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Separation and Characterization of Cholesteryl Oxo- and Hydroxy-Linoleate Isolated from Human Atherosclerotic Plaque

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In previous work we demonstrated that up to 30 % of cholesteryl linoleate in homogenates of advanced human plaque samples is present in oxidized forms. Here we show that the material from plaque hexane extracts which co-elutes with cholesteryl hydroxy-linoleate on reversed phase HPLC (Anal Biochem 1993;213:79), is composed of several isomers of cholesteryl hydroxy- and cholesteryl oxo-octadecadienoate. Enzymatic hydrolysis and measurement of liberated cholesterol and disappearance of the esters revealed that almost all of the material consisted of unoxidized cholesterol esterified to oxidized derivatives of octadecadienoate. Semi-preparative reversed-phase HPLC was used to obtain sufficient quantities of this co-eluting material to undertake normal phase HPLC separation of these components. The nature of such separated and isolated compounds was identified, by co-chromatography with authentic standards, UV spectroscopy and chemical ionization and electron impact mass spectrometry, as cholesteryl hydroxy- and cholesteryl oxo-octadecadienoate. These oxidized fatty acids have been observed previously in plaque, in agreement with our new unambiguous demonstration of their presence as cholesteryl esters. The application of the methods described for the separation of the various forms of oxidized cholesteryl octadecadienoate may aid mechanistic studies of *in vitro* and *in vivo* lipoprotein lipid oxidation.

Keywords: Atherosclerosis, lipid peroxidation, lipoxygenase, cholesteryl hydroxy-octadecadienoate, cholesteryl oxo-octadecadienoate

Abbreviations: Ch18:2, cholesteryl linoleate or cholesteryl-9*Z*, 12*Z*-octadecadienoate; Ch18:2=O, isomers of cholesteryl oxo-octadecadienoate; Ch18:2-OH, isomers of cholesteryl hydroxy-octadecadienoate; Ch13(*Z*,*E*)-HODE, cholesteryl 13-hydroxy-9*Z*,11*E*-octadecadienoate; Ch13(*E*,*E*)-HODE, cholesteryl 13-hydroxy-9*E*,11*E*-octadecadienoate; Ch9(*E*,*Z*)-HODE, cholesteryl 9-hydroxy-10*E*, 12*Z*-octadecadienoate; Ch9(*E*,*E*)-HODE, cholesteryl 9-hydroxy-10*E*,12*E*-octadecadienoate; Ch18:2[O(H)], group of compounds co-eluting with authentic standard of Ch18:2-OH in reversed-phase HPLC under the conditions described in ref, 4; Ch18:2-OOH, isomers of cholesteryl hydroperoxy-octadecadienoate; FC, free (unesterified) cholesterol; NP-HPLC, normal phase HPLC; MS, mass spectrometry; PBS, phosphate buffered saline; RP-HPLC, reversed phase HPLC.

INTRODUCTION

Increasing evidence indicates that atherogenesis is intimately associated with oxidation of both the lipid and protein moieties of lipoproteins.[1,2] We

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have recently demonstrated that lipids in homogenates prepared from advanced atherosclerotic lesions are substantially oxidized, in agreement with earlier literature, and in spite of the presence of essentially normal levels of a range of non-proteinaceous aqueous and lipophilic antioxidants.[3] Up to 30% of cholesteryl linoleate (Ch18:2) was found in oxidized forms, together with smaller amounts of other oxidized lipids such as free 7-ketocholesterol. In particular, we noted[3] that a substantial amount of the oxidized Ch18:2 co-eluted with authentic cholesteryl hydroxy-octadecadienoate (Ch18:2-OH) on reversed phase (RP)-HPLC, using the method described in.[4] However, it was noted[3] that this co-eluting material (termed 'plaque Ch18:2[O(H)]') required further characterization, as it was heterodisperse in nature.

We now report the separation of 'plaque Ch18:2[O(H)]' into several forms of oxidized Ch18:2 by normal phase (NP)-HPLC. We identify these oxidized lipids as isomers of Ch18:2-OH and cholesteryl oxo-octadecadienoate (Ch18:2=O) by enzymatic hydrolysis of 'plaque Ch18:2[O(H)]', co-chromatography with authentic standards, UV spectroscopy, and by chemical ionization and electron impact mass spectrometry (MS) of the purified and isolated isomers of Ch18:2-OH and Ch18:2=O.

MATERIAL AND METHODS

Materials

Free (unesterified) cholesterol (FC), Ch18:2, cholesterol esterase [EC 3.1.1.13] and taurocholic acid (sodium salt) were purchased from Sigma (St Louis, MO, USA). Cholesteryl 13(S)-hydroxy-9*Z*,11*E*-octadecadienoate (Ch13(*Z*,*E*)-HODE) and cholesteryl 9(S)-hydroxy-10*E*,12*Z*-octadecadienoate (Ch9(*E*,*Z*)-HODE) were obtained from Cayman Chemicals (Ann Arbor, MI, USA; purities ≥98%), whereas cholesteryl hydroperoxy-octadecadienoate (Ch18:2-OOH, referring to a mixture of 9- and 13-hydroperoxide isomers) was prepared from purified Ch18:2 by oxidation with peroxyl radicals as described.[5,6] Cholesteryl oxo-octadecadienoate (Ch18:2=O) was synthesized as follows. Briefly, Ch18:2 (0.56 mmol) was added to 3 mL of ethanol and 2 mL of methanol and dissolved by warming to 60°C. To this, $CuS0_4$ (1.6 mM) was added and the mixture incubated at 37°C for two days. The reaction mixture was then extracted with hexane (5 × 3 mL) and the flask containing a thin film of oxidized lipid stoppered and kept at room temperature for 2 weeks. This resulted in the conversion of Ch18:2 into a mixture containing Ch18:2-OOH, Ch18:2-OH and Ch18:2=O, which were separated and collected using the RP- and NP-HPLC methods described below. Ch18:2=O was idenitifed by UV and MS, the purity confirmed by reinjection into HPLC, and its concentration determined by weight.

