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Normal-phase high-performance liquid chromatography with a fluorimetric postcolumn detection system for lipid hydroperoxides

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ABSTRACT

Hydroperoxides (HPO) of triacylglycerols (TG) and cholesterol esters (ChE) were selectively determined at picomole levels with a fluorescence detector by postcolumn reaction with diphenyl-1-pyrenylphosphine. Hydroperoxides were separated on a normal-phase silica gel column with gradient elution with *n*-hexane–1-butanol. With this system, TG-HPO and ChE-HPO were separated according to their class and determined in the range 5–1000 pmol. Detection limits of hydroperoxides of cholesterol linolate and trilinolein were about 2 pmol (signal-to-noise ratio = 3) and the relative standard deviations of their peak areas were 3.4% (39.1 pmol, $n = 7$) and 1.8% (32.4 pmol, $n = 7$), respectively.

INTRODUCTION

Lipid peroxidation has attracted much attention as one of the factors that cause some diseases and ageing [1–4]. However, it has been difficult to determine lipid peroxides in biological materials because of their trace concentrations, instability and diversity. In the initial stage of lipid peroxidation, hydroperoxides are produced both enzymatically and non-enzymatically, and they decompose or polymerize gradually to various secondary products.

Lipid hydroperoxides have been determined according to their class of molecular levels by both normal- and reversed-phase high-performance liquid chromatography (HPLC) [5–9]. UV detection at 235 nm, depending on their conjugated diene systems, or refractive index detection were used. However, these methods suffer problems such as selectivity, sensitivity or interferences from co-existing compounds.

Recently, chemiluminescence has been proposed for the highly sensitive and selective postcolumn detection of lipid hydroperoxides using isoluminol [10] or luminol [11]. Electrochemical detection has also been used for this purpose [12]. With chemiluminescence methods, hydroperoxides of cholesterol esters (ChE) and triacylglycerols (TG) were separated on a reversed-phase ODS column and gave multiple peaks based on their fatty acid compositions. Howevere, some hydroperoxides of ChE and TG might not be separated from each other.

Hydroperoxides of ChE and TG were expected to separate into their class levels by normal-phase HPLC with gradient elution, as non-oxidized lipids were separated [13]. However, the system is not suitable for either chemiluminescence or electrochemical detection because of the incapability of detection with non-polar organic solvents such as *n*-hexane, which is popular solvent for normal-phase HPLC.

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We have previously reported an aryl phosphine, diphenyl-1-pyrenylphosphine (DPPP), as a fluorescence reagent for lipid hydroperoxides [14,15]. This reagent was successfully applied to the highly sensitive and selective determination of lipid hydroperoxides by methods HPLC postcolumn [16–18]. The reaction proceeded in organic solvents such as methanol, 1-butanol, acetone, benzene and *n*-hexane. This allowed us to use a normal-phase column eluted with *n*-hexane. In this paper, we describe the HPLC determination of hydroperoxides of TG and ChE separated by a gradient elution with *n*-hexane–1-butanol on a silica gel column with postcolumn fluorimetric detection with DPPP.

EXPERIMENTAL

Chemicals

DPPP was synthesized according to the method described previously [14]. Trilinolein (TLo), triolein (TOl), cholesteryl linoleate (ChLo), cholesteryl oleate (ChOl), and cholesteryl arachidonate (ChAr) were purchased from Sigma (St. Louis, MO, USA). Methylene blue and triphenylphospine were obtained from Wako (Osaka, Japan). Methanol and 1-butanol were of HPLC grade from Wako and used as received. *n*-Hexane was used after distillation. *tert.*-Butylhydroxytoluene (BHT) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Vegetable oils were obtained from Nacalai Tesque (Kyoto, Japan). The solvents for dissolving the samples contained BHT (0.5 g/l) as an antioxidant.

