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Separation of Representative Lipid Compounds of Biological Membranes and Lipid Derivatives from Peroxidized Polyunsaturated Fatty Acids by Reversed Phase High-Performance Liquid Chromatography

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A complex mixture of different lipid compounds, including phosphatidylcholine, phosphatidylserine, all-trans-retinol, 15(S)-hydroperoxyeicosatetraenoic acid, D-α-tocopherol, saturated and unsaturated fatty acids can be separated by reversed phase HPLC by using a C-18, 120 mm × 4 mm, 3 μm particle size column and a step gradient from acetonitrile/water (1:1; v:v) to 100% acetonitrile at a flow rate of 0.8 ml/min. By applying this elution condition, separation of various groups of lipid hydroperoxides and lipid derivatives, each one originating from a different in vitro peroxidized polyunsaturated fatty acid, can be obtained. Simultaneous detection is carried out by a diode array detector at a wavelength accumulation range set up between 195 and 400 nm. The possibility of simultaneously having such a large number of measurements renders this chromatographic method particularly suitable in studies concerning lipid peroxidation where, in addition to the detection of free radical-induced lipid hydroperoxides, data on some key antioxidant molecules, i.e. vitamin A and E, as well as that of structural compounds of biological membranes, i.e. phosphatidylcholine and phosphatidylserine, can be achieved.

Keywords: HPLC, phospholipids, fatty acids, vitamins, lipid hydroperoxides, lipid peroxidation

INTRODUCTION

Free radical-induced lipid peroxidation is a phenomenon occurring in several pathological conditions, such as ischemia and reperfusion of tissues, chronic inflammations, toxicity of certain classes of drugs, etc.[1-3] When lipid peroxidation is triggered on the unsaturated fatty acid moiety of membrane phospholipids it originates several unstable lipid radicals responsible for the chain propagation to other neighbouring fatty acids.[4] This chain reaction can be terminated by different possible mechanisms, including the interaction of lipid radicals with lipid soluble scavenging molecules, such as vitamin E or vitamin A.[5]

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Activation of phospholipase A_2 produces the liberation of several peroxidized lipid derivatives from modified phospholipids such as lipid hydroperoxides, cycloperoxides, endoperoxides together with non-lipid compounds including various monofunctional (hydroxynonenal, hydroxypentenal) and bifunctional (malondialdehyde) aldehydes.[6-7] Therefore, a substantial modification of most of the lipid components of biological membranes can be observed when lipid peroxidation takes place. To better evaluate the changes induced by lipid peroxidation to various lipid classes of biological membranes, it seems reasonable to monitor simultaneously the most representative lipid components. Hence, the present study describes a HPLC method for the contemporaneous separation of phosphatidylcholine, phosphatidylserine, vitamin E, vitamin A, saturated and unsaturated fatty acids by using a C-18 reversed phase column. In particular, with the aid of a diode array detector, the method also allows separation of polyunsaturated fatty acids and their respective hydroperoxides, thus rendering its application favourable in studies on lipid peroxidation.

MATERIALS AND METHODS

Phosphatidylcholine (PC), phosphatidylserine (PS), all-trans-retinol, D-α-tocopherol, 15(S)-hydroperoxyeicosatetraenoic acid (15(S)-HPETE), arachidonic acid, γ-linolenic acid, linoleic acid, oleic acid, palmitic acid and stearic acid were purchased from Sigma (St. Louis, MO, USA). Ultrapure acetonitrile for HPLC was obtained from Baker (Phillipsburg, NJ, USA). All other chemicals were of the highest purity available from commercial sources. Phospholipids, vitamins, 15(S)-HPETE and fatty acid standards, were dissolved in methanol and mixed at concentrations ranging from 6 to 400 μM. For peroxidizing polyunsaturated fatty acids, 3 mM final suspension in water of arachidonic acid, γ-linolenic acid and linoleic acid was obtained by submitting them, separately or mixed together, to 60 sec sonication in a water bath sonicator. Peroxidation was started by the addition, to each fatty acid suspension, of 1 mM ascorbic acid plus 20 μM $FeCl_3$; after 1 hr at 37 °C, the reaction was terminated by supplementing the solutions with 100 μM deferoxamine mesylate. Separation of standards and of peroxidized unsaturated fatty acids was achieved by injecting 200 μl of each sample onto a C-18, 120 mm × 4 mm, 3 μm particle size column (Macherey-Nagel, Duren, Germany) provided with its own guard column. The HPLC apparatus consisted of a Jasco PU-980 dual pump system connencted to a Jasco MD-910 diode array detector (Tokyo, Japan). Acquisition and chromatogram analysis was performed by a PC-486. The following gradient from solvent A (50% CH_3CN in water) to solvent B (100% CH_3CN) was used: 0–20 min at up to 40% solvent A; 20–30 min at up to 0% solvent A. The flow rate was 0.8 ml/min. The diode array detector was set up for a wavelength accumulation from 195 to 400 nm. Calculation of standard areas was performed at 207 nm for phospholipids, vitamin E, saturated and unsaturated fatty acids. The vitamin A area was calculated at 327 nm, while areas of 15(S)-HPETE and of other lipid hydroperoxides, originating from peroxidized polyunsaturated fatty acids, were calculated at 239 nm.

