A Simple Assay for Lipid Hydroperoxides in Serum or Plasma*

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Summary In order to develop a simple and reliable assay method for lipid hydroperoxides in serum or plasma, we sought to determine the suitable conditions for direct application of the previously reported colorimetric method using a methylene blue derivative, 10-(N-methylcarbamoyl)-3,7-(dimethylamino)-phenothiazine, for the measurement of lipid hydroperoxides in a sample without extraction of them with organic solvents. For such purpose, dissociation of lipid hydroperoxides from proteins by lipoprotein lipase was found to be necessary. Also, we found that holotransferrin, which oxidizes the methylene blue derivative, should be eliminated by its chelation with trimethylenetetraminehexaacetic acid. Pretreatment of the sample with ascorbate oxidase was also necessary to eliminate interference by ascorbic acid in a sample. Thus the recommended method is to mix the sample with lipoprotein lipase, trimethylenetetraminehexaacetic acid, ascorbate oxidase, and the detergent Triton X-100, then to incubate the mixture with the methylene blue derivative dissolved in the detergent in the presence of hemoglobin, and to measure the oxidized product, methylene blue. The amount of lipid hydroperoxides is calculated by use of an external standard, either linoleic acid hydroperoxide or cumene hydroperoxide.

Key Words: lipid hydroperoxides, serum, plasma, hemoglobin-methylene blue method

Many years ago one of us (Yagi) devised a reliable method involving the thiobarbituric acid reaction for the assay of the lipid peroxide level in serum or plasma [1]. This method gives the level of the total amount of lipid hydroperox-

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ides and lipid aldehydes derived from them. The lipid peroxide level measured by this method was found to be increased in various diseases [2], especially vascular disorders such as angiopathy in diabetes [3], atherosclerosis [4], and apoplexy [5]. To verify our hypothesis that increased lipid peroxides are causative of vascular disorders, we conducted experiments on the effects of linoleic acid hydroperoxide on blood vessels, and found injury to the endothelial cells both in vivo [6] and in vitro [7]. Recently, we demonstrated the generation of hydroxyl radicals from lipid hydroperoxides contained in low-density lipoprotein (LDL) upon addition of ferrous iron or epinephrine-iron complexes [8]. Because of the high reactivity of hydroxyl radicals, we paid special attention to the increase in the lipid hydroperoxide level and to the occurrence of epinephrine-iron complexes in the blood, both of which probably explain at least in part catecholamine-induced atherogenesis [9] or stress-induced atherosclerosis [10]. In view of the above findings, we sought to measure specifically the amount of lipid hydroperoxides in the blood by a simple procedure.

Earlier we reported the reaction of lipid hydroperoxides with a methylene blue derivative by the catalysis of hemoglobin to yield methylene blue [11]. Thereafter, we adopted another derivative, 10-(N-methylcarbamoyl)-3,7-(dimethylamino)-phenothiazine (MCDP), for the measurement of lipid hydroperoxides contained in lipids extracted from foods [12]. The basic reaction involved in this method is presented in Fig. 1. We called this method the hemoglobin-methylene blue (Hb-MB) method. In the present study, we established suitable conditions to apply the Hb-MB method directly to serum or plasma for a simple and reliable assay of their lipid hydroperoxides for clinical investigation.

**MATERIALS AND METHODS**

**Materials.** Triton X-100 and 3-(N-morpholino)propanesulfonic acid (MOPS) were purchased from Nacalai tesque Inc., Kyoto; soybean lipoxygenase and holotransferrin, from Sigma Chemical Co., St Louis, MO; triethylenetetramine-
hexaacetic acid (TTHA) and 2-methylbenzimidazole, from Wako Pure Chemical Ind., Ltd., Osaka; and Sarcosinate LN, from Nikko Chemicals, Co., Ltd., Tokyo. Ascorbate oxidase, lipoprotein lipase, and MCDP came from Kyowa Medex Co., Ltd., Tokyo.

