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Determination of triacylglycerol and cholesterol ester hydroperoxides in human plasma by high-performance liquid chromatography with fluorometric postcolumn detection

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)

Makoto Tamura

Tohoku University Hospital, Seiryoumachi, Aoba, Sendai 981 (Japan)

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ABSTRACT

Cholesterol ester (ChE) and triacylglycerol (TG) hydroperoxides in human plasma were determined by high-performance liquid chromatography with postcolumn detection with diphenyl-1-pyrenylphosphine. Human plasma was extracted once with *n*-hexane. 2,6-Di-*tert*.-butyl-4-methylphenol and N-stearylcinnamide were added to human plasma before extraction as an antioxidation agent and an internal standard, respectively. The detection limits of both ChE and TG hydroperoxides were 1 pmol. The sample size was minimized to 250 μl for each run. The recoveries of ChE and TG hydroperoxides from fresh plasma were *ca.* 90 and 80%, respectively. The relative standard deviations ($n = 8$) of their values in frozen human plasma were 5.4% (ChE hydroperoxides, 298 n*M*) and 5.7% (TG hydroperoxides, 267 n*M*). No TG hydroperoxides and 24.5 ± 9.6 n*M* ($n = 15$) ChE hydroperoxides were detected in fresh human plasma. The relative standard deviation ($n = 8$) of ChE hydroperoxides values in fresh plasma was 5.8% (27.1 n*M*).

INTRODUCTION

Lipid peroxidation has attracted much attention as one of the causes of ageing and some diseases [1–4]. However, it has been very difficult to determine lipid peroxides in biological materials such as plasma and tissues, because of their trace amounts, their instability and their diversity.

Lipid hydroperoxides have been determined at their class or individual molecular levels by both normal- and reversed-phase high-performance liquid chromatography (HPLC) [5–9]. They were detected by UV at 235 nm, depending on their conjugated diene systems, or by differential refractometer. Electrochemical detection was also useful for this purpose [10]. Some problems arose due to selectivity, sensitivity or interference by coexistent compounds.

Recently, we reported the highly sensitive and selective determination of lipid hydroperoxides with a fluorescence reagent, diphenyl-1-pyrenylphosphine (DPPP) [11–13]. This reagent can be used as an HPLC postcolumn reagent [14,15]. Chemiluminescence with isoluminol [16] or lumi-

* Corresponding author.

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nol [17] has been proposed for the highly sensitive and selective postcolumn detection of lipid hydroperoxides. These methods have been successfully used to determine phospholipid hydroperoxides in biological materials [14,18].

The hydroperoxides of cholesterol esters (ChE) and triacylglycerols (TG) were separated on a reversed-phase ODS column with chemiluminescence detection [16]. In spite of its high sensitivity when applied to human plasma samples, the method had some problems from negative peaks of radical trapping agents and a positive peak of ubiquinol-10 [19]. There is also the problem that complicated peak separations of the hydroperoxides are based on the differences in their fatty acids compositions, which makes their assignment difficult.

Recently, we reported an HPLC determination of hydroperoxides of TG and ChE separated in their class levels by gradient elution with *n*-hexane and 1-butanol on a silica gel column. They were detected at picomole levels by a postcolumn fluorometry with DPPP [20]. However, it was necessary to modify the system for the determination of these hydroperoxides in human plasma. This paper describes a practical procedure, using a minimum amount of human plasma, for the determination of ChE and TG hydroperoxides with some modification of the system.

