCOOH and PEOOH, and 2 nmol of α-tocopherol when a Finepak SIL-NH_2 column was used with hexane–2-propanol–methanol–water 5:7:2:1 (v/v/v/v) as the mobile phase. PCOOH (9 min), PEOOH (15 min), and α-tocopherol (4 min) were separated, respectively, on the chromatogram. BHT added as antioxidant in the extracting solvent also coeluted at almost the same retention time as that of α-tocopherol. The chemiluminescence peak of PEOOH was slightly broader than that of PCOOH, and the peak height of PEOOH was lower as compared with that of PCOOH even though the same amount of hydroperoxide (50 pmol) was analyzed. The chemiluminescence peaks ascribed to PCOOH and PEOOH disappeared after reduction of these mixtures

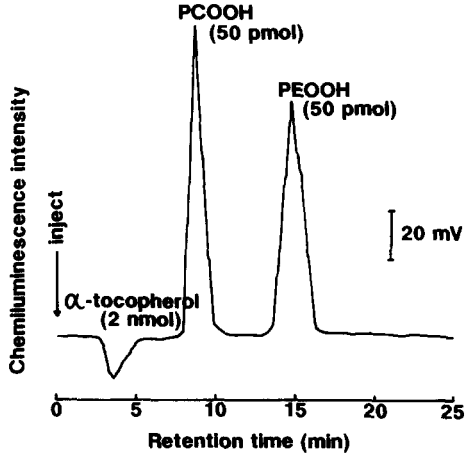

Fig. 1. Chemiluminescence chromatogram of a mixture of phosphatidylcholine hydroperoxide (PCOOH, 50 pmol), phosphatidylethanolamine hydroperoxide (PEOOH, 50 pmol), and α-tocopherol (2 nmol) with CL-HPLC. The CL-HPLC conditions were as follows: HPLC column, Finepak SIL-NH_2; mobile phase, hexane–2-propanol–methanol–water 5:7:2:1 (v/v/v/v), flow rate 1 ml/min; luminescent reagent, 10 μg/ml cytochrome *c* and 2 μg/ml luminol in 50 mM borate buffer (H_3BO_3 • KCl-Na_2CO_3, pH 10), flow rate 1.1 ml/min; mixing-joint temperature, 40°C.

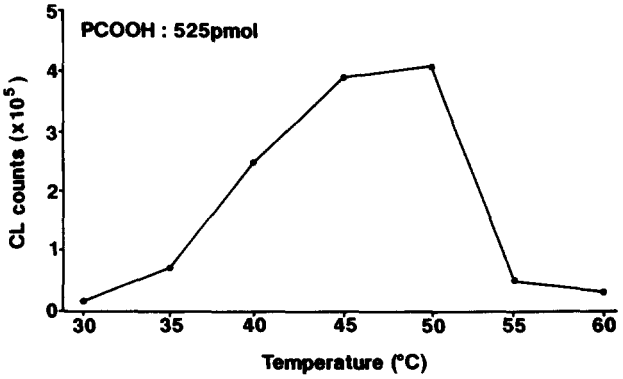

Fig. 2. Effects of mixing-joint temperatures on chemiluminescence intensity of phosphatidylcholine hydroperoxide (PCOOH). PCOOH (525 pmol) was analyzed by CL-HPLC at different mixing-joint temperatures. The CL-HPLC conditions were as follows: HPLC column, Finepak SIL-NH_2; mobile phase, hexane–2-propanol–methanol–water 5:7:2:1 (v/v/v/v), flow rate 1 ml/min; luminescent reagent, 10 μg/ml cytochrome *c* and 1 μg/ml luminol in 50 mM borate buffer (H_3BO_3 • KCl-NaOH, pH 9.3), flow rate 1 ml/min.