**Preparation of lipid hydroperoxides.** Hydroperoxides of unsaturated fatty acids were prepared by the reaction of these acids with soybean lipoxygenase as described by Ohkawa *et al.* [13] and purified by high-performance thin-layer chromatography on a Kiesel Gel<sub>50</sub>F<sub>254</sub> plate with a mixture of hexane : diethyl ether (8 : 7, v/v) as the mobile phase. Hydroperoxides of phospholipids, triglycerides, and cholesterol ester were prepared by photosensitized peroxidation as follows: 50 mg of each lipid was dissolved in 50 ml of methanol containing 0.1 mm methylene blue and incubated at 10°C for 20 h under illumination by a 30-W tungsten lamp. After the peroxidized sample had been applied to a silica gel column (1 × 4 cm) to remove the methylene blue, the crude lipid hydroperoxide was eluted with methanol. The hydroperoxide was purified by high-performance liquid chromatography on a Develosil ODS-5 column (4.6×150 mm) with methanol as the mobile phase.

**Preparation of peroxidized LDL.** LDL was purified from the sera of rabbits fed a high-cholesterol diet as described in our previous paper [14], and peroxidized in the presence of 5 mM CuSO<sub>4</sub>. After peroxidation, the LDL was applied onto a PD-10 column (Pharmacia, Uppsala) for rapid desalting.

**RESULTS**

**Reactivity of MCDP with lipid hydroperoxides**

Since MCDP is scarcely soluble in aqueous solution, but can be dissolved in the presence of a detergent like Triton X-100, the effect of this detergent on the reactivity of MCDP with linoleic acid hydroperoxide was first examined. Linoleic acid hydroperoxide solution (0.1 ml of 50 nmol/ml hydroperoxide dissolved in 10% ethanol) was mixed with 1.0 ml of 0.1 M MOPS buffer (pH 5.8) containing graded concentrations of Triton X-100 (% w/v), and then with 2.0 ml of 0.1 M MOPS buffer (pH 5.8) containing graded concentrations of Triton X-100, 0.04 mM MCDP, and 60 μg/ml hemoglobin. After having been incubated at 30°C for different periods in the presence of different concentrations of the detergent, the samples were measured for absorbance at 665 nm. The absorbance increased up to 7 min of incubation and up to 0.05% concentration of the detergent, and then reached a plateau, as shown in Figs. 2 and 3. Therefore, we adopted 10 min as the incubation time and 0.1% as the detergent concentration for further experiments.

Under the above conditions, we checked the reactivity of different lipid hydroperoxides. Hydroperoxide values of linoleic acid, oleic acid, arachidonic acid, eicosapentaenoic acid, trilinolein, dilinoleoyl phosphatidylcholine, and cholesterol linoleate hydroperoxides were measured by the above-mentioned method using linoleic acid hydroperoxide as the standard. Each hydroperoxide...
The obtained values also agreed with the peroxide values measured according to the method of Swoboda and Lea [16]. In addition, we confirmed with linoleic acid hydroperoxide that the value obtained

\[ \text{Absorbance at } 665 \text{ nm} \]

\( \begin{array}{c|c}
0 & 0.05 \\
5 & 0.10 \\
10 & 0.15 \\
15 & 0.20 \\
20 & 0.25 \\
\end{array} \]

Incubation time (min)

Fig. 2. Effect of incubation time on the reaction of MCDP with lipid hydroperoxides. One hundred microliters of linoleic acid hydroperoxide solution (50 nmol/ml in 10% ethanol) was mixed with 1.0 ml of 0.1 M MOPS buffer (pH 5.8) containing 0.1% Triton X-100, and then the reaction was started by the addition of 2.0 ml of 0.1 M MOPS buffer (pH 5.8) containing 0.1% Triton X-100, 0.04 mM MCDP, and 60 μg/ml hemoglobin. The change in absorbance at 665 nm was followed at 30°C.

