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Liquid chromatography–mass spectrometry of fatty acids including hydroxy and hydroperoxy acids as their 3-methyl-7-methoxy-1,4-benzoxazin-2-one derivatives

Takashi Kusaka*

Department of Clinical Nutrition, Kawasaki University of Medical Welfare, 288 Matsushima Kurashiki, Okayama 701-01 (Japan)

Mitsunori Ikeda

Department of Biochemistry, Kawasaki Medical School, 577 Matsushima Kurashiki, Okayama 701-01 (Japan)

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ABSTRACT

Many kinds of authentic fatty acids, including hydroperoxy and hydroxy acids, were found to be not only detectable by liquid chromatography–mass spectrometry (LC–MS) with an atmospheric pressure chemical ionization interface system, but also measurable quantitatively by LC–MS installed with a spectrophotometer. We used LC–MS with spectrophotometry to analyse fatty acids split from lecithins after phospholipase A_2 treatment. Thus, some hydroperoxy fatty acids split from photo-oxidized lecithin could be identified.

INTRODUCTION

Recently, we reported the development of a new method of analysing fatty acids [1,2]. With this method the amide derivatives of hydroxy and non-hydroxy fatty acids were sensitively detected by liquid chromatography–mass spectrometry with an atmospheric pressure chemical ionization interface system (LC–APCI-MS). The method was applied to the simultaneous analysis of hydroxy and non-hydroxy fatty acids in rat brain [2] and to the analysis of fatty acids with a wide range of carbon chain lengths (C_{14}–C_{54}) in some mycobacteria [3].

We further attempted to quantitatively analyse labile fatty acids such as polyunsaturated hydroxy and hydroperoxy fatty acids. As a result, 3-bromomethyl-7-methoxy-1,4-benzoxazin-2-one (BrMB), known as a fluorescence labelling reagent for carboxylic acid, was found to be useful for detecting hydroperoxy and hydroxy fatty acids by LC–MS.

EXPERIMENTAL

Chemicals

Heptadecanoic acid ($C_{17:0}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$), α- and γ-linolenic acids ($C_{18:3}$), eicosatetraenoic acid ($C_{20:4}$), docosadienoic acid ($C_{22:2}$) and docosahexaenoic acid ($C_{22:6}$) were purchased from Nu-Chek-Prep. through Funakoshi Pharmaceutical (Tokyo, Japan). 12-Hydroxystearic acid (12-OH-$C_{18:0}$) was obtained from Serdary Research Labs., 2-hydroxystearic acid (2-OH-$C_{18:0}$) was purchased from Larodan Fine Chemicals, 15-hydroxyeicosatetraenoic acid (15-HETE or 15-OH-

* Corresponding author.

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$C_{20:4}$), 15-hydroperoxyeicosatetraenoic acid (15-HPETE or 15-OOH-$C_{20:4}$) and 5-hydroperoxy-eicosatetraenoic acid (5-HPETE or 5-OOH-$C_{20:4}$) were obtained from Cascade Biochemistry and 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine and bovine liver lecithin were purchased from Avanti Polar Lipids through Funakoshi Pharmaceutical. A standard mixture of eight kinds of fatty acids ($C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, γ-$C_{18:3}$, $C_{20:0}$) was purchased from Funakoshi Pharmaceutical. High-performance liquid chromatography (HPLC) grades of methanol and acetonitrile and N,N-diisopropylethylamine (DPEA) were purchased from Nakarai Tesque (Kyoto, Japan). Redistilled water was used for HPLC. BrMB was obtained from Tokyo Kasei (Tokyo, Japan). Bee venom phospholipase (PLase) A_2 (E.C.3.1.1.4) (Sigma, St. Louis, MO, USA) was further purified by reversed-phase HPLC according to the method of Hara *et al.* [4].

Equipment

A Hitachi (Tokyo, Japan) M-2000-type double-focusing mass spectrometer–computer system, equipped with a Hitachi L-6200-type HPLC instrument through a Hitachi APCI interface system, was used. HPLC was performed using a reversed-phase Cosmosil $5C_{18}$-packed column with 5-μm particles (250 mm × 4.6 mm I.D., Nakarai Tesque). The column temperature was maintained at 37°C in the column oven. The eluate from the LC column was conducted to the mass spectrometer via a photometric cell and the APCI interface system. Spectrophotometric chromatograms at a wavelength (λ) of 355 nm [λ_{max} for the 7-methoxy-1,4-benzoxazin-2-one-3-methyl (MB) ester of fatty acid] were processed with a 7000-B-type chromatogram processor (System Instrument, Tokyo, Japan). The drift voltage of APCI was 100 V and the temperatures of the vaporizer and desolvator were 250 and 385°C, respectively. The multiplier voltage of the mass spectrometer was 1700 V.

