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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AS A TOOL FOR IDENTIFICATION OF LINOLENIC ACID HYDROPEROXIDE PREPARED WITH SOYBEAN LIPOXYGENASE

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SUMMARY

Linolenic acid hydroperoxide, enzymatically produced by soybean lipoxygenase from linolenic acid, was purified by high-performance liquid chromatography (HPLC) using a Supelco LC-8 (5 μm), 22 cm × 4.6 mm I.D. column with a solvent system of acetonitrile–tetrahydrofuran–0.1% phosphoric acid (50.4:21.6:28, v/v/v). The hydroperoxide was converted to several derivatives, the hydroxy, methyl, and stearate which were analyzed by HPLC and identified by gas chromatography–mass spectrometry. The 13-hydroperoxy linolenic acid was the major hydroperoxide produced by soybean lipoxygenase.

INTRODUCTION

Linolenic acid (18:3ω3) is one of the three prevalent polyunsaturated fatty acids (PUFA) found in brain tissue[1,2]. The other two PUFA are arachidonic acid (20:4:ω6) and docosohexaenoic acid (22:6:ω3). In general, PUFA have not been found free in the cell but are primarily located on the second carbon atom of the glycerol moiety of phospholipids[1]. PUFA, however, have been reported to be released from the phospholipid into the cell by stress to the brain tissue, *e.g.* ischemia, hypoxia[3–5]. Both free PUFA and phospholipid-bound PUFA have been suggested to be susceptible to peroxidation[6,7] by lipoxygenase, free radicals, hydrogen peroxide, or auto-oxidation. One product of these oxidative reactions would be lipid hydroperoxide, a compound which itself, can promote and propagate formation of more lipid hydroperoxides[8]. This continued proliferation of hydroperoxides could lead to extensive cellular damage[6–8].

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These hydroperoxides have been difficult to isolate and identify due to their lability and inadequate methodology. We decided to determine whether specific lipid hydroperoxides can be identified by high-performance liquid chromatography (HPLC) in brain tissue. HPLC is a very sensitive method and has been utilized in conjunction with gas chromatography–mass spectrometry (GC–MS) to identify lipid hydroperoxides[9–12]. We chose to utilize linolenic acid as the model fatty acid substrate because it was found to be a major constituent of the brain[1,2]. In these experiments linolenic acid was peroxidized by soybean lipoxygenase, and one major hydroperoxide was separated by HPLC. This hydroperoxide was reduced, methylated, and/or hydrogenated, and the respective derivatives analyzed by HPLC, and identified by GC–MS.

EXPERIMENTAL

Materials

Linolenic acid was purchased from NuChek, (Elysian, MN, U.S.A.). The HPLC column, a Supelcosil LC-8 (5 μm), 22 cm × 10 mm I.D. semi-preparative column, was purchased from Supelco (Bellefonte, PA, U.S.A.). Acetonitrile and tetrahydrofuran (THF) were bought from B&J, a subsidiary of American Hospital Products (Minneapolis, MN, U.S.A.). Phosphoric acid and $NaBH_4$ were purchased from J. T. Baker, and Fisher Scientific (Pittsburg, PA, U.S.A.). Soybean lipoxygenase was purchased from Sigma (St. Louis, MO, U.S.A.) and 1-methyl-3-nitro-1-nirosoguanidine (MNNG) bought from Aldrich (Milwaukee, WI, U.S.A.). [1-^{14}C]linolenic acid was obtained from DuPont New England Nuclear (Boston, MA, U.S.A.).

Methods

Linolenic acid which was 99% pure [as determined by thin-layer chromatography (TLC) and GC] according to NuChek, was further purified by HPLC (Hewlett-Packard, Model 1084) to remove any products resulting from auto-oxidation. The LC-8 semi-preparative column, was used at a flow-rate of 3.2 ml/min, with a solvent system of acetonitrile–THF–0.1% phosphoric acid (50.4:21.6:28.0, v/v/v). Linolenic acid (2 mg/ml by weight) was solubilized in the acetonitrile–THF mobile phase, and 100-μl aliquots injected ten times for a total volume of 1.0 ml. The absorbance was measured at both 215 nm and at 235 nm. The fraction which elutriated at 7.67 min (215 nm) was collected and pooled, and the linolenic acid concentrated in a rotary evaporator. This HPLC purified residue was extracted with diethyl ether three times, and the ether extract washed with water three times before drying with magnesium sulphate. The diethyl ether was removed by evaporation with nitrogen. The purified linolenic acid was oxygenated with soybean lipoxygenase according to Kaplan and Ansari's modification[13] of Hamberg and Samuelsson's method[14]. The linolenic acid hydroperoxide was purified with the LC-8 semiprep column under the same conditions as described above. The major peak, which eluted at 5.3 min, 235 nm, was collected, pooled and extracted from the mobile phase as described above for the fatty acid. This pooled fraction was used for characterization and derivatization of the hydroperoxide.

