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Journal of Chromatography B, 693 (1997) 211–217

JOURNAL OF CHROMATOGRAPHY B

Short communication

Simultaneous determination of phospholipid hydroperoxides and cholesteryl ester hydroperoxides in human plasma by high-performance liquid chromatography with chemiluminescence detection

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Received 20 May 1996; revised 26 November 1996; accepted 5 December 1996

Abstract

A method was developed for the simultaneous determination of phosphatidylcholine hydroperoxides (PCOOH) and cholesteryl ester hydroperoxides (CEOOH). Lipid hydroperoxides (LOOH) were quantitatively extracted from human plasma with a mixture of *n*-hexane and ethyl acetate, and separated by column-switching high-performance liquid chromatography using one aminopropyl column and two octyl columns followed by chemiluminescence detection. LOOHs could be completely separated from each other and detected at picomole levels. The results of method validation tests were satisfactory. This method was then applied to determine LOOH in normal human plasma; the levels of PCOOH and CEOOH found were 36.0±4.0 n*M* (mean±S.D., $n=6$) and 12.3±3.1 n*M* (mean±S.D., $n=6$), respectively.

Keywords: Phospholipid hydroperoxides; Cholesteryl ester hydroperoxides

1. Introduction

Lipid peroxidation has been suggested to play a significant role in the etiology of a number of diseases and in the aging process. For example, recent studies have suggested that the oxidation of low density lipoprotein (LDL) is likely to be the key event causing atherosclerosis [1]. This hypothesis is supported by the fact that phospholipid hydroperoxides (PLOOH) and/or cholesteryl ester hydroperoxides (CEOOH), the primary oxidation products of the major components of LDL, have been found in human plasma [2].

Increased attention to lipid peroxidation in biological systems has accelerated the development of techniques for the determination of lipid hydroperoxides (LOOH) in biological samples [3]. Among the methods proposed, a combination of chemiluminescence (CL) detection and high-performance liquid chromatography (HPLC) is one of the most promising because of its high sensitivity and selectivity [4,5]. Miyazawa et al. [6] developed an HPLC–CL method for the determination of PLOOH. In their method, after total lipid extraction from plasma, PLOOH are separated into their lipid classes by HPLC prior to CL detection. However, CEOOH

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PII S0378-4347(97)00034-0

cannot be measured under their conditions. On the other hand, Yamamoto and Niki [7] reported a method for analyzing CEOOH using a combination of hexane extraction and reversed-phase HPLC with CL detection. They could determine low levels of CEOOH in human plasma, but not PLOOH.

The rates of oxidation of phospholipids and cholesteryl esters have been reported to differ in the LDL particles [8]. Thus, analysis of PLOOH and CEOOH in plasma should afford useful information on the pathogenesis of atherosclerosis. In many cases, however, only either PLOOH or CEOOH have been measured in the study of LDL oxidation, because different HPLC–CL systems are needed to determine these LOOH.

In the present paper, we describe the simultaneous HPLC–CL determination of PLOOH and CEOOH with a convenient extraction system for pretreatment. The method was validated and then used to determine PLOOH and CEOOH in human plasma.

2. Experimental

2.1. Chemicals

Isoluminol was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Microperoxidase (MP-11), lipoxidase (from soy bean), phosphatidylcholine (PC; from bovine brain), phosphatidylethanolamine (PE; from egg yolk) and cholesteryl linoleate (Ch18:2) were purchased from Sigma (St. Louis, MO, USA). Other reagents and solvents were of HPLC or the highest grade available. All chemicals were used without further purification.

2.2. Preparation of lipid hydroperoxides

The hydroperoxides of PC and PE (PCOOH, PEOOH) were prepared by enzymatic oxidation by soy bean lipoxidase [9]. As for the preparation of standard CEOOH, Ch18:2 was autoxidized at room temperature in the dark for 3 days. The prepared lipid hydroperoxides were dissolved in a mixture of methanol and *tert.*-butanol (1:1, v/v), and stored at −20°C. Their concentrations were determined by the iodometric technique [10].