Preparation of hydroperoxides

TLo, ChLo, ChAr and vegetable oils were autoxidized at room temperature or 40°C in the dark for 12–72 h. TOl and ChOl where photooxidized in the presence of 0.1–0.3 m*M* of methylene blue in ethanol. TOl-HPo was also prepared by autoxidation at 40°C for 1 week. They were used after purification as their monohydroperoxides by silica gel column chromatography. Their hydroperoxide contents were determined by fluorimetry [19]. They were stored in a refrigerator at −25°C as a chloroform–*n*-hexane (1:1) solution.

Separation and detection of hydroperoxides

The HPLC separation was performed on a Develosil 60-3 (3 μm) column (100 mm × 4.6 mm I.D.) (Nomura Chemical, Aichi, Japan). The chromatographic mobile phase were solvent A = *n*-hexane and solvent B = *n*-hexane–1-butanol (20:1, v/v), with a linear solvent gradient from 8% to 90% B between 2 and 24 min. The flow-rate of the mobile phases was 0.6 ml/min.

The HPLC eluate was monitored by UV absorbance measurement at 235 nm prior to the postcolumn reaction with DPPP, but this was not essential for this system. The eluent was mixed with DPPP reagent [3 mg in 400 ml of methanol–1-butanol (1:1, v/v)] which was kept in an ice-bath in the dark to prevent drifting of the baseline. The flow-rate of the reagent was 0.3 ml/min and the mixture was passed through a 20 m × 0.5 mm I.D. reaction coil (stainless steel) at 80°C followed by a 0.5 m × 0.5 mm I.D. coil in a 20°C water-jacket. Detection was performed by monitoring the fluorescence intensity at 380 nm with excitation at 352 nm. The DPPP reagent was degassed by sonication under reduced pressure before use. The mobile phase solutions were also degassed and stood for 12–24 h.

Equipment

The HPLC pump used was a CCPM multipump (Tosoh, Tokyo, Japan) in the high-pressure gradient mode. The pump for the reagent solution was an LC-3A (Simadzu, Tokyo, Japan). The reaction oven was an RE-8000 reactor. The detectors used were a UV-8000 spectrophotometer and an FS-8000 spectrofluorimeter (Tosoh). An SC-8010 data processor (Tosoh) was used.

RESULTS AND DISCUSSION

The properties of DPPP and the mechanism of its reaction with hydroperoxides have been described previously [14,15].

The effect of reaction temperature on the peak height of ChLo-HPO was examined between 40 and 80°C. The peak became higher with increasing reaction temperature as shown in Fig. 1. This means that hydroperoxides were not decomposed to unreactive compounds under these conditions. No increase in the baseline noise level was observed with increase in temperature. The flow-rate of the reagent solution had no effect on the peak height in the range 0.2–0.4 ml/min, but at 0.6 ml/min the peak became smaller (about 70%) owing to short-

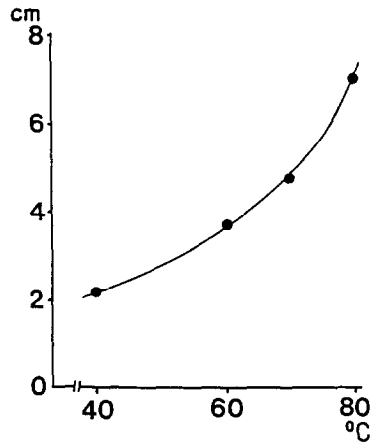

Fig. 1. Effect of reaction temperature on the peak height of ChLo-HPO. The separation column was Develosil 60-3 (100 mm × 4.6 mm I.D.) eluted isocratically with hexane–1-butanol (100:1, v/v) at 0.6 ml/min.

ening of the reaction time at higher flow-rate. The flow-rate of reagent solution adopted in subsequent work was 0.3 ml/min. After the reaction, the mixture was cooled to room temperature by passing it through a 0.5-m coil in a water-jacket to prevent foaming in the fluorimeter cell. This coil also prevented the methanol and *n*-hexane from boiling in the reaction coil by increasing the pressure.