RESULTS

Figure 1 reports a chromatogram at two different wavelengths (207 and 239 nm) showing the simultaneous separation of PS, PC, 15(S)-HPETE, γ-linolenic acid, arachidonic acid, linoleic acid, oleic acid, palmitic acid, D-α-tocopherol and stearic acid. The use of appropriate eluents and gradient, as well as that of a diode array detector, allows to discriminate simultaneously several naturally occurring hydrophobic substances that are fundamental components of biological membranes. In addition, compounds like 15(S)-HPETE, which are

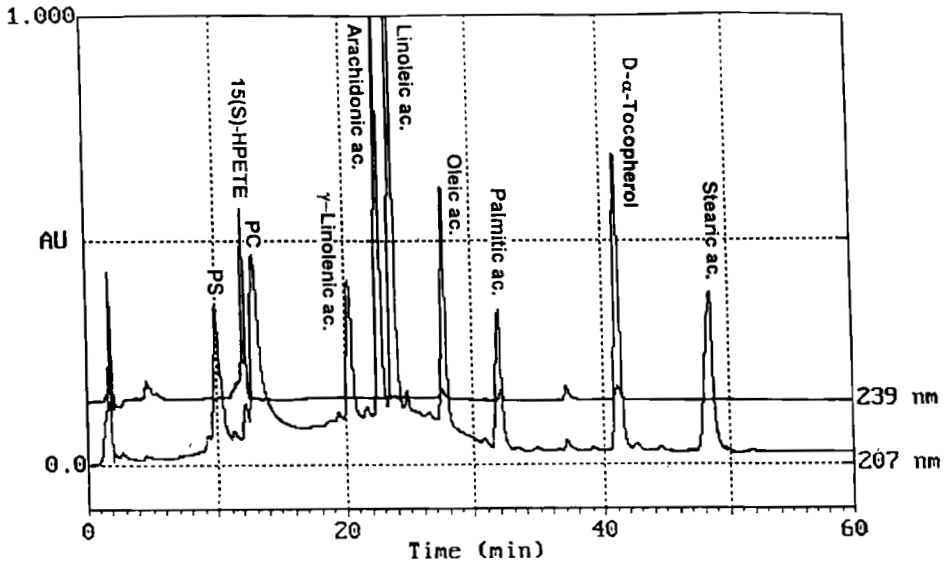

FIGURE 1 Separation by reversed phase HPLC of a standard mixture of several lipid-soluble compounds including 15(S)-HPETE. Elution profile of the same chromatographic run is reported at 207 and 239 nm. This latter wavelength corresponds to the maximum of absorption of 15(S)-HPETE. Chromatographic conditions are described under Materials and Methods.

related to lipid peroxidation, are also detected. It is worth noting that PC and PS do not present a significant absorption at 239 nm so that, although 15(S)-HPETE elutes after 12.12 min, as a shoulder of PC when observed at 207 nm, 15(S)-HPETE itself can be easily detected and calculated at 239 nm.

Concerning vitamin A, since it exactly coelutes with linoleic acid, a separate run (reported in Fig. 2) was performed. As linoleic acid does not show any significant absorption at 327 nm, concentration of vitamin A in a complex mixture can be calculated at this wavelength. On the other hand,