The linolenic acid hydroperoxide was reduced to hydroxylinolenic acid with $NaBH_4$ using the method described by Lindstrom and Aust[15]: The hydroperoxide

was solubilized in 5 ml methanol, and 1.5 ml of $NaBH_4$ (300 mg per 5 ml methanol) added to this solution. The reaction was allowed to proceed for 20 min in an ice bath, and then for an additional 40 min at room temperature. At the end of this time period the pH of the reaction solution was acidified to pH <3.0 with 5 *M* hydrochloric acid and the hydroxy derivative extracted with diethyl ether as described above for extraction of the fatty acid.

The unsaturated fatty acid hydroperoxide and its hydroxy derivative were reduced to the corresponding fatty acid, stearate, by hydrogenation using the PAAR hydrogenation apparatus: the hydroperoxide and hydroxylinolenic acid were each individually solubilized in a minimum volume (10 ml) of 95% alcohol. A minimum of 10 mg of platinum oxide was added to the solution, and the unsaturated fatty acid hydroperoxide and hydroxy fatty acid were individually hydrogenated for 24 h. The platinum residue was removed by filtration, and the stearate derivative recovered by removal of the solvent in a rotary evaporator. This residue was solubilized in ether and divided into three aliquots for HPLC, GC–MS and methylation by the diazomethane method.

The derivatives of hydroperoxy and hydroxylinolenate and/or stearate were methylated using diazomethane. Diazomethane was generated from MNNG in a Macro-Diazomethane generator using the method described for the apparatus[16]. The diazomethane, generated after addition of 5 *M* sodium hydroxide to MNNG (133 mg in water), reacted with the fatty acid derivative which had been solubilized in diethyl ether and was in the outer chamber of the generator. This ether fraction was divided into two aliquots, one for HPLC and the other for GC–MS. The HPLC fraction yielded two peaks which were collected and analyzed by GC–MS.

In order to confirm the fact that linolenic acid was oxidized to a hydroperoxide, we used labeled linolenic acid as a substrate for soybean lipoxygenase. [1-^{14}C]linolenic acid (10 μCi, Spec. activ. 54.8 mCi/mmol) was added to cold linolenic acid and the solution purified by HPLC. The labeled compound was then treated with soybean lipoxygenase as described above in the synthesis of linolenic acid hydroperoxide. Samples of effluent from the HPLC column, five drops or 0.12 ml each, were collected in a scintillation vial and counted on a Packard Scintillation counter. The starting material, the labeled linolenic acid, had a retention time of 7.6 min at 215 nm. After treatment with lipoxygenase two peaks containing labeled compounds were observed at 235 nm with retention times of 6.05 and 6.35 min. The similarity in retention time and absorbance at 215 and at 235 nm between ^{14}C-labeled and non-labeled linolenic acid, and between the peaks of labeled and non-labeled hydroperoxide supported the fact that linolenic acid was converted to two separate compounds, one of which we have identified as linolenic acid hydroperoxide as described in this paper.

GC–MS analyses of linolenic acid hydroperoxide and its derivatives were performed with bis(trimethyl-silyl)trifluoroacetamide–1% trimethylchlorosilane treated samples[17] (Krick, personal communication) on a 4 ft. × 2 mm I.D. glass column packed with 3% OV-1 on Supelcoport (100/120 mesh) programmed from 170 to 290°C with a 10°C increase per min. The carrier gas was helium and the flow-rate was 28 ml/min. Electron impact (23 eV) mass spectra were obtained on a LKB 9000 instrument equipped with a digital PDP 8/E data system.

RESULTS

Even though the linolenic acid from NuChek was >99% pure, we further purified the fatty acid before synthesis of the hydroperoxide because the unsaturated fatty acid can be readily auto-oxidized upon standing. Examining the compound at both 215 and 235 nm, Fig. 1A and B, we observed only one peak at 215 nm (Fig. 1B), that at 7.67 min (89%) whereas two peaks were seen at 235 nm, one at 5.37 min (20%) and the other at 7.11 min (67%) (Fig. 1A). The peak at 5.37 min (235 nm) was probably linolenic acid hydroperoxide as described in the next paragraph. The peak at 7.67 min (215 nm) was utilized for subsequent analysis.

