*Journal of Chromatography*, 622 (1993) 153–159
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 7160

# Simultaneous determination of hydroperoxides of phosphatidylcholine, cholesterol esters and triacylglycerols by column-switching high-performance liquid chromatography with a post-column detection system

Kazuaki Akasaka*, Hiroshi Ohrui and Hiroshi Meguro

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

(First received August 17th, 1993; revised manuscript received October 18th, 1993)

ABSTRACT

A method for the simultaneous determination of hydroperoxides of phosphatidylcholines (PC), triacylglycerols (TG) and cholesterol esters (CE) has been developed. A sample was separated into a combined TG and CE hydroperoxides fraction and a PC hydroperoxides fraction on a short silica column. The fractions were introduced into an ODS column and another silica column by a valve-switching device. The PC hydroperoxides were monitored by a post-column detection system with diphenyl-1-pyrenylphosphine, and the TG and CE hydroperoxides were monitored by another switching device. With this system, the hydroperoxides were determined at the picomole level within 32 min. Their detection limits were 2–4 pmol at a signal-to-noise ratio of 3, and the relative standard deviations of the peak areas were 1.6–3.1%. This method was successfully applied to determine lipid hydroperoxides in human plasma.

## INTRODUCTION

Previously, we reported the determination of lipid hydroperoxides by high-performance liquid chromatography (HPLC) with post-column detection using diphenyl-1-pyrenylphosphine (DPPP) [1–4]. With these methods, we showed the presence of hydroperoxides of phosphatidylcholine (PC) [1,2] and cholesterol ester (CE) [4] in human plasma. However, it was necessary to determine these compounds using different HPLC systems, because the hydroperoxides of phospholipids and of neutral lipids have different polarities. The methods thus required the use of double the amount of sample, complicated sample preparation procedures, and two HPLC systems. We thus attempted to develop a simultaneous determination of all lipid hydroperoxides.

Recently, column-switching has been widely used to heart-cut the chromatographic zone containing the peaks of interest from the first column [5–8]. This method is useful for the on-line clean-up of biological samples. In this paper, we report an HPLC system for the simultaneous determination of the hydroperoxides of PC, CE, and triacylglycerol (TG). The system has two silica columns (one for the

* Corresponding author.

class separation and another for the analytical separation of PC hydroperoxide), an ODS column for the separation of CE and TG hydroperoxides, two column-switching valves, and a sensitive and selective post-column detection system with DPPP.

## EXPERIMENTAL

### *Chemicals*

DPPP was prepared by our previous method [9]. Phosphatidylcholine (from egg yolk), dipalmitoyl PC, trilinolein (TLo), triolein (TOl), cholesteryl linolate (CLo), cholesteryl olate (COl), and cholesteryl cinnamate for the internal standard (I.S.) were purchased from Sigma (St. Louis, MO, USA). 2,6-Di-*tert.*-butyl-4-methylphenol (BHT) was purchased 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.

### *Preparation of lipid hydroperoxides*

Both TLo and CLo were autoxidized at 50°C for 2–4 h under an oxygen atmosphere. Hydroperoxides of PC, COl, and TOl were prepared by photo-oxidation with methyleneblue (0.2–0.5 m*M*) in ethanol-chloroform (2:1, v/v) for 6–12 h at ambient temperature. Each hydroperoxide prepared was purified by silica gel column chromatography. They were dissolved in chloroform–methanol (1:1, v/v) and stored at −20°C. Their hydroperoxide contents were determined by fluorimetry with DPPP, as described previously [10].

Dipalmitoyl PC was added to the standard hydroperoxide solutions that contained PC hydroperoxide so as to inject 30–100 μg of dipalmitoyl PC on-column.

The structures of the hydroperoxides, such as the positional locations on the glycerol molecule, and the carbon locations on the fatty acid, were not elucidated, but they had one hydroperoxy group in the molecule. In cholesterol ester, most hydroperoxy groups should be located on a fatty acid moiety because that part was autoxidized much more easily than the sterol moiety.

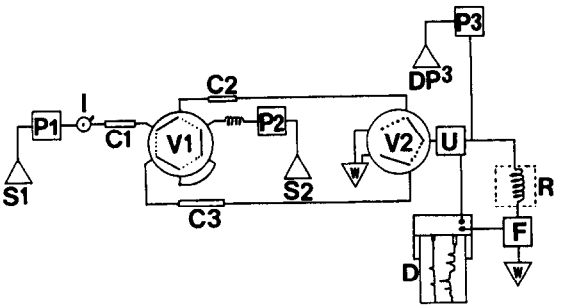


Fig. 1. Diagram of the HPLC system.