Fig. 3. Effect of Triton X-100 on the reaction of MCDP with lipid hydroperoxides. Reaction conditions of linoleic acid hydroperoxide with MCDP were same as described in the legend for Fig. 2, except that the concentration of Triton X-100 was varied and the incubation time was 10 min.

value was equivalent to the amount of the conjugated diene of the respective lipid hydroperoxide determined by spectrophotometry on the basis of the molar absorptivity reported by Tappel et al. [15]. The obtained values also agreed with the peroxide values measured according to the method of Swoboda and Lea [16]. In addition, we confirmed with linoleic acid hydroperoxide that the value obtained
by this method agreed well with that determined by the glutathione peroxidase reaction according to the method of Heath and Tappel [17].

These results show that the reaction can be applied to a mixture of lipid hydroperoxides to obtain their total value by use of linoleic acid hydroperoxide as the external standard.

**Dissociation of lipid hydroperoxides from proteins**

Since preliminary experiments showed that lipid hydroperoxides contained in LDL cannot easily react with MCDP, we considered that lipid hydroperoxides in serum or plasma should be dissociated from serum or plasma proteins for this assay. To check this problem, we examined the effect of lipoprotein lipase on the reaction of lipid hydroperoxides contained in LDL with MCDP as follows: 0.1 ml of peroxidized LDL (2 mg protein/ml) was mixed with 1.0 ml of 0.1 M MOPS buffer (pH 5.8) containing 0.1% Triton X-100 and graded concentrations of lipoprotein lipase, and the mixture was preincubated at 30°C for 5 min. Then 2.0 ml of 0.1 M MOPS buffer (pH 5.8) containing 0.1% Triton X-100, 0.04 mM MCDP, and 60 µg/ml hemoglobin was added. Further incubation was carried out at 30°C for 10 min, and the absorbance at 665 nm was measured. As shown in Fig. 4, more than 1.0 unit/ml lipoprotein lipase was necessary to obtain the maximum value.

For the preincubation time period, we adopted 5 min based on the results of time course experiments.

Next, we examined whether this condition is valid for serum or plasma. In

![Graph](image.png)

**Fig. 4.** Effect of lipoprotein lipase on the reaction of lipid hydroperoxides contained in LDL with MCDP. Peroxidized LDL was prepared as described in the text. One hundred microliters of peroxidized LDL (2 mg protein/ml) was mixed with 1.0 ml of 0.1 M MOPS buffer (pH 5.8) containing various concentrations of lipoprotein lipase, and the mixture was incubated at 30°C for 5 min. Then the reaction with MCDP was carried out as described in the legend for Fig. 2.
serum or plasma, holotransferrin and ascorbic acid were found to disturb the measurement, and so their elimination became necessary as mentioned below.

Determination of the concentration of lipoprotein lipase necessary for the assay of serum was performed in the presence of TTHA, which chelates with holotransferrin, and ascorbate oxidase, which oxidizes ascorbic acid. Thus, 0.1 ml of serum was mixed with 1.0 ml of 0.1 M MOPS buffer (pH 5.8) containing 0.1% Triton X-100, 9 mM TTHA, 15 units/ml ascorbate oxidase, and graded concentrations of lipoprotein lipase; and the mixture was incubated at 30°C for 5 min. Then 2.0 ml of 0.1 M MOPS buffer (pH 5.8) containing 0.1% Triton X-100, 0.04 mM MCDP, and 60 µg/ml hemoglobin was added to the mixture, further incubation was carried out at 30°C for 10 min, and the absorbance at 665 nm was measured. The results are shown in Fig. 5. As can be seen from the figure, the addition of more than 0.5 unit/ml of lipoprotein lipase was necessary to obtain the maximum value.