with sodium borohydride, because the chemiluminescent hydroperoxide group was converted to the corresponding nonluminescent hydroxyl group after reduction. Hydroperoxides of neutral lipids such as these of cholesterol, cholesteryl esters, triglycerides, and free fatty acids were eluted within 4 min under these analytical conditions. Both α-tocopherol and BHT, which are known to interfere with the hydroperoxide-dependent chemiluminescent reaction, were eluted around 4 min as a negative peak (Fig. 1). The chemiluminescent intensity of PCOOH and PEOOH was not affected by the presence of exogenous α-tocopherol and BHT added to the liver total lipid. Other possible interfering substances for the chemiluminescent reaction such as ascorbic acid and uric acid were not recovered in the chloroform layer under the present extraction procedure. These substances were not included in the total lipid fraction that was subjected to CL-HPLC assay. Therefore, under these analytical conditions, antioxidants that are known to interfere with the chemiluminescence reaction of a hydroperoxide group did not influence quantification of phospholipid hydroperoxides by CL-HPLC.

Optimal assay conditions

Fig. 2 shows the effects of mixing-joint temperatures on hydroperoxide-dependent chemiluminescence when CL-HPLC conditions were set as follows: HPLC column, Finepak SIL-NH_2; flow rate of column mobile phase (hexane–2-propanol–methanol–water 5:7:2:1), 1 ml/min; and flow rate of luminescent reagent (10 μg/ml of cytochrome *c* and 1 μg/ml of luminol in 50 mM borate buffer at pH 9.3), 1 ml/min. The chemiluminescence intensity of PCOOH (525 pmol) expressed as integrated chemiluminescence peak counts was increased by increasing the mixing-joint temperature from 30°C to 50°C and

was decreased above 55°C. We selected 40°C as the mixing-joint temperature taking into account the stability of the hydroperoxide group. No changes of chemiluminescence responses of authentic PCOOH and PEOOH and rat liver total lipid were noted when these hydroperoxides and lipids were dissolved in the HPLC column eluant for up to 30 min at 40°C. Because no positive chemiluminescence peak response was observed when the rat liver total lipid, after reduction by sodium borohydride, was subjected to CL-HPLC at these assay conditions, it could be also concluded that no hydroperoxide is artificially formed during the assay procedures in the column transit and in the mixing-joint.

Fig. 3 shows the effects of pH of luminescent reagent when the mixing-joint temperature was set at 40°C. Other conditions were the same as those described in the figure legend. Hydroperoxide-dependent chemiluminescence of PCOOH (131 and 262 pmol) was detected at pH 9 and above and showed a maximum intensity at pH 10.5. We selected pH 10 of 50 mM H_3BO_3 • KCl-Na_2CO_3 buffer as the optimum luminescent solution because of pH stability and chemiluminescence reproducibility. This buffer solution was stable at pH 10 for at least 3 days even at room temperature.

Fig. 4 shows the effect of flow rate (from 0.6 to 1.6 ml/min) of luminescent reagent. The luminescent reagent was dissolved in 50 mM H_3BO_3 • KCl-Na_2CO_3 buffer, pH 10. Other conditions were the same as these described in the figure legend. At a flow rate of 1.2–1.4 ml/min, PCOOH (26 pmol) showed a maximum chemiluminescence response. By considering this and the signal-to-noise ratios, the flow rate of luminescent reagent was determined as 1.1 ml/min.

Table 1 shows the effects of luminol and cytochrome *c* concentrations in the luminescent reagent. The flow rate of luminescent reagent was set at 1.1 ml/min. Other conditions were the same as described in the table legend. Chemiluminescence peak intensity of PCOOH (100 pmol) was enhanced as concentrations of cytochrome *c* and luminol increased. However, the chemiluminescence base line (background counts) became too unstable and the signal-to-noise ratios decreased at higher concentrations of cytochrome *c* and luminol. Therefore, cytochrome *c* and luminol concentrations of 10 μg/ml and 2 μg/ml, respectively, were selected, considering the above-mentioned factors and solubilities of the reagents in borate buffer.

