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Journal of Pharmaceutical and Biomedical Analysis
23 (2000) 515–520

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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A chemiluminescent assay for hydroperoxide level of phosphatidylcholine hydroperoxide fraction purified by two Sep-Pak cartridges in biological samples

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Received 17 November 1999; received in revised form 18 February 2000; accepted 26 February 2000

Abstract

A chemiluminescent assay for hydroperoxide level of phosphatidylcholine hydroperoxide (PCOOH) fraction purified from biological samples was presented. This method utilized of two Sep-Pak cartridges. A lipid soluble fraction was isolated from each homogenized tissue or blood by Folch's method. The mixture of phosphatidylcholine (PC) and PCOOH was separated from the lipid soluble fraction by a Sep-Pak silica cartridge. A Sep-Pak tC_{18} cartridge made complete separation of both PCOOH and PC possible. The hydroperoxide level of PCOOH fraction was quantified by the reaction with ferrous ion using 2-methyl-6-[*p*-methoxyphenyl]-3,7-dihydroimidazo[1,2-a]pyrazin-3-one as a chemiluminescent dye. The mixture of positional isomers, 1-hexadecanoyl-2-[9, or 10-hydroperoxyl octadecanoyl]-*sn*-glycero-3-phosphocholine was used as an authentic standard. The good recovery rate for authentic PCOOH of 87.1 ± 11.6% (mean ± S.E., $n = 4$) was obtained by using two Sep-Pak cartridges. Linear calibration curve was obtained in the range from 2.5 to 20 nmol, and the detection limit of the standard was 10 pmol (signal-to-noise ratio > 3). This method was applied to the investigation of the lipid peroxidation induced by reperfusion of the liver with cold preservation, mimicking liver transplantation in rats. The effect of liposome-encapsulated dichloromethylene diphosphonate (LEDD), which eliminate of Kupffer cells to prevent the generation of oxygen radicals on the lipid peroxidation, was compared with the untreated group as a control. After 1 h reperfusion at 37°C the hydroperoxide level obtained the liver without preservation in the untreated group was 12.4 ± 2.4 nmol/100 mg lipid ($n = 4$) and levels increased significantly by prolongation of the preservation time. On the other hand, the hydroperoxide level in the LEDD treated group did not change up to 24 h preservation. These results suggest that this improved assay for hydroperoxide level of PCOOH fraction in biological samples can be applied to investigations involving lipid peroxidation because of its simplicity and accuracy.

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0731-7085/00/$ - see front matter
PII: S0731-7085(00)00316-2

Keywords: Chemiluminescent assay; Phosphatidylcholine hydroperoxide; 2-Methyl-6-[*p*-methoxyphenyl]-3,7-dihydroimidazo[1,2-a]pyrazin-3-one; 1-Hexadecanoyl-2-[9, or 10-hydroperoxyl octadecanoyl]-*sn*-glycero-3-phosphocholine

1. Introduction

It is well-known that phospholipid hydroperoxide, which is produced by the reaction of reactive oxygen species and unsaturated fatty acyl chains of lipids in biomembranes, induces cell damage and various diseases by changing the conformations of cell membranes [1,2]. Phosphatidylcholine (PC) is a main component of phospholipids in cell membranes and includes many unsaturated fatty acyl chains, causing the production of phosphatidylcholine hydroperoxide (PCOOH) than other phospholipids [1]. For these reasons, the determination of PCOOH is very important clinically.

Currently, some determination methods for PCOOH were proposed by high performance liquid chromatography (HPLC) using chemiluminescent or fluorescent probe [3–5]. Recently, by development of the HPLC method, we demonstrated that superoxide anion radicals caused the lipid peroxidation that results from liver transplantation [6,7]. However, because more than half of PC is composed of various unsaturated acyl chains on the *sn*-2 position, it is difficult to accurately determine PCOOH level by HPLC method. Therefore, it is necessary to develop a more reliable quantitative method for determining the hydroperoxide level of the PCOOH fraction in biological samples.

