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CP-HPLC separation of hydroperoxyoctadecenoic acids and their biosynthesis by fatty acid dioxygenases

By

Ernst H. Oliw and Anneli Wennman

From the Division of Biochemical Pharmacology, Department of Pharmaceutical Biosciences, Uppsala Biomedical Center, Uppsala University, SE-75124, Uppsala, Sweden

Address correspondence to: Ernst H. Oliw, Division of Biochemical Pharmacology, Department of Pharmaceutical Biosciences, Uppsala University, P.O Box 591, SE-751 24 Uppsala, Sweden; Tel. +46-18-4714455; Email: Ernst.Oliw@farmbio.uu.se

Running head: Chiral Separation of Hydroperoxyoctadecenoates

Abstract

Fatty acid oxygenases are often characterized by steric analysis of their hydroxy or hydroperoxy metabolites. Chiral phase-HPLC (CP-HPLC) can be used to separate enantiomeric hydroperoxyoctadecenoic acids. This method is based on analysis of seven octadecenoic fatty acids with double bonds at positions 6Z to 13Z, which were oxidized to hydroperoxides by photooxidation.

A stationary phase, Reprosil Chiral NR, was found to resolve these hydroperoxy fatty acids with 1-hydroperoxy-2-propene and with 3-hydroperoxy-1-propene elements so that the *S* hydroperoxy fatty acids consistently eluted before the *R* stereoisomers. The chiral selector has not been disclosed, but it is described as an aromatic chiral phase with π -donor and π -acceptor groups of Pirkle type. The MS³ spectra of the hydroperoxides showed characteristic fragments, which were influenced by the distance between the hydroperoxy and the carboxyl groups and the relative position of the double bond. Octadecenoic fatty acids can be oxidized by fungal and bacterial dioxygenases to hydroperoxides with *cis* or *trans* double bond configuration. Steric analysis of the hydroperoxy metabolites can be performed by this method, and it can also be used for preparative purposes.

Key words: CP-HPLC, fatty acid dioxygenases, fatty acid hydroperoxides, photooxidation, mass spectrometry.

1. Introduction

Polyunsaturated fatty acids are oxygenated to hydroperoxides by lipoxygenases and by heme-dependent fatty acid dioxygenases in animals, plants, and fungi (1-3). Enzymatic oxidation of octadecenoic acids has been reported in fungi and a few bacteria. *Pseudomonas aeruginosa* expresses a specific oleate 10*S*-dioxygenase and a diol synthase in the periplasm, which sequentially form (10*S*)-hydroperoxy-(8*E*)-octadecenoic acid (10*S*-HPOME) and (7*S*,10*S*)-dihydroxy-(8*E*)-octadecenoic acid (4). The take all fungus of wheat and several aspergilli oxidize oleic acid sequentially to (8*R*)-hydroperoxyoleic acid (8*R*-HPOME) and diols, e.g., (7*S*,8*S*)-dihydroxyoleic acid (7*S*,8*S*-DiHOME), 5*S*,8*R*-DiHOME, or 8*R*,11*S*-DiHOME (3, 5-7). The key intermediate in biosynthesis of the latter diols is 8*R*-HPOME, which is formed by the heme-dependent dioxygenase domains of linoleate diol synthases with both sequence and catalytic similarities to cyclooxygenases and α -dioxygenases of plants (8, 9). Lipoxygenases with catalytic iron or manganese oxidize monoenoic fatty acids at low rates compared to these 8*R*- and 10*S*-dioxygenases (10).

CP-HPLC is now routinely used for analysis of polyunsaturated hydroxy- and hydroperoxy fatty acids, e.g., hydroxyeicosatetraenoic and hydroperoxyeicosatetraenoic acids (11). Versatile matrices contain chiral selectors linked to cellulose or amylose, which are coated on silica, e.g., 3,5-dimethylphenyl carbamate (Chiralcel OD, Chiralpak AD, Reprosil Chiral-AM) and benzoate (Chiralcel OB). With different alcoholic modifiers, these matrices can separate a large number of hydroxy and hydroperoxy fatty acid metabolites (11). An alternative to cellulose and amylose is an

aromatic chiral selector of “Pirkle type”, coated on silica (Reprosil Chiral NR (see Note 1). The latter can separate enantiomers of hydroperoxyeicosatetraenoic and hydroperoxyoctadecadienoic acids (12). Unfortunately, there is little information on chromatographic separation of enantiomers of HOME and HPOME with derivatized cellulose and amylose as chiral selectors.