Derivatization of fatty acids with BrMB

Derivatization of fatty acids with BrMB was performed according to a slight modification of the method of Naganuma *et al.* [5] (Fig. 1). To 0.3 ml of acetonitrile containing 10–50 nmol of fatty acids were added 0.2 ml of 2.0 m*M* BrMB in acetonitrile and 30 nmol of DPEA as a catalyser. The reaction mixture was left at room temperature for 30 min, and then evaporated to dryness *in vacuo*. The residue was dissolved in 20–50 μl of acetonitrile. It was measured by the LC–MS immediately or stored at −70°C until use.

Photo-oxidation of unsaturated fatty acids and lecithin

Photo-oxidation of unsaturated fatty acids and lecithin was performed according to the method of Terao *et al.* [6]. Briefly, 100–200 μg of each authentic fatty acid ($C_{18:1}$, $C_{18:2}$, α- and γ-$C_{18:3}$, $C_{20:2}$, $C_{20:4}$, $C_{22:2}$, $C_{22:6}$) or bovine liver lecithin were dissolved in 0.5 ml of chloroform–methanol (1:1, v/v) containing 20 μg of methylene blue in test tubes. Each tube was irradiated with a tungsten lamp (40 W) at a distance of 10 cm for 2–3 h. The temperature of the reaction mixture was maintained at 20°C during the reaction. After the irradiation, the methylene blue in the medium was removed by column chromatog-

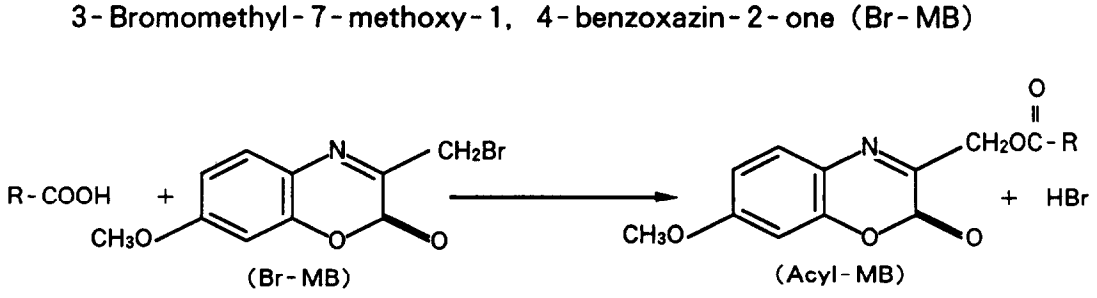

Fig. 1. Equation for the acyl MB preparation.

raphy with silica gel G (Merck, Darmstadt, Germany) using a mixture of chloroform and methanol (1:1, v/v) as the elution system.

Splitting of fatty acids from lecithin with PLase A_2

Splitting of fatty acids from lecithin with PLase A_2 was performed according to the method of Hara *et al.* [4]. Briefly, 100–200 μg of lecithin from bovine liver or 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine were incubated with 10 units of purified PLase A_2 for 10 min at room temperature in 1 ml of 10 m*M* Tris–HCl (pH 8.9) containing 0.1 ml of diethyl ether, 0.1 m*M* EDTA, 2 m*M* calcium chloride and 20 m*M* sodium chloride. The split fatty acids were extracted with hexane–diethyl ether (1:1, v/v).