Various determination methods for hydroperoxide level of PCOOH have been developed, for example, iodine methods, TBA method, and enzymatic methods [8–11]. Although these methods have been useful for measurement of hydroperoxide levels, they are all performed in the aqueous medium. However, almost all phospholipids including PC are difficult to dissolve in the aqueous medium. Recently, we developed the chemiluminescent measurement method for determining the hydroperoxide level of PCOOH. In this method, the reaction of hydroperoxide and ferrous ion is performed and the level of radicals produced is measured in the methanol medium by using 2-methyl-6-[*p*-methoxyphenyl]-3,7-dihydroimidazo[1,2-a]pyrazin-3-one (MCLA), a *Cypridina* luciferin analog, as a chemiluminescent probe [12]. However, it was unclear whether this method could be applied for the quantification of hydroperoxide level of PCOOH fraction in biological samples.

In this report, we present a more reliable method for measuring the hydroperoxide level of the PCOOH fraction using rat liver tissues. To clarify the usefulness of our method, we investigated the lipid peroxidation by reperfusion of the liver with cold preservation, in which the cause of lipid peroxidation was reactive oxygen species induced by Kupffer cells and activated neutrophiles [7].

2. Materials and methods

2.1. Materials

L-α-Phosphatidylcholine 1-palmitate 2-oleate, Methylene Blue tetrahydrate, and analytical grades of methanol and chloroform were obtained from Wako Pure Chemical Company (Japan). Pentobarbital sodium was purchased from Tokyo Kasei (Japan). Ringer's lactate and University of Wisconsin cold-storage solution were obtained from Du Point Pharmaceuticals (Wilmington, DE). Male Lewis rats were allocated to two groups: (1) the control group, in which rats were not pretreated; (2) the liposome encapsulated dichloromethylene diphosphonate (LEDD) treated group, in which rats were administered 5 mg/kg (i. v.) of LEDD to inactivate Kupffer cells 24 h before the procurement.

2.2. Cold preservation and reperfusion experiments [7]

Under anesthesia with pentobarbital sodium (50 mg/kg, intraperitoneally), the livers of male

Lewis rats weighing 220–280 g were flushed with cold Ringer's lactate and then University of Wisconsin (UW) cold-storage solution via the portal vein. Those livers were preserved in the UW solution for 0, 12 and 24 h at 4°C. After cold preservation, each liver was connected to a perfusion circuit via the portal vein and flushed with 10 ml of Ringer's lactate. Thereafter, the liver was perfused for 60 min at a pressure of 10 cm H_2O with Krebs–Henseleit bicarbonate buffer (pH 7.4, 37°C) saturated with a 95% O_2–5% CO_2 air mixture in a non-recirculating system as described previously [7]. After reperfusion, the liver was immediately frozen with liquid nitrogen and stored at −80°C until analysis.

2.3. Preparation of tissue extracts

Approximately 1 g of the frozen liver was homogenized in 2 ml of saline for 1 min at 4°C. The homogenate was extracted three times with 4 ml of chloroform–methanol (2:1) containing 0.002% 2,6-dibutyl-4-methylphenol as an antioxidant [13]. The lower phase was concentrated under reduced pressure and the residue termed lipid soluble fraction was dissolved with 2 ml of chloroform–acetic acid (100:1).

The lipid soluble fraction was absorbed into Sep-Pak silica solid-phase extraction cartridge (Waters, Milford, MA) containing 1 g of absorbent, which was conditioned with 10 ml methanol followed by 10 ml of chloroform–acetic acid solution (100:1). After washing with 12 ml of chloroform–acetic acid solution (100:1) and then 5 ml of methanol–chloroform (2:1) solution, PC fraction was eluted with 5 ml of methanol–chloroform–water (2:1:0.8) solution. PC fraction was concentrated under reduced pressure and redissolved with 0.5 ml of methanol–chloroform–water (9:1:0.5) solution [14].

The PC fraction was absorbed into Sep-Pak tC_{18} cartridge (0.5 g), which was conditioned with 10 ml methanol followed by 10 ml of methanol–chloroform–water (9:1:0.5). After washing with 0.5 ml of methanol–chloroform–water (9:1:0.5) solution, PCOOH fraction was eluted with 4 ml of methanol–chloroform–water (9:1:0.5) solution. PCOOH fraction was concentrated under reduced pressure and redissolved with 2 ml of methanol, which was offered to the chemiluminescent assay.