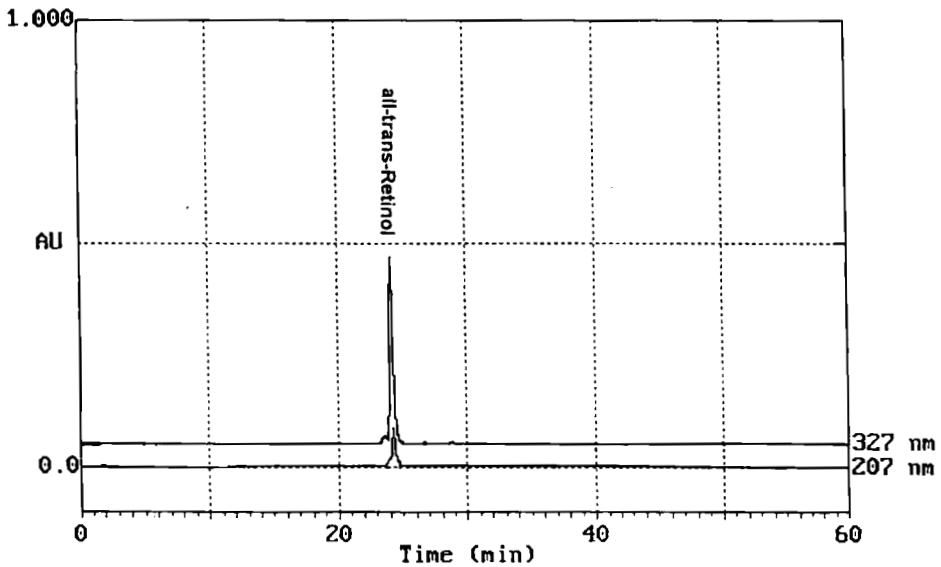

FIGURE 2 Identification at two different wavelengths of all-trans-retinol using the same chromatographic conditions adopted for separating the standard mixture reported in Figure 1. Calculation of vitamin A concentration was performed at 327 nm, i.e. at its maximum of absorption.

since vitamin A absorbs also at 207 nm, its contribution to the area of linoleic acid peak in a complex mixture can be subtracted by means of two calibration curves of all-trans-retinol performed at 207 and 327 nm. This also allows to calculate the concentration of linoleic acid. The detection limits of the compounds reported in Figures 1 and 2 (expressed as pmol/200 µl injected) are as follows: PS 100, 15(S)-HPETE 20, PC 80, γ-linolenic acid 50, arachidonic acid 20, linoleic acid 20, all-trans-retinol 30, oleic acid 400, palmitic acid 500, D-α-tocopherol 9 and stearic acid 250. Chromatograms reported in Figures 3, 4 and 5 show the simultaneous separation of products originating from the peroxidation of linoleic acid, γ-linolenic acid and arachidonic acid, respectively. Panel A of each Figure shows the recording at 207 nm of the fatty acids peroxidized in vitro, for 1 hr at 37 °C, by 1 mM ascorbate + 20 µM $FeCl_3$. Elution profiles of the same chromatographic runs at 239 and 280 nm, respectively, are shown in Panels B and C of Figures 3, 4 and 5. As evidenced by the absorption spectra between 195 and 300 nm in Panel B of each Figure, several lipid hydroperoxides, with a maximum of absorption between 235 and 239 nm, can be separated from the peroxidation of each fatty acid, under the present chromatographic conditions. In particular, linoleic acid shows a main lipid hydroperoxide with a retention time of 12.82 min while peroxidation of γ-linolenic acid produces three major lipid hydroperoxides with retention times of 6.56, 10.04 and 10.91 min, respectively. The challenge of prooxidant with arachidonic acid generates different lipid hydroperoxides, one of which corresponds to 15(S)-HPETE on the basis of comparison both of retention time and absorption spectrum of 15(S)-HPETE ultrapure standard. Analysis of the same runs at 280 nm, as reported in Panel C of Figures 3, 4 and 5, revealed that each peroxidized fatty acid generates a number of lipid derivates characterized by absorption spectra with a maximum at 280 nm. It is worth noting that, as for the various lipid hydroperoxides, these lipid derivatives have different positions in the chromatograms, depending on the nature of the polyunsaturated fatty acid considered. In order to verify if a more complex mixture formed by compounds originating from the three fatty acids peroxidized simultaneously, could be chromatographically resolved by the present reversed phase HPLC method, equal concentrations of linoleic, γ-linolenic and arachidonic acid were challenged together in the presence of a ferric iron-ascorbic acid prooxidant mixture. Panels A, B and C of Figure 6 report the same chromatographic separation of mixed peroxidized fatty acids recorded at 207, 239 and 280 nm, respectively. The main lipid hydroperoxides (Fig. 6, Panel B) deriving from each fatty acid, including 15(S)-HPETE, can be resolved by this HPLC method. Similarly, also different lipid derivatives absorbing at 280 nm can be determined in a complex mixture of simultaneously peroxidized polyunsaturated free fatty acids (Fig. 6, Panel C). By using the calibration curve determined for 15(S)-HPETE, concentrations of total lipid hydroperoxides, produced both by separated and mixed fatty acids, were calculated by cumulating areas of the peaks showing absorption spectra similar to that of 15(S)-HPETE.[6] The results reported in the Table I show that, as could be expected from the number of double bonds of each fatty acid molecule, linoleic and arachidonic acid produced the lowest and highest concentration of lipid hydroperoxides, respectively. It is worth underlining that the amount of lipid hydroperoxides, produced by the three peroxidized polyunsaturated fatty acid mixture, corresponds to the sum of lipid hydroperoxides determined for each fatty acid peroxydized separately.