Methanol and acetonitrile were obtained from Mallinckrodt (Paris, KY, USA), *n*-heptane from Ajax Chemicals (Auburn, NSW, Australia), and diethyl ether from Fluka (Buchs, Switzerland). All solvents were of HPLC or the highest quality available. Phosphate-buffered (50 mM), isotonic saline pH 7.4 (PBS) was prepared in nanopure water and stored overnight at 4°C over Chelex-100 (Bio-Rad, Hercules, CA, USA) to remove contaminating transition metals, as verified regularly by the ascorbate autoxidation method.[7] A teflon-in-glass homogenizer (55 mL, Wheaton, Millville, NJ, USA) was used with a piston rotor (Heidolph, Heidelberg, Germany).

Chemical ionization mass spectra were obtained on a Finnigan MAT spectrometer (San Jose, CA, USA), using a desorption probe with methane as reactant gas at 100 eV and an ion source temperature of 140°C. Electron impact mass spectra were obtained on a Fisons AutospecQ (Beverly, CA, USA) at 70 eV, using 8 kV accelerating voltage, an insertion probe and programmed to 400°C.

Extraction of Plaque Lipids

Human carotid plaques (3–5 g wet tissue each) were collected into PBS from patients under-

going carotid endarterectomy at the Royal Prince Alfred Hospital, Sydney.[3] Within approximately one hour of obtaining the material, plaques were minced with scissors and homogenized for 5 min in PBS supplemented with butylated hydroxytoluene (100 μM), EDTA (1 mM), α-tocotrienol (1 μM) and isoascorbic acid (5 μM) using a teflon-to-glass homogenizer.[3] The homogenate (1 vol) was mixed vigorously with 1 vol of methanol for about 10 seconds, diluted with 1 vol of nanopure water before 5 vol of hexane were added and the mixture shaken vigorously for about 30 sec. The mixture was then centrifuged at 1500 × g for 5 min, the hexane layer collected and the remaining material extracted a further two times with hexane. The combined hexane extracts were evaporated, redissolved in isopropanol and subjected to HPLC analysis as described below. This extraction procedure, while slightly different from that employed previoulsy,[3] allowed the work-up of larger quantities of plaque material needed for characterization, and yielded qualitatively similar hexane extracts as judged by the first RP-HPLC analysis (see below).

HPLC Separation of Oxidized Plaque Lipids

Three chromatographic steps were used to separate and purify isomers of Ch18:2-OH and Ch18:2 = O. First, RP-HPLC[4] was used to isolate 'plaque Ch18:2[O(H)]', defined here and previously[3] as the group of compounds coeluting with authentic cholesteryl 13-hydroxy-9*Z*,11*E*-octadecadienoate (Ch13(*Z*,*E*)-HODE) and cholesteryl 9-hydroxy-10*E*,12*Z*-octadecadienoate (Ch9 (*E*,*Z*)-HODE). To obtain sufficient material for subsequent further characterization, a semipreparative C_{18} column (25 × 1.0 cm, 5 μm particle size, 2 cm guard column, Supelco, Bellefonte, PA, USA) was used with acetonitrile:isopropanol:water = 44:54:2 (vol/vol/vol) as the eluant at a flow rate of 3.0 mL/min. The eluant was monitored at 210 and 234 nm using a 1000S Diode Array Detector (Applied Biosystems, Foster City, CA, USA). The detection limit for Ch18:2[O(H)] was ≈ 10 pmol per injection. Where indicated, RP-HPLC with post-column chemiluminescence detection[6] was used to distinguish Ch18:2-OOH from Ch18:2-OH. The second chromatographic step was to subject the collected 'plaque Ch18:2[O(H)]' to NP-HPLC using an analytical silica column (25 × 0.46 cm, 5 μm particle size, 2 cm guard column, Supleco) eluted with heptane:diethyl ether:isopropanol = 100:0.5:0.175 (vol/vol/vol) (solvent A) at a flow rate of 2.0 mL/ min and monitored by the diode array detector at 234 and 270 nm. The third step was to separate and purify overlapping peaks of Ch18: 2=O by NP-HPLC using the analytical and guard columns described above and the less polar solvent heptane:diethyl ether:isopropanol = 100:0.06:0.075 (vol/vol/vol) (solvent B) at 3.0 mL/min, with detection at 234 and 270 nm.

Hydrolysis of 'Plaque Ch18:2[O(H)]'

An aliquot of isolated 'plaque Ch18:2[O(H)]' obtained after semi-preparative RP-HPLC was evaporated and resuspended in PBS containing 6.0 mM taurocholate so that the final concentration of 'plaque Ch18:2[O(H)]' was ≈ 20 μM. Cholesterol esterase (2.9 mg or 1.4 U) was added and the mixture incubated at 37°C. At various times 200 μL aliquots were removed, acidified to pH 3.0 and extracted with 2 mL of ether as described.[8] The ether layer (1.7 mL) was then removed, evaporated, and the residue resuspended in isopropanol and analyzed for FC (RP-HPLC) and Ch18:2-OH and Ch18:2=O (NP-HPLC, solvent A).