2.4. Synthesis of authentic PCOOH

The synthesis of the positional isomer of 1-hexadecanoyl-2-[9 or 10-hydroperoxyl octadecanoyl]-*sn*-glycero-3-phosphocholine which was used as standard in our method was performed as described in a prior report Seya et al. [12]. Briefly, a solution of L-α-phosphatidylcholine 1-palmitate 2-oleate (200 mg) in methanol and 1 mM methylene blue (1 ml) in methanol was irradiated for 5 h. The suspension was chromatographed on a reverse phase glass column (50×8 mm^2) prepacked with Lichroprep RP-8 gel (20 g, Merck, Darmstadt, silica gel powder binding octane, 40–63 μm size), which was eluted with methanol–chloroform–water (300:30:15). Solvent flow was maintained at 0.5 ml/min. A fraction of monooxygenated products was obtained 128.3 mg (61.6%) as the 1:1 mixture of positional isomer. Monooxygenated products termed authentic PCOOH, were diluted with methanol (5 ml) and then stored at −20°C. Stock solution of authentic PCOOH was stable for 1 month at 4°C.

2.5. Measurement of the hydroperoxide

The PCOOH fraction in methanol (0.5 ml) was transferred to a test tube. A total of 4 mM MCLA in methanol (0.5 ml) was added to the solution and the mixture was stirred. Then, 10 mM $FeSO_4$ in methanol (0.5 ml) was added to the mixture and the number of generated photon was measured at room temperature by a luminescence reader BLR-301 (Aloca, Japan). MCLA solution was stored in 1.0 ml aliquots at 20°C, until needed.

3. Results

3.1. Purification of PCOOH

Fig. 1A shows the typical elution profile of the authentic PCOOH and PC using Sep-Pak silica

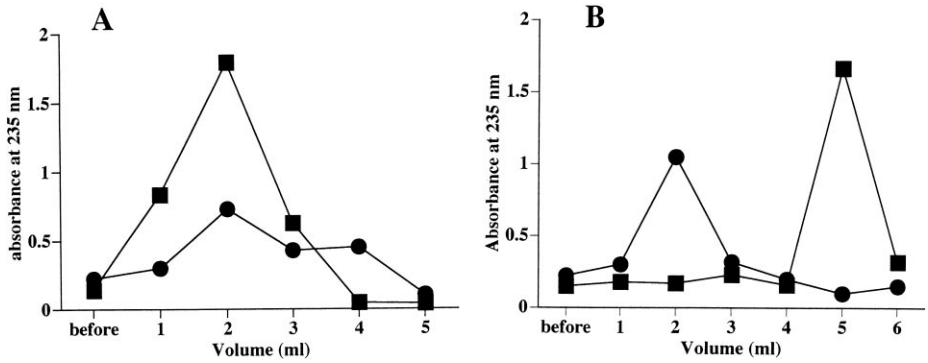

Fig. 1. Typical elution profile of PC (●) and PCOOH (▲) through Sep Pak silica cartridges (A) fractionating with methanol–chloroform–water (2:1:0.8) and Sep-Pak tC_{18} cartridges (B) fractionating with methanol–chloroform–water (9:1:0.5). The level of eluted PC and PCOOH was spectrophotometrically measured at 235 nm.

cartridge. The authentic PCOOH eluted was measured using an ultra violet detector at 235 nm. The authentic PCOOH as well as PC was sufficiently eluted by methanol–chloroform–water (2:1:0.8) solution. The recovery ratio of the authentic PCOOH fraction was $109.7 \pm 26.3\%$. The mixture of PCOOH and PC does not allow the measurement of hydroperoxide level because when the PC reacted with MCLA and ferrous ion, a weak chemiluminescence was generated (Fig. 2). Fig. 1B shows the typical elution profile of the authentic PCOOH and PC using Sep-Pak tC_{18} cartridge. PCOOH was sufficiently separated from PC by eluting with methanol–chloroform–water (9:1:0.5) solution. The recovery ratio of PCOOH fraction was $87.1 \pm 11.6\%$ over two cartridges.

3.2. Chemiluminescent assay

The present assay is based on a measurement of the chemiluminescence from MCLA [12], which reacted with hydroxy radicals released from PCOOH by ferrous ion induced decomposition. Luminescence curve for reaction of 20 nmol authentic PCOOH and $FeSO_4$ with MCLA is described in Fig. 3. After administration of $FeSO_4$, chemiluminescence intensity increased flashily. However, the chemiluminescence intensity increase did not return to basal line after reaching plateau. We defined the flush peak as the range of intensity from $FeSO_4$ administration to the time of achieving a plateau level, and then calculated the area of flush peak per second. The plateau trace in the chemiluminescence intensity revealed the constant rate of the chemiluminescence reaction, which may be increased another nonspecific reaction when the Fenton-like reaction with PCOOH was started by adding $FeSO_4$.