EXPERIMENTAL

Chemicals

DPPP was prepared according to the method described previously [11]. Cholesteryl linolate (ChLo), trilinolein (TLo) and cholesterol esterase (EC 3.1.1.13, from bovine pancreas) were purchased from Sigma (St. Louis, MO, USA). As standards, their hydroperoxides were prepared by autoxidation as described previously [20], and stored in a refrigerator at −25°C as *n*-hexane solutions. Methanol of HPLC grade from Kanto Chem (Tokyo, Japan) and 1-butanol from Wako (Osaka, Japan) were used as received. *n*-Hexane was used after distillation. 2,6-Di-*tert*.-butyl-4-methylphenol (BHT) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Human blood was drawn from healthy volunteers, aged 21–50, who fasted for at least 9 h before collection; sodium heparin was used as an anticoagulant. Plasma was separated by centrifugation at 1500 *g* for 30 min at 4°C. Frozen plasma for transfusion was stored at −30°C for two to eight months in Tohoku University Hospital. They were subdivided to test-tubes after defrosting, and then refrozen in dry ice–acetone and stored at −30°C. They were thawed by standing at room temperature just before use.

Separation and detection of hydroperoxides

The HPLC conditions in our previous report [20] were slightly modified for the present method. The separation was performed on a Develosil 60-3 column (100 mm × 4.6 mm I.D., 3 μm particle size). The chromatographic mobile phases were: solvent A, *n*-hexane; solvent B, *n*-hexane–1-butanol (500:25, v/v). An 8% solution of B in A was used as the initial solvent. Linear solvent gradients were run from 8 to 67% B in A between 2 and 20 min, and then from 67 to 90% for 1 min. The solvent was returned linearly to the initial composition at 22–23 min. After more than 2 min elution with the initial solvent, the next sample was injected into the system. The flow-rate of the mobile phase was 0.6 ml/min, and the HPLC eluent was monitored by UV absorbance at 268 nm prior to the postcolumn reaction with DPPP. The reagent solution (3 mg/400 ml in 1-butanol–methanol, 1:1, to which 0.5 mg/ml BHT was added) was added to eluent from UV detector at the rate of 0.3 ml/min. The mixture reacted in a stainless-steel coil (20 m × 0.5 mm I.D.) at 80°C, and was then cooled by passing through short stainless-steel coil (0.5 m × 0.5 mm I.D.) in a water jacket. Detection was performed by monitoring the fluorescence intensity at 380 nm (excitation at 352 nm).

Equipment

The HPLC pump used was a CCPM multipump (Tosoh, Tokyo, Japan) in high-pressure gradient mode. An LC-3A pump (Shimadzu, Tokyo, Japan) was used for the reagent solution. The RE-8000 reaction oven, the UV-8000 spec-

trophotometer, the FS-8000 spectrofluorometer and the SC-8010 data processor were all from Tosoh.

Preparation of N-stearylcinnamide

N-Stearylcinnamide (NSC) was prepared from stearylamine (1.0 g) and cinnamoyl chloride (0.6 g) by refluxing them in 30 ml of pyridine. After the reaction, the mixture was poured into a 300 ml of hexane–ethyl acetate (1:1) and washed twice with aqueous phosphoric acid solution and with distilled water. After drying, it was recrystallized from ethyl acetate–hexane (1:1) in a yield of 70%. Physical data: m.p. 89.8°C; MS *m/z* 399 (M^+); UV λ_{max} 268 nm ($\varepsilon = 2.19 \cdot 10^4$ in hexane). Elemental analysis: calculated for $C_{27}H_{45}NO$: C, 81.14%; H, 11.35%; N, 3.50%; O, 4.00%; found: C, 80.97%; H, 11.39%, N, 3.49%.

Sample preparation procedure

A human plasma sample (250 μl) was mixed with 50 μl of NSC solution (131 μ*M* in hexane), 50 μl of freshly prepared BHT solution (20 mg/ml in *n*-hexane), 1 ml of methanol and 2 ml of *n*-hexane. The mixture was vigorously shaken for 1 min, and then centrifuged at 1000 *g* for 5 min at 4°C. The upper layer was evaporated under reduced pressure, and then the pressure was returned to ambient with nitrogen gas. The residue was dissolved in 100 μl of *n*-hexane, and 70–100 μl aliquots were injected into the HPLC system.