Reduction of hydroperoxy fatty acids with sodium borohydride

Reduction of hydroperoxy fatty acids with sodium borohydride was performed according to the method of Van Rollins and Murphy [7]. An aliquot of 10–50 nmol of authentic 15-HPETE, 5-HPETE or photo-oxidized unsaturated fatty acids prepared as described above was dissolved in 1 ml of methanol, after which sodium borohydride (1 mg) was added. The solutions were incubated on ice for 15 min and at room temperature for 10 min. Then 1 ml of water was

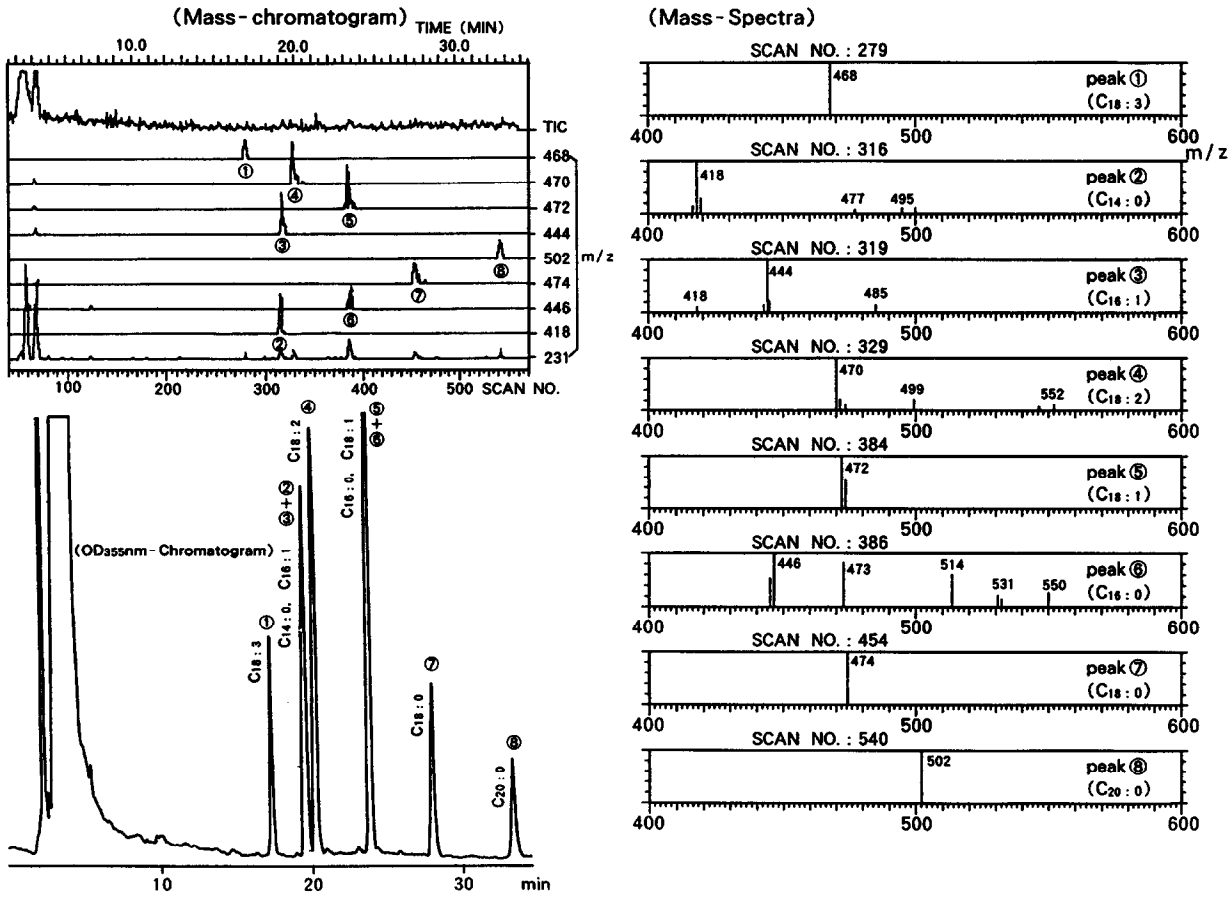

Fig. 2. LC–MS of the MB derivatives of standard fatty acids. A mixture of eight kinds of fatty acids (approximately 10 nmol each of $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$, $C_{20:0}$) was the starting material. The mobile phase was acetonitrile–water (80:20, v/v) with acetonitrile increasing linearly at the rate of 1%/min.