Racemic HPOME with *trans* configuration can conveniently be obtained by photooxidation and serve as racemic standards. Photooxidation generates singlet oxygen (13). Addition of singlet oxygen to a double bond and hydrogen transfer from one of the neighbouring methylen groups leads to formation of a hydroperoxide. Autoxidation will also yield HPOME with retained *cis* configuration of the double bond (14).

In this chapter we describe a method for CP-HPLC separation of enantiomers of HPOME and their identification by LC-MS³ analysis. This method is based on hydroperoxides of seven octadecenoic acids, which were separated by CP-HPLC on Reprosil Chiral NR (15), and identified by chemical methods described by Hamberg (16). We also illustrate that this CP-HPLC method can be used for analysis of HPOME formed by dioxygenases of bacteria and fungi. The method can also be used for preparative isolation of stereoisomers of HPOME.

2. Materials

2.1 Equipment

1. Photooxidation lamp (MIRA 63, Malmbergs Elektriska AB, Kumla, Sweden; 400 W, E40 high pressure sodium bulb) was obtained locally.
2. Analytical HPLC-MS systems for in-line UV and/or MS³ analysis.

The HPLC pumps were from Thermo Separation Products (P 2000) or from Spectra-Physics Analytical (ConstaMetric 3200). The effluent was mixed post-column with the Surveyor MS pump (Thermo) for in-line MS³ analysis in a linear ion trap mass-spectrometer (LTQ, Thermo). The effluent was subject to UV analysis in a photodiode array detector (Surveyor PDA plus, Thermo) and fractions were collected (Gilson FC 203).

3. MS and UV data were analyzed by the Xcalibur software (Thermo).

2.2 Reagents

1. Octadecenoic acids: (9Z)-18:1 (99%) and (11Z)-18:1 (99%) were from Larodan, and (12Z)-18:1 (99%) was from Lipidox.
2. Methylene blue, N-hydroxyphthalimide, ceric ammonium nitrate, and α -tocopherol were from Sigma-Aldrich.
3. Kieselgel-60 F254 thin layer chromatographic plates were from Merck (Darmstadt, Germany) and cartridges with silica (~0.9 g silica; Sep-Pak Classic) were from Waters.
4. Reprosil Chiral NR (8 μ m; 250 x 2 mm) and Reprosil Chiral NR-R (8 μ m; 250x4 mm; the opposite enantiomeric chiral form to the “NR” chiral selector) columns were from Dr. Maisch GmbH (Beim Brückle 14, 72119 Ammerbuch-Entringen, Germany; See Note 1).
5. Fatty acid dioxygenases of bacterial and fungal origin were prepared as follows. Native diol synthase of *P. aeruginosa* was prepared as described (4). Recombinant Mn-lipoxygenase was expressed in *Pichia pastoris* (strain X-33) as a secreted protein and purified as described (17, 18). Recombinant 8-dioxygenase domain (amino acids 1-673) of 7,8-linoleate diol synthase of the take-all fungus, *Geaumannomyces graminis*, was expressed in *Escherichia coli* as described (19).

3. Methods

3.1 Photooxidation of octadecenoic acids

20 mM octadecenoic acids (2-5 mg) in CHCl_3 with 20 μ M methylene blue in a sealed glass tube were placed ~20 cm from the nitrogen lamp (cf. Ref. (13)). The tube was cooled by a fan to reduce heating from the lamp. Additional methylene blue was added from a concentrated stock solution (8 mM in methanol) when the dye was consumed, as indicated by a shift of colour from blue to brownish. The reaction was followed by TLC (hexane/ethyl acetate/acetic acid, 50/50/0.1) and the fatty acids were visualized by spraying with molybdatophosphoric acid solution for TLC (Merck) before heating on a ceramic plate. About 50% of the octadecenoic acids were usually oxidized after a few hours. The reaction was continued until at least 75% of the acids were oxidized (see Note 2). The products were purified as described in Section 3.3.