RESULTS AND DISCUSSION

Effect of sample injection volume

In the previous method [20], the peak separation of ChE hydroperoxides meant that their identification was a problem. Their sensitive and reproducible determination in human plasma was difficult because of their diversity and trace concentrations in plasma. To solve these problems, the effect of the injection volume of a sample on the peaks of ChE hydroperoxides was studied. The gradient pattern described in our previous report was slightly modified to shorten the analysis time, so that samples could be injected at 25-min intervals.

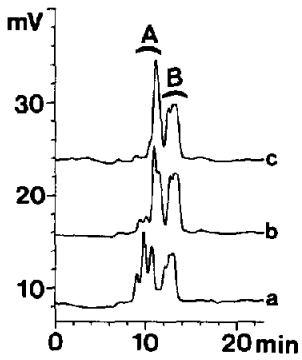

Fig. 1. Chromatograms of hydroperoxides of cholesteryl linolate (ChLo) and trilinolein (TLo). The injection volumes were (a) 10 μl, (b) 50 μl and (c) 100 μl. The concentrations of ChLo hydroperoxides (A) and TLo hydroperoxides (B) were (a) 3280 and 2690 n*M*, (b) 656 and 538 n*M* and (c) 328 and 269 n*M*, respectively.

Fig. 1 shows typical chromatograms of standard hydroperoxides: the different injection volumes of ChE and TG hydroperoxides made no significant difference. The largest injection volume (c) made the peaks of ChE hydroperoxides sharper than in (a) and (b), but did not influence the total peak areas of the ChE hydroperoxides or the retention times of the TG hydroperoxides. This effect might be due to the focusing caused by using a larger volume of *n*-hexane. ChE hydroperoxides were adsorbed on the silica gel relatively strongly by *n*-hexane, and they were eluted at the same time with a more polar solvent by using the gradient elution. The injection of a larger volume of *n*-hexane had an effect on ChE hydroperoxides but not on TG hydroperoxides. Because the peaks of ChE hydroperoxides were never eluted after 13 min, even when the injection volume was increased to 100 μl, the peaks between 8 and 13 min were assigned to ChE hydroperoxides and those between 13.8 and 14.5 min to TG hydroperoxides, as described in our previous report [20]. Because the sensitivity was enhanced by the larger injection volume, 70–100 μl of a plasma extract were injected into the system.

Elimination of negative peaks in human plasma extract

As shown in Fig. 2a, some negative peaks interfered with the determination of hydroperox-

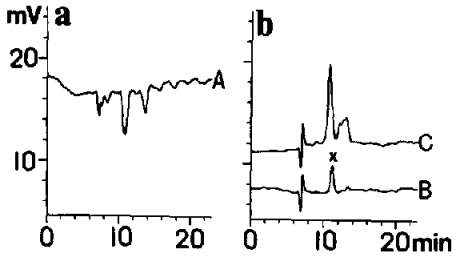

Fig. 2. Typical chromatograms of human plasma extracts. The DPPP solutions were used without BHT (a) and with BHT (b). (A and B) fresh plasma extracts; (C) an extract from fresh plasma to which standard ChE hydroperoxides (63.4 n*M*) and TG hydroperoxides (53.1 n*M*) were added before extraction. Peak x was assigned as ChE hydroperoxides and corresponded to 4.7 pmol on column (27.4 n*M* in plasma).

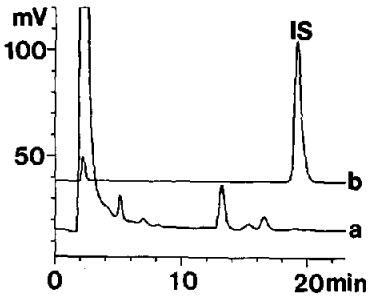

Fig. 3. Chromatograms of (a) a human plasma extract and (b) the internal standard (IS, NSC) solution. The injection volume of the extract was 85 μl. The NSC solution (0.11 m*M* in hexane) was used as IS solution, and 30 μl were injected.

ides in a fresh plasma extract. These interferences were eliminated by the addition of BHT to the reagent solution, as shown in Fig. 2b. This suggests that the production of some oxidants during the reaction was restrained by antioxidation agents in a sample to give negative peaks and it was cancelled by the addition of excess BHT. The addition of BHT also decreased the baseline noise level without interfering with the determination. It enhanced the detection limits of both ChLo and TLo hydroperoxides to 1 pmol (at a signal-to-noise ratio of 3).