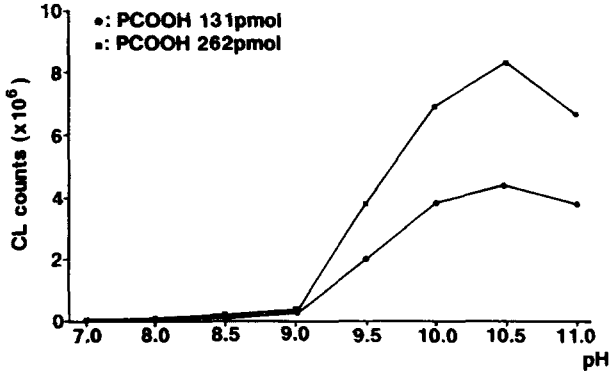

Fig. 3. Effects of pH of luminescent reagent on chemiluminescence intensity of phosphatidylcholine hydroperoxide (PCOOH, 131 and 262 pmol). The CL-HPLC conditions were as follows: HPLC column, Finepak SIL-NH_2; mobile phase, hexane-2-propanol-methanol-water 5:7:2:1 (v/v/v/v), flow rate 1 ml/min; luminescent reagent, 10 μg/ml cytochrome *c* and 1 μg/ml luminol in borate buffer, flow rate 1 ml/min; mixing-joint temperature, 40°C.

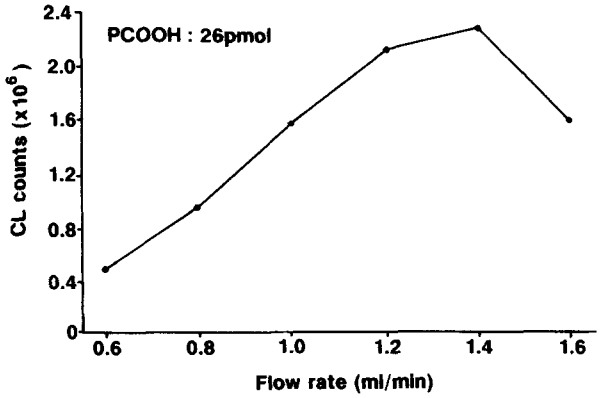

Fig. 4. Effects of flow rate of post-column luminescent reagent on chemiluminescence intensity of phosphatidylcholine hydroperoxide (PCOOH, 26 pmol). The CL-HPLC conditions were as follows: HPLC column, Finepak SIL-NH_2; mobile phase, hexane-2-propanol-methanol-water 5:7:2:1 (v/v/v/v), flow rate 1 ml/min; luminescent reagent, 10 μg/ml cytochrome *c* and 1 μg/ml luminol in 50 mM borate buffer (H_3BO_3 • KCl-Na_2CO_3, pH 10); mixing-joint temperature, 40°C.

TABLE 1. Effects of luminol and cytochrome *c* concentrations of luminescent reagent on chemiluminescence intensity

Luminescent Reagent	Integrated Chemiluminescence Counts of PCOOH Peak
Cytochrome *c*, 10 μg/ml	
+ Luminol 1 μg/ml	254,134
+ Luminol 2 μg/ml	1,221,325
+ Luminol 5 μg/ml	6,277,735
Luminol, 1 μg/ml	
+ Cytochrome *c* 2 μg/ml	10,881
+ Cytochrome *c* 5 μg/ml	50,650
+ Cytochrome *c* 10 μg/ml	245,593
+ Cytochrome *c* 50 μg/ml	2,096,656

Chemiluminescence counts are mean of three experiments when 100 pmol of phosphatidylcholine hydroperoxide (PCOOH) was analyzed by CL-HPLC. The CL-HPLC conditions were as follows: HPLC column, Finepak SIL-NH_2; mobile phase, hexane-2-propanol-methanol-water 5:7:2:1 (v/v/v/v), flow rate 1 ml/min; luminescent reagent, 50 mM borate (H_3BO_3 • KCl-Na_2CO_3) buffer, pH 10, flow rate was 1.1 ml/min; mixing-joint temperature, 40°C.