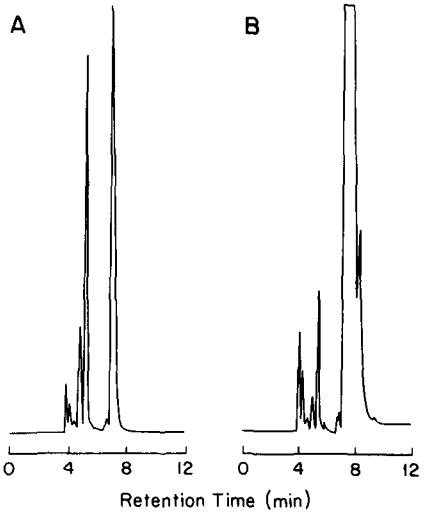

Fig. 1. HPLC of linolenic acid at 235 nm (A) and 215 nm (B).

Peroxidation of linolenic acid with soybean lipoxygenase yielded three components at 235 nm (Fig. 2A) peaking at 4.6, 5.33 and 6.10 min, being 16, 24, and 8% of the total peaks, respectively. The major peak, that at 5.33 min, was collected and upon re-chromatography had a retention time at 6.01 min (90%) (Fig. 3A). Another component with an area of 17% appeared of 4.95 min when the absorbance was measured at 215 nm (Fig. 3B). The major peak did not absorb as well at 215 nm compared to 235 nm. It is known that conjugated dienes, such as hydroperoxy fatty acids, absorb better at 235 nm.

The hydroperoxide was reduced with $NaBH_4$ to form hydroxylinolenate. The retention time of this reduced fatty acid was 5.45 min (Fig. 4A and B) which constituted 86% of the total area at both wave lengths, 215 and 235 nm.

The methylated purified hydroperoxide indicated only one peak at 235 nm, that with a retention time of 6.54 min (76%), Fig. 5A whereas two peaks were observed at 215 nm, one at 5.17 (21%) and the other at 6.39 (64%), (Fig. 5B). The absorbance of the methylated hydroperoxide was only slightly greater at 235, Fig. 5A than that at 215 nm (Fig. 5B).

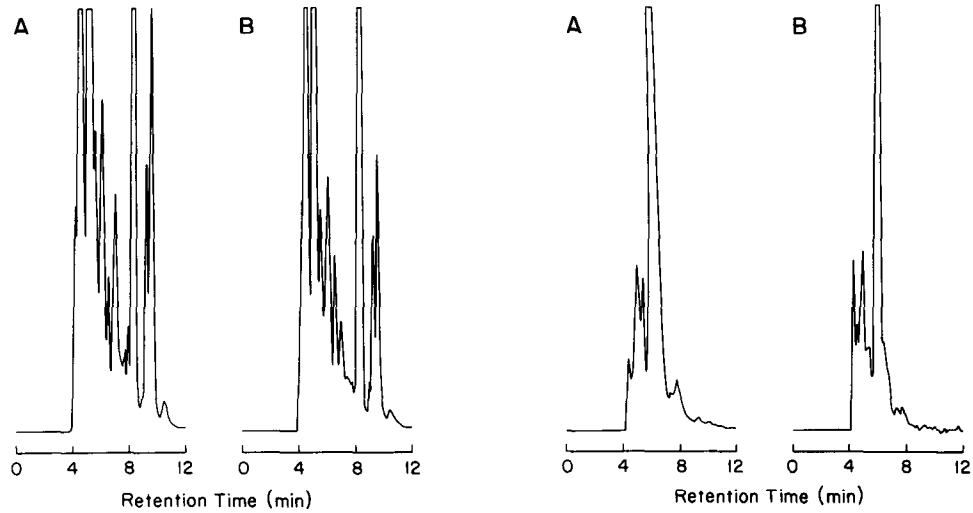

Fig. 2. HPLC of original linolenic acid hydroperoxide at 235 nm (A) and 215 nm (B).

Fig. 3. HPLC of purified linolenic acid hydroperoxide at 235 nm (A) and 215 nm (B).

The methylated hydroxy compound showed two major peaks at 235 nm with the retention time of one peak being 4.75 min (47%) and the other at 5.16 min (45%) (Fig. 6A). The differences in absorbance between 215 or 235 nm (Fig. 6A and B) were small with this group of derivatives.