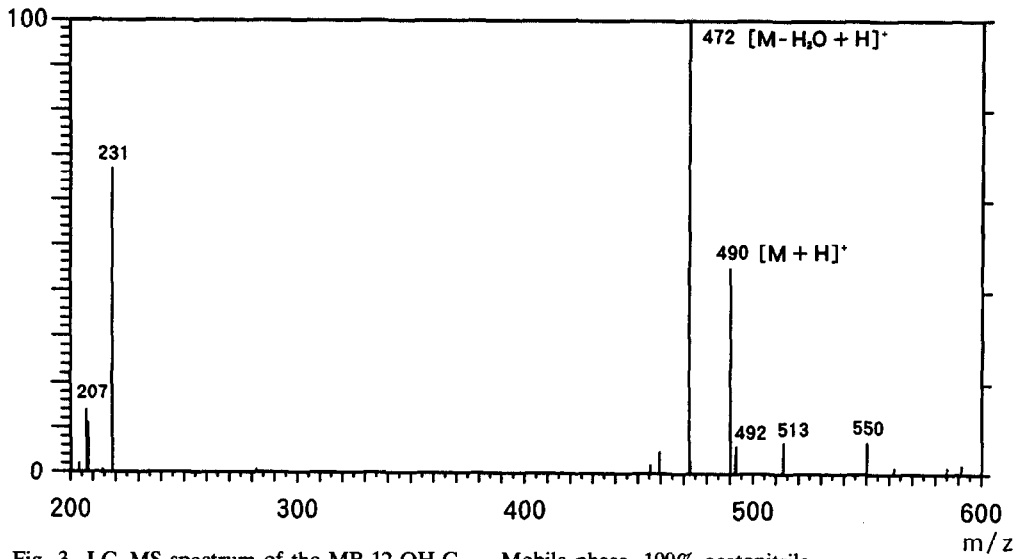

Fig. 3. LC–MS spectrum of the MB-12-OH-$C_{18:0}$. Mobile phase, 100% acetonitrile.

added and the solutions were adjusted to pH 4 with 5% hydrochloric acid. The fatty acids in the solutions were extracted with methylene chloride.

RESULTS

LC–MS of MB derivatives of authentic fatty acids

First, we examined whether the MB derivatives of fatty acids could be detected by LC–MS.

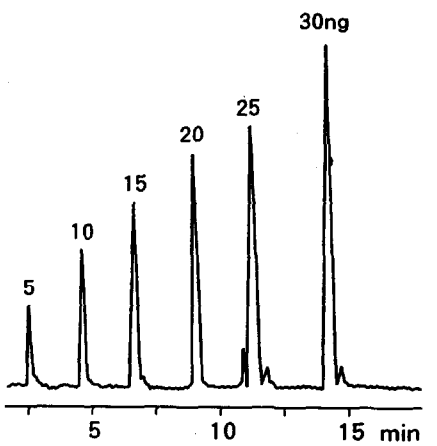

Fig. 4. Sensitivity of the MB derivative of palmitic acid. The mass spectrometer–computer system was set to detect a single ion $[M + H]^+$ with the maximum sensitivity for the derivative.

The MB derivatives of a mixture of eight kinds of authentic fatty acids were measured by the LC–MS with spectrophotometry. As shown in Fig. 2, we observed the mass spectra and mass chromatograms of MB derivatives of fatty acids. The pseudomolecular ions $[M + H]^+$ in these derivatives are the base peaks. Ions observed at

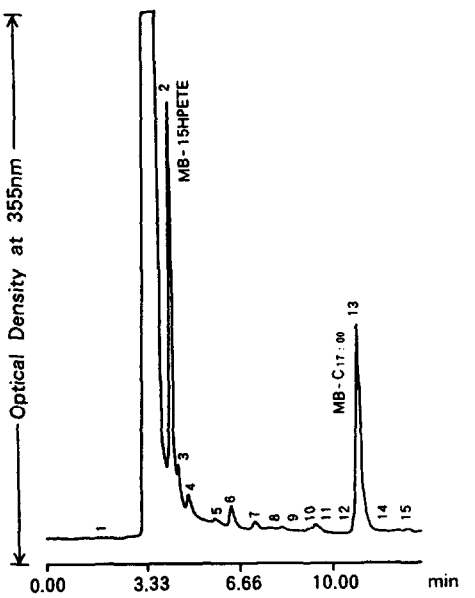

Fig. 5. HPLC of MB-15-HPETE and $C_{17:0}$. A mixture of 3 nmol each of 15-HPETE and $C_{17:0}$ was derivatized with BrMB. Mobile phase, 100% acetonitrile.

m/z 231 in these MB derivatives are assumed to be ions of [7-methoxy-1,4-benzoxazin-2-one-3-methyl + CH_3CN]. No significant difference could be observed between the spectra of saturated and unsaturated fatty acids. Peaks in the spectrophotometric chromatogram at 355 nm synchronized with those in the mass chromatogram. The mass spectrum of the MB derivative of authentic 12-OH-$C_{18:0}$ was also observed (Fig. 3). In this spectrum, an ion $[M - H_2O + H]^+$ (base peak) accompanying a smaller pseudomolecular ion $[M + H]^+$ was observed. The spectrum of the MB derivative of 2-OH-$C_{18:0}$ was similar (data not shown).