**Elimination of disturbing substances**

**Elimination of disturbance due to holotransferrin.** When we fractionated human serum lipoproteins on a Superose 6 column in an FPLC system and determined the lipid hydroperoxides in each lipoprotein fraction by the Hb-MB method, we found most of them in very low-density lipoprotein, LDL, and high-density lipoprotein fractions, but also noticed a large unexpected peak containing the Hb-MB reaction positive substance(s) that appeared after the high-density lipoprotein fraction. Judging from the elution position of the unexpected peak, we considered that this peak fraction contained transferrin and that MCDP might

![Graph](image-url)
react with transferrin besides its reaction with lipid hydroperoxides. To check this point, we preincubated 0.2 ml of human holotransferrin solution (5 mg/ml) with 1.0 ml of 0.1 M MOPS buffer (pH 5.8) containing 0.1% Triton X-100 and various concentrations of TTHA at 30°C for 5 min, incubated this mixture with 2.0 ml of 0.1 M MOPS buffer (pH 5.8) containing 0.1% Triton X-100, 0.04 mM MCDP, and 60 µg/ml hemoglobin at 30°C for 10 min, and measured the absorbance at 665 nm. As shown in Fig. 6, a significant amount of the product methylene blue was found in the absence of the chelator. However, this reaction was inhibited by TTHA in a concentration-dependent manner, and become nil in the presence of the chelator at its concentration over 6 mM.

Then, we checked the effect of TTHA on the value obtained by the Hb-MB method with the above fractions in the serum, and found that the unexpected peak observed without TTHA disappeared in the presence of TTHA, and that TTHA had no effect on the reactivity of each lipoprotein fraction with MCDP. Accordingly, it is clear that the reaction of holotransferrin with MCDP can be eliminated by the addition of TTHA.

Since the concentration of transferrin in the human serum is about 4 mg/ml, the addition of more than a 6-mM concentration of the iron-chelator TTHA to the preincubation mixture can eliminate the effect of transferrin. To confirm this point, we checked the effect of TTHA on the reaction of serum lipid hydroperoxides with MCDP, and found that the above-mentioned conclusion was valid for the serum (data not shown).

Elimination of disturbance due to ascorbic acid. Since we noticed that

![Fig. 6. Effect of the iron-chelator TTHA on the Hb-MB reaction of human holotransferrin. To 0.2 ml of human holotransferrin solution (5 mg/ml), 1.0 ml of 0.1 M MOPS buffer (pH 5.8) containing 0.1% Triton X-100 and graded concentrations of TTHA was added, and the mixture was then incubated at 30°C for 5 min. After the preincubation, the Hb-MB reaction was carried out as described in the legend for Fig. 2.](image-url)
ascorbic acid inhibited the Hb-MB reaction, we intended to eliminate the inhibitory effect of ascorbic acid by treatment of the sample with ascorbate oxidase. As shown in Fig. 7, ascorbic acid inhibited the Hb-MB reaction of linoleic acid hydroperoxide in a concentration-dependent manner. However, this inhibitory effect was completely eliminated by the addition of ascorbate oxidase to the preincubation mixture at its concentration of 15 units/ml.

Then the effect of addition of ascorbic acid on the Hb-MB reaction of lipid hydroperoxides in serum and its elimination by ascorbate oxidase were examined. As shown in Fig. 8, the addition of ascorbic acid inhibited the reaction. However, simultaneous addition of ascorbate oxidase to the preincubation mixture at its concentration of 15 units/ml eliminated the effect of ascorbic acid.

Since it was reported that the concentration of ascorbic acid in normal serum is approximately 11 µg/ml and does not increase much by intake of ascorbic acid, i.e., approximately 13 µg/ml after intake of over 200 mg of the acid [18], the concentration of ascorbic acid in the reaction mixture for pretreatment could be below 1.2 µg/ml. Accordingly, the addition of 15 units/ml of ascorbate oxidase to the preincubation mixture seems to be sufficient to eliminate the inhibitory effect of ascorbic acid on the reaction. To confirm this point with serum, we checked the reaction of lipid hydroperoxides in human serum in the presence of graded concentrations of ascorbate oxidase, and found that the amount of reaction product increased with increasing concentration of ascorbate oxidase in the