### *High-performance liquid chromatography*

Fig. 1 shows the HPLC system. The HPLC pump (P1 and P2) was a CCPM multi-pump (Tosoh, Tokyo, Japan), which pumped two mobile phases (S1 and S2) with a single apparatus. The six-port switching-valve device (V1) used was VL-611 (Japan Spectroscopic, Tokyo, Japan), and two Rheodyne type 7125 injectors were used, one as a sample injector (I) with a 100-μl loop and another as a five-port switching-valve device (V2). The pump (P3) for the reagent solution ($DP^3$) was a Model LC-3A (Shimadzu, Tokyo, Japan). The reaction oven was an RE-8000 reactor (R) (Tosoh). The detectors used were a UV-8000 spectrophotometer (U) and an FS-8000 spectrofluorometer (F) (Tosoh). The data processor used was a Model SC-8010 (D) (Tosoh). An isothermal water-bath, NTT-1100 (EYELA, Tokyo, Japan), was used to keep the ODS column temperature constant. HPLC columns used were Develosil 60-3 (C1, 30 mm × 4.6 mm I.D., 3 μm particle size; C2, 50 mm × 4.6 mm I.D., 3 μm particle size) and Develosil ODS HG-5 (C3, 100 mm × 4.6 mm I.D., 5 μm particle size) (Nomura Chem., Aichi, Japan). *n*-Hexane–1-butanol–methanol–water (40:100:500:35, v/v) was used for both mobile phases (S1 and S2), at the same flow-rate of 0.8 ml/min. The column temperature of C3 was kept at 30°C, and those of C1 and C2 were ambient.

### *Column-switching procedure*

Fig. 2 shows the column-switching time program. The initial positions of column-switching devices are shown by bold lines in Fig. 1. An injected sample was first separated on a silica column (C1). Between 0.40 and 1.45 min, the

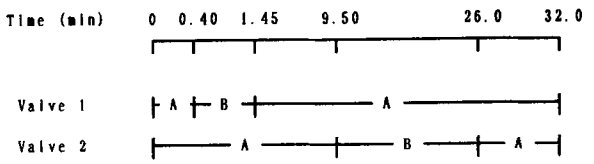


Fig. 2. Valve-switching time programme: A and B are the valve positions shown in Fig. 1 by bold lines and broken lines, respectively.

eluate from it was transferred to a reversed-phase column (C3) by switching valve V1. The later eluate from C1 was transferred to another silica column (C2) by switching V1. The eluate from C2 between 0 and 9.5 min was transferred to the post-column detection system through V2, and that from C3 between 9.5 and 26 min was transferred to the detection system by switching V2. After 26 min, the valves were returned to the initial positions.

*Post-column detection of hydroperoxides*

The eluent from the C2 or C3 column was monitored by a UV detector at 268 nm. The DPPP solution (3 mg in 400 ml of 1-butanol–methanol 1:1, v/v) was mixed with the eluate at a flow-rate of 0.3 ml/min. It reacted at 80°C when passed through a stainless steel coil (30 m × 0.5 mm I.D.) followed by a cooling coil (0.5 m × 0.5 mm I.D.) in a water-jacket, and the fluorescence intensity was monitored at 380 nm (excitation at 352 nm). The DPPP solution was degassed by ultrasonic treatment under reduced pressure and kept for more than 5 h below −10°C before use, and it was kept in an ice-bath during the determination.

*Extraction from plasma*

An aliquot of plasma (0.75 ml) was mixed with 50 μl of BHT (10 mg/ml in dichloromethane), 50 μl of the I.S. (0.15 mg/ml in dichloromethane), methanol (0.75 ml) and dichloromethane (2 ml) in a 16 × 100 mm screw-cap test-tube. After being tightly capped, the tube was shaken vigorously for 1 min and centrifuged at 1000 *g* for 5 min. The organic phase was transferred to another test-tube. Dichloromethane (2 ml) was added to the water phase, and the extraction was carried out again. The organic phases were combined, and the solvent was evaporated under reduced pressure at 20°C. The pressure was restored to ambient with nitrogen gas, and the sample was dissolved in 50 μl of chloroform–methanol (1:1, v/v). The whole sample was injected into the HPLC system.

Here, fresh human plasma was sampled with sodium heparine as an anticoagulant from a healthy male volunteer, who had fasted for more than 12 h.

## RESULTS AND DISCUSSION

*Separation of hydroperoxides*

Following separation on a reversed-phase column, the PC hydroperoxides gave a broad peak or split peaks near the solvent front, which made their assignment difficult and lowered the sensitivities. In most cases, they have been separated on a silica or an aminopropyl silica column. TG and CE hydroperoxides are not retained at all by such separation systems.

