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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF HYDROPEROXY DERIVATIVES OF STEAROYLLINOLEOYLPHOSPHATIDYLCHOLINE AND OF THEIR ENZYMATIC REDUCTION PRODUCTS

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SUMMARY

Two high-performance liquid chromatographic methods are presented to identify hydroperoxy and hydroxy derivatives of stearoyllinoleoylphosphatidylcholine (SLPC). When SLPC was peroxidized by Fe^{3+}–ascorbate, the peroxidation products were mainly 9- and 13-hydroperoxylinoleic acid derivatives of SLPC. The corresponding 9- and 13-hydroxy derivatives were formed if glutathione and a recently discovered phospholipid hydroperoxide glutathione peroxidase were also present. Analyses of the β-position fatty acids released by phospholipase A_2 were carried out on an aminic column and of the whole phospholipid following acetic anhydride treatment on a reversed-phase column.

INTRODUCTION

The reduction of lipid hydroperoxides to the corresponding hydroxy derivatives plays an important role both in the defence reactions against lipid peroxidation and in the repair of peroxidized membranes[1]. The enzymatic reduction of hydroperoxides leads to inhibition of lipid peroxidation by removing the centres of secondary initiation, from which free radicals can otherwise easily be generated by molecule-induced homolysis or by reductive activation of hydroperoxides[2].

The selenium enzyme glutathione peroxidase (E.C. 1.11.1.9) reduces hydrogen peroxide and fatty acid hydroperoxides[3], but it is not reactive towards phospholipid hydroperoxides[4]. The latter are reduced by another glutathione peroxidase, identified in this laboratory and purified from pig liver[5], heart[6] and brain[7], and provisionally called "peroxidation inhibiting protein" (PIP). The peroxidase activity of PIP was demonstrated by titration of the hydroperoxide groups in an oxidized preparation of soy bean phosphatidylcholine. The amount of hydroperoxide groups consumed is stoichiometrically equivalent to the amount of oxidized glutathione formed in the reaction[5]. In addition, mass spectrometric (MS) evidence has been obtained of the enzymatic reduction of a hydroperoxy group of intact oxidized dilinoleoylphosphatidylcholine to a hydroxy group[8].

To learn more about the mechanism of action of PIP, we needed a quantitative

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evaluation of the relative content of hydroperoxide and hydroxy groups in the oxidized phosphatidylcholine acted upon by the enzyme, as well as a discrimination between the positional isomers of the oxidized derivatives of phosphatidylcholine. The main approach to study oxidized fatty acids of phospholipids employed by previous authors entailed gas chromatography–mass spectrometry (GC–MS) of derivatized fatty acid hydroperoxides[9,10]. However, all the hydroperoxide groups were converted into hydroxy groups, and so such methods were not completely adequate for our purposes. The problem was solved by resorting to an high-performance liquid chromatographic (HPLC) analysis on a polar bonded phase column, which allows a clean resolution of underivatized oxidized derivatives of fatty acids, released from phosphatidylcholine by enzymatic hydrolysis with phospholipase A_2. Moreover, we took advantage of an acetic anhydride treatment of a peroxidized phospholipid to separate on a reversed-phase column the hydroperoxy and the hydroxy derivatives of the whole phospholipid molecule.

MATERIALS AND METHODS

The 1-stearoyl-2-linoleoylphosphatidylcholine (SLPC) was purchased from P.L. Biochemical; linoleic acid and soy bean lipoxidase were from Sigma. All solvents used were HPLC grade and were filtered and degassed before use. SLPC liposomes were prepared according to ref. 10. The peroxidation-inhibiting protein (PIP) was purified from pig heart as previously described[5]. Glutathione peroxidase and phospholipase A_2 were from Boehringer Mannheim.

SLPC was peroxidized by the Fe^{3+}–ascorbate system in an oxygraph vial and the oxygen consumption was measured polarographically in the following medium: 0.1 *M* Tris–HCl buffer pH 7.5; 0.5 m*M* SLPC; 0.16 m*M* ascorbic acid; 0.05 m*M* ADP; 0.005 m*M* $FeCl_3$; when present, reduced glutathione was 5 m*M* and pure PIP was 0.05 mg/ml.