2.3. Equipment and HPLC–CL conditions

The HPLC–CL system consisted of three pumps (LC-10AD, Shimadzu, Kyoto, Japan), a CL detector (CLD-10A, Shimadzu), a column oven (CTO-10A, Shimadzu), an auto injector with a sample cooler (SIL-10A, Shimadzu), a system controller (SCL-10A, Shimadzu), a degasser (DGU-3A, Shimadzu) and a column switching valve controller (PT-8000, Tosoh, Tokyo, Japan). The data processor used was a Chromatopac C-R7A (Shimadzu). A CLD-10A was used after removing the inner reaction coil (4 m length) for peroxyoxalate CL detection. The HPLC columns used were an aminopropyl (NH2) column (LiChrospher 100 NH2, 250×4 mm I.D., 5 μm; Kanto Chemical, Tokyo, Japan) and two octyl (C8) columns (LiChrospher 100 RP-8, 75×4 mm I.D., 5 μm; Kanto Chemical). The column temperature was kept at 33°C. The eluent was acetonitrile–methanol–distilled water–glacial acetic acid (65:30:5:0.01, v/v). The CL reagent was prepared by dissolving 100 μ*M* isoluminol and 5 μ*M* microperoxidase in a mixture of 50 m*M* borate buffer, pH 10, and methanol (3:7, v/v), and left overnight at room temperature before use. The flow-rate of the CL reagent was 1.0 ml/min.

2.4. Analytical system and procedure

Fig. 1 shows the schematic diagrams of the HPLC–CL system. The time program of the system

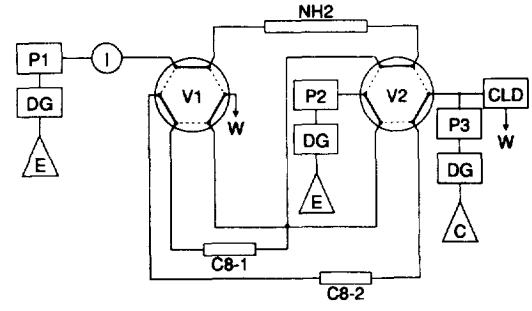

Fig. 1. Schematic diagram of the HPLC–CL system. E=Eluent; DG=degasser; P1, P2, P3=pump-1, 2, 3; I=auto injector; NH2=aminopropyl column; C8-1, C8-2=octyl column-1, 2; C=chemiluminescence reagent; CLD=chemiluminescence detector; V1, V2=switching valves 1 and 2; W=waste. Bold solid lines in V1 and V2 show initial positions.

Table 1
Time program of the HPLC system

Time (min)		0	4.5	17.5	30
Valve position	V1	S		B	
	V2	S	B	S	
Flow-rate (ml/min)	P1	1			
	P2	0	0.6	1.5	

"S" and "B" represent the solid and broken lines shown in Fig. 1, respectively.

is described in Table 1. The sample injected was introduced into the NH2 column and PLOOH were retained on it, while CEOOH passed through to the C8-1 column. By switching V2 from the solid lines to the broken lines, PLOOH were eluted from the NH2 column and transferred to the CL detector between 4.5 and 17.5 min. By switching V1 and V2, CEOOH were eluted from the C8-2 column and detected by CL, while the NH2 column and C8-1 column were back-flushed between 17.5 and 30 min. After 30 min, the valves were returned to the initial positions.

2.5. Preparation of human plasma

Human blood was collected with sodium heparin as an anticoagulant from healthy volunteers who had fasted overnight. The blood was centrifuged at 1500 *g* for 15 min at 4°C to obtain plasma. To determine the levels of LOOH, the plasma was kept at 0°C and used within a day. For method validation, plasma stored at −20°C was used. For the stability test of LOOH in the plasma on storage, the plasma was stored at −20°C with/without butylated hydroxytoluene (BHT) and *N*-ethylmaleimide (NEM).

2.6. Assay method

To 500 μl of plasma, 100 μl of 10 m*M* NEM aqueous solution was added and vortex-mixed in an amber centrifuge tube (screw cap with PTFE liner). After addition of 500 μl of methanol and mixing, 3 ml of ethyl acetate–*n*-hexane (1:1, v/v) containing 0.02% BHT as an antioxidant was added to the sample solution. The tube was shaken vigorously for 3 min and centrifuged at 2000 *g* for 10 min at 4°C. The upper layer was collected and stored in another amber test tube (stoppered) at 0°C. To the aqueous layer, 3 ml of ethyl acetate–*n*-hexane (1:1, v/v) containing 0.02% BHT was added, and the same extraction was repeated. The collected organic solutions were combined and evaporated to dryness under reduced pressure. The vacuum was broken with a nitrogen stream, and the residue was dissolved in 200 μl of methanol–ethyl acetate–*n*-hexane (1:1:1, v/v). After centrifugation at 2000 *g* for 5 min at 4°C, 100 μl of the supernatant was injected into the HPLC–CL system.