The effects of the solvent of the reagent solution on the peak height of ChLo-HPO were examined. *n*-Hexane alone was not suitable because of the instability of the baseline. *n*-Hexane–1-butanol (1:1, v/v) solution gave the highest peak. 1-Butanol and 1-butanol–methanol (1:1, v/v) gave 85% and 92% peak heights, respectively, of that of the *n*-hexane–1-butanol. This might be attributed to the viscosity of the reaction solution. Although 1-butanol–methanol gave a smaller peak than *n*-hexane–1-butanol, it was selected as the solvent in this method because the baseline became unstable with *n*-hexane–1-butanol with prolonged operation.

The most significant characteristic of the method is that gradient elution with mobile phase from *n*-hexane to *n*-hexane–1-butanol (5:1, v/v) is usable without any effect on the peak area of ChLo-HPO. The mobile phase solutions were degassed by sonication under reduced pressure. It was recommended that they be used after standing for 12–24 h to minimize baseline drift and to improve the reproducibilities of the peak areas.

Fig. 2 shows the chromatograms obtained with gradient elution. ChLo-HPO and TLo-HPO were separated from each other. The baseline drift was negligibly small for determination in this range. Table I shows the retention times and relative peak areas of ChE-HPOs and TG-HPOs detected by fluorimetry. ChLo-HPO, ChAr-HPO and ChOl-HPO gave three or two peaks. This might be attributed to the separation of the structural isomers. With the exception of the last peak of ChAr-HPO, which was eluted at 13.75 min, ChE-HPOs and TG-HPOs were eluted between 8.5 and 12.5 min and between 13.8 and 14.5 min, respectively, independent of their structural differences. Although there was only one exception tested, this allowed us to assign generally the peaks eluted between 8 and 13 min as ChE-HPO and those between 13.8 and 14.5 min as TG-HPO. Although unoxidized ChE and TG gave no peaks in this system, they eluted at 7.5 min.

The peak areas of ChOl-HPO and TOl-HPO were smaller than those of other HPOs. This could be attributed to the lower reactivities of the HPO of oleic acid derivatives with DPPP [19] or less probably to the much loweer reactivity of the ChOl-HPOs with a sterically hindered hydroperoxy group on the cholesterol moiety. These HPOs could not be detected by the UV method because they do not contain a conjugated diene system. This means that the present method is much superior to the UV method in selectivity for hydroperoxides.

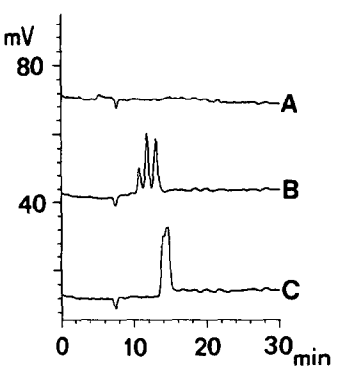

Fig. 2. HPLC of ChLo-HPO and TLo-HPO. (A) No injection (baseline); (B) ChLo-HPO (39.2 pmol); (C) TLo-HPO (42.5 pmol.

TABLE I

RETENTION TIMES AND RELATIVE PEAK AREAS OF LIPID HYDROPEROXIDES

Hydroperoxide	Oxidation method[a]	Retention time (min)	Relative peak area
ChOl-HPO	P	8.50, 12.30	0.57
ChLo-HPO	A	10.37, 11.50, 12.37	1.00
ChAr-HPO	A	9.24, 12.33, 13.75	1.06
TOl-HPO	A	13.87, 14.23	0.79
	P	13.83	0.73
TLo-HPO	A	14.23	1.11
TLn-HPO	A	14.25	0.92
Olive oil-HPO	A	13.83	0.98
Soybean oil-HPO	A	13.80	1.07
Linseed oil-HPO	A	14.07	1.04

[a] Hydroperoxides were prepared by (A) autoxidation and (P) photooxidation with methylene blue.