Preparation of human plasma extract

NSC was used as the internal standard (I.S.) because it had relatively strong UV absorbance at 268 nm and it was separated from the peaks in the human plasma by the present separation system (Fig. 3). The UV detection was performed prior to the mixing with the DPPP reagent. Because NSC did not influence the fluorometry, it did not complicate the assignment of the hydroperoxide peaks.

Table I shows the peak-area ratios of ChE hydroperoxides and TG hydroperoxides to NSC, in various volumes of the extraction solvent. In this case, a frozen plasma was used as a test sample because fresh plasma samples contained only trace amounts of ChE hydroperoxides and no detectable TG hydroperoxides, but some frozen plasma samples contained detectable amounts of them (Fig. 4).

Although the peak areas obtained following extraction with 2 ml of *n*-hexane (C) were the

TABLE I

EFFECTS OF THE VOLUME OF *n*-HEXANE AND NUMBER OF EXTRACTION TIMES

The values in parentheses are relative to A.

	Volume of *n*-hexane (ml)	N.E.T.[a]	Peak area of I.S. ($\times 10^3$)	Peak-area ratio	
				ChE-HPO[b]/I.S.	TG-HPO[c]/I.S.
A	2	3	6.58 (1.00)	0.772 (1.00)	1.51 (1.00)
B	4	1	5.86 (0.89)	0.762 (0.99)	1.29 (0.85)
C	2	1	5.58 (0.85)	0.787 (1.02)	1.54 (1.02)

[a] Number of extraction times.
[b] Cholesterol ester hydroperoxides.
[c] Triacylglycerol hydroperoxides.

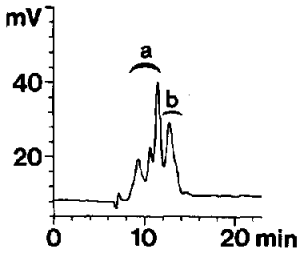

Fig. 4. Chromatogram of a frozen plasma extract. Peaks: a = ChE hydroperoxides (130 pmol on column, 713 n*M* in plasma); b = TG hydroperoxides (72.0 pmol on column, 394 n*M* in plasma).

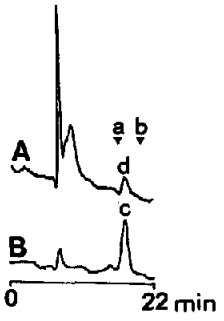

Fig. 5. Chromatograms of (A) a human plasma extract, and (B) ChLo hydroperoxides separated on an ODS column. The extract was prepared by the four times scale procedure described in Experimental, and the whole extract was dissolved in 50 μl of chloroform–methanol (1:1, v/v) for injection. The points a and b were the times where the hydroperoxides of cholesteryl arachidonate (16.4 min) and olate (19.6 min) should be eluted, respectively. Both c (ChLo hydroperoxide) and d were eluted at 17.5 min.

smallest among the test results [*ca.* 85% of the extraction repeated three times with 2 ml of *n*-hexane (A)], there was no significant difference between A and C in the ratios of hydroperoxides to the I.S. The extraction with 4 ml of *n*-hexane (B) gave the smallest ratios of TG hydroperoxides, but the reason for this was not clear. These results mean that TG and ChE hydroperoxides are extracted in the same ratio to the I.S. by extraction with 2 ml of *n*-hexane. Therefore, in the developed method, the extraction was performed once with 2 ml of *n*-hexane.