The assay was validated using four point calibration curves in the concentration ranges from 5 to 40 nmol. The standard curve of the peak area of authentic PCOOH was linear. The correlation coefficient (r^2) was 0.9892 ± 0.0108 for the standard curves of authentic PCOOH. Equation of standard curves was: $y = 3181.8x$, where y represents the peak area of analyte and x represents the

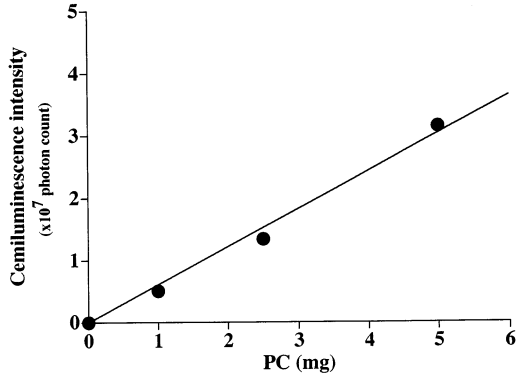

Fig. 2. Change in the chemiluminescence intensity of MCLA, reacted with various levels of PC using ferrous ion.

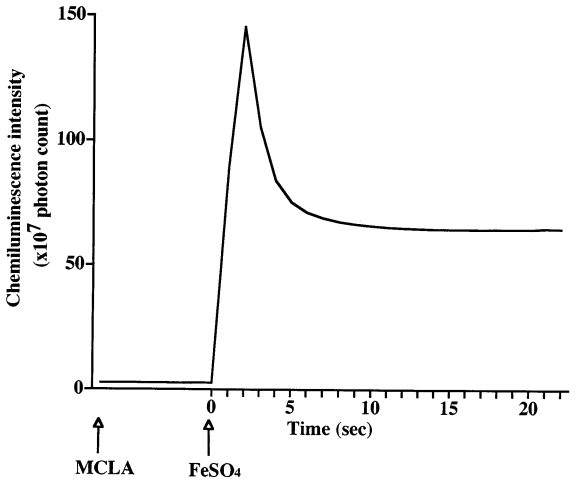

Fig. 3. Typical time course change in the chemiluminescence intensity produced by the standard procedure using 20 nmol of authentic PCOOH.

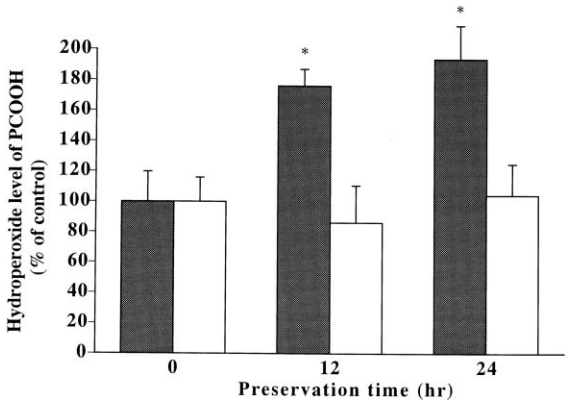

Fig. 4. Hydroperoxide level of PCOOH after 1 h reperfusion at 37°C of 0, 12, or 24 h cold preserved liver in the untreated (closed bar) and LEDD treated groups (open bar). Values were presented as mean ± S.E. Statistical analysis was performed by Wilkoxon test and $P < 0.05$ was considered to be statistically significant. * $P < 0.05$ vs. without preservation.

added concentration of the analyte. The detection limit of authentic PCOOH was 10 pmol (signal-to-noise ratio > 3). The intra-day variability was determined by analyzing four sets of standards on the same day. For the determination of inter-day variability, a single set of standards was assayed on four consecutive days. Over the range of concentrations of authentic PCOOH, the intra-day relative standard deviations (RSDs) were 0.5–13%. The inter-day RSDs were 0.5–17.0%. Analytical accuracy was evaluated by measuring the variation between the added concentration and the measured concentration for the three analytes. The intra-day accuracies over the range of concentrations were 98.9–122.2%. The inter-day accuracies were 95.4–114.4%.