DISCUSSION

Lipid peroxidation is a complex phenomenon in which several important components of biological membranes, such as phospholipids and vitamin E, are involved. Although some aspects of the process driven by oxygen-derived free radicals, are still poorly understood, i.e. the nature of the radical

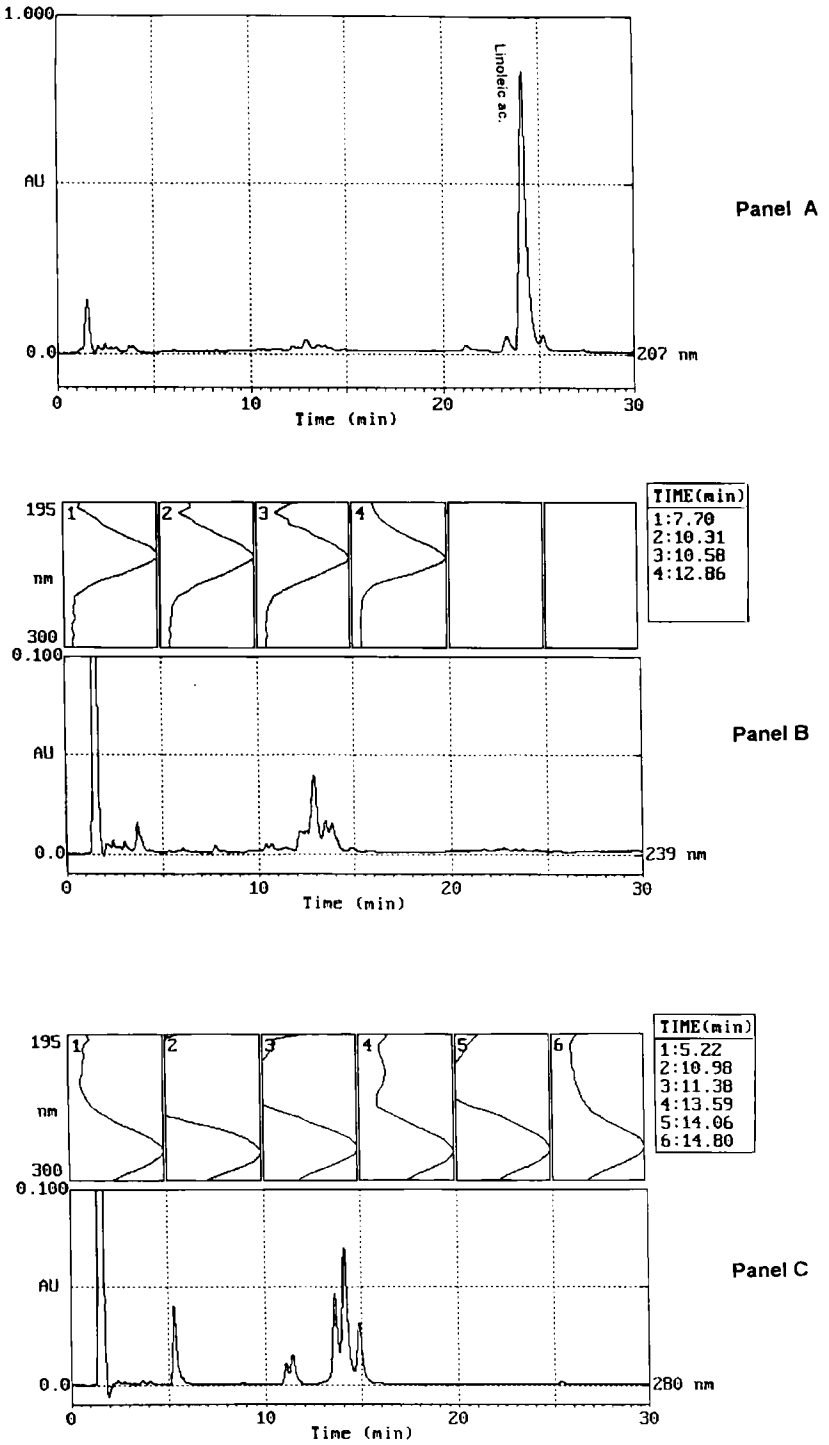

FIGURE 3 Separation of lipid derivatives originated by peroxidizing 3 mM linoleic acid in presence of ferric iron-ascorbic acid. The elution profile is reported at: 207 nm (Panel A), 239 nm (Panel B), and 280 nm (Panel C). A main lipid hydroperoxide is identified as the peak at 12.86 min (Panel B). The chromatogram obtained at 280 nm shows the presence of several characteristic peaks of lipid derivatives which are undetectable at other wavelengths.

