Femtomole analysis of 9-oxononanoyl cholesterol by high performance liquid chromatography[1]

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Abstract **9-Oxononanoyl cholesterol, a cholesterol core-aldehyde formed during lipoprotein oxidation, was recently identified in advanced human atherosclerotic lesions. Here we present a rapid and sensitive HPLC method for 9-oxononanoyl cholesterol analysis. 9-Oxononanoyl cholesterol was converted to the corresponding fluorescent decahydroacridine derivative by reaction with 1,3-cyclohexanedione. The derivatives formed were purified by solid-phase extraction on C-18 columns, separated by reversed phase HPLC with isocratic elution, and detected by their fluorescence. Decahydroacridine derivatives of 9-oxononanoyl cholesterol were stable for at least 160 h. The limit of quantitation of the method presented is at the low (≈50) femtomole level, with an absolute limit of detection (signal: noise = 6) of 15 fmol. Intra-assay variation was ≤5%, while inter-assay variations were between 5 and 15%, depending on the concentration of the analyte. Standard curves were linear over nearly three orders of magnitude (50 fmol–12.5 pmol). 9-Oxononanoyl cholesterol proved to be the major cholesterol core-aldehyde formed during t-BuOOH/$FeSO_4$ oxidation of cholesteryl linoleate and Cu^{2+}-induced LDL oxidation, findings confirmed by atmospheric pressure chemical ionization–mass spectrometry. Analysis of lipid extracts obtained from advanced human atherosclerotic lesions revealed the presence of 9-oxononanoyl cholesterol in all tissue samples analyzed (28 ± 14 μmol/mol cholesterol, n = 9) despite the presence of α-tocopherol (4 ± 1.2 mmol/mol cholesterol, n = 9).**—Karten, B., H. Boechzelt, P. M. Abuja, M. Mittelbach, K. Oettl, and W. Sattler. **Femtomole analysis of 9-oxononanoyl by high performance liquid chromatography. *J. Lipid Res.* 1998.** 39: **1508–1519.**

Supplementary key words LDL • atherosclerosis • plaque lipids • reactive aldehydes • lipid peroxidation • core-aldehydes

Oxidation of low density lipoproteins (LDL) is now commonly implicated as an initiator of atherosclerosis (1). Depending on the nature of the oxidant, LDL oxidation might be a sequential process, with oxidation of the lipid moiety preceding the oxidation/modification of the apolipoprotein domain (2), an effect that can ultimately result in the uncontrolled uptake of these lipoproteins by cells (3). Lipid peroxidation leads to the formation of a broad array of different products with diverse and powerful biological activities, among them are a variety of different aldehydes (4). The primary products of lipid peroxidation, lipid hydroperoxides (5), undergo carbon–carbon bond cleavage in the presence of transition metals giving rise to the formation of short chain, unesterified aldehydes (6, 7) and aldehydes still esterified to the parent lipid, termed core-aldehydes (8–11). The properties of the aliphatic, unesterified aldehydes were extensively investigated (12) while the properties of core-aldehydes have received much less attention.

The formation of cholesterol core-aldehydes during copper-mediated oxidation of LDL has been reported by Kamido et al. (8–10), and their presence in atherosclerotic plaque material has been shown, but not quantified (13). As cholesterol core-aldehydes represent a class of cholesterol ester-derived secondary lipid peroxidation products, they might be interesting marker molecules to monitor oxidation in the hydrophobic lipid domain of lipoproteins. The physiological role of these aldehydic cholesteryl esters is presently not clear. In analogy to cholesteryl esters, phospholipid oxidation can also result in the formation of core-aldehydes with the aldehydic group in the *sn*-2 position. These hydrophilic core-aldehydes have been identified in lipid extracts from atherosclerotic plaques (14) as well as in minimally oxidized LDL (15) and in the plasma of burn patients (16). Due to their structural similarity to the platelet activating factor (PAF) (reviewed in: ref. 17) these oxidized phospholipids can promote platelet aggregation (18), neutrophil activation (19), and leukocyte adhesion (20).

Abbreviations: apCI, atmospheric pressure chemical ionization; BHT, tert-butyl hydroxytoluene; Ch18:2, cholesteryl linoleate; CHD, 1,3-cyclohexanedione; DNPH, dinitrophenyl hydrazine; GC–FID, gas chromatography–flame ionization detection; LC–MS, liquid chromatography–mass spectrometry; LDL, low density lipoprotein; LPO, lipid peroxidation; RP-HPLC, reversed phase high-performance liquid chromatography; SPE, solid-phase extraction; t-BuOOH, tert-butylhydroperoxide.