3. Results and discussion

3.1. Extraction of LOOH from human plasma

In an earlier stage of this work, LOOH were extracted from human plasma with a mixture of methanol and chloroform according to the method of Folch et al. [11] which has been extensively used for total lipid extraction. After some trials, however, we found that this method was unsuitable for microanalysis of LOOH because they were distributed in a chloroform layer under an aqueous layer and a precipitated protein layer. This made it very difficult to collect the organic layer without contamination. We therefore devised an alternative extraction system.

In our new extraction system, after treatment of the plasma sample with NEM, an inhibitor of glutathione peroxidase, followed by addition of methanol, LOOH were extracted with a mixture of ethyl acetate and *n*-hexane into the upper layer. The optimum ratio of ethyl acetate and *n*-hexane was 1:1. When NEM treatment was omitted, the recovery of standard PLOOH added to plasma was quite low while CEOOH recovery was quantitative (data not shown). Since Yamamoto et al. [12] have claimed that plasma glutathione peroxidase (pGSH-Px) reduces PLOOH in plasma to the corresponding hy-

droxide, this phenomenon was considered to be mainly caused by pGSH-Px.

As the preliminary test of LOOH stability in the extraction solvent showed PEOOH was relatively unstable (data not shown), the recovery test was done for PCOOH and CEOOH, with recoveries of 92% and 90%, respectively. PEOOH was analyzed qualitatively in this assay.

Our extraction method before LOOH microanalysis proved to be easy to perform and prevented contamination. Another positive factor was that it avoided the use of chloroform which can be harmful to the environment.

3.2. Separation of LOOH

For simultaneous analysis of PLOOH and CEOOH of quite different polarities, we could not use a gradient HPLC system with CL detection using luminol derivatives. The chromatographic conditions could not be changed; e.g., any change in eluent composition or flow-rate seriously disturbed detection. Therefore, a column switching-HPLC system using the same eluent was employed in combination with columns of different characteristics, namely, an NH2 column and two C8 columns and the flow-rate was kept constant at the detection site. A mixture of acetonitrile, methanol, water and glacial acetic acid was selected as the eluent. This solvent system could be used to separate both PLOOH by an NH2 column and CEOOH by a C8 column. Furthermore, it could be easily mixed with a CL reagent solution, a mixture of borate buffer and methanol, containing isoluminol and microperoxidase. Glacial acetic acid was required as a solvent component to control PEOOH retention and to stabilize the base line after column switching. As long as its concentration was kept low, glacial acetic acid improved the signal-to-noise ratio rather than attenuated the CL intensity by decreasing the pH of the CL reagent. The HPLC conditions established gave complete separation of the different types of LOOH from each other and from plasma components and also maintained the high sensitivity of the CL detection.

Separation of PLOOH and CEOOH is schematically illustrated in Fig. 2. The injected sample was introduced to an NH2 column where PLOOH were retained and separated into PCOOH and PEOOH,

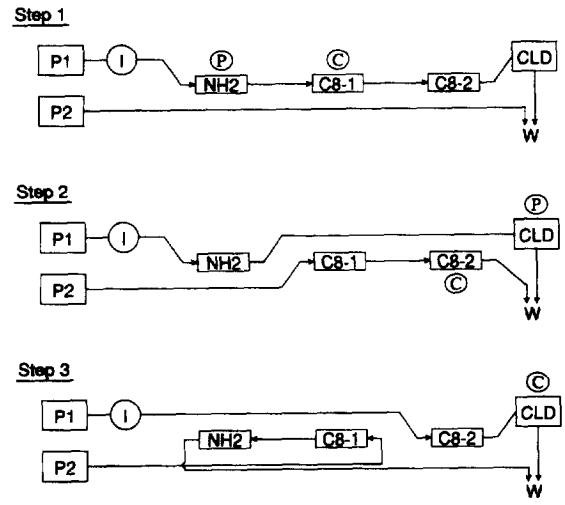

Fig. 2. Schematic illustrations of separation of PLOOH and CEOOH. Step 1: Injection and separation of PLOOH and CEOOH; Step 2: detection of PLOOH; Step 3: detection of CEOOH and back-flushing. P=PLOOH; C=CEOOH; P1, P2= pump-1, 2; I=auto injector; NH2=aminopropyl column; C8-1, C8-2=octyl column-1, 2; CLD=chemiluminescence detector; W= waste.