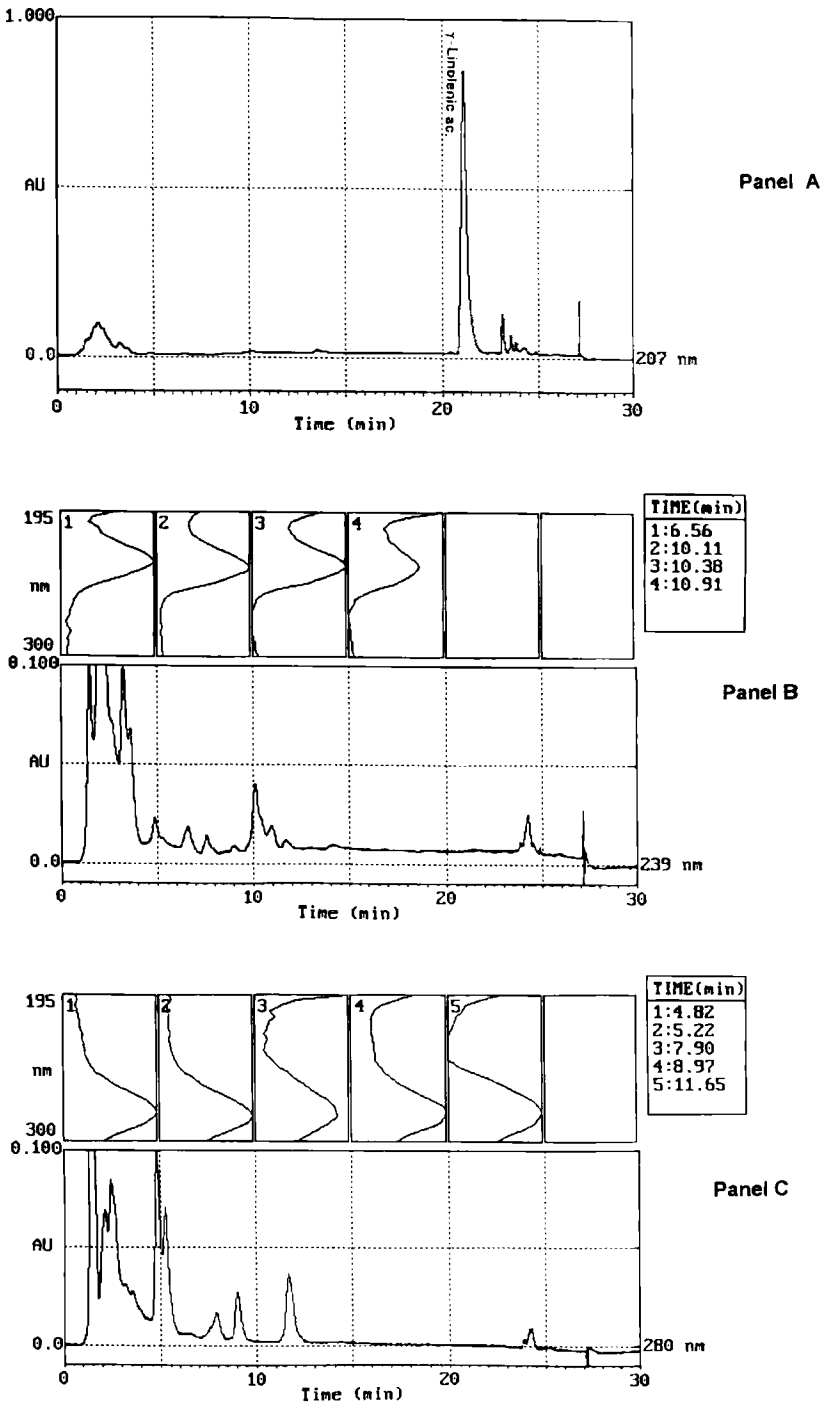

FIGURE 4 Separation of lipid derivatives originated by peroxidizing 3 mM γ-linolenic acid in presence of ferric iron-ascorbic acid. The elution profile is reported at: 207 nm (Panel A), 239 nm (Panel B), and 280 nm (Panel C). Four main lipid hydroperoxides have been identified as the peaks at 6.56, 10.11, 10.38 and 10.91 min, respectively (Panel B). The chromatogram obtained at 280 nm shows the presence of several characteristic peaks of lipid derivatives, different from those of linoleic acid (Figure 3), which are undetectable at other wavelengths.