The lipoxidase-catalyzed oxidation of linoleic acid was carried out in the following medium: 0.1 *M* Tris–HCl pH 7.5; 0.15 *M* potassium chloride; 10 m*M* magnesium chloride; 0.3 m*M* linoleic acid and 0.05 mg/ml of lipoxidase; when present, reduced glutathione was 5 m*M* and glutathione peroxidase 0.05 mg/ml. The stock solution of linoleic acid was 25 m*M* in 0.1 m*M* Tris–HCl, pH 8.5, and was deoxygenated by purging with a nitrogen stream. Auto-oxidation of linoleic acid was performed at room temperature by exposing a sample from the stock solution to air in a shaker for 3 days at room temperature.

SLPC, linoleic acid and their oxidation products were extracted from the incubation mixtures using a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.). The reaction mixtures were applied to the cartridge equilibrated with water; after washing off the hydrophilic components with 5 ml of water, the lipophilic compounds were eluted with methanol. Previous studies on soy bean lecithin indicated that this procedure does not cause losses or degradation of peroxidation products.

Reversed-phase HPLC of SLPC was carried out on a 4.6 × 250 mm Ultrasphere ODS column (Beckman) eluted with a linear gradient of methanol–acetonitrile–water from 90.5:2.5:7 to 90.5:8.8:0.7; both solutions contained 20 m*M* choline. The gradient was generated over a 20-min period, after 5 min under isocratic conditions. The flow-rate was 1 ml/min. The detection was carried out simultaneously

at two wavelengths by a Beckman Model 165 variable wavelength UV detector. The samples obtained from several chromatographic runs were concentrated to a small volume under reduced pressure, and choline was eliminated using the cartridge procedure previously described.

The hydrolysis of β-position fatty acid derivatives of peroxidized SLPC, isolated by HPLC as described, was carried out by adding to the residue 5 ml diethyl ether, 0.1 mg phospholipase A_2 and 25 μmoles calcium chloride. The reaction mixture was shaken overnight and then centrifuged at 3000 g to eliminate the insoluble lysophosphatidylcholine. The pellet and the ether phase were then washed with ether and water, respectively, and the ether phases were pooled. Thin-layer chromatographic (TLC) analysis, accomplished according to ref. 11, demonstrated that the hydrolysis was complete and that the ether phase contained only fatty acid derivatives.

The polar bonded phase chromatography of linoleic acid derivatives was carried out on a Ultrasil-NH_2 column (4.6 × 250 mm) (Altex, Santa Clara, CA, U.S.A.). The column needed "activation", which was accomplished by washing with ten column volumes of water containing 0.1% orthophosphoric acid, followed by three column volumes of isopropanol containing 0.1% orthophosphoric acid and ten column volumes of solvent B (hexane-10% orthophosphoric acid in isopropanol–isopropanol–water, 85.1:1.2:13.4:0.3). The analysis was carried out using either a gradient from 5 to 20% of solvent B or isocratically at 6% of B in solvent A (hexane–10% orthophosphoric acid in acetonitrile, 99:1). After several runs the retention times progressively increased and the column needed reactivation. The presence of hydroperoxide groups in the isolated peaks was evaluated by the iron–thiocyanate method according to ref. 12.

RESULTS

When SLPC liposomes were peroxidized by the Fe^{3+}–ascorbate system (Fig. 1a, trace A–B), HPLC analysis of lipids contained in the reaction mixture showed a decrease of the major peak of native SLPC detected at 210 nm with a retention time of 47 min and the formation of a more polar product with a retention time of 26 min, absorbing at 233 nm, the absorption maximum of conjugated dienes (Fig. 1b and c). This product could be identified as a hydroperoxy derivative of SLPC by its chemical reactivity as a hydroperoxide and by analogy of the HPLC elution pattern with those of oxidized soy bean lecithin[9] and oxidized dilinoleoylphosphatidylcholine (DLPC) obtained in our laboratory[8].