Sensitivity of MB derivative for detection by LC–MS

The sensitivity of the MB derivative of palmitic acid for detection by LC–MS was determined by monitoring a single ion $[M + H]^+$ (Fig. 4). Even 5 ng of palmitic acid could be observed.

Derivatization of hydroperoxy fatty acids with BrMB

Derivatization of 15-HPETE with BrMB was examined. The MB derivative of a mixture of 15-HPETE and $C_{17:0}$ was prepared according to the method described in the Experimental sec-

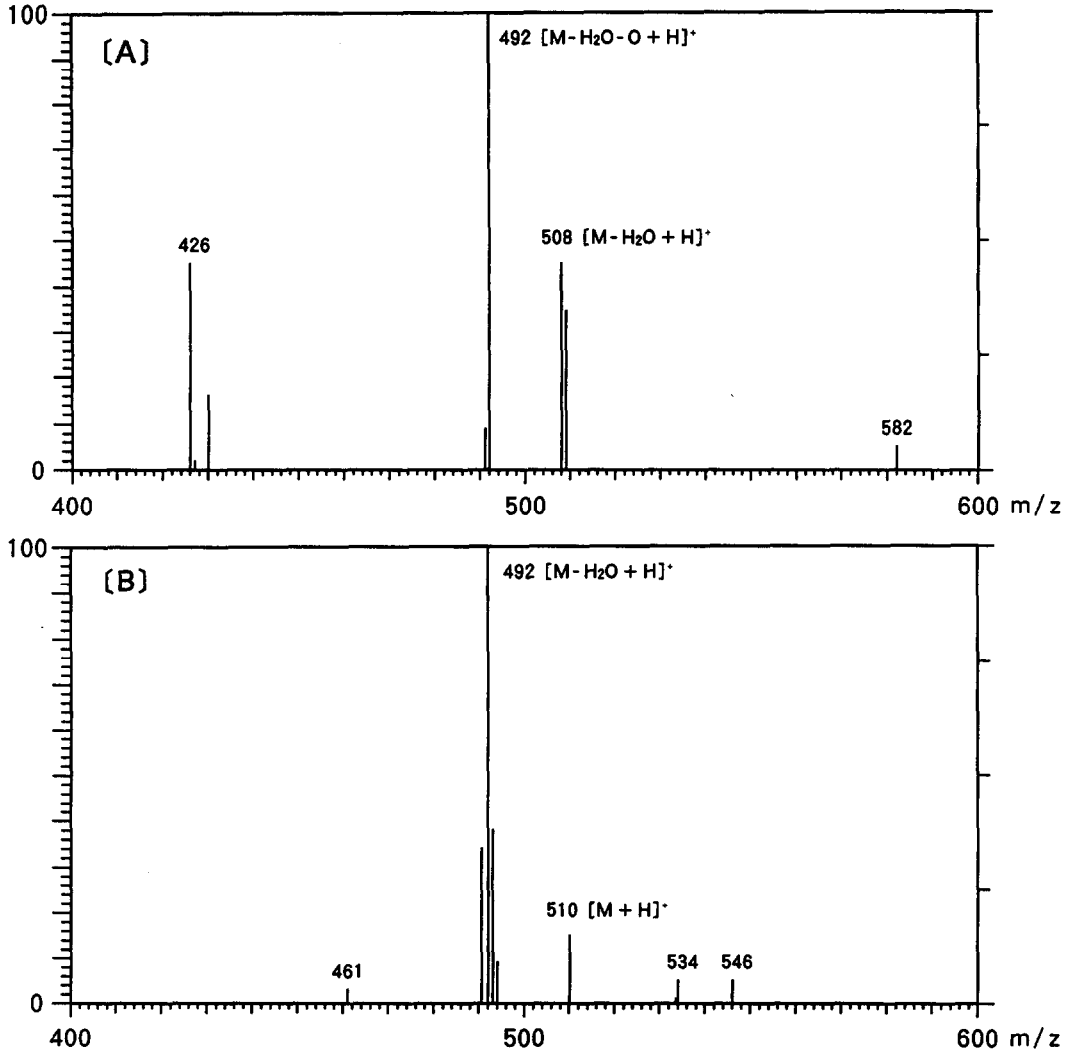

Fig. 6. LC–MS spectra of MB derivatives of 15-HPETE (A) and 15-HETE (B).