3.2 Autoxidation of oleic acid

Photooxidation will produce HPOME with *trans* configuration of the double bond, whereas autoxidation also yields HPOME with *cis* configuration. Autoxidation of oleic acid was modified from the method of Punta et al. (20). Oleic acid (10-30 mg; 0.2 M in CH_3CN with 5-10% α -tocopherol

(w/w) in some experiments) reacted under oxygen with 40 μ M N-hydroxyphthalimide and 20 μ M ceric ammonium nitrate in CH_3CN at 37 °C for a few days under oxygen (see Note 2) . The conversion was followed by TLC.

3.3 Separation of HPOME from octadecenoic acids on silica

The photooxidized solution was evaporated to dryness under a stream of nitrogen and dissolved in 10 ml hexane/diethyl ether/acetic acid, 95/5/0.1, and applied to a silica cartridge in the same solvent. The cartridge was washed with 40 ml of this solvent to elute remaining octadecenoic acid. The HPOME were eluted in 10 ml fractions with 50 ml hexane/diethyl ether/acetic acid, 80/20/0.1. An aliquot of each fraction was assayed on TLC. The peak fractions were combined, reduced to dryness, dissolved in a small volume of hexane/isopropyl alcohol, 90/10, and diluted with hexane to 2% isopropyl alcohol. Products from autoxidation of oleic were purified in the same way (about 5 mg per silica cartridge).

3.4 Analytical CP-HPLC separations with in-line LC-MS analysis

CP-HPLC-MS analysis of HPOME was performed with the Reprosil Chiral NR column (8 μ ; 250x2 mm), which was eluted at 0.5 ml/min with hexane/isopropyl alcohol/acetic acid, 98.8/1.2/0.01. The effluents from the column were combined with isopropyl alcohol/water (3/2) in a ratio of ~2:1 from a second HPLC pump (21) and then introduced by electrospray into a linear ion trap mass spectrometer (LTQ, Thermo) with analysis of carboxylate anions. The ion isolation width was set at 5 for anions of HPOME (m/z 313 $\rightarrow$ full scan) in the first selection and 1.5 at the final selection of MS^3 analysis of HPOME (m/z 313 $\rightarrow$ 295 $\rightarrow$ full scan). The collision energy was set at 1.7 V, and the ion tube lens at -110 to -130 V. We recorded five microscans and used the Gaussian algorithm for peak smoothing (Xcalibur Software, Thermo). Prostaglandin $\text{F}_{1\alpha}$ was infused for tuning.

3.5 Oxidation of octadecenoic acids by fatty acid dioxygenases

10S-Dioxygenase was obtained as described and incubates with oleic acid for 5 min at room temperature. Mn-lipoxygenase (7 μ g) in 0.1 M NaBO_3 (pH 9.0) was activated with 10 eq. 13R-hydroperoxyoctadecatrienoic acid and then incubated with 100 μ M (12Z)-18:1 (40 min, 21°C). Recombinant 8R-dioxygenase (the N-terminal 673 amino acids constituting the 8R-dioxygenase domain of 7,8-linoleate diol synthase) was incubated with 100 μ M (9Z)-18:1 for 30 min on ice. The products were extracted on a SepPak/ C_{18} cartridge and analyzed by LC-MS.

3.6 Preparative CP-HPLC with UV analysis

Preparative CP-HPLC was performed with the Reprosil Chiral NR-R column (8 μ ; 250x4 mm; eluted at 2 ml/min) and HPOME were analyzed with in-line UV detection (210 nm; PDA plus detector, Thermo). Fractions (1-2 ml) were collected, and an aliquot (2 μ l) of fractions with UV absorbance was analyzed for HPOME by direct injection to the mass spectrometer in a solvent stream (0.3 ml/min) of methanol/water/acetic acid, 800/200/0.1. This method can also be used for separation of stereoisomers of 9- and 13-HPODE obtained by photooxidation of linoleic acid (12), and with UV analysis at 235 nm for detection of HPODE.