3.3. Cold preservation and reperfusion study of the liver (Fig. 4)

The quantitation limit of hydroperoxide level of PCOOH fraction in the rat liver was ~ 50 pmol/ml. The basal hydroperoxide level of PCOOH fraction in the rat liver was 12.4 ± 2.4 (nmol/100 mg lipid), which was obtained from the liver without preservation. The hydroperoxide level of PCOOH had increased by 176.1 ± 11.0% ($n = 4$, $P < 0.05$ vs. without preservation) after 12 h preservation. This increment was augmented to 193.7 ± 21.7% ($n = 4$, $P < 0.05$ vs. without preservation) by lengthening the time of preservation to 24 h. On the other hand, in the LEDD group, hydroperoxide level of the liver without preservation was 18.2 ± 2.9 ($n = 4$). However, this level was maintained constantly at 86.2 ± 24.5 and 104.2 ± 20.7% at 12 and 24 h preservation, respectively.

4. Discussion

We improved the chemiluminescent assay to quantify the hydroperoxide level of the PCOOH fraction in the biological samples and applied this method to the organ preservation and reperfusion study using the rat liver tissues. Using this method, we demonstrated that the inactivation of Kupffer cells by LEDD completely inhibits the lipid peroxidation of liver during reperfusion after cold preservation.

The most popular measurement method for PCOOH is HPLC, which quantifies one peak area corresponding to PCOOH spectrophotometrically [3]. However, this peak area is composed of various unsaturated acyl chains on the *sn*-2 position. In addition, there is no the authentic samples for quantification of PCOOH. In the presented method,

we were able to quantify the hydroperoxide level of PCOOH fraction by using a synthesized monohydroperoxide, 1-hexadecanoyl-2-[9, or 10-hydroperoxyl octadecanoyl]-*sn*-glycero-3-phosphocholine as an authentic PCOOH. Thus, the chemiluminescent values obtained by the reaction of hydroperoxide with MCLA and ferrous ion can be regarded as the net level of the peroxidation of PC.

The purification method for PC using Sep-Pak silica cartridge is well known and can separate PC and other phospholipids, selectively [14]. However, it is unclear whether it is possible to purify PCOOH by using same condition that was used for purification of PC. In this study, we confirmed that PCOOH was also sufficiently eluted together with PC using Sep-Pak silica cartridge. Furthermore we investigated the separation of PC and PCOOH. If PC itself does not react with ferrous ion, it is not necessary to separate PCOOH and PC. However, PC, which includes choline residue, reacted with ferrous ion and generated weak chemiluminescence (Fig. 2). In this study, we confirmed that the Sep-Pak tC_{18} cartridge allows the separation of PC and PCOOH. However, the recovery ratio of authentic PCOOH through Sep-Pak tC_{18} cartridge is somewhat below 80%. When the volume of tC_{18} resin was increased to 2 g, the recovery ratio of PCOOH decreased fewer than 30%. On the other hand, a resin volume of less than 500 mg was insufficient to separate these substances. Thus, we concluded that 500 mg of Sep-Pak tC_{18} cartridge is the best volume to separate PC and PCOOH. Procedurally, it is noteworthy that the authentic PCOOH that is being used as internal standard should measured in parallel when making determinations of PCOOH over Sep-Pak silica cartridges.

We recently demonstrated that lipid peroxidation of the liver after reperfusion, which was facilitated after 24 h cold preservation, was completely inhibited by treating with gadolinium chloride, the eliminator of the Kupffer cells [10]. We also confirmed that reactive oxygen species generated during reperfusion were completely inhibited by treating with superoxide dismutase, the quencher of superoxide anion radicals [13]. In this study, we further demonstrated that LEDD, another inactivator of Kupffer cells, also inhibited the lipid peroxidation during reperfusion of the liver with cold preservation. These results suggest that superoxide anion radicals cause directly lipid peroxidation. Recently, we also confirmed that electron leak from mitochondria cannot generate superoxide anion radicals [15], but another sources of superoxide anion radicals may still remains at reperfusion. To determine the root of superoxide anion radicals generated during the reperfusion of the liver with cold preservation, it will be necessary to further investigate the hydroperoxide level of PCOOH more accurately using our developed method.

Acknowledgements

This study was supported in part by the Grant-in-Aid for Scientific Research (09670086) from the Minister of Education, Science and Culture, Japan, and by the Transplantation Research from Aomori Bank.

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