RESULTS

The RP-HPLC assay described by Kritharides *et al.*[4] separated oxidized cholesteryl esters (detected at 234 nm) from unoxidized free cholesterol (FC) and cholesteryl esters (detected at 210 nm) in human plaque homogenates (Fig. 1). In addition to the various identified com-

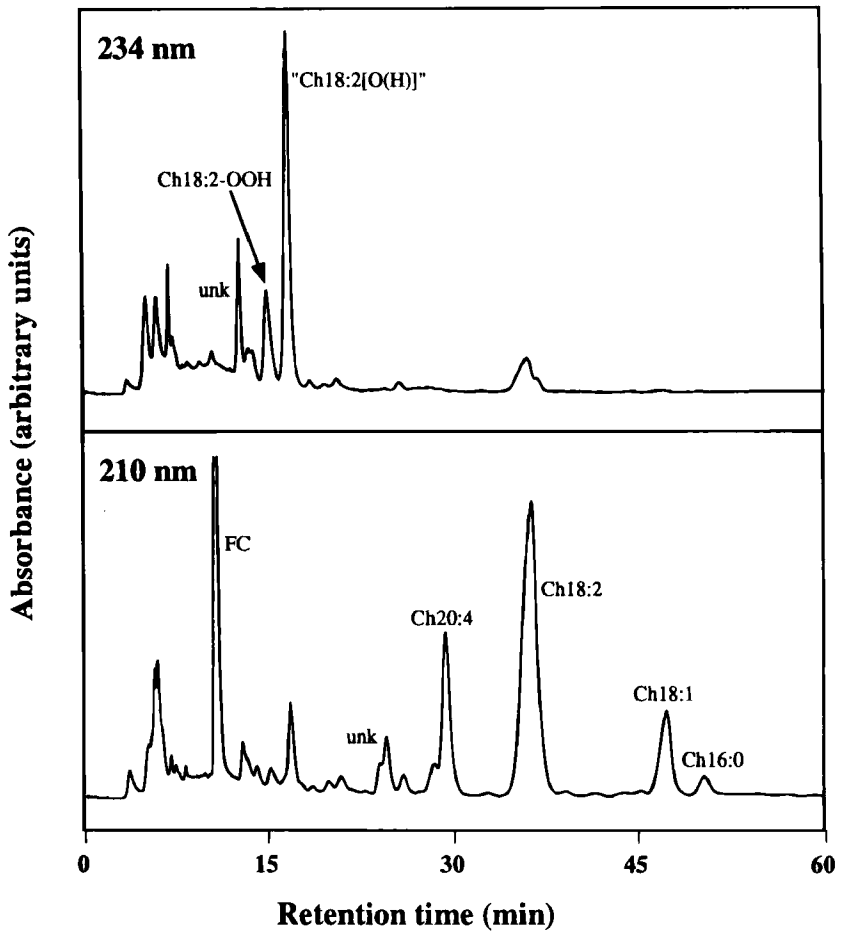


FIGURE 1 Typical RP-HPLC traces of unoxidized and oxidized lipids from human atherosclerotic plaques. For HPLC conditions and extraction procedure see the Materials and methods section. Top panel: UV_{234nm} detection of oxidized lipids, including Ch18:2-OOH and 'plaque Ch18:2[O(H)]' eluting at ≈ 14.3 and 16.0 min, respectively. Lower panel: UV_{210nm} detection for unoxidized lipids including free cholesterol (FC), cholesteryl arachidonate (Ch20:4), cholesteryl linoleate (Ch18:2), cholesteryl oleate (Ch18:1), and cholesteryl palmitate (Ch16:0); unk, unknown.

pounds (see legend to Fig. 1), human plaque extracts also contained a number of unknown compounds, eluting e.g. at ≈ 13 (top panel) and 24 min (bottom panel). We did not attempt to characterize the compounds responsible for these peaks even though we cannot exclude that they represented major components of plaque. Hexane extracts of human plaque homogenates contained compounds that co-eluted with standards of Ch18:2-OOH (all isomers eluting at 14.9 min) and Ch13(*Z*,*E*)-HODE and Ch9(*E*,*Z*)-HODE (co-eluting at ≈ 16.7 min) (Fig. 1, top panel). Spectral analysis of the peaks present in the plaque extract and co-eluting with authentic Ch18:2-OH showed strong absorbance at both 234 and 270 nm (not shown), indicating the presence of compound(s) in addition to Ch18:2-OH, which shows no absorbance peak at 270 nm. We therefore termed this compound 'plaque Ch18:2[O(H)]' and characterized it further by treatment with cholesterol esterase, NP-HPLC, UV and mass spectrometry (MS).