When the peroxidation mixture contained also glutathione and PIP, the oxygen consumption decreased (Fig. 1a, trace A–C). However, the experiment was carried out until the oxygen consumption reached the level observed in the absence of PIP. At this point, the peroxidation products were extracted and the HPLC analysis exhibited a pattern (Fig. 1d) identical to that seen in Fig. 1c. Since PIP reduces the phospholipid hydroperoxides to alcohols[5–8], it is apparent that, as in the case of DLPC[8], the hydroperoxy and the hydroxy derivatives of SLPC are indistinguishable by the adopted procedure because they were eluted with identical relative retention times.

Hydrolysis of the β-position linoleic acid was carried out by phospholipase A_2 treatment of the major peak of SLPC peroxidized by the Fe^{3+}–ascorbate system in

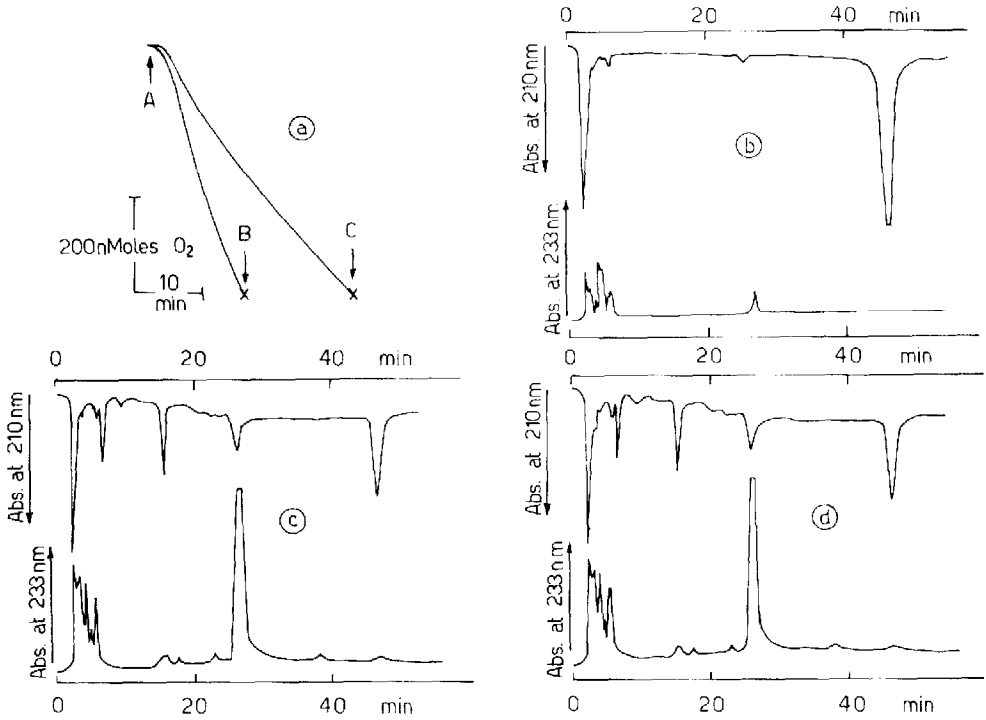

Fig. 1. Peroxidation of SLPC and reversed-phase HPLC analysis of SLPC and its peroxidation products. a, Oxygen uptake due to peroxidation of SLPC by the Fe^{3+}-ascorbate system (trace A–B); trace A C was obtained in the presence of Fe^{3+} ascorbate plus reduced glutathione and PIP. b, HPLC analysis of native SLPC. c, Analysis of lipid extracted from the peroxidation mixture at the time indicated by arrow B. d, Analysis of lipid extracted from the peroxidation mixture, containing also glutathione and PIP, at the time indicated by arrow C.

the absence or in the presence of PIP. When SLPC was peroxidized in the absence of PIP, HPLC analysis of the β-position fatty acid gave the pattern in Fig. 2a. There were essentially four peaks, A, B, C and D, of which B and D were dominant. When SLPC was peroxidized in the presence of glutathione and PIP (Fig. 2b), the HPLC analysis showed that the peaks A and C were dominant and there were only traces of B and D.