The BHT solution, which was used as an antioxidation agent in the extraction, should be freshly prepared before use because the solution gave a peak at 13 min after it was stored for several weeks at room temperature. This peak interfered with the determination of both ChE and TG hydroperoxides because it eluted between their peaks. No peak was detected from the BHT solutions freshly prepared or stored for a week at −30°C.

TABLE II

RECOVERIES OF HYDROPEROXIDES FROM FRESH HUMAN PLASMA

Added (n*M*)	Found (n*M*)	Total (n*M*)	Recovery (%)
ChE hydroperoxides			
34.2	15	47.1	93.4
68.4		74.7	87.3
TG hydroperoxides			
23.2	N.D.	19.1	82.3
46.4		36.6	78.9

Presence of ChE hydroperoxides in fresh human plasma

Although there were several peaks at 8–15 min in the chromatograms of frozen plasma (Fig. 4) and a single peak at 11 min in fresh plasma chromatograms (Fig. 2b), no peak was detected between 8 and 15 min from either type of plasma samples after treatment with sodium borohydride. This indicates that they were hydroperoxides. On the other hand, no detectable amounts of hydroperoxides were produced during the extraction, because there was no significant difference in the amount of hydroperoxides between the extract and the reextract. The reextract was prepared as follows. The extract was mixed with 0.25 ml of 0.85% NaCl solution and then extracted again. These results suggest that the hydroperoxides were not artifacts but originated from human plasma. This conclusion is supported by the absence of TG hydroperoxides in fresh plasma.

The peak at 11 min in fresh human plasma was assigned to ChE hydroperoxides, from the retention time and the result of the standard addition

test (Fig. 2b). This peak was not detected after treatment of the plasma sample with cholesterol ester hydrolase (4.7 U/ml) in 10 m*M* phosphate buffer (pH 7.0) in the presence of sodium deoxytaurocholate (10 mg/ml) at 37°C for 2 h, but it was detected after the similar treatment without the enzyme.

It was possible to separate ChE hydroperoxides on an ODS column, depending on their fatty acid components. Fig. 5 shows a chromatogram of human plasma separated on a Develosil ODS P-5 column (50 mm × 4.6 mm I.D., Nomura Chem) eluted with methanol at 0.6 ml/min and 30°C. Here, the detection was carried out by the same postcolumn system described in Experimental. The retention time of peak A (17.5 min) agreed perfectly with that of cholesterol linolate hydroperoxide. Under these conditions, cholesterol arachidonate and olate hydroperoxides were eluted at 16.4 and 19.6 min, respectively. This result suggested that ChE hydroperoxides in fresh human plasma was mainly cholesterol linolate hydroperoxides.

Reproducibilities and recoveries of TG and ChE hydroperoxides

The relative standard deviations ($n = 8$) of TG and ChE hydroperoxides using frozen plasma were 5.4% (289 n*M*) and 5.7% (267 n*M*), respectively. For ChE hydroperoxides in fresh plasma the value was 5.8% (27.1 n*M*, *ca.* 4 pmol on column, $n = 8$). The recoveries of TG and ChE hydroperoxides from fresh plasma are shown in Table II. About 90% of ChE hydroperoxides and 80% of TG hydroperoxides were recovered from fresh plasma.

Determination of ChE hydroperoxides in human plasma

By the present method, no TG hydroperoxides and 24.5 ± 9.6 n*M* (fifteen plasma samples drawn from twelve people) of ChE hydroperoxides were detected in fresh plasma. Yamamoto and Niki [21] reported the presence of 3.4 ± 2 n*M* ChE hydroperoxides in human plasma by their chemiluminescence method: our results showed the level of ChE hydroperoxides in plasma to be about seven times higher. In our method, ChE hydroperoxides gave a single peak with no interfering peak, and radical-trapping agents did not interfere with the determination.