4. Results

4.1 Separation of enantiomers of HPOME by CP-HPLC

All pairs of investigated enantiomers of HPOME were resolved on Reprosil Chiral NR. The relative retention times of HPOME with *trans* double bonds are summarized in Table 1. The absolute configuration of enantiomers of HPOME obtained following chromatography on Reprosil Chiral NR was determined by chemical methods (15, 16). This analysis was performed with (-)-menthoxycarbonyl derivatives of the corresponding HOME methyl esters, followed by ozonolysis of the double bond, methylation, and separation and identification of the diastereoisomers by gas chromatography-mass spectrometry (16). The results showed that the *S* stereoisomers of 14 regioisomeric HPOME with the double bond in *trans* configuration consistently eluted before the *R* stereoisomers on Reprosil Chiral NR. As far known, this elution order also applies to HPOME with *cis* double bonds (Table 1).

As illustrated in Table 1, the HPOME formed by photooxidation typically eluted from the Reprosil Chiral NR with an *S* stereoisomer with the shortest retention time and an *R* isomer with the largest, whereas the middle peaks contained *R* and *S* stereoisomers, eg., 10*S*-HPOME eluting first and 9*R*-HPOME last, and elution of a 9*S*- and 10*R*-HPOME in between. The Reprosil Chiral NR-R column separates the isomers in the reverse order with 10*S*- and 9*R*-HPOME in the middle. By the selection of matrix, the desired 10-HPOME isomer can be eluted in the first peak and the desired 9-HPOME isomer in the last.

The separation of the 12-HPOME and 13-HPOME enantiomers is shown in Fig. 1A. 13-HPOME is formed from (12*Z*)-18:1 by Mn-lipoxygenase, and it has the same retention time as 13*R*-HPOME (Fig. 1B). 10*S*-Dioxygenase of *P. aeruginosa* oxidizes oleic acid to 10*S*-HPODE (Fig. 2A). Addition of this stereoisomer to photooxidized oleic acid with analysis of 10-HPOME by selected ion monitoring confirmed the elution order, *S* before *R* (Fig. 2A). This column also appeared to separate enantiomers of HPOME with *cis* double bond configuration. Autoxidation of oleic acid thus also yielded 8- and 11-HPOME(*Z*) as well as 9- and 10-HPOME. The separation of 9-HPOME is

shown in Fig. 2B as well as the separation of 8-HPOME(9Z). 8*R*-HPOME(9Z) is formed by linoleate diol synthases, and addition of this compound to racemic 8-HPODE(9Z) confirmed the elution order, *S* before *R* (Fig 2).

Chiral separation requires the interaction of the analyte to the chiral selector at a minimum of three positions with at least one position dependent on stereochemistry (22). The two oxygen atoms of the hydroperoxide group and the double bond seem to interact with the chiral selector of Reprosil Chiral NR in this way (cf. the insets in Figs. 1-2). Interestingly, chiral separation was lost when the hydroperoxide group of HPOME was reduced to an alcohol. Enantiomers of 10-HOME(8Z) thus interacted with the chiral NR selector without separation, but the stereoisomers of 10-hydroxy-(8*E*,12*Z*)-octadecadienoic acid, with an additional double bond at 12*Z* compared with 10-HOME(8Z), were resolved (23), presumably by three-point interaction of the two double bonds and the hydroxyl group with the chiral selector. Enantiomers of polyunsaturated fatty acid hydroperoxides with 1-hydroperoxy-(2*E*,4*Z*)-pentene elements and additional double bonds (e.g., hydroperoxyeicosatetraenoic acids) may not elute in the same “*S*” and “*R*” order as HPOME and 9- and 13-HPODE (cf. Ref. (12)).

4.2 Mass-spectrometric fragmentation of HPOME and KOMe

The MS/MS spectra (m/z 313 $\rightarrow$ full scan) confirmed that HPOME were dehydrated to KOMe as judged from the main ion at m/z 295 (313-18) in analogy with dehydration of other hydroperoxy fatty acids (12, 24). Important ions in the MS³ spectra (m/z 313 $\rightarrow$ 295 $\rightarrow$ full scan) of HPOME are summarized in Tables 2 and 3.