The identification of peaks A–D was done on the basis of the following evidence: (a) peaks B and D had the chemical reactivity of hydroperoxides; (b) peak B had the same relative retention time as the product of the soy bean lipoxidase reaction with linoleic acid, namely 13-hydroperoxylinoleic acid (Fig. 2c); (c) peak A had the same relative retention time as the product of the glutathione peroxidase reaction with 13-hydroperoxylinoleic acid, namely 13-hydroxylinoleic acid (Fig. 2d); (d) peaks B and D had the same relative retention times as the major peroxidation products of free linoleic acid auto-oxidized in water (Fig. 2e), identified as the 9- and 13-hydroperoxy derivatives, respectively, on the basis of analysis on silica gel[3] and of their chemical reactivity as hydroperoxides; (e) peaks A and C had the same relative retention times as the products of the reduction catalyzed by glutathione peroxidase of the linoleic acid hydroperoxides (Fig. 2f). So the peaks of Fig. 2 are identified as:

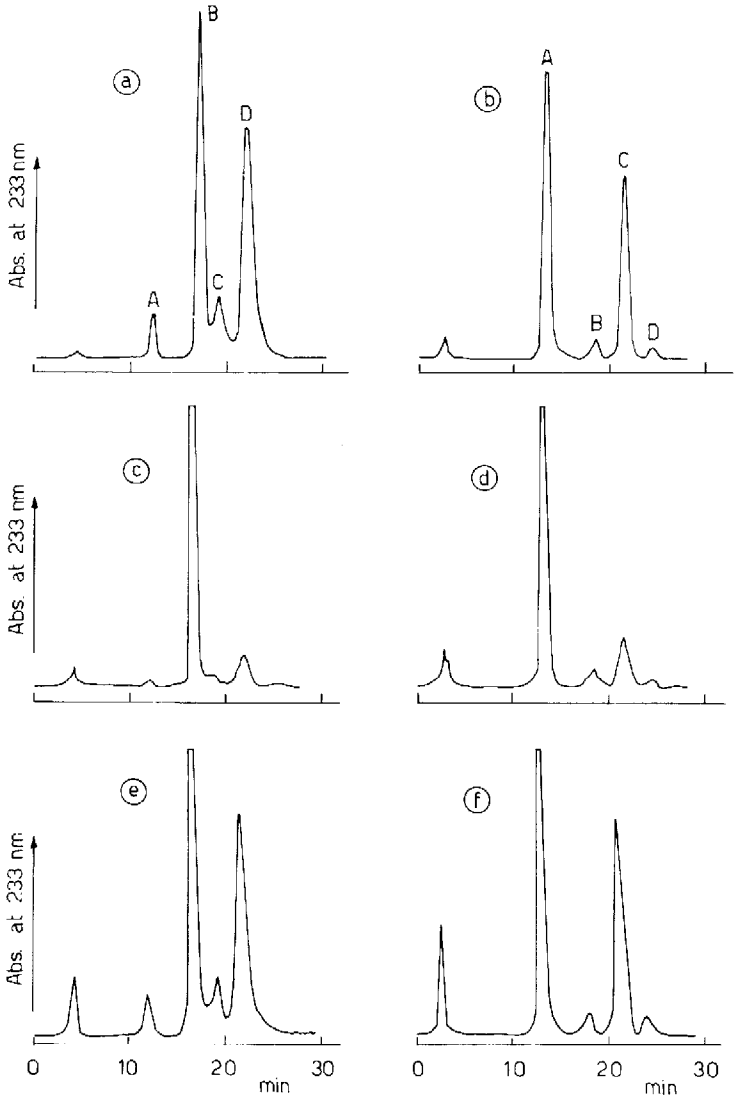

Fig. 2. Polar bonded phase HPLC of β-position acid derivatives of peroxidized SLPC and of linoleic acid. a, Analysis of fatty acid released by phospholipase A_2 treatment from SLPC peroxidized by Fe^{3+} ascorbate. b, Analysis of fatty acid released by phospholipase A_2 treatment from SLPC peroxidized as before but in the presence of the reducing system glutathione and PIP. c, Linoleic acid peroxidized by soy bean lipoxidase. d, Linoleic acid peroxidized by soy bean lipoxidase and reduced by glutathione and glutathione peroxidase. e, Linoleic acid auto-oxidized in water. f, Linoleic acid auto-oxidized in water and reduced by glutathione and glutathione peroxidase. See text for details.