[1] This paper is dedicated to the memory of Prof. Hermann Esterbauer who initiated this project.

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Due to their powerful physiological activities, the analysis of aldehydic lipid peroxidation products has received much attention. Aliphatic aldehydes can be converted to fluorescent decahydroacridine derivatives by reaction with 1,3-cyclohexanedione (CHD) in the presence of ammonium sulfate (21) or ammonium acetate (22). These derivatives have good chromatographic properties and excellent fluorescence yields. The separation of a variety of different aliphatic aldehydes up to decanal with a gradient solvent system has been reported (23, 24).

The most frequently used technique for the detection of the whole spectrum of aliphatic aldehydes is the formation of corresponding dinitrophenyl hydrazine (DNPH)-derivatives (25). However, analysis of DNPH-derivatives is time-consuming and tedious, as this procedure involves a combination of preparative thin layer chromatography, elution of bands and subsequent analysis of pre-separated aldehyde classes by HPLC (26).

In analogy to aliphatic aldehydes, analysis of cholesteryl ester- and 7-ketocholesteryl ester core-aldehydes as the corresponding dinitrophenyl hydrazones has been reported (11). Identification of the DNPH derivatives was accomplished by LC-MS and 9-oxononanoyl cholesterol was found to be the most prominent cholesteryl ester core-aldehyde in copper-oxidized LDL (8–10). Recently, several cholesteryl ester core-aldehydes were found in human atherosclerotic lesions after derivatization with DNPH. Identification was carried out by means of HPLC–electrospray ionization–MS (13).

In order to circumvent the time-consuming DNPH-derivatization, we have developed a rapid and highly sensitive method for the analysis of 9-oxononanoyl cholesterol as a fluorescent decahydroacridine derivative by reversed phase HPLC and isocratic elution. The method presented allows the detection of 9-oxononanoyl cholesterol in biological specimens at the low femtomole level and requires only simple pre-purification of the crude derivatization mixture on C-18 solid-phase extraction columns. The method presented was used to quantitate 9-oxononanoyl cholesterol in oxidized cholesteryl linoleate, copper-oxidized LDL, and advanced human atherosclerotic lesions.

MATERIALS AND METHODS

Chemicals

Chemicals were obtained from Sigma (Vienna, Austria) or Merck (Vienna, Austria) unless indicated otherwise. 9-Oxononanoyl cholesterol was synthesized as described in ref 27. Solid-phase extraction cartridges were obtained from Isolute (ICT, Vienna, Austria). 1,3-Cyclohexanedione (stabilized with 3% w/w NaCl) was purchased from Sigma and used without further purification. Ethanol used during the derivatization procedure was distilled over DNPH and ortho-phosphoric acid to remove contaminating carbonyls. PD-10 size exclusion columns were obtained from Pharmacia (Uppsala, Sweden).

Isolation and oxidation of LDL

Plasma from overnight fasted normolipemic donors was obtained by venipuncture with EDTA as anticoagulant (1 mg/ml). LDL was prepared using single-step discontinuous gradient ultracentrifugation in a Beckman NVT65 rotor (60,000 rpm, 2 h, 10°C) as described (28). LDL was recovered by direct syringe aspiration, filtered through a 0.2-μm sterile filter (Corning Glass Works, Corning, NY), transferred to evacuated glass vials (Techne Vial, Mallinckrodt-Diagnostica, Vienna, Austria), and stored under argon at 4°C in the dark.