while CEOOH passed through and were transferred to a C8 column (Step 1). While CEOOH on the C8 column were separated from plasma components and transferred to another C8 column, PCOOH and PEOOH were eluted from the NH2 column and detected by CL (Step 2). After detection of PLOOH, CEOOH were eluted from the second C8 column and detected while the NH2 column and the first C8 column were back-flushed because unknown substance(s) giving a large CL peak remained on these columns (Step 3). The retention of CEOOH was adjusted with the length of C8 columns and the flow-rate in order to elute CEOOH after PEOOH. It was possible to inject samples at 40-min intervals.

Fig. 3 shows a typical chromatogram of standard LOOH. On an NH2 column with eluent for reversed-phase HPLC, PLOOH are generally separated into their lipid classes, i.e., PCOOH and PEOOH, regardless of their fatty acid moieties. Under the chromatographic conditions employed in this work, however, the PEOOH peak was split into two peaks.

The unknown CL peak mentioned above was not due to the peroxide(s) because it did not disappear after treatment of plasma with sodium borohydride

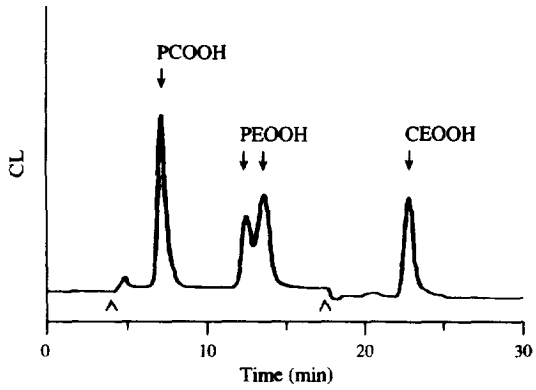

Fig. 3. Chromatogram of standard hydroperoxides. 1.8 pmol of PCOOH, 5.7 pmol of PEOOH, and 3.0 pmol of Ch18:2OOH were injected. "^" shows the switching time.

which is widely used as a reducing reagent for hydroperoxides. It is known that some ubiquinols can generate CL under the conditions of the heme iron metal catalyzed-isoluminol system [13], but this CL peak was unlikely to be those compounds as judged by their retention behaviors and intensities reported [7,13]. The identification of the peak is under investigation.

3.3. Method validation

The reliability of the established method was evaluated using human plasma spiked with standard PCOOH and CEOOH. As shown in Table 2, the accuracy, precision and reproducibility were satisfactory except for the precision at low CEOOH concentrations. The calibration curves showed good relationships between LOOH concentrations and peak areas with good reproducibility in the range tested (Table 3).

3.4. Stability of LOOH in human plasma

The stability of LOOH with/without additions in human plasma were tested by storage for one week at −20°C. As shown in Table 4, both PCOOH and CEOOH levels were almost the same on day 1 and day 7, but were higher than their initial values. This result suggested that the artificial formation of LOOH should occur during the freeze–thaw cycle while LOOH were stable for at least one week at −20°C and that additions did not have any effects on

Table 2
Validation of the method

	Added (n*M*)	Found[a] (n*M*)	Precision (%)	Accuracy (%)
Within-run				
PCOOH	0	46.2±6.9	15.0	
(*n*=5)	23.4	67.3±7.2	10.7	−9.9
	93.7	134.2±5.2	3.9	−6.0
	187.4	212.0±22.7	10.7	−11.5
CEOOH	0	11.1±3.3	30.1	
(*n*=5)	4.8	16.1±1.6	9.7	2.6
	19.4	29.7±1.7	5.8	−3.7
	38.7	47.0±4.3	9.1	−7.2
Between-run				
PCOOH	0	40.7±6.5	16.0	
(*n*=5)	23.4	60.3±4.9	8.2	−16.4
	93.7	131.9±5.1	3.9	−2.7
	187.4	226.8±14.6	6.4	−0.7
CEOOH	0	9.1±3.1	34.4	
(*n*=5)	4.8	13.4±3.7	27.9	−9.6
	19.4	26.9±4.0	14.7	−7.9
	38.7	45.9±4.1	9.0	−4.8

[a] Mean±S.D.