To determine whether 'plaque Ch18:2[O(H)]' contained cholesteryl esters and, if so, whether

the oxidized group was part of the cholesterol or the octadecadienoic acid moieties, we subjected 'plaque Ch18:2[O(H)]' to cholesterol esterase treatment and determined the formation of unoxidized FC by RP-HPLC with acetonitrile/isopropanol/water (44/54/2, vol/vol/vol) as the eluant monitored at 210 and 234 nm.[4] Treatment of 'plaque Ch18:2[O(H)]' with the enzyme resulted in a time-dependent release of unoxidized FC and concomitant decrease in 'plaque Ch18:2[O(H)]' (not shown). As 'plaque Ch18:2-[O(H)]' contains cholesteryl hydroxy-octadecadienoate (Ch18:2-OH) and Ch18:2=O as the major components (see below), we selectively monitored for the disappearance of these oxidized lipids during incubation of 'plaque Ch18:2[O(H)]' with cholesterol esterase. Figure 2 shows that nearly stoichiometric amounts of FC were formed as Ch18:2-OH and Ch18:2=O disappeared during the hydrolysis. In two separate experiments using different starting concentrations of 'plaque Ch18:2[O(H)]' isolated from two different plaques, the amounts of FC formed corresponded to 101.9 and 102.7% of the esterified lipids lost, indicating that the great majority of 'plaque Ch18:2[O(H)]' contained an unoxidized cholesterol moiety. The fact that the levels of FC formed somewhat exceeded the loss of Ch18:2-OH and CH18:2 = O may be explained by the small amounts of Ch18:2-OOH present in 'plaque Ch18:2-[O(H)]' (see below). The results in Figure 2 also show that the starting cholesterol value was significantly above zero, possible due to non-enzymic hydrolysis occurring during sample work-up and/or 'trailing' of some FC into the 'plaque Ch18:2-[O(H)]' peak.

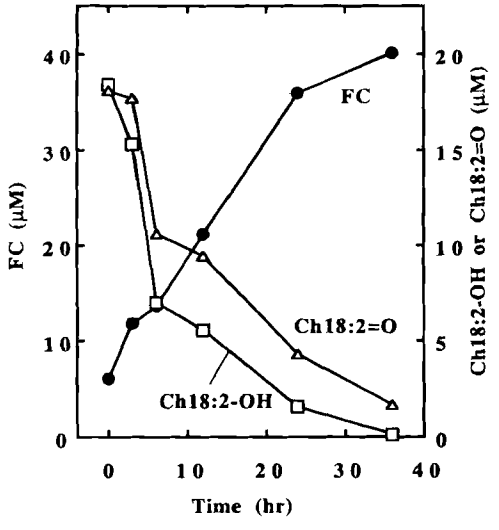


FIGURE 2 Time-dependent hydrolysis of isolated 'plaque Ch18:2[O(H)]'. Isolated 'plaque Ch18:2[O(H)]' was hydrolyzed using cholesterol esterase as described in the Materials and methods section, and the loss of Ch18:2-OH and Ch18:2=O together with the formation of free cholesterol (FC) measured using NP- and RP-HPLC, respectively. Similar results were obtained in two separate experiments carried out with preparations of 'plaque Ch18:2[O(H)]' prepared from two different plaque samples. Note the difference in scales fro FC and Ch18:2-OH and Ch18:2=O.

NP-HPLC (using solvent A) separated 'plaque Ch18:2[O(H)]' into a number of different compounds detected at 234 or 270 nm and numbered consecutively in order of elution (Fig. 3). Chromatography of 'plaque Ch18:2[O(H)]' obtained from homogenates of six separate human plaque samples gave comparable results. Among the major compounds, peaks 2–5 (Fig. 3 top panel) showed stronger absorbance at 270 than 234 nm, whereas peaks, 7, 9, 11 and 12 (bottom panel) absorbed at 234 but not 270 nm (Figs. 3 & 4). Indeed, spectral analyses of the individual eluting compounds using a diode array detector revealed a single absorption maximum at 268–274 nm for peaks 2–5 (Fig. 4A) and at 230–234 nm for peaks 7, 9, 11, and 12 (Fig. 4B). These spectra are typical, respectively, of oxo, and hydroxy or hydroperoxy, derivatives of polyenoic fatty acids containing the 1,4-pentadiene structure (see e.g. ref. 9).

To distinguish between putative hydroxy and hydroperoxy derivatives, peaks 7, 9, 11, and 12 were collected separately and re-injected onto RP-HPLC with post-column chemiluminescence detection.[6] This was done because lipid hydroperoxides, but not lipid hydroxides, are chemiluminescence active.[5,10] Peaks 7, 9, 11, and 12 where chemiluminescence inactive (not shown). In addition, peaks 7 and 11 co-chromatographed on NP-HPLC (solvent A) with authentic standards of Ch13(Z,E-HODE and Ch9(E,Z)-HODE, respec-

