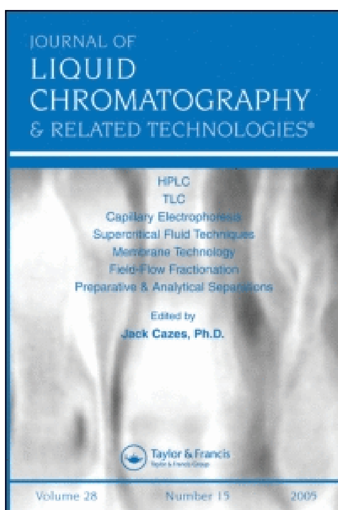
This article was downloaded by:
On: *24 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

High Performance Liquid Chromatographic Separation of Hydroperoxy-Phospholipids and Their Corresponding Hydroxy-Phospholipid Derivatives

Yongping Bao[a]; Rachel Hurst[a]; Gary Williamson[a]
[a] Department of Biochemistry, Institute of Food Research, Norwich, UK

To cite this Article Bao, Yongping , Hurst, Rachel and Williamson, Gary(1998) 'High Performance Liquid Chromatographic Separation of Hydroperoxy-Phospholipids and Their Corresponding Hydroxy-Phospholipid Derivatives', Journal of Liquid Chromatography & Related Technologies, 21: 13, 2061 — 2068

To link to this Article: DOI: 10.1080/10826079808006605

URL: http://dx.doi.org/10.1080/10826079808006605

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

J. LIQ. CHROM. & REL. TECHNOL., 21(13), 2061–2068 (1998)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF HYDROPEROXY-PHOSPHOLIPIDS AND THEIR CORRESPONDING HYDROXY-PHOSPHOLIPID DERIVATIVES

Yongping Bao, Rachel Hurst, Gary Williamson

Department of Biochemistry
Institute of Food Research,
Norwich Research Park
Colney, Norwich NR4 7UA, UK

Downloaded At: 10:57 24 January 2011

ABSTRACT

This paper describes high performance liquid chromatography (HPLC) conditions suitable for separating hydroperoxy-phospholipids from hydroxy-phospholipids. 1-stearoyl-2-(13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoyl)-L-3-phosphatidylcholine, 1, 2 di (13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoyl)-L-3-phosphatidylcholine, 1-palmitoyl-2-(13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoyl)-L-3-phosphatidylethanolamine and their corresponding hydroxy-phospholipid derivatives were resolved by HPLC on an Ultracarb 5 ODS (20) column at 30°C. Each pair of these phospholipids required different conditions for optimal separation but all used a mobile phase of acetonitrile-methanol-water mixture containing 10 mM choline chloride. The separation described allows accurate measurement of phospholipid hydroperoxide peroxidase activities.

Copyright © 1998 by Marcel Dekker, Inc.

INTRODUCTION

Lipid peroxidation in biological membranes has been implicated in the pathogenesis of various degenerative diseases.[1] The primary products of lipid peroxidation are lipid hydroperoxides, which in the presence of transition metals are degraded into secondary oxidation products, such as hydrocarbons and carbonyl compounds, some of which are cytotoxic.[2] Glutathione-dependent factors including glutathione transferases and selenium-dependent phospholipid hydroperoxide glutathione peroxidase (PHGPx) prevent lipid peroxidation.[3-6] The reduction of hydroperoxy-phospholipids to their corresponding hydroxy-phospholipids plays an important role both in the defense reactions against lipid peroxidation and in the repair of peroxidized membranes, and it is the major protective pathway in mammalian cells.[7,8]