Fig. 7. Effect of ascorbic acid on the Hb-MB reaction of linoleic acid hydroperoxide and its elimination by ascorbate oxidase. One hundred microliters of linoleic acid hydroperoxide solution (50 nmol/ml in 10% ethanol) was incubated with 1.0 ml of 0.1 M MOPS buffer (pH 5.8) containing 0.1% Triton X-100 and various concentrations of ascorbic acid in the absence (○) or presence (●) of 15 units/ml ascorbate oxidase at 30 °C for 5 min. After the preincubation, the Hb-MB reaction was carried out as described in the legend for Fig. 2.

preincubation mixture and reached a plateau at its concentration of 5 units/ml, indicating that 15 units/ml of ascorbate oxidase in the preincubation mixture is sufficient for the assay of serum lipid hydroperoxides (data not shown).

Effect of anti-coagulant on the Hb-MB method

To apply the Hb-MB method to plasma samples, we had to examine the effects of various anti-coagulants. None of the anti-coagulants tested, viz., heparin, ethylenediaminetetraacetic acid, citrate, or oxalate, had any influence on the Hb-MB reaction when examined at their concentration normally used to prevent coagulation. When serum and plasma obtained from the same blood were checked, they gave essentially the same value (data not shown).

Check for applicability of cumene hydroperoxide as the external standard

From the results described above, we can establish a standard procedure for the assay of the total amount of lipid hydroperoxides in serum or plasma by use of linoleic acid hydroperoxide as the external standard. We are convinced that this standard procedure can be used for clinical investigation.

However, if we want to apply this method for diagnosis of diseases in clinical medicine, a much more stable standard would be convenient. From this point of view, we checked cumene hydroperoxide, which is stable and commercially

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available, for its reactivity with MCDP. As in the case of linoleic acid hydroperoxide described in the legend for Fig. 2, 0.1 ml of cumene hydroperoxide solution (50 nmol/ml of the hydroperoxide in water) was mixed with 1.0 ml of 0.1 M MOPS buffer (pH 5.8) containing 0.1% Triton X-100, and with 2.0 ml of 0.1 M MOPS buffer (pH 5.8) containing 0.1% Triton X-100, 0.04 mM MCDP, and 60 μg/ml hemoglobin, and then absorbance at 665 nm was followed at 30°C. Under the above conditions, the reaction proceeded slowly (data not shown). To improve the reactivity, we tested the effect of many substances, and found that the reaction proceeded as fast as in the case of linoleic acid hydroperoxide, when 2-methylbenzimidazole and Sarcosinate LN were present (Fig. 9). We further found that these two substances had no effect on the other reactions involved in the present assay.

**Standard procedure**

On the basis of all the data described in the previous sections, we constructed the following standard procedures for the assay of lipid hydroperoxides in serum or plasma by the Hb-MB method.

**Procedure using linoleic acid hydroperoxide as the external standard.** To 0.1 ml of serum or plasma, 1.0 ml of the pretreatment reagent (0.1 M MOPS buffer, pH 5.8, containing 0.1% Triton X-100, 1.5 units/ml lipoprotein lipase, 9 mM TTHA, and 15 units/ml ascorbate oxidase) is added, and mixed; then the mixture is incubated at 30°C for 5 min. Next, 2.0 ml of the MCDP reagent (0.1 M MOPS buffer, pH 5.8, containing 0.1% Triton X-100, 0.04 mM MCDP, and 60 μg/ml hemoglobin) is added, and mixed. After incubation of the mixture at 30°C for 10