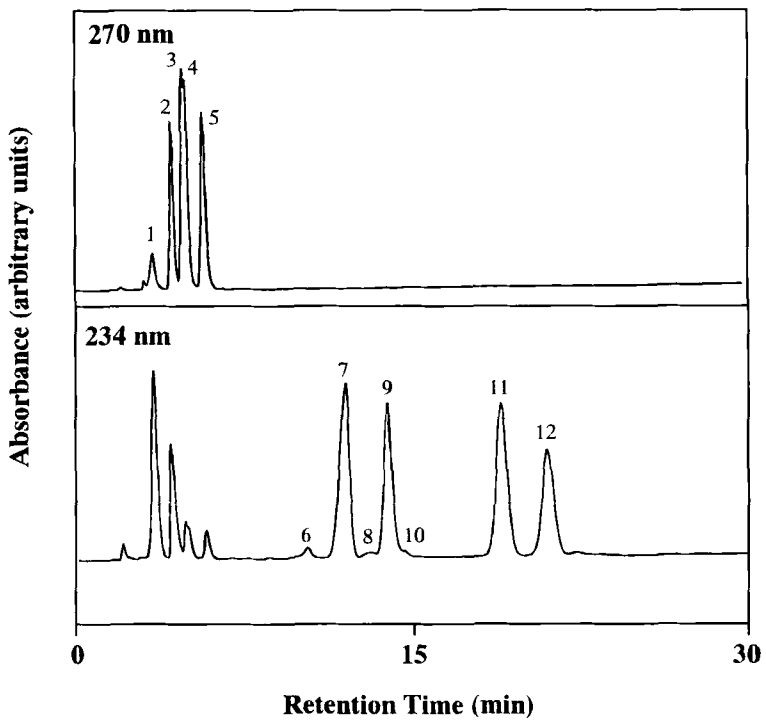


FIGURE 3 Typical NP-HPLC traces of the isolated 'plaque Ch18:2[O(H)]' eluting at 15.8 min in Figure 1. For HPLC conditions see the Materials and methods section. Upper panel: UV_{270nm} detection for isomers of Ch18:2=O (peaks 2, 3, 4 and 5 eluting at 4.3, 4.8, 4.9 and 5.7 min, respectively). Lower panel: UV_{234nm} detection for isomers of Ch18:2-OH (peaks 7, 9, 11 and 12 eluting at 12.0, 13.9, 19.0 and 21.1 min, respectively). The compounds giving rise to the small signals 6, 8, and 10 correspond to isomers of Ch18:2-OOH (see text).

tively. A Ch18:2-OOH standard, prepared by free radical-mediated oxidation of purified Ch18:2, revealed 3 peaks co-eluting with peaks 6, 8, and 10 of 'plaque Ch18:2[O(H)]' when analyzed by NP-HPLC (sovent A) (not shown). When this Ch18:2-OOH standard was reduced with $NaBH_4$ prior to NP-HPLC analysis, 4 compounds absorbing at 234 nm were observed that co-eluted with compounds 7, 9, 11, and 12 present in 'plaque Ch18:2-[O(H)]' (not shown). These findings, together with the MS data (see below), identify compounds 7 and 11 as Ch13(*Z*,*E*)-HODE and Ch9(*E*,*Z*)-HODE, and strongly suggest that compounds 9 and 12 are their corresponding *trans*, *trans* isomers, i.e., cholesteryl 13-hydroxy-9*E*, 11*E*-octadecadienoate (Ch13(*E*,*E*)-HODE) and cholesteryl 9-hydroxy-10*E*,12*E*-octadecadienoate (Ch9(*E*,*E*)-HODE), respectively. The results also suggest that compounds 6, 8, and 10 are isomers of Ch18:2-OOH. We did not attempt to characterize further peak 1 (Fig 3, top panel).

As can be seen in Figure 3 (top panel), the components of 'plaque Ch18:2[O(H)]' absorbing at 270 nm eluted close to each other and not all were baseline-separated. We therefore collected peaks, 2, 5, and (3 plus 4), and subjected them to an additional NP-HPLC step using a less polar eluant (solvent B). In this way peak 2 was separated into 6 components of which four (eluting at 3.0, 9.3, 16.9, and 17.8 min) and two (21.5 and 25.1 min) absorbed maximally at 234 and 270 nm, respectively; the peak eluting at 21.5 min, termed 2′ was clearly the major component (not shown). Similarly, using solvent B peaks 3 plus 4 were separated into 5 components of which peaks 3′ and 4′ eluting at 21.5 and 22.9 min absorbed maximally at 270 nm and were clearly the major