Downloaded At: 10:57 24 January 2011

The separation of hydroperoxy- and hydroxy derivatives of membrane phospholipids is required not only for studies on the damaging effects of lipid peroxidation products, but also for studies on the cellular defense mechanism against lipid peroxidation. However, hydroperoxy- and hydroxy-phospholipids are not easily resolved by HPLC as reported in many publications.[7,9-11] Previously we succeeded in separating 1-palmitoyl-2-(13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoyl)-L-3-phosphatidylcholine (PLPC-OOH) and 1-palmitoyl-2-(13-hydroxy-*cis*-9, *trans*-11-octadecadienoyl)-L-3-phosphatidylcholine (PLPC-OH) on an Ultracarb ODS column.[11] In this paper, we show that three other major hydroperoxy-phospholipids, 1-stearoyl-2-(13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoyl)-L-3-phosphatidylcholine (SLPC-OOH), 1, 2 di (13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoyl)-L-3-phosphatidylcholine (DLPC-$(OOH)_2$), 1-palmitoyl-2-(13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoyl)-L-3-phosphatidylethanolamine (PLPE-OOH), and their corresponding hydroxy-phospholipid derivatives, 1-stearoyl-2-(13-hydroxy-*cis*-9, *trans*-11-octadecadienoyl)-L-3-phosphatidylcholine (SLPC-OH), 1, 2 di (13-hydroxy-*cis*-9, *trans*-11-octadecadienoyl)-L-3-phosphatidylcholine (DLPC-$(OH)_2$), 1-palmitoyl-2-(13-hydroxy-*cis*-9, *trans*-11-octadecadienoyl)-L-3-phosphatidylethanolamine (PLPE-OH), were all baseline separated using modified conditions of the separation of PLPC-OOH and PLPC-OH.[12]

EXPERIMENTAL

Materials

1-Stearoyl-2-linoleoyl-L-3-phosphatidylcholine (SLPC), 1, 2-dilinoleoyl-L-3-phosphatidylcholine (DLPC), 1-palmitoyl-2-linoleoyl-L-3-phosphatidyl-

ethanolamine (PLPE), soybean lipoxidase (EC 1.13.11.12, type IV), choline chloride, reduced glutathione (GSH), sodium borohydride ($NaBH_4$) and Triton X-100 (peroxide and carbonyl free) were purchased from Sigma. Methanol and acetonitrile, used for HPLC, were filtered and degassed. HPLC quality water was obtained by purification of distilled water through a Milli Q water purifier (Millipore Corp., Bedford, MA, USA). Ultracarb 5 ODS (20) column (250 × 4.6 mm) was from Phenomenex, UK.

Preparation and Purification of Hydroperoxy-Phospholipids

SLPC-OOH, DLPC-$(OOH)_2$, and PLPE-OOH were prepared from SLPC, DLPC and PLPE respectively using soybean lipoxidase as described by Maiorino et al.[13] Hydroperoxy-phospholipid was separated from unoxidized phospholipid by fast protein liquid chromatography (FPLC) on a PepRPC H5/5 column with a mobile phase of methanol/water gradient. Hydroperoxy-phospholipid was eluted with 100% methanol and concentration of a hydroperoxide solution was determined by absorbance at 232 nm (ε = 25,000 $M^{-1}cm^{-1}$).[14]

The molecular ions of DLPC-$(OOH)_2$, SLPC-OOH, and PLPE-OOH were confirmed by fast atom bombardment mass spectrometry (Fab-MS) or electrospray mass spectrometry (ES-MS). The protonated molecular ions of DLPC-$(OOH)_2$, SLPC-OOH, and PLPE-OOH occurred at *m/z* 846, 818, and 748. Human liver PHGPx was purified as described previously.[12]

Downloaded At: 10:57 24 January 2011

Conversion of Hydroperoxy- to Hydroxy-phospholipids

SLPC-OOH was converted to SLPC-OH using PHGPx as follows: A mixture, containing 0.1 M Tris-HCl, pH 7.4, 2 mM EDTA, 1 mM NaN_3, 3 mM GSH, 0.12% Triton X-100, and 10 μL (0.03 μg protein) PHGPx preparation in a final volume of 500 μL, was incubated at 37°C for about 3 min and the reaction was started by addition of SLPC-OOH to 25 μM.