In spite of the difference between our value and the previously reported one for the level of ChE hydroperoxides, their presence in human plasma was definitively proved because they were detected by the two methods that are different in their separation modes and their detection principles. The absence of TG hydroperoxides might be partly due to the faster turnover of TG than of ChE in living bodies, because some frozen plasma samples contained large amounts of TG and ChE hydroperoxides. This shows that both ChE and TG in the frozen plasma samples were produced by gradual autoxidation at −30°C.

CONCLUSION

A highly sensitive and selective determination of ChE and TG hydroperoxides in human plasma was developed. The problems of negative peaks and peak separations of ChE hydroperoxides were solved by the addition of BHT to the reagent solution and by the larger volume of the extract injected into the system, respectively. The simple and quick extraction procedure made it possible to extract the hydroperoxides with high reproducibility and without artificial formation or degradation of the hydroperoxides. By the method, 24.5 ± 9.4 n*M* ChE hydroperoxides were detected in fresh human plasma using 250 μl of plasma, but no TG hydroperoxides were detected.

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REFERENCES

1 J. Glavind, S. Hartmann, J. Clemmesen, K. E. Jessen and H. Dam, *Acta Pathol. Microbiol. Scand.*, 30 (1952) 1.
2 T. Yoshikawa, K. Yamaguchi, M. Kondo, N. Mizukawa, T. Ohta and K. Hirakawa, *Arch. Gerontol. Geriatr.*, 1 (1982) 209.

3 T. Nakayama, M. Kodama and C. Nagata, *Agric. Biol. Chem.*, 48 (1984) 571.
4 D. Harman, in W. A. Pryor (Editor), *Free Radicals in Biology*, Vol. V, Academic Press, New York, 1982, pp. 255–275.
5 H. W. S. Chan, G. Levett and J. A. Matthew, *Chem. Phys. Lipids*, 24 (1979) 245.
6 D. K. Park, J. Terao and S. Matsusita, *Agric. Biol. Chem.*, 45 (1981) 2448.
7 J. Terao, I. Asano and S. Matsusita, *Lipids*, 20 (1985) 312.
8 C. G. Crawford, R. D. Plattner, D. J. Sessa and J. J. Rackis, *Lipids*, 15 (1984) 325.
9 S. Hara, K. Nemoto, H. Yamaya and Y. Totani, *J. Jpn. Oil Chem. Soc.*, 37 (1988) 541.
10 K. Yamada, J. Terao and S. Matsushita, *Lipids*, 22 (1987) 125.
11 K. Akasaka, T. Suzuki, H. Ohrui and H. Meguro, *Anal. Lett.*, 20 (1987) 731.
12 K. Akasaka, T. Suzuki, H. Ohrui and H. Meguro, *Anal. Lett.*, 20 (1987) 797.
13 K. Akasaka, I. Sasaki, H. Ohrui and H. Meguro, *Biosci. Biotech. Biochem.*, 56 (1992) 605.
14 K. Akasaka, H. Ohrui and H. Meguro, *Anal. Lett.*, 21 (1988) 965.
15 K. Akasaka, S. Ijichi, K. Watanabe, H. Ohrui and H. Meguro, *J. Chromatogr.*, 596 (1992) 197.
16 Y. Yamamoto, M. H. Brodsky, J. C. Baker and B. N. Ames, *Anal. Biochem.*, 160 (1987) 7.
17 T. Miyazawa, K. Yasuda and K. Fujimoto, *Anal. Lett.*, 21 (1988) 1033.
18 T. Miyazawa, T. Suzuki, K. Fujimoto and K. Yasuda, *J. Lipid Res.*, 33 (1992) 1051.
19 B. Frei, Y. Yamamoto, D. Niclas and B. N. Ames, *Proc. Natl. Acad. Sci. U.S.A.*, 85 (1988) 9748.
20 K. Akasaka, H. Ohrui and H. Meguro, *J. Chromatogr.*, 628 (1993) 31.
21 Y. Yamamoto and E. Niki, *Biochem. Biophys. Res. Commun.*, 165 (1989) 988.