The fragmentation pattern was influenced by the position of the hydroperoxide group in relation to the double bond and the carboxyl group. The MS³ spectra of HPOME with their hydroperoxide groups located at the carboxylic side of a *trans* or *cis* double bond and relatively near the carboxyl group could only be explained by ions formed by a rearrangement mechanism. MS³ analysis of 8-HPOME(9Z) thus yielded a rather intense signal pair at m/z 183 and 165 (Table 2). A possible fragmentation mechanism is suggested in Fig. 3A. It seems likely that the characteristic ions at m/z 183 of 8-HPOME could be due to rearrangement with loss of an uncharged species containing part of the carboxyl end with transfer of O⁻ to the ω end. In contrast, the MS³ spectra of 12-HPOME(13*E*) can be interpreted with aid of keto-enol tautomerism and fragmentation by α -cleavage at the oxidized carbons as illustrated in Fig. 3B. This mechanism could also explain the fragmentation of 13-HPOME(11*E*), as illustrated in Fig. 3C. Other examples of these fragmentation mechanisms are found in Tables 2 and 3.

5. Notes

1. References to the separation of drugs on the Reprosil Chiral NR column and its chemical properties can be found at the home page of Dr. Maisch (http://www.dr-maisch.com/xedin.php?point=chiral_nr.html). This column can be operated both in reverse and normal phase mode, but separation of enantiomers of oxygenated fatty acids has so far only been reported in normal phase mode (12, 25).
2. Photooxidation and autoxidation under an atmosphere of oxygen will increase the reaction rate, and this is of practical value for autoxidation.

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TABLES

Table 1. Relative retention times of HPOME on the Reprosil Chiral NR column.

18:1		HPOME(<i>E</i>) ^a				HPOME(<i>Z</i>) ^b	
Position and configuration of the hydroperoxide group							
(9 <i>Z</i>)-18:1	10 <i>S</i>	10 <i>R</i>	9 <i>S</i>	9 <i>R</i>	8 <i>S</i>	8 <i>R</i>	
	0.76	0.90	0.88	1	0.84	0.91	
(11 <i>Z</i>)-18:1	12 <i>S</i>	12 <i>R</i>	11 <i>S</i>	11 <i>R</i>	10 <i>S</i>	10 <i>R</i>	
	0.71	0.82	0.86	1	0.76	0.85	
(12 <i>Z</i>)-18:1	13 <i>S</i>	13 <i>R</i>	12 <i>S</i>	12 <i>R</i>	11 <i>S</i>	11 <i>R</i>	
	0.82	0.95	0.87	1	ND	0.87	

^aHPOME(*E*) were obtained by photooxidation, and the absolute configurations of HPOME(*E*) were determined by chemical methods after separation of enantiomers on a preparative Reprosil Chiral NR-R column. ^bHPOME(*Z*) were obtained by oxidation with the 8*R*-dioxygenase of linoleate diol synthase and Mn-lipoxygenase.

Table 2. Structurally important ions in the MS³ spectra of hydroperoxides of regioisomeric octadecenoic acids with 1-hydroperoxy-2-propene functional groups.

HPOME ^a	X-44	X-18	X ^b	Y-44	Y ^c
8-HPOME(9 <i>E</i>)	111 (35)	137 (ND)	155 (4)	127 (30)	171 (25)
9-HPOME(10 <i>E</i>)	125 (55)	151 (90) ^e	169 (10) ^e	141 (40)	185 (45)
10-HPOME(11 <i>Z</i>)	139 (90)	165 (90) ^e	183 (<1)	155 (55) ^e	199 (70)
11-HPOME(12 <i>E</i>)	153 (50)	179 (100)	193 (<1)	169 (20)	213 (30)
12-HPOME(13 <i>E</i>)	167 (40)	193 (100)	211 (<1)	183 (10)	227 (12)
HPOME	Z-18	Z ^d			
8-HPOME(9 <i>E</i>)	165 (100)	183 (15)			
9-HPOME(10 <i>E</i>)	151 (90) ^e	169 (10) ^e			
10-HPOME(11 <i>Z</i>)	165 (90) ^e	155 (55) ^e			
11-HPOME(12 <i>E</i>)	123 (6)	151 (1)			
12-HPOME(13 <i>E</i>)	109 (1)	137 (0)			