Table 3
Calibration data for the validation

	PCOOH	CEOOH
Range (n*M*)	11.7–375	4.84–77.4
Equation[a] ($n=5$)	$y=1.19(\pm0.02)x+3.30(\pm0.03)$	$y=1.12(\pm0.10)x+3.03(\pm0.16)$
r[b]	0.999±0.000	0.989±0.011

[a] y=log(peak area); x=log(concentration); mean values±S.D. of the intercept and the slope.
[b] r=correlation coefficient, mean±S.D..

their stability. Then, the assay was carried out within a day after blood was drawn.

3.5. Determination of PCOOH and CEOOH in human plasma

The method was applied to the analysis of LOOH in human plasma. A typical chromatogram of healthy human plasma extract is shown in Fig. 4. Peaks on the chromatogram were assigned as PCOOH, PEOOH and CEOOH from the retention times of their standard samples. These peaks disappeared with sodium borohydride pretreatment.

Table 5 shows the levels of PCOOH and CEOOH in plasma of healthy male adults. The PCOOH levels obtained in this work were almost the same as those reported by Akasaka et al. [14] but much lower than those reported by Miyazawa et al. [6]. On the other hand, the levels of CEOOH were slightly higher than those reported by Yamamoto and Niki [7] and Akasaka et al. [14]. Certain levels of PEOOH were detected but not determined quantitatively. Our

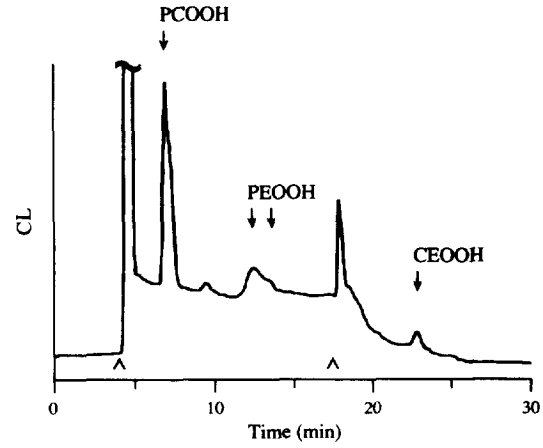

Fig. 4. Chromatogram of healthy human plasma extract. "^" shows the switching time.

results indicated that both PLOOH and CEOOH exist in human plasma of healthy adults though several antioxidant systems are employed.

In conclusion, the HPLC–CL method established can be used to simultaneously determine PCOOH and CEOOH in human plasma at the picomole level and to detect PEOOH qualitatively. The method is accurate, precise and reliable. It should be useful for studying the pathogenesis of diseases involving lipid

Table 4
Stability of LOOH in human plasma at −20°C

Day	Addition	PCOOH[a] (n*M*)	CEOOH[a] (n*M*)
0		27.8±3.9	5.4±0.8
1		37.1±1.8[b,c]	7.0±0.5[b,c]
	+BHT[d]	37.2±5.3[b]	7.0±1.6
	+BHT[d], NEM[e]	34.5±2.2[b]	6.9±0.5[b]
7		34.3±5.8	6.4±2.0
	+BHT[d]	38.2±6.4[b,c]	7.0±2.1[c]
	+BHT[d], NEM[e]	37.2±5.2[b]	6.8±1.8

$n=5$; [a] mean±S.D..
[b] $p<0.05$ vs. day 0.
[c] $n=4$.
[d] 0.01%.
[e] 1.7 m*M* as final concentration.

Table 5
Lipid hydroperoxide levels in human plasma

Subject	Age	PCOOH (n*M*)	CEOOH (n*M*)
H.F.	45	32.4	10.2
S.N.	41	38.6	15.3
K.U.	36	36.0	10.3
T.N.	35	42.1	17.2
A.M.	30	35.7	10.1
S.M.	29	31.3	10.9
Mean±S.D.		36.0±4.0	12.3±3.1

peroxidation and for the developmental research of drugs.

Acknowledgments

We thank Drs. Takayasu Kitagawa and Tsuneji Umeda of our laboratories for their valuable advice and discussions throughout this study.

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