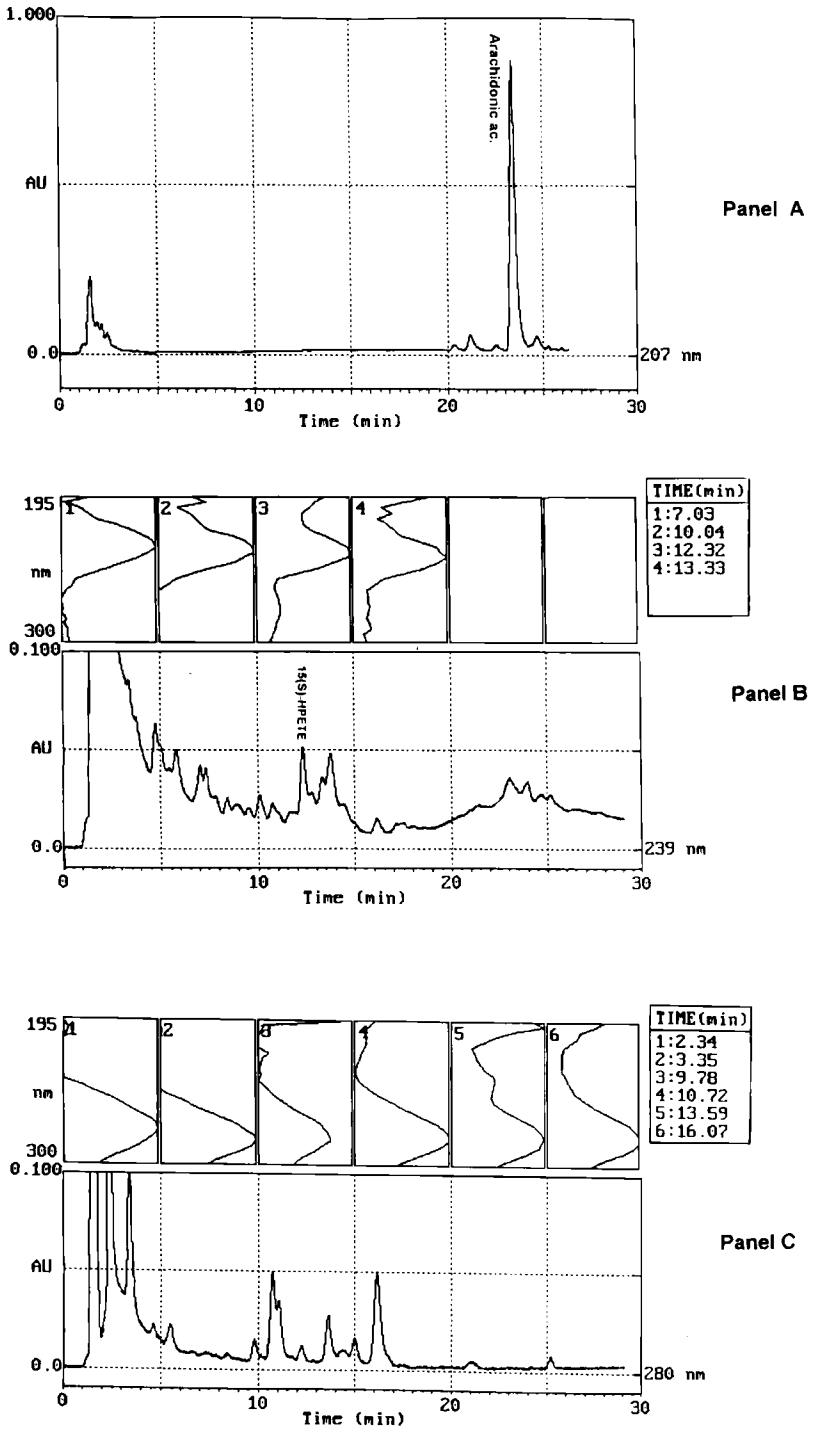

FIGURE 5 Separation of lipid derivatives originated by peroxidizing 3 mM arachidonic acid in presence of ferric iron-ascorbic acid. The elution profile is reported at: 207 nm (Panel A), 239 nm (Panel B), and 280 nm (Panel C). Four main lipid hydroperoxides are identified as the peaks at 7.03, 10.04, 12.32 and 13.33 min, respectively (Panel B). On the basis of the comparison of both retention time and absorption spectrum, the peak at 12.32 min has been identified as 15(S)-HPETE. The chromatogram obtained at 280 nm shows the presence of several characteristic peaks of lipid derivatives, different from those of linoleic and γ-linolenic acid (Figure 3 and 4), which are undetectable at other wavelengths.