The reaction was stopped by adding 500 μL ice-cold acetonitrile, then centrifuged at 11,600g for 2 min to remove any particulate matter prior to HPLC analysis. One unit of PHGPx activity is defined as the amount of enzyme of producing 1 μmol hydroxy-phospholipid per minute at 37°C. The reductions of PLPE-OOH to PLPE-OH and DLPC-$(OOH)_2$ to DLPC-$(OH)_2$ were catalyzed non-enzymatically by $NaBH_4$ in methanol at 4°C.[10,15]

Apparatus and Chromatographic Conditions for HPLC

The liquid chromatographic system consisted of one model 306 pump (Gilson), a 231 autosampler with 410 dilutor, and 621 data master. The column oven temperature was set at 30°C. The system was controlled by a Gilson 715 HPLC controller.

Separation of SLPC-OOH and SLPC-OH was achieved with the same conditions as for separation of PLPC-OOH and PLPC-OH in a previous publication.[12] The column was an Ultracarb 5 ODS (20) column (250 × 4.6 mm) and the mobile phase was a mixture of acetonitrile-methanol-water at a ratio of 50:49.5:0.5, v/v/v containing 10 mM choline chloride. The retention times were 24.0 and 26.2 min for SLPC-OOH and SLPC-OH respectively, compared to 17.5 and 19.2 min for PLPC-OOH and PLPC-OH as described previously.[12] For the separation of DLPC-$(OOH)_2$ and DLPC-$(OH)_2$, the mobile phase mixture was acetonitrile-methanol-water at 50:45:5, v/v/v and for separation of PLPE-OOH and PLPE-OH the ratio was 55:35:10, v/v/v. The flow rate was 0.5 mL/min and the UV detector wavelength was 232 nm (0.02 AUFS). The injection volume was 20 μL.

Downloaded At: 10:57 24 January 2011

RESULTS AND DISCUSSION

The separations of three pairs of hydroperoxy- and hydroxy-phospholipids: SLPC-OOH and SLPC-OH, DLPC-$(OOH)_2$ and DLPC-$(OH)_2$, PLPE-OOH and PLPE-OH are shown in Fig. 1-3. DLPC-$(OOH)_2$ and PLPE-OOH have been used as model compounds in several studies but no conditions were found which could separate them from the corresponding compounds, DLPC-$(OH)_2$ and PLPE-OH, by either normal phase or reverse phase HPLC.[10,11,16] DLPC-$(OOH)_2$ and DLPC-$(OH)_2$, and PLPE-OOH and PLPE-OH were separated under the conditions described in the Experimental section. SLPC-OOH and SLPC-OH were reported to be eluted with identical retention time using an Ultrasphere ODS column with a linear gradient of methanol-acetonitrile-water from 90.5:2.5:7 to 90.5:8.8:0.7 as a mobile phase.[7] However, in this paper, we used an Ultracarb ODS (20) which has been widely used for analyzing moderately polar compounds. The mobile phase composition of methanol-acetonitrile-water was modified to 50: 49.5:0.5, and then baseline separation of SLPC-OOH and SLPC-OH was achieved (Fig. 3). According to the mobile phase used in these reverse phase HPLC conditions and the retention time obtained, we predict that the polarity order is: DLPC-$(OOH)_2$ > DLPC-$(OH)_2$ > PLPE-OOH > PLPE-OH > PLPC-OOH > PLPC-OH > SLPC-OOH > SLPC-OH (most polar first).