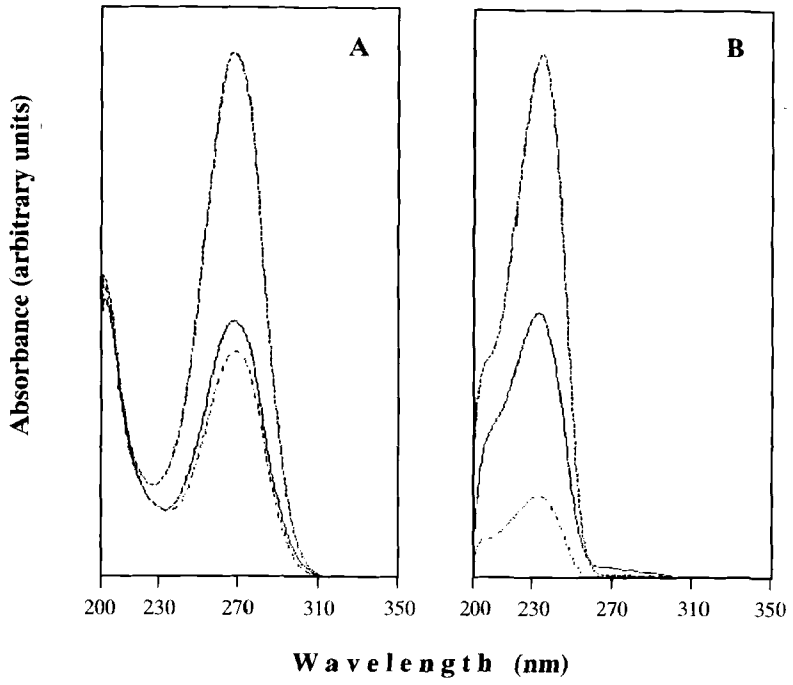


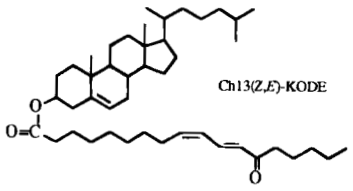
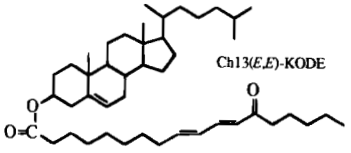
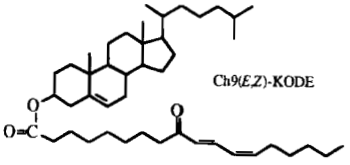
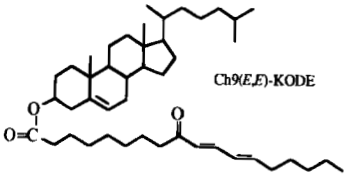
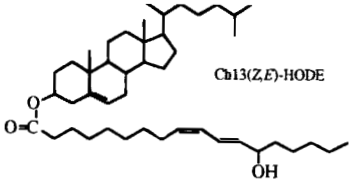
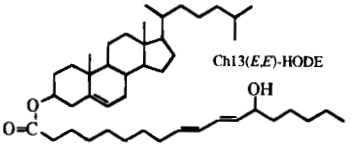
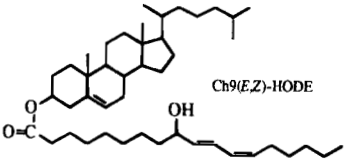
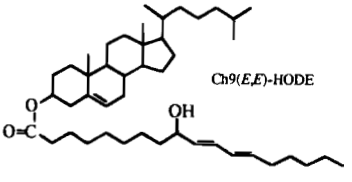
FIGURE 4 Representative ultraviolet spectra Ch18:2=O (A) and Ch18:2-OH (B) isomers separated by NP-HPLC (see Fig. 2). UV spectra of the individual eluting Ch18:2=O (compounds 2–5) and Ch18:2-OH (compounds 7, 9, 11, and 12) were recorded on-line at approximately mid ascending, maximal, and mid descending peak heights using the diode array detector. A, maximal UV absorption for the individual Ch18:2=O isomers 2, 3, 4, and 5 were 273, 268, 274 and 268 nm, respectively. B, maximal UV absorption for the individual Ch18:2-OH isomers 7, 9, 11, and 12 were 234, 230, 234 and 230 nm, respectively.

peaks (not shown). Peak 5 of the first NP-HPLC gave rise to a single, 270 nm absorbing peak when solvent B was used (not shown). Re-injection of the isolated peaks 2′, 3′, 4′ and 5 onto NP-HPLC (solvent A or solvent B) gave single peaks, indicating that these compounds were pure, and suitable for subsequent MS analysis. Peaks, 7, 9, 11, and 12 purified once using the NP-HPLC (solvent A) gave single peaks on chromatography using solvent B, and were used for subsequent MS analysis.

Compounds 2′, 3′, 4′ and 5 (conjugated dienones, λ_{max} 270 nm) all showed quasi molecular ions $(M + 1)^+$ at 663 by chemical ionization (methane) (Table I) and M^+ at 662 by electron impact (not shown), in agreement with the introduction of a keto group into Ch18:2. The major fragments were observed at m/z 369 (base peak) and 295 (Table I), corresponding to protonated anhydrocholesterol and protonated oxo-octadecadienoic acid, respectively. This suggests that oxygen was present in the fatty acid rather than cholesterol moiety, and this is supported further by the major fragment at m/z 295. From the MS and UV data shown here, and based on analogy with the retention times of previously observed isomers of oxo-octadecadienate,[11] we conclude that the components 2′, 3′, 4′ and 5 of 'plaque Ch18:2[O(H)]' are isomers of cholesteryl oxo-octadecadienoate (Ch-KODE in Table I), possibly cholesteryl 13-oxo-9*Z*,11*E*-, 13-oxo-9*E*, 11*E*, 9-oxo-10*E*,12*Z*-, and 9-oxo-10*E*,12*E*-octadecadienoate, respectively. We note however, our data do not allow unequivocal determination of the position of the keto group and the geometry of the conjugated double bonds in the four isomers of Ch18:2 = O.

Compounds 7, 9, 11 and 12 (conjugated dienes, λ_{max} 234 nm) all showed molecular ions $(M + 1)^+$

TABLE I Chemical ionization (methane) mass spectra of hydroxy- and keto- derivatives of cholesteryl linoleate