From these experiments, optimum conditions of CL-HPLC for the simultaneous assay of PCOOH and PEOOH were as follows; Finepak SIL-NH_2 HPLC column, hexane-2-propanol-methanol-water 5:7:2:1 (v/v/v/v) as the mobile phase, 1.0 ml/min as a mobile phase flow rate, 40°C as mixing-joint temperature, 10 μg cytochrome *c*/ml and 2 μg luminol/ml in 50 mM borate buffer (H_3BO_3 • KCl-Na_2CO_3, pH 10) as the luminescent reagent, and 1.1 ml/min as the flow rate of luminescent reagent.

Fig. 5 shows the calibration curves of PCOOH and PEOOH as determined by CL-HPLC under the optimal assay conditions. The integrated chemiluminescence counts of PCOOH and PEOOH were shown to be proportional to their hydroperoxide concentrations, at least in the range of 10-1000 pmol. Each calibration line showed a good proportional correlation between the chemiluminescence peak area calculated by integrating the chemiluminescence counts and the hydroperoxide concentration (correlation coefficient was 0.995 for PCOOH and 0.989 for PEOOH). The chemiluminescence of PCOOH was higher (130%) than that of PEOOH. The coefficient of validation was within 3% (n = 10) when 100 pmol of PCOOH and PEOOH was analyzed. The validity of the lines was also confirmed when known amounts of PCOOH and PEOOH (each from 100 to 1000 pmol) were added to the rat liver total lipids as a typical lipid mixture.

Phospholipid hydroperoxides in liver and brain

Fig. 6 shows chemiluminescent chromatograms of phospholipid hydroperoxide; 20 μl of liver total lipid diluted with 300 μl of chloroform-methanol 2:1 (v/v) and

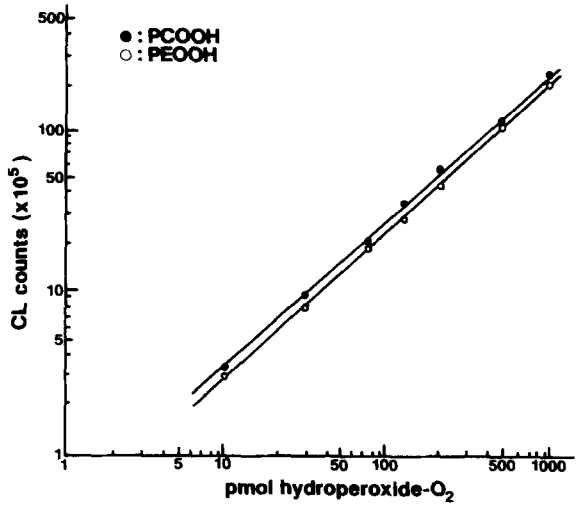

Fig. 5. Log-log calibration curves of phosphatidylcholine hydroperoxide (PCOOH) and phosphatidylethanolamine hydroperoxide (PEOOH) with CL-HPLC. Each spot is the average of five determinations. The CL-HPLC conditions were as described in the legend to Fig. 1.