Hydrogenation of linolenic acid hydroperoxide and hydroxylinolenic acid to stearic acid derivatives both yielded one peak with a retention time of 4.29 min. Both

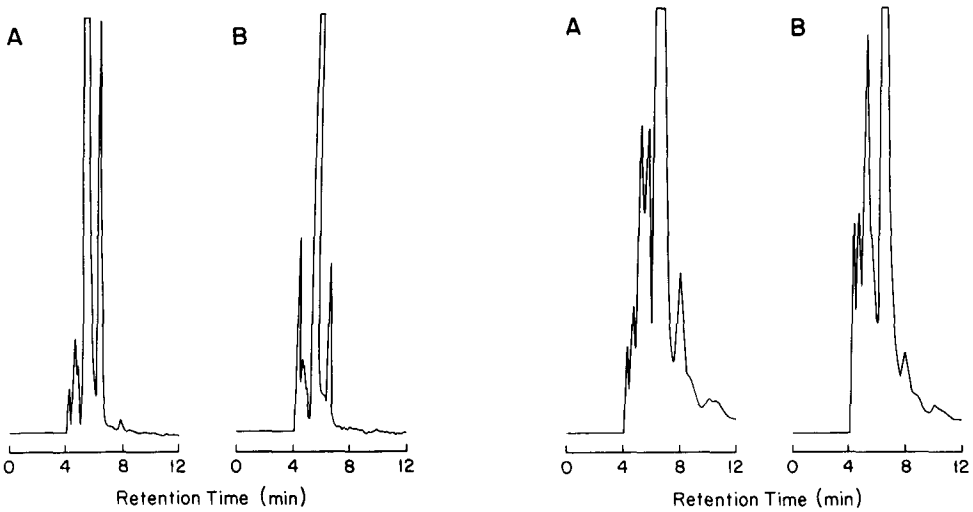

Fig. 4. HPLC of hydroxylinolenic acid at 235 nm (A) and 215 nm (B).

Fig. 5. HPLC of methyllinolenic acid hydroperoxide at 235 nm (A) and 215 nm (B).

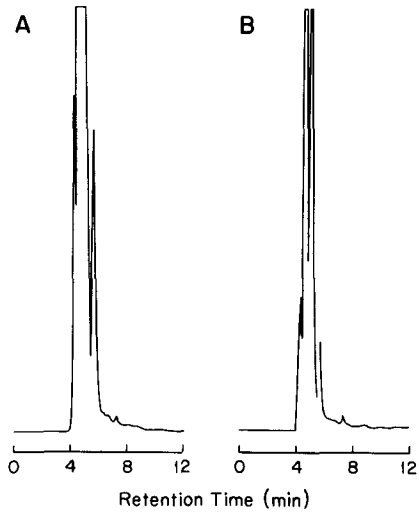

Fig. 6. HPLC of methylhydroxylinolenic acid at 235 nm (A) and 215 nm (B).

derivatives had low absorbance at 215 and 235 nm. Methylation of stearate hydroperoxide produced two peaks, Fig. 7A, with retention times of 4.23 (24%) and 4.94 min (31%) whereas three peaks were observed at 215 nm with retention times of 4.23 (24%), 4.92 (19%) and 5.15 (15%) (Fig. 7B). The methylation of hydroxystearate yielded two components with a retention time of 4.15, (20%), and 4.23 (44%) (Fig. 8A and B). The peak at 4.23 min is methyl hydroxy stearic acid.

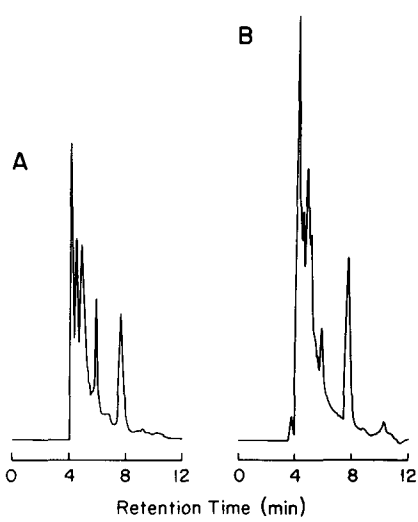

Fig. 7. HPLC of methylstearic acid hydroperoxide at 235 nm (A) and 215 nm (B).

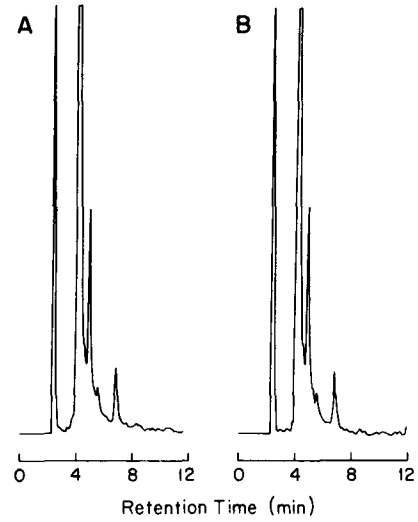

Fig. 8. HPLC of methylhydroxy stearic acid at 235 nm (A) and 215 nm (B).