Compound	Fragmentation Pattern [m/z (relative intensity)]	Probable Structure
2'	663 (24), 648 (5), 623 (3), 601 (16), 577 (16), 575 (22), 550 (20), 521 (16), 507 (4), 495 (7), 475 (1), 417 (1), 411 (1), 385 (1), 369 (100), 353 (9), 323 (4), 295 (53), 277 (3), 255 (2), 117 (12)	 Ch13(*Z,E*)-KODE
3'	663 (10), 650 (5), 648 (5), 626 (4), 623 (4), 599 (18), 575 (17), 549 (18), 524 (8), 521 (6), 507 (16), 495 (5), 475 (8), 411 (5), 385 (2), 369 (100), 353 (10), 295 (19), 277 (1), 255 (5), 117 (15)	 Ch13(*E,E*)-KODE
4'	663 (6), 650 (1), 607 (2), 648 (1), 577 (4), 549 (4), 547 (4), 532 (5), 505 (4), 491 (4), 475 (12), 385 (2), 369 (100), 353 (9), 295 (26), 277 (2), 117 (12)	 Ch9(*E,Z*)-KODE
5	663 (9), 648 (2), 603 (3), 577 (3), 552 (1), 549 (1), 521 (1), 507 (1), 385 (1), 369 (100), 353 (9),323 (4), 295 (50), 277 (3)	 Ch9(*E,E*)-KODE
7	665 (2), 664 (3), 648 (14), 647 (6), 646 (12), 632 (4), 608 (3), 577 (7), 549 (8), 523 (7), 495 (5), 467 (5), 439 (3), 411 (4), 385 (2), 369 (100), 353 (8), 313 (4), 297 (1), 279 (3)	 Ch13(*Z,E*)-HODE
9	665 (6), 648 (12), 647 (5), 646 (10), 632 (4), 607 (4), 605 (4), 603 (4), 577 (13), 550 (13), 535 (9), 524 (9), 521 (9), 507 (4), 495 (4), 467 (4), 439 (4), 411 (3), 385 (46), 369 (100), 353 (7), 313 (3), 297 (1), 279 (3)	 Ch13(*E,E*)-HODE
11	665 (1), 664 (2), 648 (13), 647 (4), 646 (10), 632 (4), 608 (3), 578 (4), 549 (4), 524 (3), 521 (3), 385 (8), 369 (100), 353 (6), 313 (3), 297 (1), 279 (2)	 Ch9(*E,Z*)-HODE
12	665 (1), 664 (1), 648 (7), 647 (3), 646 (6), 632 (2), 603 (3), 577 (5), 549 (4), 524 (2), 496 (1), 467 (1), 385 (1), 369 (100), 353 (6), 313 (1), 297 (1), 279 (3)	 Ch9(*E,E*)-HODE

at 665 by chemical ionization (Table 1) and M^+ at 664 by electron impact (not shown), in agreement with the introduction of a hydroxy group into Ch18:2. In each case, the loss of OH from the $(M + 1)^+$ and loss of H_2O from the $(M + 1)^+$ and M^+ gave rise to a group of fragments with m/z at 646, 647 and 648. In contrast to Ch18:2 = O which gave strong peaks for acid fragments, the hydroxy analogs showed only weak ions at 297 for the protonated hydroxy octadecadienate with derivative ions at 279 corresponding to the loss of water (Table I). Again, the mass spectral data are consistent with addition of oxygen to the fatty acid rather than the cholesterol moiety of the molecule, clearly identifying this group of isomers as Ch18:2-OH. This was supported further by mass spectra of authentic Ch13(*Z,E*)-HODE and Ch9(*E,Z*)-HODE which produced the same molecular ions and fragmentation pattern (not shown).

DISCUSSION

A central observation in this study is that the previously described 'Ch18:2[O(H)]' group of compounds[3] is constituted predominantly of cholesteryl esters in which the octadecadienoic acid moiety is oxidized in the form of hydroxy- and oxo-derivatives while the cholesterol residue is not oxidized. As 'Ch18:2[O(H)]' is not detectable in extracts of control arteries,[3] human plaque therefore specifically contains these oxidized fatty acids esterified to unoxidized cholesterol. Other studies have shown that, in addition, human plaque also contains free and esterified oxidized cholesterol (see ref. 3).

Several groups have previously identified different forms of hydroxy- and oxo-octadecadienate as significant components of human atherosclerotic plaque (see e.g. refs. 11–14). Their analyses largely focussed on the free fatty acids obtained by saponification of the samples and therefore did not address the amounts of esterified oxidized fatty acids, although the studies of Brooks and colleagues (12–13) were very detailed and provided substantial molecular characterization. By contrast, our analyses used plaque lipids without saponification, in their native, esterified forms. This provides additional useful information, since esterification is an important function of foam cells in plaque (reviewed refs. 1–2). The only data in the work of Brooks et al. concerning esters was obtained by TLC, and the compounds of interest were not well resolved in comparison with that achieved now by HPLC, and were not fully characterized as esters. In contrast, their subsequent GC analysis concerned the hydrolyzed components, after saponification. Both approaches, avoiding or including saponification, have advantages and disadvantages, but in order to understand the role of sterol ester metabolism in plaque, our approach is one essential component. Together, our previous[3] and present findings, while supporting the above-mentioned previous studies, demonstrate that in advanced human lesions up to 30% of linoleate in Ch18:2 is oxidized and present as either hydroxy-, keto-, or hydroperoxy-derivatives.

We separated Ch18:2-OH and Ch18:2=O using a combination of RP- and NP-HPLC methods. In the case of Ch18:2=O, two separate NP-HPLC methods were required following RP-HPLC to separate the pure compounds, highlighting the need for extensive chromatography to separate individual oxidation products present in a complex matrix such as advanced human atherosclerotic plaque. Four isomers of each Ch18:2-OH (single absorbance peak around 234nm) and conjugated Ch18:2=O (single absorbance peak around 270nm) were separated and identified by means of co-migration with authentic standards (where available) and their optical properties. The mass spectra confirmed the presence of the characteristic cholesterol and oxidized octadecadienate fragments, and clearly distinguished between the hydroxides and the oxones (Table I). Together, this allowed assignment of compounds 7, 9, 11, and 12 in Figure 3 to Ch13(*Z,E*)-HODE, Ch13(*E,E*)-HODE, Ch9(*E,Z*)-HODE, and Ch9-

(*E,E*)-HODE, respectively. Thus, the combination of RP-HPLC [4] with NP-HPLC (solvent A) described here is suitable for the separation of these four isomers of Ch18:2-OH, although the methods do not distinguish between the S- and R-enantiomers. The latter separation requires either chiral HPLC[15] or chiral gas chromatography of hydrogenated lipids,[13] both of which methods are available for free, but not for esterified hydroxy fatty acids. The combination of RP-HPLC with two consecutive NP-HPLC (solvent A followed by solvent B) also allowed baseline separation of four isomers of Ch18:2=O, the precise regio- and stereo-specificity of which requires further characterization.