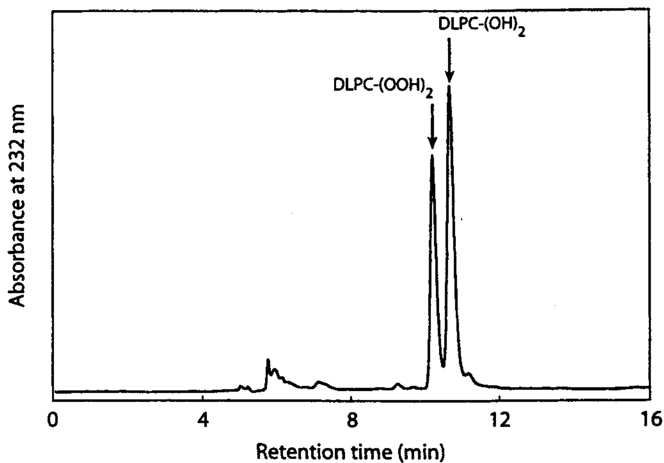


Figure 1. The separation of a mixture of DLPC-$(OOH)_2$ and DLPC-$(OH)_2$: DLPC-$(OH)_2$ was synthesized from DLPC-$(OOH)_2$ by $NaBH_4$ reduction.

Downloaded At: 10:57 24 January 2011

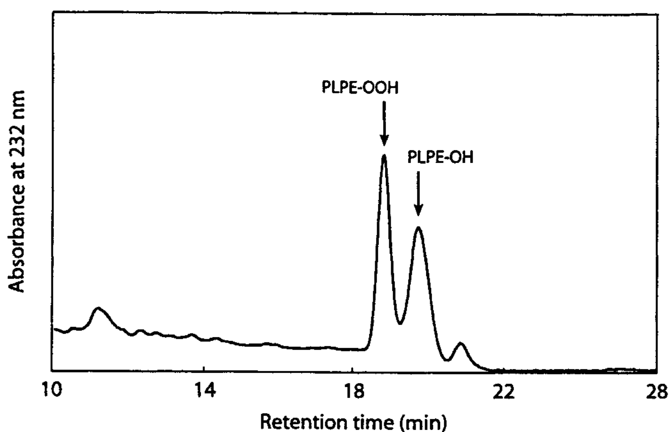


Figure 2. The separation of a mixture of PLPE-OOH and PLPE-OH: PLPE-OH was synthesized from PLPE-OOH by $NaBH_4$ reduction.

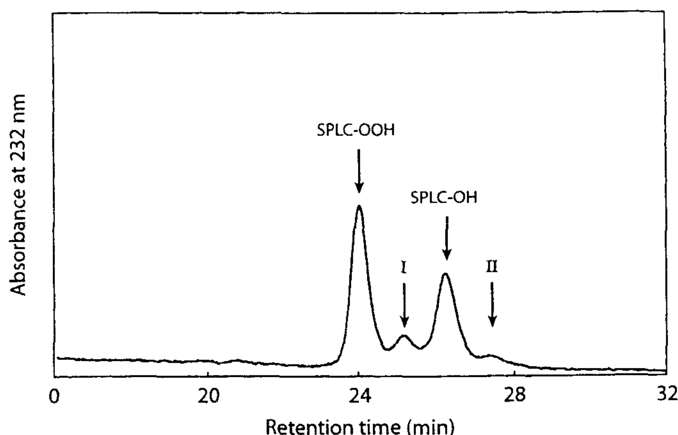


Figure 3. The separation of SLPC-OOH and SLPC-OH. SLPC-OOH was converted to SLPC-OH using PHGPx (0.03 μg protein in total 500μl reaction mixture at pH 7.4, 37°C, 15 min). The minor peaks indicated by arrows (I and II) are probably the 9-OOH and 9-OH isomers of SLPC-OOH and SLPC-OH.

Downloaded At: 10:57 24 January 2011

The assay for measuring phospholipid hydroperoxide glutathione peroxidase activity, based on the separation of hydroperoxy- and hydroxy-phospholipids, is superior to a coupled spectrophotometric assay[13] and this HPLC assay is especially superior for measuring PHGPx activity in crude extracts.[12] The separation of hydroperoxy- and hydroxy- phospholipids allows evaluation of the extent of lipid peroxidation. It also overcomes some drawbacks of the derivatization methods.[1,2,17] The HPLC conditions for separation of hydroperoxy- and hydroxy- phospholipids described in this paper may be also applicable to the study of low density lipoprotein (LDL) oxidation by analysis of the individual oxidized phospholipid species.