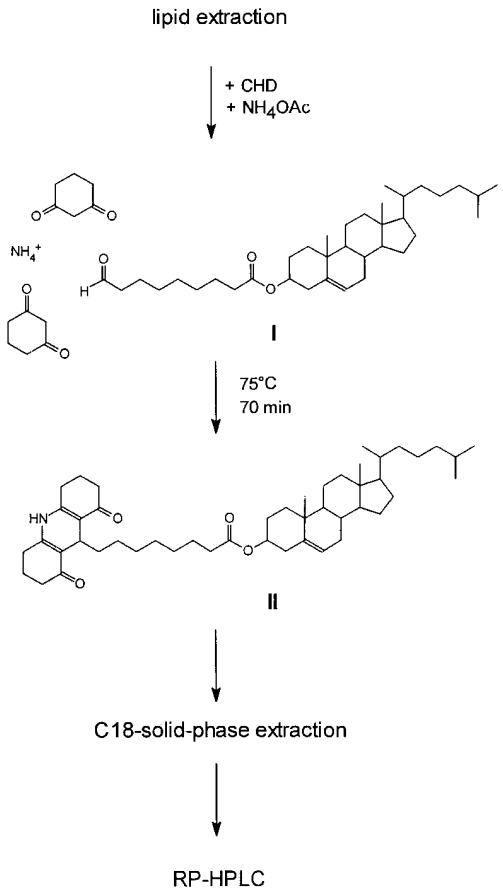
Prior to oxidation experiments, LDL was desalted by size exclusion chromatography on PD-10 columns. LDL oxidation was initiated by the addition of 1.6 μm $CuSO_4$ to a solution containing 0.25 mg/ml LDL in phosphate-buffered saline (PBS) as described (29). The progress of oxidation was followed by monitoring the formation of conjugated dienes as the increase in absorption at 234 nm. Aliquots of the reaction mixture were removed at the time points indicated and EDTA (20 μl; 100 mg/ml) and butyl hydroxytoluene (BHT, 20 μl; 2 mg/ml in EtOH) were added. The samples were kept at −20°C under argon until further analysis.

Preparation of the 1,3-cyclohexanedione (CHD) reagent

To a solution of 1,3-cyclohexanedione in ethanol (200 mg/ml) an equal volume of ammonium acetate (60%, w/v) in acetic acid (20%, v/v) was added. The derivatization reagent was kept at 4°C in the dark.

Preparation of aldehyde-CHD derivatives

The procedure for the preparation of fluorescent CHD-derivatives of 9-oxononanoyl cholesterol, oxidized cholesteryl linoleate, or oxidized LDL is outlined in Scheme 1. To estimate



Scheme 1. Sample work-up and derivatization of 9-oxononanoyl cholesterol with 1,3-cyclohexanedione. I: 9-oxononanoyl cholesterol; II: 9-(1,2,3,4,5,6,7,8,9,10-decahydroacridine-1,8-dione)-nonanoyl cholesterol.

the recovery of 9-oxononanoyl cholesterol from an LDL matrix, the following experiments were performed. LDL solutions (1.3 mg/ml in PBS) were spiked with increasing amounts of 9-oxononanoyl cholesterol (10 μl of a $CHCl_3$–MeOH solution 2:1, v/v) resulting in final analyte concentrations of 0.01 to 10 μm. Spiked LDL samples (200 μl) were shaken under argon for 20 min at 25°C. One ml of water was added to the core-aldehyde containing LDL sample and the mixture was extracted three times with $CHCl_3$–MeOH 2:1 (v/v). The organic extracts were combined and evaporated under vacuum. The dried residue was redissolved in 200 μl $CHCl_3$–MeOH 2:1 (v/v). Forty μl of the extracts was transferred to an Eppendorf tube, combined with 20 μl of the CHD-reagent, and incubated at 75°C for 70 min. As control, 9-oxononanoyl cholesterol at the concentrations indicated above was added to PBS and carried through the same extraction and derivatization procedure.

The optimum time and temperature for CHD derivatization was assessed as follows. Three ml of an LDL lipid extract (1 mg/ml total lipid) was spiked with 30 μl of 9-oxononanoyl cholesterol (100 μm). For each analysis, 40 μl of the spiked lipid extract was mixed with 20 μl CHD-reagent (100 mg/ml) and incubated for 30–180 min at the indicated temperatures.

Optimum CHD concentrations for core-aldehyde analysis in copper-oxidized LDL were determined by derivatization of the