A, 13-hydroxylinoleic acid; B, 13-hydroperoxylinoleic acid; C, 9-hydroxylinoleic acid; D, 9-hydroperoxylinoleic acid.

In a second set of peroxidation experiments, the SLPC was peroxidized in the presence or in the absence of PIP, and the lipid contents of the reaction mixtures were extracted after identical oxygen consumption, taken to drynness and resus-

pended in a small volume of acetic anhydride–pyridine (1:1). After incubation for 6 h, the samples were extracted and analyzed by the HPLC procedure described above for the untreated phospholipids. When SLPC was peroxidized in the presence of glutathione and PIP and thus the sample contained the hydroxy derivatives of SLPC, the acetic anhydride treatment increased the relative retention time of the major peak of the peroxidation products detected at 233 nm, as expected on the basis of the decrease in polarity, due to esterification of the hydroxy groups (Fig. 3a). At 277 nm, the absorption maximum of conjugated ketones[3,13], a small peak could be detected with a retention time close to that of underivatized hydroperoxides (Fig. 3a). However, when the acetic anhydride treatment was carried out on the sample containing hydroperoxides (*i.e.*, when SLPC was peroxidized in the absence of PIP), HPLC analysis monitored at 233 nm showed only a small peak at the relative retention time

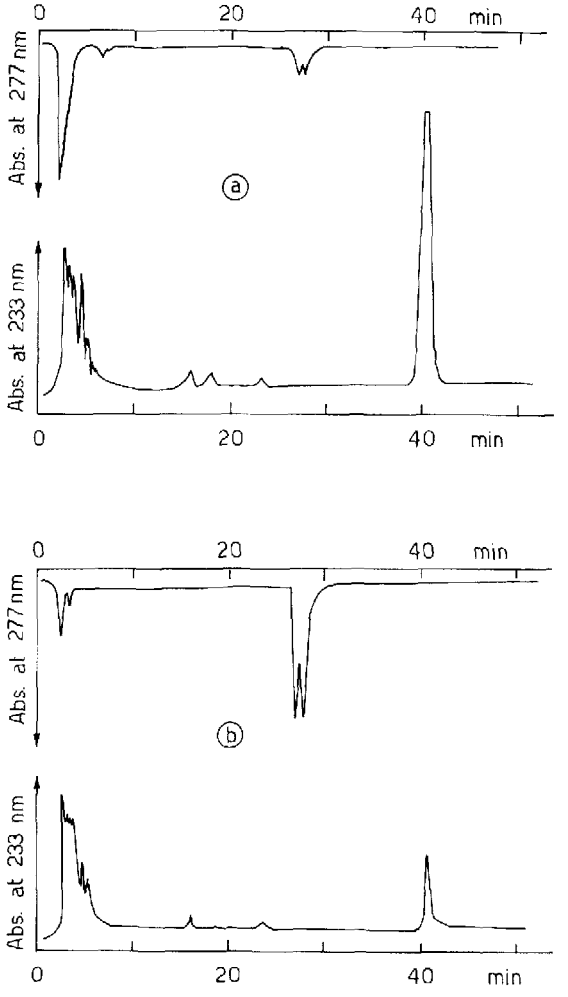

Fig. 3. Reversed-phase HPLC analysis of peroxidized SLPC treated with acetic anhydride. a, Elution profile of SLPC peroxidized by Fe^{3+}–ascorbate in the presence of the reducing system glutathione and PIP. b, SLPC peroxidized in the absence of the reducing system.

of the acetylated hydroxy derivatives, while a major double peak was observed at 277 nm with the retention time of the small peak described above (Fig. 3b). The double peak was attributable to the conjugated ketone derivatives of the SLPC generated by dehydration of the hydroperoxides. The double nature of the peak was probably due to limited separation of positional isomers.