In the proposed method, the PC hydroperoxide fraction is separated from the TG and CE hydroperoxides fraction by passage through a short silica column, followed by further separations on two analytical columns. The first fraction from the first silica column (C1), which contained TG and CE hydroperoxides, were transferred to an ODS column (C3), and the later fraction, which contained PC hydroperoxides, were transferred to another silica column (C2).

On a silica column PC hydroperoxides gave a single peak, which became sharper and eluted faster when injected with a larger amount of unoxidized PC, as described previously [2]. Because the retention times of the PC hydroperoxides were almost the same when they were injected with more than 30 μg of dipalmitoyl PC, the PC hydroperoxide standard solution used contained sufficient dipalmitoyl PC so as to inject 30–100 μg of it on column. It is of no use to add unoxidized PC to samples extracted from food-stuffs and biological materials, because they contain much larger amounts of unoxidized PC than PC hydroperoxides.

Figs. 3 and 4 show the effects of the column temperature and mobile phase composition on

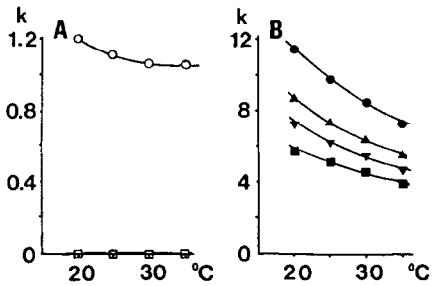


Fig. 3. Effect of column temperatures on capacity factors ($k$) of lipid hydroperoxides. The columns used were Develosil 60-3 (A) and ODS-HG-5 (B). Samples were hydroperoxides of PC (○), TLo (■), CLo (▼), COl (▲), TOl (●), and the mixture of neutral lipids (□), TLo, CLo, COl, and TOl.

the capacity factors ($k$) of the hydroperoxides, respectively. The capacity factors of PC hydroperoxides on the silica column slightly increased when the column temperature decreased, and were unaffected by increased $n$-hexane content in the mobile phase. On the other hand, the capacity factors of the TG and CE hydroperoxides on the ODS column decreased markedly when either the $n$-hexane content or the column temperature increased. It was possible to control the retention times of TG and CE hydroperoxides on the C3 column by controlling the $n$-hexane content without altering the separation on the C1 and C2 columns. This permitted us to use the same solvent system as S1 and S2. Here, we selected the mobile phase so as to elute TG and CE hydroperoxides from the C3 column after PC hydroperoxides eluted from the C2 column. The temperature of the C3 column was kept at 30°C because the temperature markedly influenced the retention times of the hydroperoxides.

Fig. 5 shows a typical chromatogram of hydroperoxides on the C1 column. The eluate between 0.40 and 1.45 min (fraction A) from the C1 column was transferred to an ODS column, and the later eluate (fraction B) was transferred to another silica column, because the TG and CE hydroperoxides were eluted between 0.50 and 1.38 min (peak a). The PC hydroperoxides were eluted after 1.55 min (peak b) at the earliest. It was possible to inject at least 50 $\mu$l of samples in chloroform–methanol (1:0 to 1:3, v/v), because they gave no significant difference in the separations on the C1 column. The second valve device (V2) was switched at 9.5 min because the PC hydroperoxides were completely eluted from the C2 column within 9.5 min, and the TG and CE hydroperoxides tested were eluted after 11 min from an ODS column. By this system, five hydroperoxides tested were separated within 32 min, as shown in Fig. 6. It was possible to inject samples at 26-min intervals because no peak was observed and the baseline was very stable up to 6.5 min after the injection. The baseline levels were shifted by switching the

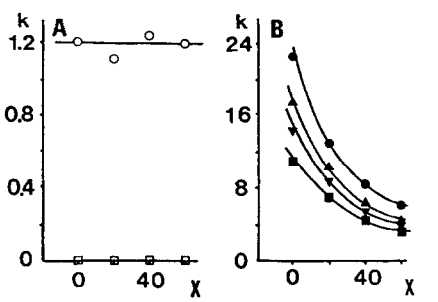


Fig. 4. Effect of $n$-hexane content in the mobile phase on capacity factors ($k$) of lipid hydroperoxides. Mobile phases were $n$-hexane–methanol–water–1-butanol ($x$:540 − $x$:35:100, v/v). The columns were Develosil 60-3 (A) and ODS-HG-5 (B). Samples were hydroperoxides of PC (○), TLo (■), CLo (▼), COl (▲), TOl (●), and the mixture of neutral lipids (□), TLo, CLo, COl, and TOl.