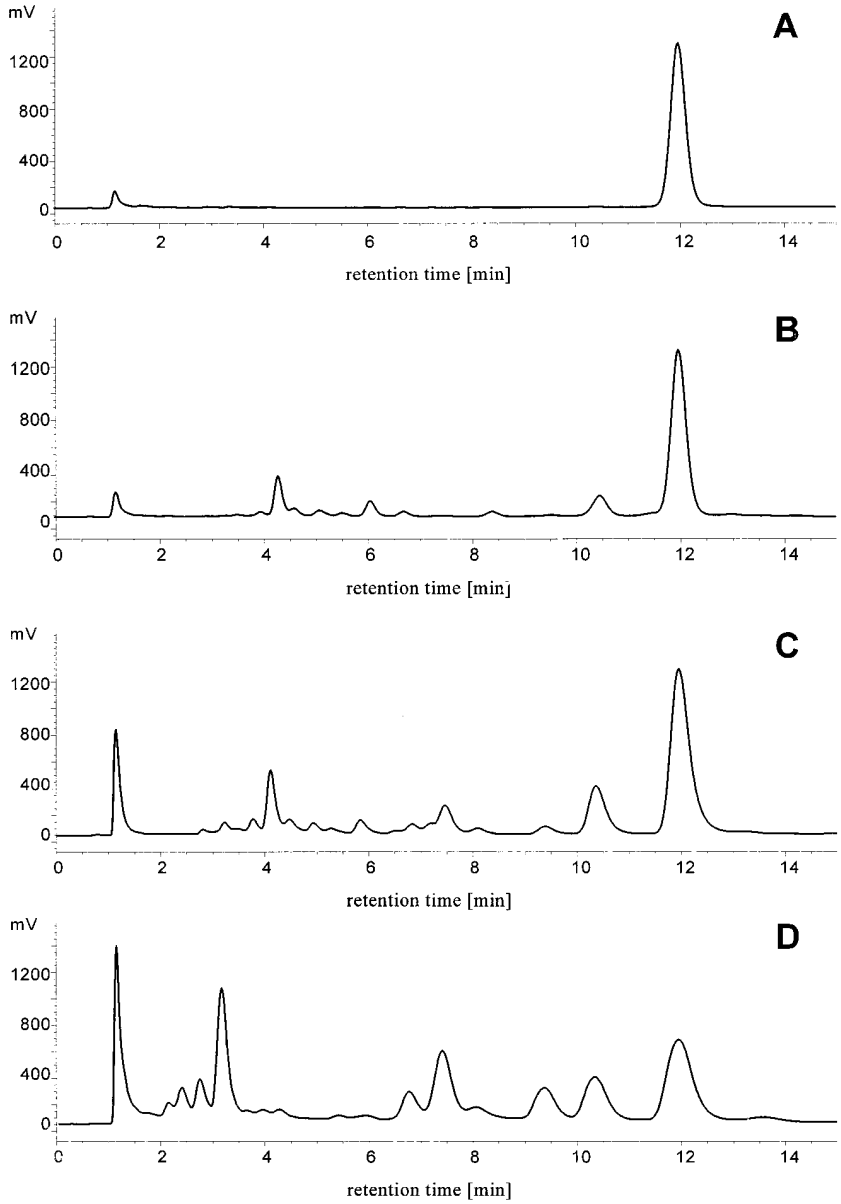


Fig. 1. HPLC traces of 9-oxononanoyl cholesterol (A), oxidized Ch18:2 (B), oxidized LDL (C), and atherosclerotic lesion (D) lipid extracts. Forty μl of standard (3 μm 9-oxononanoyl cholesterol, A) and lipid extracts of t-BuOOH/Fe^{2+} oxidized Ch18:2 (40 μg/ml Ch18:2, B), 3 h Cu^{2+}-oxidized oxLDL (0.2 mg/ml total lipid, C) and atherosclerotic plaque (sample 2, 72 mg/ml total lipid, D) were derivatized with 20 μl CHD-reagent (100 mg CHD/ml, 75°C for 70 min). Excess derivatization reagent was removed by solid-phase extraction on Isolute MF C-18 cartridges and the decahydroacridine derivatives were analyzed by reversed phase chromatography (Ultrasphere ODS, 4.6 × 75 mm) with acetonitrile–methanol–2-propanol 68:17:15 (v/v/v; 1 ml/min; 25°C) as the mobile phase and fluorescence detection at 366/455 nm ex/em.

lipid extracts of Cu^{2+}-oxidized LDL (3 h; as described above). Forty μl of the lipid extracts was derivatized with 20 μl of the CHD reagent containing final CHD concentrations of 20 to 200 mg/ml.