DISCUSSION

The characterization of hydroperoxide and alcohol derivatives of a phospholipid was previously accomplished for DLPC by mass spectroscopy[8]. However, this analysis did not allow a conclusive distinction between positional isomers and the detection of small amounts of hydroxy derivatives when the sample contained mainly hydroperoxides. To fulfil these requirements we set up two HPLC analyses, the first for the β-position fatty acid derivatives of the peroxidized phospholipid and the second for the intact phospholipid, for which a derivatization procedure allowed the distinction between hydroperoxy and hydroxy forms of the whole phospholipid molecule.

Preliminary experiments had shown that the conditions of enzymatic hydrolysis necessary for analysis of the peroxidized β-position fatty acids did not convert hydroperoxides into alcohols, as in the case of chemical hydrolysis[10]. Chromatographic separation of peroxidized fatty acids was at first attempted using reversed-phase chromatography under the conditions described[14]. The peroxidized linoleic acid (detected at 233 nm) was well separated from the unperoxidized linoleic acid (detected at 210 nm), but, as in the case of the whole phospholipid[8], the relative retention time was identical for the hydroxy and the hydroperoxy derivatives (results not shown). For this reason we resorted to an HPLC analysis on a polar bonded phase column (Ultrasil-NH_2) used in a normal phase mode. This analysis was suggested by the chromatographic approach described[15] for the separation of positional isomers of hydroperoxylinoleic acid on a silica column. The resolution was improved by generating gradients and by using water and phosphoric acid. Phosphoric acid was employed to suppres the dissociation of the carboxylic groups of fatty acids and thereby to avoid the esterification procedure. By this method, the β-position fatty acid derivatives of peroxidized and reduced SLPC could clearly be distinguished and identified (Fig. 2).

It is worth noting that in all these experiments SLPC was peroxidized at a low level of conversion onto peroxidation products (less that 30%) and that under these conditions essentially hydroperoxides were produced[9,10]. Many additional peaks could be detected both under these chromatographic conditions and under the conditions described by Capdevila *et al.*[14] when phosphatidylcholine is peroxidized by more than 30%, and when care is not taken to avoid drying and exposure of the samples to light during manipulations.

The acetic anhydride treatment was chosen to modify through esterification the polarity of the hydroxy derivatives of SLPC and to separate them from the hydroperoxy derivatives which under the same conditions are dehydrated to conjugated ketones[3,13]. The procedure was suggested by that used by Christophersen[3] to identify the hydroxy- and the hydroperoxylinoleic acid by TLC.

From the above experiments the following conclusions can be drawn:

(1) The β-position fatty acid derivatives from a peroxidized phospholipid can be analyzed without derivatization, and the positional isomers of the hydroperoxy and hydroxy derivatives can be resolved by HPLC on a polar bonded phase column.

(2) The major products of peroxidation of SLPC at a low level of conversion comprise a mixture in approximately equal amounts of 9- and 13-hydroperoxy derivatives of linoleic acid and small amounts of hydroxy derivatives.

(3) The PIP-catalyzed reduction of SPLC hydroperoxides gives rise to two isomeric hydroxy derivatives of β-position linoleic acid, which can be released through phospholipase A_2 treatment and appear to be identical to those produced by glutathione peroxidase on hydroperoxides of free linoleic acid. So PIP acts on both positional isomers of hydroperoxylinoleic acid present in SLPC.

(4) The relative content of hydroperoxy and hydroxy derivatives of a peroxidized phospholipid can easily be measured by derivatizing the sample with acetic anhydride before HPLC analysis on a reversed-phase column.

A precise quantitation of hydroxy and hydroperoxy derivatives of a phospholipid can be achieved by these HPLC methods on the basis of a calibration curve obtained from known amounts of hydroperoxy derivatives, determined colorimetrically, and of hydroxy derivatives, formed in controlled amounts from hydroperoxides by PIP and glutathione. The evaluation of the ratio of hydroperoxy to hydroxy derivatives from a phospholipid appears to be of interest in the study of lipid peroxidation and can be accomplished by the two approaches described for a peroxidized phospholipid previously isolated by HPLC.

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