These retention times and relative areas at the two wavelengths are summarized in Table I. The total area does not always add up to 100% because of the appearance of many small peaks of less than 10%. Some of these small peaks have been identified by GC–MS to be impurities such as phthalates.

TABLE I

RETENTION TIME AT 215 AND 235 NM OF LINOLENIC ACID AND ITS DERIVATIVES

Compound	*Retention time (min)*	
	Wavelength (nm)	
	215 (area)*	*235 (area*)*
Linolenic acid		5.37 (20)
		7.11 (67)
	7.64 (87)	
Linolenic acid hydroperoxide	4.95 (17)	
	6.02 (73)	6.01 (90)
Hydroxylinolenic acid	5.44 (86)	5.45 (86)
Methyllinolenic acid hydroperoxide	5.17 (21)	
	6.39 (64)	6.54 (76)
Methylhydroxylinolenic acid	4.69 (54)	4.75 (47)
	5.12 (38)	5.16 (45)
Methyl stearic acid hydroperoxide	4.23 (24)	4.23 (24)
	4.92 (19)	4.94 (31)
	5.15 (15)	
Methylhydroxy stearic acid	4.15 (20)	4.14 (19)
	4.23 (44)	4.23 (45)

* area = percentage of total area of all peaks at that wavelength.

Mass spectra of the tetramethylsilane (TMS) derivatives of the linolenic acid hydroperoxide supported the fact that the hydroperoxide and the various derivatives were produced under our conditions. The mass spectrum of the TMS derivative of methylhydroxystearate and TMS derivative of the methylhydroperoxide stearate indicated that the peroxide was located on C-13. The relative intensity of the characteristic peak at *m/e* was 396, 381, 364, 314 and 298 for both methylhydroperoxy and hydroxystearate. The non-methyl TMS derivatives of hydroperoxy and hydroxystearate had characteristic peaks with intensity of *m/e* 438, 423, 369 and 354. The non-TMS derivatives were not detected without TMS derivatization.

DISCUSSION

Linolenic acid (18:3ω3) can be peroxidized by soybean lipoxygenase to form linolenic acid hydroperoxide. Our data indicated that several hydroperoxides were produced. In this investigation we concentrated on (1) identifying the major hydroperoxide, and (2) the location of hydroperoxide which we have found to be located on C-13 of the unsaturated fatty acid.

Christophersen[18] had prepared five hydroperoxides from linolenic acid by au-

to-oxidation in air, and he isolated and identified by GC–MS the hydroxy derivatives of these hydroperoxides. From the GC–MS data he concluded that the 8-hydroperoxide, 9-hydroperoxide, 12-hydroperoxide, 13-hydroperoxide and the 16-hydroperoxide were produced by auto-oxidation of linolenic acid. The hydroxy compounds utilized for GC–MS were produced metabolically by glutathione peroxidase found in liver supernatant. In a previous publication we also have shown that brain glutathione peroxidase system is capable of reducing several lipid hydroperoxides including linolenic acid hydroperoxide[13].

Linolenic acid hydroperoxide formed by soybean lipoxygenase appeared to represent less than 50% of the total area. Hydroperoxides are considered to be unstable, and it may be that the linolenic acid hydroperoxide, which was initially isolated, was partially reduced to hydroxylinolenic acid since the retention times of some of the compounds of the hydroperoxy and hydroxy derivatives were similar. For example, the peak at 5.17 min in methyllinolenic acid hydroperoxide, that at 5.12 min in methylhydroxylinolenic acid, and that at 5.15 min, in methylstearic acid hydroperoxide, Table I, may represent the same compound. These retention times correspond to that of unreacted hydroxylinolenic acid (5.45 min). It is conceivable that these retention times may represent separate compounds. Our data suggest that compounds and/or their derivatives should be evaluated at two different wavelengths because a compound which appears to be highly purified compound at one wavelength may be found to have to have two components at the second wavelength.

Brain has been analyzed for lipid hydroperoxide formation by the TBA assay[19–21]. This method primarily assays the breakdown product of lipid hydroperoxides, malonyldialdehyde (MDA). Furthermore this MDA assay provides no direct evidence that lipid hydroperoxides are formed. Data presented here indicate that HPLC can be used to detect and eventually identify specific lipid hydroperoxides. This technique may serve as a valuable tool for direct demonstration of lipid hydroperoxides in tissues such ass brain where these peroxides are suspected of causing cellular damage[6–8].

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