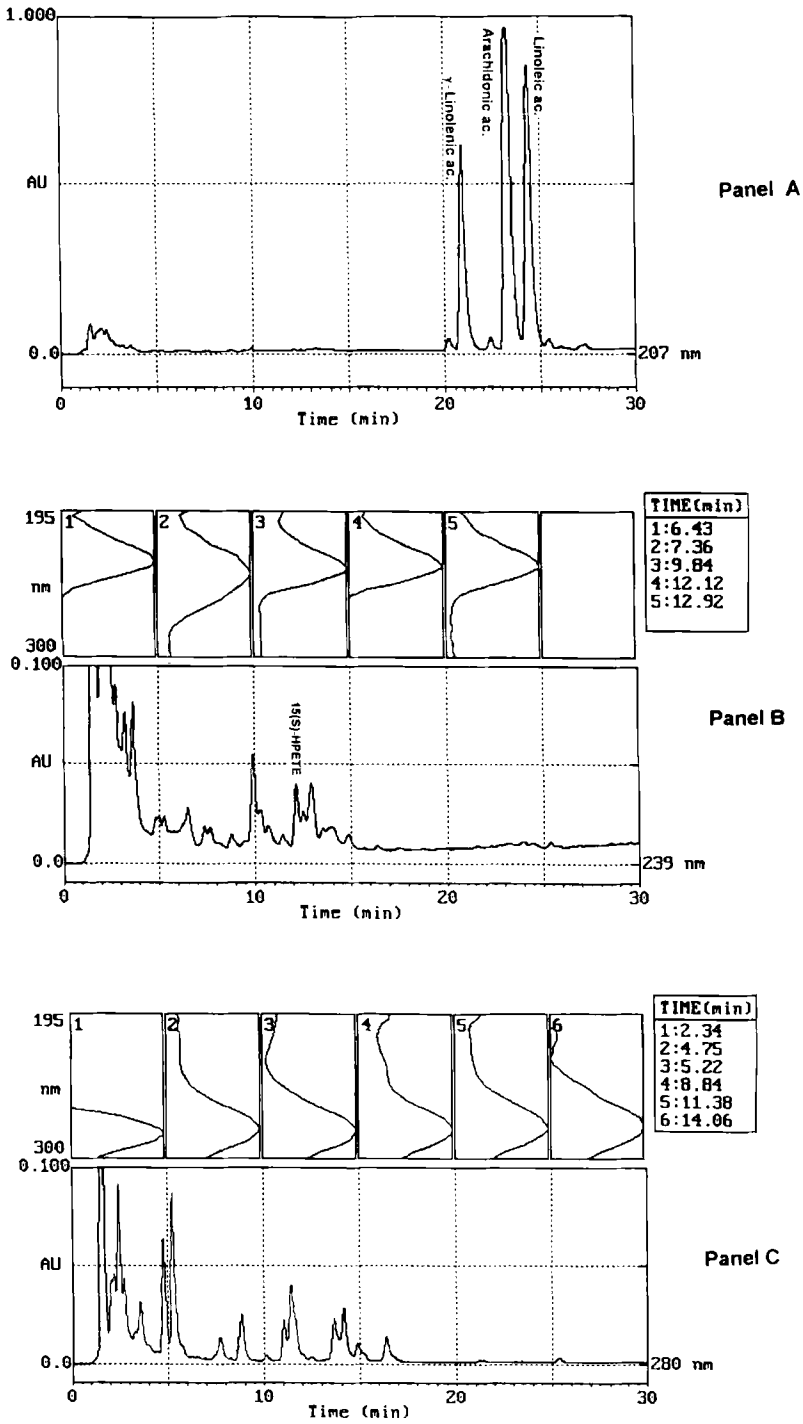

FIGURE 6 Separation of lipid derivatives originated by peroxidizing linoleic, γ-linolenic and arachidonic acid (3 mM each) in presence of ferric iron-ascorbic acid. The elution profile is reported at: 207 nm (Panel A), 239 nm (Panel B), and 280 nm (Panel C). The main lipid hydroperoxides deriving from linoleic, γ-linolenic or arachidonic acid are identified as the peaks at 9.84, 12.12 and 12.92 min, respectively (Panel B). The peak at 12.12 min has been identified as 15(S)-HPETE. The chromatogram obtained at 280 nm shows the presence of several characteristic peaks of lipid derivatives which are undetectable at other wavelengths.