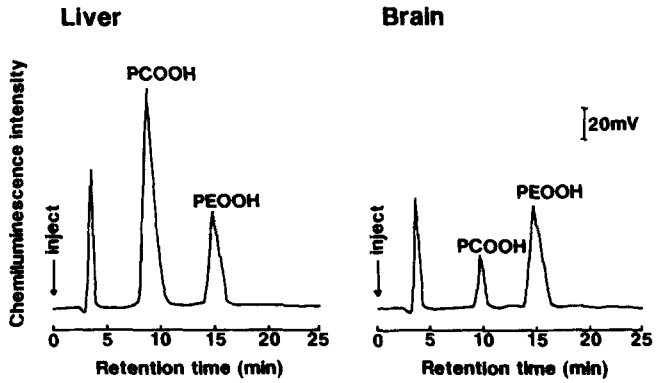

Fig. 6. Chemiluminescence chromatograms of phosphatidylcholine hydroperoxide (PCOOH) and phosphatidylethanolamine hydroperoxide (PEOOH) obtained when the liver and brain total lipids of a rat that received the fish oil diet were injected into CL-HPLC. The CL-HPLC conditions were as described in the legend to Fig. 1. The hydroperoxide concentrations of phospholipid hydroperoxides were 1626 pmol PCOOH/g and 925 pmol PEOOH/g for liver and 85 pmol PCOOH/g and 286 pmol PEOOH/g for brain, respectively, by using the calibration curves shown in Fig. 5, after correction for the sample dilution.

20 μl of brain total lipid diluted with 100 μl l of chloroform-methanol 2:1 (v/v) were analyzed by CL-HPLC under the optimal conditions described. The total lipid was extracted from 400 mg of liver or brain. Three chemiluminescence peaks were observed for both liver and brain lipids; the first component with a retention time around 4 min was not identified, but was considered to be a mixture of neutral lipid hydroperoxides that would also contain endogenous α-tocopherol. The second component was identified as PCOOH (9 min) and the third as PEOOH (14 min) by comparing their retention times with those of authentic PCOOH and PEOOH, by cochromatography, and by disappearance of the chemiluminescence peak after chemical reduction of total lipid by sodium borohydride as an indication of hydroperoxide group. Generally, as shown in Fig. 6, the liver phospholipid hydroperoxide concentration was higher than that of the brain, and the liver was found to contain more PCOOH than PEOOH, while the brain contained more PEOOH than PCOOH. There was no difference in body weight gain or organ weight between the rats fed the 10% safflower oil diet or the 10% fish oil diet over the 3-month period. For the rats fed the safflower oil diet, PCOOH contents were 1324 pmol/g liver and 114 pmol/g brain and PEOOH contents were 728 pmol/g liver and 349 pmol/g brain. For the rats fed the fish oil diet, PCOOH contents were 2109 pmol/g liver and 135 pmol/g brain and PEOOH contents were 1044 pmol/g liver and 308 pmol/g brain (**Table 2**).

Recovery of phospholipid hydroperoxide

The recovery of exogenous PCOOH (200 pmol) and PEOOH (200 pmol) added to the liver homogenate and

TABLE 2. Phospholipid hydroperoxide contents of liver and brain of rats fed diets containing safflower oil or fish oil for 3 months as determined by CL-HPLC

Diet	Liver		Brain	
	PCOOH	PEOOH	PCOOH	PEOOH
	pmol/g		*pmol/g*	
Safflower oil	1324 ± 122	728 ± 89	114 ± 18	349 ± 60
Fish oil	2109 ± 328[a]	1044 ± 343	135 ± 25	308 ± 69

Data are means ± SD of six rats for each diet group. PCOOH, phosphatidylcholine hydroperoxide; PEOOH, phosphatidylethanolamine hydroperoxide.

[a]Significantly different ($P < 0.005$) from the rats fed safflower oil diet.

then extracted with chloroform–methanol 2:1 (v/v) was 73 ± 7% for PCOOH and 82 ± 13% for PEOOH (mean ± SD, n = 8). Recovery of exogenous PCOOH and PEOOH added to the liver total lipid as a typical lipid mixture was 95 ± 5% (mean ± SD, n = 6) for both PCOOH and PEOOH, and was not influenced by the diet.

DISCUSSION

In the present study, we have demonstrated the optimal chemiluminescence-HPLC conditions for the simultaneous determination of phospholipid hydroperoxides such as PCOOH and PEOOH. Both PC and PE are important functional lipids in membranes and are also the major structural constituents of tissue lipids. Hydroperoxy derivatives of membrane phospholipids are formed as a result of free radical-mediated peroxidation. Therefore, the simultaneous determination of PCOOH and PEOOH is important to assess the degree of membrane lipid damage.