APPENDIX 1

Abbreviations

PHGPx	Phospholipid hydroperoxide glutathione peroxidase
FPLC	Fast protein liquid chromatography
PLPC-OOH	1-palmitoyl-2-(13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoyl)-L-3-phosphatidylcholine

PLPC-OH 1-palmitoyl-2-(13-hydroxy-*cis*-9, *trans*-11-octadecadienoyl)- L-3-phosphatidylcholine

SLPC-OOH 1-stearoyl-2-(13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoyl)-L-3-phosphatidylcholine

SLPC-OH 1-stearoyl-2-(13-hydroxy-*cis*-9, *trans*-11-octadecadienoyl)-L-3-phosphatidylcholine

DLPC-$(OOH)_2$ 1, 2 di (13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoyl)-L-3-phosphatidylcholine

DLPC-$(OH)_2$ 1, 2 di (13-hydroxy-*cis*-9, *trans*-11-octadecadienoyl)-L-3-phosphatidylcholine

PLPE-OOH 1-palmitoyl-2-(13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoyl)-L-3-phosphatidylethanolamine

PLPE-OH 1-palmitoyl-2-(13-hydroxy-*cis*-9, *trans*-11-octadecadienoyl)-L-3-phosphatidylethanolamine

Downloaded At: 10:57 24 January 2011

ACKNOWLEDGMENTS

We wish to thank the Biotechnology and Biological Sciences Research Council for financial support, John Eagles (Institute of Food Research, Norwich, UK) for Fab-MS support, and Dr Corinne M. Spickett (University of Strathclyde, Glasgow, UK) for ES-MS support.

REFERENCES

1. T. Nakamura, H. Maeda, Lipids, **26**, 765-768 (1991).

2. A. Mullertz, A. Schmedes, G. Holmer, Lipids, **25**, 415-418 (1990).

3. S. S. Singhal, M. Saxena, H. Ahmad, S. Awasthi, A. K. Haque, Y.C. Awasthi, Arch. Biochem. Biophys., **299**, 232-241 (1992).

4. E. Mosialou, C. Andersson, G. Lundqvist, G. Andersson, T. Bergman, H. Jornavall, R. Morgenstern, FEBS, **315**, 77-80 (1993).

5. M. Maiorino, J. P. Thomas, A. W. Girotti, F. Ursini, Free Rad. Res. Commun., **12-13**, 131-135 (1991).

6. J. D. Hayes, D. J. Pulford, Crit. Rev. Biochem. Mol. Biol., **30**, 445-600 (1995).

7. F. Ursini, L. Bonaldo. M. Maiorino, C. Gregolin, J. Chromatogr., **270**, 301-308 (1983).

8. Y.-P. Bao, G. Williamson, J. Lipid Res., **37**, 2351-2360 (1996).

9. K. Yamada, J. Terao, S. Matsushita, Lipids, **22**, 125-128 (1987).

10. J. Terao, I. Asano, S. Matsushita, Lipids, **20**, 312-317 (1985).

11. K. Schnurr, J. Belkner, F. Ursini, T. Schewe, H. Kühn, J. Biol. Chem., **271**, 4653-4658 (1996).

12. Y.-P. Bao, G. Williamson, Anal. Biochem., **224**, 395-399 (1995).

13. M. Maiorino, C. Gregolin, F. Ursini, Methods Enzymol., **186**, 448-457 (1990).

14. H. W.-S. Chan, **Autoxidation of Unsaturated Lipids**, Food Science and Technology Academic Press Inc., London. (1984).

15. T. Miyazawa, T. Suzuki, K. Fujimoto, Lipids, **28**, 789-793 (1993).

16. Y.-J. Duan, S. Komura, B. Fiszer-Szafarz, D. Szafarz, K. Yagi, J. Biol. Chem., **263**, 19003-19008 (1988).

17. T. Miyazawa, T. Suzuki, K. Fujimoto, Lipids, **28**, 789-793 (1993).

Received September 19, 1997
Accepted October 20, 1997
Manuscript 4633

Downloaded At: 10:57 24 January 2011