tion. Then, LC–MS with spectrophotometry was performed (Fig. 5). Fig. 6A shows a mass spectrum from peak 2 in Fig. 5. In the spectrum, no pseudomolecular ion could be observed, but ions $[M - H_2O + H]^+$ and $[M - H_2O - O + H]^+$ (base peak) were noted. This spectrum was confirmed to be distinguishable from that of 15-HETE representing a pseudomolecular ion $[M + H]^+$ and an ion $[M - H_2O + H]^+$ (base peak) (Fig. 6B). The time courses for the preparation of the MB derivatives of 15-HPETE and $C_{17:0}$ were examined by HPLC with spectrophotometry (Fig. 7). $C_{17:0}$ was derivatized quantitatively after 30 min, and derivatization of 15-HPETE showed the best yield at 30 min. Therefore, derivatization was thereafter carried out by incubation for 30 min. The derivatization yield of hydroperoxy fatty acids was determined using various amounts (2–8 nmol) of 15-HPETE and 3 nmol of $C_{17:0}$ as an internal standard. Then, 15-HPETE was found to be derivatized quantitatively (data not shown).

To confirm further MB derivatization of hydroperoxy fatty acids, several kinds of unsaturated fatty acids ($C_{18:1}$, $C_{18:2}$, α-$C_{18:3}$, γ-$C_{18:3}$, $C_{20:2}$, $C_{20:4}$, and $C_{22:6}$) were photo-oxidized separately to prepare hydroperoxy fatty acids and were derivatized after or without reduction to hydroxy fatty acids with sodium borohydride as described in the Experimental section. Then,

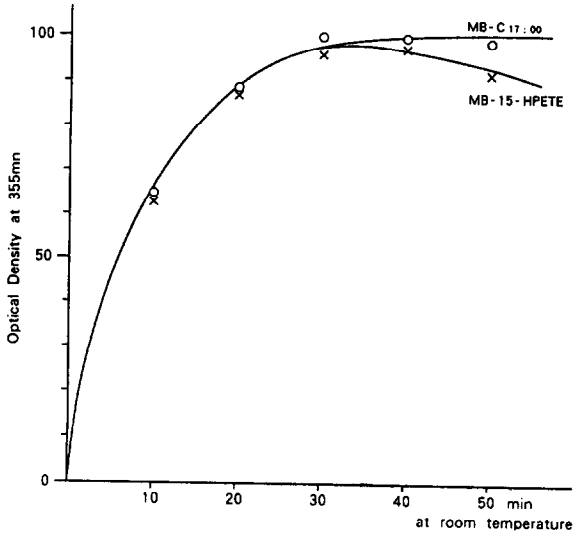

Fig. 7. Time course for MB derivatization. Aliquots of 20 nmol each of $C_{17:0}$ (○) and 15-HPETE (×) were used.

LC–MS with spectrophotometry was performed. Fig. 8 shows data thus obtained from $C_{20:2}$ and $C_{20:4}$. Similar data were obtained from the other acids (data not shown). Clusters of peaks appeared in the spectrophotometric chromatograms of photo-oxidized and reduced photo-oxidized fatty acids. These clusters of peaks should be from the isomers of each hydroperoxy or hydroxy fatty acid, because the LC–MS spectra of individual peaks in the clusters were similar to each other. The spectra from the MB derivatives of photo-oxidized fatty acids showed a characteristic combination of ions $[M - H_2O - O + H]^+$ (base peak) and $[M - H_2O + H]^+$. On the other hand, spectra from the MB derivatives of reduced photo-oxidized fatty acids showed peaks of ions $[M - H_2O + H]^+$ (base peak) and $[M + H]^+$ which are characteristic of the MB derivatives of hydroxy fatty acids.