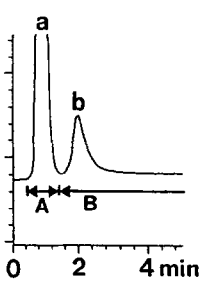


Fig. 5. Typical chromatogram on the C1 column; peaks: a = mixture of solvent, TG hydroperoxides, and CE hydroperoxides; b = mixture of PC hydroperoxides and unoxidized PC. Detection was carried out by UV at 235 nm. A and B indicate the TG and CE hydroperoxides fraction and the PC hydroperoxides fraction, respectively.

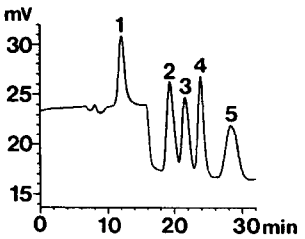


Fig. 6. Typical chromatogram of lipid hydroperoxides obtained using the proposed system. Peaks: 1 = PC hydroperoxide (45.2 pmol); 2 = TLo hydroperoxide (47.7 pmol); 3 = CLo hydroperoxide (44.6 pmol); 4 = COl hydroperoxide (54.6 pmol); 5 = TOl hydroperoxide (61.6 pmol).

TABLE I

EFFECTS OF FLOW-RATE ON THE PEAK AREAS AND HEIGHTS OF HYDROPEROXIDES

| Flow-rate (ml/ml) | Retention time (min) | Relative peak area | Relative peak height |
|---|---|---|---|
| *PC hydroperoxide* | | | |
| 0.6 | 14.5 | 1.00 | 1.00 |
| 0.8 | 11.3 | 0.65 | 0.80 |
| 1.0 | 9.4 | 0.45 | 0.67 |
| *CLo hydroperoxide* | | | |
| 0.6 | 26.4 | 1.00 | 1.00 |
| 0.8 | 20.4 | 0.56 | 0.71 |
| 1.0 | 16.7 | 0.35 | 0.53 |
| *COl hydroperoxide* | | | |
| 0.6 | 29.2 | 1.00 | 1.00 |
| 0.8 | 22.5 | 0.56 | 0.69 |
| 1.0 | 18.4 | 0.35 | 0.52 |

V2 valve, probably owing to the difference in pressure between the C2 and C3 columns.

*Detection of lipid hydroperoxides*

Table I shows the effect of flow-rate on the peak heights and areas of the PC and CE hydroperoxides. The higher flow-rate decreased both the peak areas and heights, because of their greater dilution and the shorter reaction time. At 1.0 ml/min, the sensitivities were 30–50% lower than at 0.6 ml/min. At the lower flow-rate, however, separation was prolonged, so we selected 0.8 ml/min as the flow-rate for both S1 and S2. We used 30 m of stainless steel coil as a reaction coil, because the sensitivities of the hydroperoxides of PC, CLo, and COl were 1.16, 1.58, and 1.43 greater with this coil than with a 20-m coil. The reaction temperature selected was 80°C, to give higher peaks without increased baseline noise.

*Calibration curves, detection limits and reproducibilities*

The relative peak areas of lipid hydroperoxides tested are shown in Table II. Table II also shows the determination ranges, detection limits [signal-to-noise ratio ($S/N$) = 3], and relative standard deviations of the peak areas of the hydroperoxides of PC, TLo, CLo, COl, and TOl. The calibration curves showed good coefficients of correlation, at least in the ranges tested, and their determination ranges are wider than the tested ones. The relative peak areas

TABLE II

REPRODUCIBILITIES, CALIBRATION CURVES, AND DETECTION LIMITS OF HYDROPEROXIDES

| Peak No. | HPO[a] | R.S.D. ($n$ = 6) | | Relative peak area | Calibration curve | | Detection limit[b] (pmol) |
|---|---|---|---|---|---|---|---|
| | | % | pmol | | Range (pmol) | $r$ | |
| 1 | PC-HPO | 2.08 | (45.2) | 1.00 | 4.5–1810 | 0.9996 | 2 |
| 2 | TLo-HPO | 1.98 | (47.7) | 1.45 | 6.8–2724 | 0.9998 | 2 |
| 3 | CLo-HPO | 2.18 | (44.6) | 1.25 | 2.1– 892 | 0.9993 | 2 |
| 4 | COl-HPO | 3.09 | (54.6) | 1.08 | 2.7–1090 | 0.9991 | 2 |
| 5 | TOl-HPO | 1.67 | (61.6) | 1.04 | 4.4–1760 | 0.9997 | 4 |

[a] HPO = hydroperoxide.
[b] Signal-to-noise ratio = 3.