Our findings have potentially interesting implications and applications for the assessment of the mechanism(s) of lipid oxidation occurring during atherogenesis. At present, it is not clear whether these oxidations occur largely extra- or intracellularly, are cell-mediated or cell-independent, or mediated by radical or enzymic oxidants. The present and previous[3] findings demonstrate that in advanced plaques up to 30% of linoleate in Ch18:2 is present in oxidized forms. The determination of the corresponding oxidized fraction in unesterified linoleate could give useful information. If it were much smaller than that of the esterified fatty acid, oxidation might take place extracellularly within lipid-protein particles (however modified from plasma lipoproteins), because during re-esterification oxidized linoleate would face overwhelming competition from non-oxidized linoleate. Such an oxidation might of course still be cell-mediated, and perhaps operative at the cell surface.

A 15-lipoxygenase-mediated formation of oxidized fatty acids[15–18] might be expected to be distinguished from a non-enzymic, radical oxidation by a high proportion of regio- and stereo-specific products, particularly Ch13(S)-9*Z*, 11*E*-HODE and/or its corresponding hydroperoxide. While it was not the aim of the present study to precisely quantitate the various isomers of Ch18:2-OH (or Ch18:2=O), our results do not indicate a highly 15-lipoxygenase specific oxidation product pattern, as Ch13(*Z,E*)-HODE, (Ch13(*E,E*)-HODE), Ch9(*E,Z*)-HODE and (Ch9-(*E,E*)-HODE) were all present to comparable extents in 'plaque Ch18:2[O(H)]'. These findings are in agreement with studies on *post mortem*,[11,13] and freshly obtained human samples,[15] but potentially differ from results of Folcik and co-workers[18] who reported *S* to *R* enantiomer ratios of 1.12–1.49 for H(P)ODE isolated from atherosclerotic plaque. The fact that the oxidation proceeds beyond the hydroxide to the keto compounds may be due to either enzymic[19] or non-enzymic reactions. The difficulty in interpreting these results further is that several different lipoxygenases may be active *in vivo*, the specificity of which (notably 12-lipoxygenase) in several complex biological systems is less restricted than might be expected from *in vitro* studies with defined substrates (see e.g. ref. 20). Furthermore, an initial enzymic and specific oxidation may sometimes be followed by a non-specific process, facilitated by the hydroperoxides produced by the enzymic steps.[15,21] In each case, the complex product distribution cannot be predicted readily, and hence an involvement of lipoxygenase(s) cannot be ruled out immediately. Further studies on the quantities and/or stereochemistry of Ch18:2-OH, particularly those present at the various stages of atherogenesis are needed (*cf. e.g.* ref. 22 *vs* ref. 15 for the situation in cholesterol-fed rabbits).

Despite these complications in data interpretation, the separation of the (*E,E*) from the (*Z,E*) and (*E,Z*) isomers of Ch18:2-OH described here may provide useful insight into the mechanism of *in vitro* and *in vivo* lipid peroxidation, particularly when analysis of plaque homogenate is combined and compared with that of plaque lipoproteins. For example, it is well established that during the non-enzymic oxidation of polyenoic fatty acids the presence of α-tocopherol causes exclusive production of the *Z,E* and *E,Z* conjugated diene monohydroperoxides,[23] whilst effectively suppressing the formation of (*E,E*) isomers.[24] This is

also the case for fatty acids and cholesteryl esters in LDL oxidized by peroxyl radicals[25] and recombinant human 15-lipoxygenase,[26] respectively. The fact that advanced human plaque appears to contain comparable amounts of *trans,trans* and *cis,trans* isomers of Ch18:2-OH, suggests that significant lipid peroxidation may have occured in the absence of accessible α-tocopherol (or other phenols), although more work is required to verify this. Because such advanced lesions contain, on average, normal levels of α-tocopherol per polyenoic acid,[3] this could indicate that intimal lipid oxidation occurred separated either physically (perhaps in microenvironments) or in time (different developmental stages) from the presence of vitamin E and other hydrogen-donating antioxidants. It will be interesting to test the relative occurence of *trans,trans* vs *cis,trans* isomers of Ch18:2-OH in freshly obtained lesion lipoproteins, as this may help in directly assessing the potential relevance of tocopherol-mediated peroxidation of LDL lipids[25] and its inhibition[26] in atherogenesis.

Another question for future studies is also whether these oxidized cholesterol-fatty acids may be generated in man in circumstances other than atherogenesis. Little data seems to pertain to this, although fatty acids are known minor components of the diet and of tissues of some other species. This also raises the question of possible dietary origin of oxidized fatty acids (see *e.g.*, ref. 29). Furthermore, the biological activities of these fatty acids, free or esterified, have not been studied in detail. Clearly, these areas are worthy of further study.

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