The HPOs of polyunsaturated fatty acid derivatives gave almost the same peak area (R.S.D. = 6.4%, $n = 7$). The polyunsaturated fatty acid parts are much more sensitive to peroxidation than the monoenoic acid and cholesterol parts, and therefore it may be expected that most of the HPOs in foods and biological materials were those of polyunsaturated fatty acid derivatives.

With the proposed method, the calibration graphs for ChLo-HPO and TLo-HPO showed good linearity in the range 5–1000 pmol (ChLo-HPO, $y = 13.09x + 44.4$, $r = 0.9999$; TLo-HPO, $y = 13.66x - 12.4$, $r = 0.9997$; x = concentration of hydroperoxide; y = peak area). From two calibration graphs, the ChE-HPO/TG-HPO peak-area ratios were 1.04 ($x = 50$ pmol) and 0.966 ($x = 500$ pmol). This allowed us to use the calibration graph of either ChLo-HPO or TLo-HPO for the determination of HPOs in these ranges. The R.S.D.s of their peak areas were 3.4% (ChLo-HPO, 39.1 pmol, $n = 7$) and 1.8% (TLo-HPO, 32.4 pmol, $n = 7$). At lower concentrations, ChE-HPO gave larger errors than TG-HPO because ChE-HPO separated into a few peaks and the concentration was calculated from their total peak areas. Although the peak area of ChE-HPO was larger than that of TG-HPO at lower concentrations, for both the detection limits were about 2 pmol (signal-to-noise ratio = 3). This was also attributed to the peak separation of ChE-HPO.

Lipid hydroperoxides with a wider range of polarities can also be determined by this method. Fig. 3 shows the HPLC of hydroperoxides produced by autoxidation of trilinolein at 40°C in air. At the beginning, only TLo-HPO (monohydroperoxides) was detected at 14 min and it increased gradually. After incubation for 48 h, very small peaks other than TLo-HPO were also detected at about 18 min. The peaks were considered as bishydroperoxides because of their polarities and reaction times. The more polar hydroperoxides, at 20–28 min, were detected after autoxidation for 120 h. Some of them

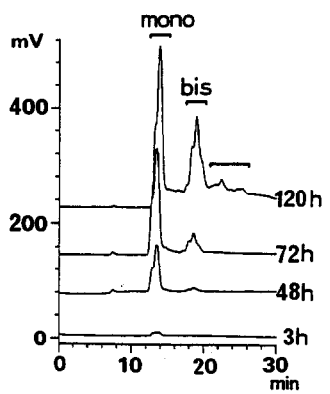

Fig. 3. Development of hydroperoxides of TLo by autoxidation at 40°C. TLo was sampled after autoxidation for 3 h (POV = 7.1), 48 h (POV = 91.4), 72 h (POV = 440) and 120 h (POV = 1470), and aliquots (5.4, 4.8, 3.0 and 2.8 μg, respectively) were injected on to the HPLC column as *n*-hexane solutions. POV = Peroxide values determined by our previous method.

gave no peaks with UV monitoring at 235 nm. They were probably decomposed products of trilinolein mono- and bishydroperoxides because they had no conjugated diene system and the products of the later stages of oxidation.

CONCLUSIONS

ChE-HPO, TG-HPO and hydroperoxides with higher polarity were separated by gradient elution with a normal-phase column. The postcolumn detection system with DPPP was successfully combined with this separation system, and the combination made it possible to determine lipid hydroperoxides with high sensitivity and selectivity. The present system should be useful for studying lipid peroxidation in complex systems such as foods and biological materials. The 1-butanol content of the mobile phase could be raised at least to 16.7% without influencing the postcolumn reaction. This suggests the possibility of the determination lipid hydroperoxides with a wider range of polarities using this system.

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