We report here HPLC conditions that take into account the complete separation of phospholipid hydroperoxides (PCOOH and PEOOH) from α-tocopherol and BHT (Fig. 1); these antioxidants inhibit the post-column hydroperoxide-dependent chemiluminescence reaction, and this may lead to errors in the hydroperoxide determination. Up to now, separation and detection of phospholipids by HPLC with a normal phase silica column (20, 26, 27), ion exchange column (28, 29), and n-propylamine-bound column (30–32) have been reported. With a normal silica column, separation of α-tocopherol and PE was difficult when rat liver lipids are assayed with the isocratic system such as chloroform–methanol (13, 14), acetonitrile–methanol–phosphoric acid (26), or hexane–2-propanol–water (27) as column eluant. The isocratic system as column eluant is preferred for hydroperoxide assay rather than the gradient system, because the chemiluminescence base line (background count) is significantly influenced by eluant composition. It has been reported that PC, PE, and α-tocopherol can be separated using an n-propylamine-bound column with an isocratic eluant (30–32). In the present study, simultaneous determination of PCOOH and PEOOH in the rat liver and brain lipids was first performed by using an n-propylamine column with hexane–2-propanol–methanol–water as an isocratic eluant.

The HPLC column eluant (hexane–2-propanol–methanol–water) solubilized α-tocopherol and BHT, but these interfering antioxidants were immediately removed from the chemiluminescence flow cell. PCOOH and PEOOH were separated from each other even with acetonitrile–methanol–water, but α-tocopherol was not satisfactorily dissolved by this solvent. The use of this acetonitrile eluant caused α-tocopherol to adhere to the flow cell and as a result decreased the chemiluminescence counts.

Although a detailed examination of optimal chemiluminescence conditions for the hydroperoxide assay was needed to obtain high sensitivity, reproducibility, and stability, little was known about the factors that affect the lipid hydroperoxide-dependent chemiluminescence. The chemiluminescence was influenced by many analytical factors, e.g., column eluant composition, post-column mixing-joint temperatures (Fig. 2), and pH (Fig. 3), flow rates (Fig. 4), and concentrations of luminol and cytochrome *c* (Table 1) in the luminescence reagent, as shown in this report. The detection limit of phospholipid hydroperoxide could be improved to as little as 0.1 pmol of phospholipid hydroperoxides, but for the assay of hydroperoxide in biological samples, a 10-pmol detection limit is enough considering the hydroperoxide content of the tissue lipids (7, 14–17).

Up to now, conjugated diene measurement at 234 nm has been used to evaluate the hydroperoxide content of authentic peroxidized lipids. But, as has been reported (33), peroxidized lipid contains hydroxyl products that possess a 234 nm chromophore (reduced form of hydroperoxide) other than the hydroperoxides. Therefore, the conjugated diene method is not recommended for estimation of phospholipid hydroperoxides because these derivatives are not chromatographically separated from hydroxyl derivatives. We also used an iodometric method, although a large amount of peroxidized lipid is needed for this assay. Iodometric measurement expresses the correct values for the hydroperoxide concentration, not being affected by the presence of hydroxyl derivatives. These are the main reasons why we used the iodometric measurement and not the conjugated diene method, in the determination of hydroperoxide concentration of authentic phospholipid hydroperoxide. The conjugated diene detection incorporated in the present CL-HPLC system was used only to detect phospholipid classes that were separated on HPLC, and not used to detect hydroperoxide.