Analysis of hydroperoxy fatty acids in photo-oxidized lecithin

Fatty acids split from lecithin by PLase A_2 with or without photo-oxidation were analysed by the above method. The splitting yield of fatty acids from the 2-position of authentic 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine was about 95 ± 5% (mean ± S.D., $n = 5$) (HPLC with spectrophotometry, $C_{17:0}$ as an internal standard). The MB derivatives of the fatty acids were measured by LC–MS with spectrophotometry and several kinds of hydroperoxy fatty acids could be identified (Fig. 9).

DISCUSSION

We successfully analysed labile fatty acids such as hydroperoxy and hydroxy polyunsaturated fatty acids quantitatively and qualitatively by LC–MS with spectrophotometry. However, the sensitivity of MB derivatives of fatty acids for detection by LC–MS was not as high as that of the amide derivatives [1,2] (Fig. 4). Therefore, a choice between the derivatization methods has to be made depending on the purpose.

There have been numerous studies on lipid hydroperoxides in relation to the possible cause of ageing, adult diseases, etc. [8,9], but there seem to have been only a few studies aimed at improving the analysis of lipid hydroperoxides. It

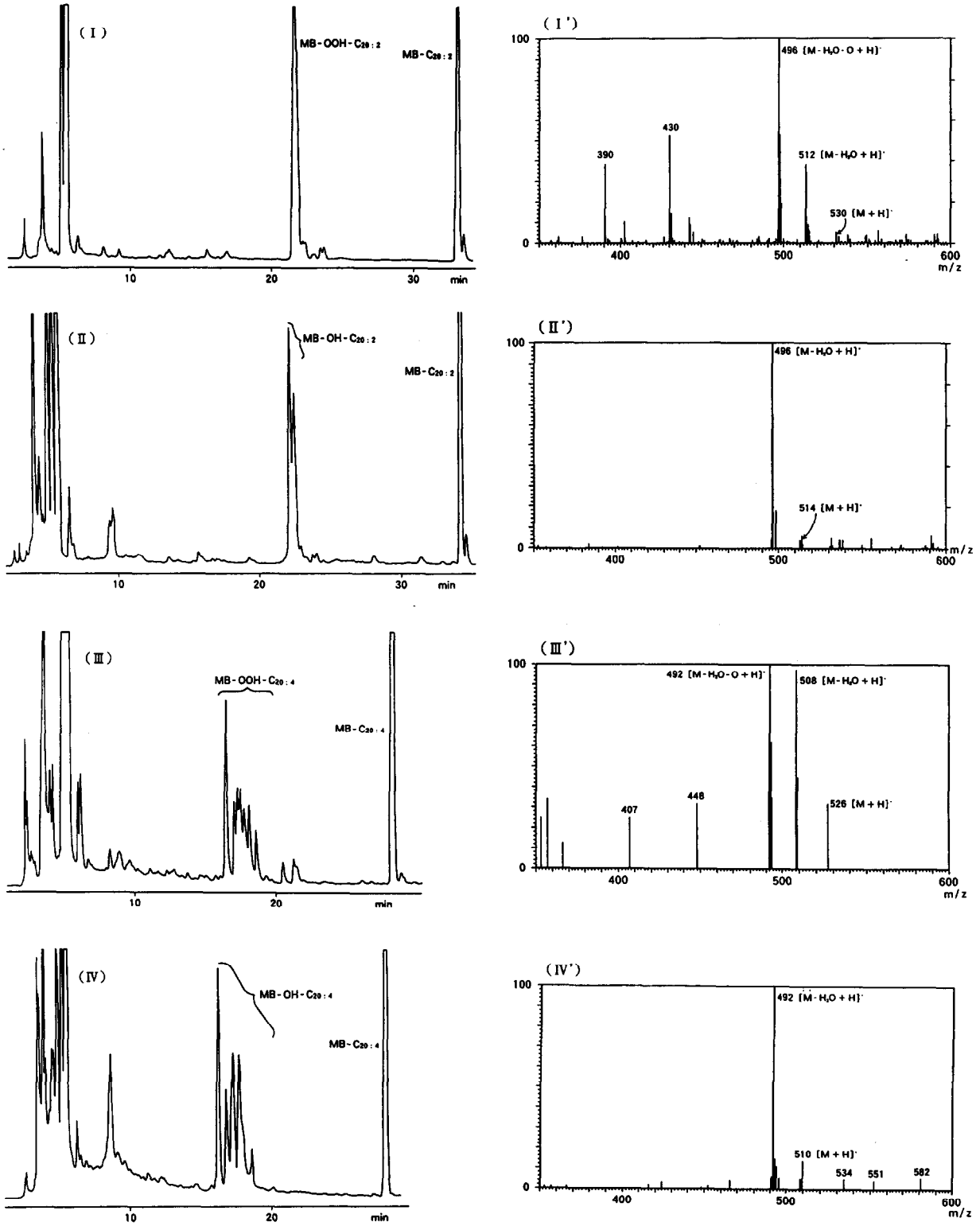

Fig. 8. HPLC profiles by spectrophotometric detection at 355 nm and LC–MS spectra of MB derivatives of photo-oxidized and reduced photo-oxidized fatty acids. I–IV: Spectrophotometric chromatograms at 355 nm. I′–IV′: LC–MS spectra. The mobile phase was acetonitrile–water (70:30, v/v) with acetonitrile increasing linearly at the rate of 1%/min.

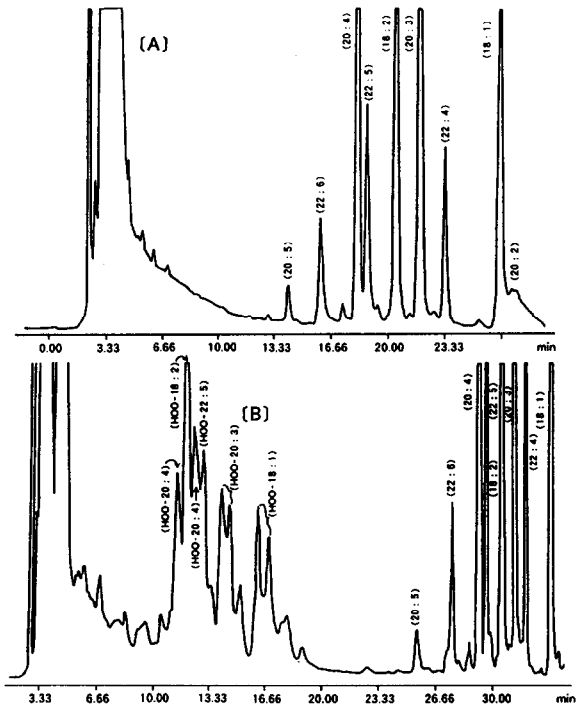