TABLE I Concentration of lipid hydroperoxides in peroxidized polyunsaturated fatty acids

CONCENTRATION OF LIPID HYDROPEROXIDES (μM)	
Linoleic acid	1.83
	(0.12)
γ-Linolenic acid	2.72
	(0.18)
Arachidonic acid	4.19
	(0.26)
Polyunsaturated fatty acid mixture	8.04
	(0.46)

Separated and mixed polyunsaturated fatty acids were peroxidized by 1 mM ascorbic acid + 20 μM $FeCl_3$ as described in Materials and Methods. Calculation of lipid hydroperoxides was performed by cumulating areas of peaks showing absorption spectra similar to 15(S)-HPETE (Panel C of Figs. 3, 4, 5 and 6). Each value is the mean (S.D.) of four different experiments.

species responsible for the initiation of the phenomenon,[8,9] its occurrence in several pathological conditions have determined the appearance of a large number of studies. Particular attention has been given to oxygen radical-mediated lipid peroxidation due to its implication in ischemia-reperfusion phenomena.[10-12] Several studies have demonstrated that unsaturated fatty acids of membrane phospholipids undergo a free radical attack to their double bonds causing a hydrogen atom abstraction that transforms fatty acids into unstable lipid radicals.[13,14] This is thought to be the starting point for the propagation of the so called "lipid peroxidation reaction chain". Molecular rearrangement of the double bonds of fatty acids, with formation of conjugated dienes, and reaction with molecular oxygen to form lipid hydroperoxides, produces a class of modified phospholipids which have characteristic absorption spectra ranging from 233 to 239 nm. Since these altered molecules induce significant perturbation of biological membrane structure, they are degraded by specific enzymes, such as phospholipase A_2,[6] thus generating free lipid hydroperoxides. Termination of lipid peroxidation may be provoked by the interaction of lipid radicals with some lipid soluble electron donor within the membrane structure such as D-α-tocopherol, which is itself transformed into the relatively unreactive D-α-tocopheroxyl radical.[15] Activation of phospholipase A_2, change of membrane phospholipid composition, increase of lipid hydroperoxides, increase of peroxidized phospholipids and fatty acid degradation products on one hand, and decrease of vitamin E concentration on the other, have been reported to occur during both in vivo and in vitro as a consequence of oxygen radical-induced lipid peroxidation.[16-21] Consequently, it seems important to have the possibility of using an analytical method that allows us to monitor simultaneously the aforementioned lipid compounds. Unfortunately, data reported in literature do not offer, to the best of our knowledge, such a possibility: in fact, HPLC methods for phospholipids and their corresponding hydroperoxides,[22-23] or for unsaturated fatty acids and their hydroperoxides,[24] or for lipid soluble vitamins,[25-26] are available. The results presented in this study show that, by using a conventional reversed phase C-18 column and a simple binary gradient, it is possible to separate at the same time phospholipids, vitamin A and E, saturated and unsaturated fatty acids. In addition, the utilization of a diode array detector allows the simultaneous determination of lipid hydroperoxides, as well as that of other lipid derivatives absorbing at 280 nm, different from lipid hydroperoxides themselves. It seems particularly interesting to obtain data on both products of lipid peroxidation and on molecules capable of scavenging oxygen-derived free radicals and of terminating lipid peroxidation reaction chain, i.e. vitamins A and E. Under the present chromatographic conditions, vitamin E is easily separated in the standard mixture reported in Figure 1, showing a retention time of 41.5 min. Differently, although vitamin A coelutes with linoleic acid, it was nevertheless determined by using a diode array detector and quantified by calculating the respective contribution of all-trans-retinol and linoleic acid at 207 and 327 nm, this last wavelength being the maximum of absorption of vitamin A. Concerning the products originating from peroxidation of polyunsaturated fatty acids with ferric iron-ascorbic acid, besides separating the residual unreacted fatty acids (Panel A of Fig. 3, 4, 5 and 6), the chromatographic conditions described in the present study, allow us to separate different

lipid hydroperoxides characteristic of each different peroxidized lipid. In particular, 15(S)-HPETE was identified either in samples from peroxidized arachidonic acid only or in samples from a more complex mixture composed of peroxidized linoleic, γ-linolenic and arachidonic acid. Referring to other products of lipid peroxidation, it is worth underlining that several compounds with a maximum of absorption at 280 nm wavelength could be detected from any polyunsaturated fatty acid considered. Some of these compounds have been reported to originate from the peroxidation of ω-3 fatty acids and were indicated as different long-chain aldehydes.[27] Our results suggest that several compounds with such spectroscopic properties are also generated from the peroxidation of linoleic and γ-linolenic acid, thus suggesting that their separation and identification might be used as a biochemical index of lipid peroxidation.

In conclusion, the high resolution and the possibility of determining such a large number of products deriving from peroxidized polyunsaturated fatty acids, render this reversed phase HPLC method particularly suitable in studies of lipid peroxidation for monitoring the variations of the main lipid compounds of biological membranes, all of them involved in the processes of tissue damage in several pathological conditions.

Acknowledgments

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