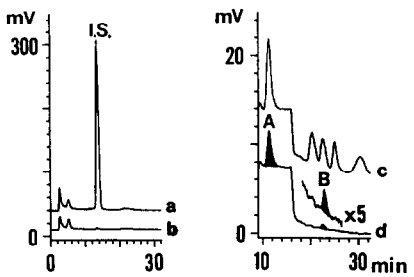


Fig. 7. Typical chromatograms of human plasma extracts Chromatograms a and b were detected by UV at 268 nm, and the samples were extracts with and without the addition of internal standard (I.S.), respectively. Chromatograms c and d were detected by fluorimetry, and the samples were extracts with and without spiking of the mixture of standard hydroperoxides of PC (60.3 pmol), TLo (63.6 pmol), CLo (59.5 pmol), COl (72.8 pmol), and TOl (82.1 pmol) to 1 ml of fresh plasma before the extraction, respectively. Peaks A and B were assigned to hydroperoxides of PC and CLo, respectively.

differed between the hydroperoxides tested. This might be partly due to their different reactivities with DPPP. This system made it possible to determine simultaneously picomolar to nanomolar levels of PC, TG, and CE hydroperoxides, with high reproducibilities.

*Determination of lipid hydroperoxides in human plasma*

This method was successfully applied to the determination of lipid hydroperoxides in a human plasma sample: Fig. 7 shows typical chromatograms of extracts from a fresh sample. Cholesteryl cinnamate was used as an internal standard, monitored by UV detection at 268 nm prior to the post-column reaction. By UV monitoring, no peak was detected in the plasma extract at 14.2 min, where cholesteryl cinnamate eluted, as shown in Fig. 7a and b. In Fig. 7d, peaks A and B were assigned as PC and CLo hydroperoxides from their retention times. These peaks disappeared following treatment with triphenylphosphine, which is widely used as a reducing reagent for lipid hydroperoxides. Although some TG hydroperoxides were eluted near CLo hydroperoxide, we previously showed the presence of CE hydroperoxide in fresh human plasma while TG hydroperoxides were absent [4]. As shown in Table III, this method gave good recoveries of the hydroperoxides tested with good reproducibilities.

## CONCLUSIONS

This method made it possible to separate the hydroperoxides PC, TLo, CLo, COl, and TOl. They were determined with high sensitivities and selectivities. The separations on two analytical columns were performed at the same time independently by the same solvent system, thus saving time and sample volume.

## ACKNOWLEDGEMENT

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

TABLE III

RECOVERY OF HYDROPEROXIDES FROM HUMAN PLASMA

| Hydroperoxide | Concentration (mean ± S.D.) (n*M*) | | | Recovery (%) |
|---|---|---|---|---|
| | Found ($n = 6$) | Added | Total ($n = 5$) | |
| PC | 44.8 ± 3.2 | 60.3 | 97.5 ± 6.7 | 92.4 |
| TLo | N.D.[a] | 63.6 | 49.1 ± 3.1 | 77.2 |
| CLo | 3.8 ± 0.7 | 59.5 | 49.3 ± 4.5 | 76.5 |
| COl | N.D. | 72.8 | 60.2 ± 5.7 | 82.7 |
| TOl | N.D. | 82.1 | 77.8 ± 3.6 | 94.8 |

[a] N.D. = not detected.

REFERENCES

1 K. Akasaka, H. Ohrui and H. Meguro, *Anal. Lett.*, 21 (1988) 965.
2 K. Akasaka, K. Morimune, H. Ohrui and H. Meguro, *Bunseki Kagaku*, 42 (1993) 27.
3 K. Akasaka, S. Ijichi, K. Watanabe, H. Ohrui and H. Meguro, *J. Chromatogr.*, 596 (1992) 197.
4 K. Akasaka, H. Ohrui, H. Meguro and M. Tamura, *J. Chromatogr.*, 617 (1993) 205.
5 J.C. Gfeller and M. Stockmeyer, *J. Chromatogr.*, 198 (1980) 162.
6 L.R. Snyder, J.W. Dolan and S. van der Wal, *J. Chromatogr.*, 203 (1981) 3.
7 W.C. Pickett and M.B. Douglas, *Prostaglandins*, 29 (1985) 83.
8 I.W. Wainer, *J. Pharm. Biomed. Anal.*, 544 (1989) 13.
9 K. Akasaka, T. Suzuki, H. Ohrui and H. Meguro, *Anal. Lett.*, 20 (1987) 797.
10 K. Akasaka, I. Sasaki, H. Ohrui and H. Meguro, *Biosci. Biotech. Biochem.*, 56 (1992) 605.