The influence of dietary fats and oils on membrane lipid composition has been well investigated, and it has been demonstrated that membrane lipid composition can be rapidly and profoundly modified by diet and that many membrane-associated functions can be altered by diet-induced changes in lipid composition (34–39). Changes in phospholipid class distribution have been also associated with various physiological processes (40). The biochemistry and nutritional role of polyunsaturated fatty acids of dietary fats and oils have been extensively studied (11, 41–43). As shown in Table 2, our results obtained by the CL-HPLC assay showed that the change of dietary oil affects tissue phospholipid hydroperoxide concentrations of the rodents, although further studies are needed to explain the biochemical implication of the hydroperoxide increments that were observed. The molar ratio of PCOOH/PC in the liver was estimated to be $9.6 \pm 0.4 \times 10^{-5}$ (mean ± SD) and $14.4 \pm 1.4 \times 10^{-5}$ (mean ± SD) for rats fed safflower oil and fish oil, respectively, when the ratio was calculated from Table 2 for 1-palmitoyl-2-linoleoyl-PC and its monohydroperoxide. For brain, the molar ratios of PCOOH/PC were $0.8 \pm 0.1 \times 10^{-5}$ (mean ± SD) and $1.0 \pm 0.1 \times 10^{-5}$ (mean ± SD) for rats fed the safflower oil and fish oil diets, respectively.

Since the first reports of high-sensitive chemiluminescence detection (44) and CL-HPLC (13, 45), we have applied the method for measuring PCOOH in human blood plasma (14, 15), plasma lipoproteins (17), and mouse and rat liver (7, 16). Our previous method (7, 13–17) used a normal phase silica column to detect PCOOH, but determination of PEOOH, which had the same retention time as α-tocopherol, was difficult.

Independently from us, Ames, Yamamoto et al. (46–48) have reported a similar chemiluminescence assay of hydroperoxides, in which a mixture of isoluminol and microperoxidase (heme peptide of cytochrome *c*) was used as luminescent reagent. However, they failed to detect any phospholipid hydroperoxides in biological samples, such as human blood plasma. There are several differences between the two methods. The first is extraction solvent; we used a mixture of chloroform and methanol and Yamamoto et al. used a hexane–methanol–water system that needs a more complex extraction procedure. With the hexane–methanol–water extraction, phospholipid recovery was very low, and during the analytical procedure phospholipid hydroperoxide might have decomposed and artificial H_2O_2 was measured (46–49) by their method. The chloroform–methanol system that we used as extraction solvent did not cause any artificial hydroperoxidation in the presence of BHT as antioxidant (14–17). It is likely that the methanol aqueous layer used by Yamamoto et al. (46–48) may have contained many hydrophilic components as contaminants (e.g., sugar, amino acid, ascorbic acid, uric acid, ubiquinols, etc.). They may have quenched or enhanced the chemiluminescence, making the identification difficult, and significantly affecting chemiluminescence intensity and thus quantification of hydroperoxides.

The second difference is the composition of luminescent reagent. Cytochrome *c* that we used is more hydrophobic than microperoxidase, and shows high reactivity and chemiluminescence yield for lipid hydroperoxides. Microperoxidase is rather hydrophilic and shows high reactivity when the substrate is H_2O_2. Luminol that we used is preferred because of its high sensitivity compared to isoluminol. The concentration of microperoxidase and isoluminol was about 20 times higher than those of cytochrome *c* and luminol reagent, causes a large increase in background counts, and decreases the signal-to-noise ratios which make the hydroperoxide determination inaccurate.

Further studies, for example on elucidation of lipid peroxidation in subcellular components such as microsomes, mitochondria, and nuclear membranes in connection with their modified functions in many diseases and aging, could be carried out using the present method. Furthermore, by application of the present method to the quantitative survey of hydroperoxides in plasma, lipoproteins, erythrocytes, and tissue organs in healthy human donors and in patients, as well as in epidemiologic studies, and in experiments using rodents, the biochemical significance of membrane and tissue lipid peroxidation could be elucidated.

We believe that the CL-HPLC method for simultaneous assay of PCOOH and PEOOH will shed light on in vivo lipid peroxidation, especially in membranes.

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