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**Fig. 9.** Effect of incubation time on the reaction of MCDP with cumene hydroperoxide. One hundred microliters of cumene hydroperoxide solution (50 nmol/ml in water) was mixed with 1.0 ml of 0.1 M MOPS buffer (pH 5.8) containing 0.1% Triton X-100, and then the reaction was started by the addition of 2.0 ml of 0.1 M MOPS buffer (pH 5.8) containing 0.1% Triton X-100, 6 mg/ml 2-methylbenzimidazole, 0.75 mg/ml Sarcosinate LN, 0.04 mM MCDP, and 60 μg/ml hemoglobin. The change in absorbance at 665 nm was followed at 30°C.

min, the absorbance at 665 nm is measured. As the external standard, 0.1 ml of 50 nmol/ml linoleic acid hydroperoxide is used, and treated in the same way. Then the total amount of lipid hydroperoxides (nmol/ml) in the serum or plasma is calculated.

Procedure using cumene hydroperoxide as the external standard. To 0.1 ml of serum or plasma, 1.0 ml of the pretreatment reagent mentioned above is added, and the mixture is incubated at 30°C for 5 min. Then 2.0 ml of the MCDP reagent supplemented with 6 mg/ml 2-methylbenzimidazole and 0.75 mg/ml Sarcosinate LN is added, and mixed. After incubation of the mixture at 30°C for 10 min, the absorbance at 665 nm is measured. As the external standard, 0.1 ml of 50 nmol/ml of cumene hydroperoxide is used; and the total amount of lipid hydroperoxides in the serum or plasma is calculated.

Reliability of the standard procedure

To check the reliability of this method, we carried out the following experiments: When lipid hydroperoxides in serum were extracted with a mixture of chloroform–methanol (2:1, v/v) according to Folch et al. [19], and measured by the Hb-MB method, the value was more than 80% of the value obtained with the serum measured by the above standard procedure. This seems to indicate that the value obtained by the standard procedure gives a reliable measure of the lipid hydroperoxides contained in the serum.

![Graph showing reactivity of lipid hydroperoxides in peroxidized LDL added to human serum.](image)

**Fig. 10.** Reactivity of lipid hydroperoxides in peroxidized LDL added to human serum. One hundred microliters of human serum was mixed with 1.0 ml of 0.1 M MOPS buffer (pH 5.8) containing 0.1% Triton X-100, 1.5 units/ml lipoprotein lipase, 9 mM TTHA, 15 units/ml ascorbate oxidase, and different volumes of peroxidized LDL (1 mg protein/ml; 100 nmol hydroperoxides/ml) and incubated at 30°C for 5 min. After the preincubation, the Hb-MB reaction was carried out as described in the legend for Fig. 2.

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To check further the reliability of this method, we carried out an addition test as follows: 0.1 ml of serum was mixed with different volumes of peroxidized LDL (100 nmol hydroperoxides/ml) and subjected to the standard procedure. As shown in Fig. 10, lipid hydroperoxides contained in the peroxidized LDL were quantitatively recovered.

DISCUSSION

Using this method, we obtained preliminary data on normal subjects, indicating a lipid hydroperoxide concentration of approximately 1 nmol/ml or less. Extensive measurements are now being made in our laboratory. Preliminary measurement also showed that a significant increase in the amount of lipid hydroperoxides was found in diabetic patients. A large-scale clinical survey is also in progress.

For the selective determination of lipid hydroperoxides in human serum or plasma, several methods have been developed. One of them is a method based upon the activation of prostaglandin H synthetase by hydroperoxides [20, 21]. By use of this method, the plasma concentration of lipid hydroperoxides in normal subjects was reported to be 0.5 nmol/ml of plasma. When a chemiluminescence-high performance liquid chromatography system was employed, the concentrations of hydroperoxides of free fatty acids and of cholesterol esters in human plasma were 0.05 and 0.317 nmol/ml, respectively, [22] and that of phosphatidylcholine hydroperoxides was in the range from 0.05 to 0.43 nmol/ml [23]. These data indicate that the amount of lipid hydroperoxides in normal human serum or plasma is less than 1 nmol/ml. These data are not much different from our results.

REFERENCES