Fig. 9. Identification of fatty acids split from bovine liver lecithin (A) without or (B) with 2.5 h photo-oxidation by PLase A_2. The numerals in parentheses in (A) indicate the numbers of carbon atoms and unsaturated bonds in the fatty acids. The HOO numerals in parentheses in (B) indicate monohydroperoxy fatty acids and the numbers of their carbon atoms and unsaturated bonds. The mobile phase in (A) was acetonitrile–water (85:15, v/v) with acetonitrile increasing linearly at the rate of 0.5%/min. That of (B) was acetonitrile–water (80:20, v/v) and it was fixed during the first 15 min, then acetonitrile increased linearly at a rate of 2%/min.

is almost impossible to detect hydroperoxy fatty acids by gas chromatography (GC) or GC–MS, tools which are still very widely used in the analysis of common fatty acids. Some studies on the analysis of oxygenated products of docosahexaenoic acid and other polyunsaturates by LC–MS have been developed [10,11], but these analyses are unquantitative or only useful for a limited number of species of fatty acids. Although other kinds of approach to the analysis of lipid peroxides, such as HPLC with chemiluminescence detection [12,13] and HPLC with UV and electrochemical detection [14,15] are highly specific and sensitive to hydroperoxy phospholipids or hydroperoxy cholesterol esters, they are not useful for the identification of individual hydroperoxy fatty acids in lipids.

This study was also concerned with analysis of hydroperoxy fatty acids split from photo-oxidized lecithin by PLase A_2. Since polyenoic fatty acids in the biomembrane are preferentially located at the 2-position of the *sn*-glycero-3-phosphor skeleton of phospholipids [16,17] and are thought to be prominent targets of naturally occurring radical or non-radical peroxidation, the method in this paper may be useful for experimental studies on lipid peroxidation in biomembranes.

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