^aThe mass spectra of the corresponding hydroperoxides with a *cis* double bond were virtually identical (10). ^bThese ions (X series) were postulated as intermediates, which formed strong fragment ions after loss of water or CO₂ in some of the spectra (cf. Fig. 3A). ^cThese ions (Y series) were formed by loss of the omega end. ^dThese ions (Z series) contained the omega end and gave rise to intense signals after loss of water in some spectra. ^eThis ion occurs in two columns and could be formed by two different fragmentation mechanisms. The hypothetical fragmentation mechanisms of 8- and 12-HPODE are

shown in Fig. 3A and B. MS³ spectra (m/z 311→295→full scan) and the base-peaks (100%) were found at m/z 277 (295-18) unless listed in this Table.

Table 3. Structurally important fragmentation ions in the MS³ spectra of hydroperoxides of regioisomeric octadecenoic acids with 3-hydroperoxy-1-propene functional groups.

HPOME	X-18	X ^a	Z ^b
10-HPOME(8 <i>E</i>) ^d	137 (10)	155 (100) ^c	155 (100) ^c
11-HPOME(9 <i>Z</i>)	151 (50)	169 (4)	141 (70)
12-HPOME(10 <i>E</i>)	165 (100)	183 (4)	127 (30)
13-HPOME(11 <i>E</i>)	179 (100)	197 (2)	113 (10)

^aThese ions (X series) contained the carboxyl group and formed fragments ions after loss of water, but only weak ions after loss of CO₂. ^bThese ions (Z series) contained the omega end (cf. Fig. 3C for details). ^cThis ion occurs in two columns, and could be formed by both fragmentation mechanisms. A hypothetical fragmentation mechanism of 13-HPOME is shown in Fig. 3C. MS³ spectra (m/z 311→295→full scan) and the base-peaks were found at m/z 277 (295-18) unless listed in this Table.

Figure Captations

- Fig. 1.** CP-HPLC separation of 12- and 13-HPOME obtained by photooxidation of (12Z)-18:1. A, The top chromatogram shows selective ion monitoring of the characteristic ion m/z 193 of 12-HPOME and the bottom chromatogram shows the characteristic ion at m/z 179 of 13-HPOME. The elution order was deduced by chemical methods (15). The inset to the right shows in bold the three structural elements of HPOME likely recognized by the chiral selector. B, Analysis of 13-HPOME formed by Mn-lipoxygenase. The latter oxidizes (12Z)-18:1 at C-11 and at C-13 with double bond migration to 13-HPOME (bottom chromatogram) with the same retention time as 13*R*-HPOME (top chromatogram).
- Fig. 2.** Separation by CP-HPLC of stereoisomers of 10-HPOME formed by photooxidation and 10*S*-dioxygenase and separation of 9- and 8-HPOME formed by autooxidation of oleic acid and 8-HPOME formed by 8*R*-dioxygenase. A, The top chromatogram show selected ion monitoring of characteristic fragment ions of 10-HPOME, formed by 10*S*-dioxygenase, and the bottom chromatogram shows elution of racemic 10-HPOME with addition of 10*S*-HPOME (cf. Tables 2 and 3). B, Separation of the stereoisomers of 9-HPOME (top) and 8-HPOME(9*Z*) (bottom). 8*R*-HPOME was added to identify this enantiomer. The insets to the right in A and B show in bold the three structural elements of HPOME likely recognized by the chiral selector.
- Fig. 3.** Overview of the variable MS³ fragmentation of hydroperoxides, illustrated by dehydration of 8-HPOME and 12-HPOME to keto compounds (KOME) and their subsequent fragmentation. A, fragmentation of 8-KOME with formation of two characteristic ions by rearrangement (cf. Table 2). B, fragmentation of 12-KOME with formation of characteristic ions after keto-enol tautomerism (cf. Table 2). C, fragmentation of 13-KOME with formation of characteristic ions (cf. Table 3).