Solid-phase extraction (SPE) of the crude derivatization mixture was performed on Isolute MF C18 cartridges (100 mg). The columns were equilibrated with acetonitrile–water 9:1 (v/v), followed by the application of the derivatization mixture in 1 ml of acetonitrile–water 9:1 (v/v). The cartridge was then washed with 2 ml acetonitrile–water 9:1 (v/v) followed by 500 μl acetonitrile and 100 μl 2-propanol. The CHD-derivatives of cholesteryl ester core-aldehydes were then eluted with 2.5 ml $CHCl_3$. The $CHCl_3$ extract was dried in a SpeedVac concentrator (Bachofer, Reutlingen, Germany) and the dry residue was redissolved in 50 μl $CHCl_3$–MeOH 2:1 (v/v) and 150 μl acetonitrile–methanol–2-propanol 68:17:15, (v/v/v) as the mobile phase. Recovery of 9-oxononanoyl cholesterol was measured by incubating standards with CHD-reagent and peak area comparison after SPE work-up or chloroform extraction of the fluorescent derivative.

HPLC analysis of the 9-oxononanoyl cholesterol CHD derivative

The decahydroacridine derivatives were separated on a 3-μm Ultrasphere ODS column (4.6 × 75 mm, Beckman, Vienna, Austria). The isocratic solvent system consisted of acetonitrile–methanol–2-propanol 68:17:15, (v/v/v; 1 ml/min; 25°C). UV absorption was monitored at 220 nm and fluorescence at 366/455 nm (excitation/emission). All separations were performed on an HP1100 HPLC (Hewlett Packard, Palo Alto, CA) equipped with fluorescence and UV detector (Jasco, Vienna, Austria). Concentrations of the analyte were calculated by peak area comparison with external standards of known concentration.

Preparative HPLC separations were performed on an Ultrasphere ODS column (5 μm material; 10 × 250 mm; Beckman, Vienna, Austria). For isolation of the CHD-derivatives, the solvent system described above was used at a flow of 5 ml/min.

Atmospheric pressure chemical ionization–mass spectrometry

MS^n analyses were performed using an LC–MS system consisting of a Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA) and a flux Rheos 4000 binary gradient pump (Munich, Germany). The mass spectrometer was equipped with an apCI interface. Nitrogen was used as nebulization gas. The vaporizer and the capillary temperature were 450° and 150°C, respectively. Discharge current was 5 μA, capillary voltage and tube lens offset were 12 and 25 V. Samples were introduced by direct flow injection via a 5-μl sample loop. Methanol at a flow rate of 0.1 ml/min was used as the mobile phase. Mass spectra were obtained in the positive ion mode with a scan range from 100 to 800 amu. For the MS^2 (tandem in time) experiment the mother ion (*m/z* 728) was selected with an isolation width of 5 amu. A collision energy of 25% was found to cleave the mother ion completely. In the MS^3 experiment the collision energies were 25 and 20% for the mother and the first daughter ion. In the MS^4 experiment the mother, first- and second-daughter ions were generated with collision energies of 25, 20, and 25%, respectively.

Association of 9-oxononanoyl cholesterol with LDL

For each time point of the incubation, 2 μl of 1 mm 9-oxononanoyl cholesterol in ethanol was mixed with 30 μl PBS and briefly sonicated. Then 50 μl LDL in PBS was added to a final concentration of 1.3 mg/ml. After incubation for the time indicated at 37°C under argon, 1 ml distilled water was added and the aliquots were extracted twice with 2 ml of $CHCl_3$–MeOH 2:1 (v/v). The organic layer was evaporated under vacuum and the residue was redissolved in 200 μl of $CHCl_3$–MeOH 2:1 (v/v). Forty μl of this solution was derivatized with 20 μl CHD reagent and analyzed for 9-oxononanoyl cholesterol content as described above. LDL or 9-oxononanoyl cholesterol alone incubated in PBS exactly as described above served as controls.

Assessment of oxidation parameters during Cu^{2+}-initiated LDL oxidation

During the time course of oxidation of LDL, the formation of 9-oxyononanoyl cholesterol was followed by HPLC, while the consumption of fatty acids and the formation of oxysterols was analyzed by gas chromatography. At the time points indicated, 200 μl of the oxidation mixture was diluted with 1 ml distilled water and the aliquots were extracted twice with 2 ml of $CHCl_3$–MeOH 2:1 (v/v). The organic layer was evaporated under vacuum and the residue was redissolved in 200 μl of $CHCl_3$–MeOH 2:1 (v/v). Forty μl of this solution was derivatized with 20 μl CHD reagent and analyzed for 9-oxononanoyl cholesterol content as described above. For fatty acid analysis, 2.5 ml of the oxidation mixture (see above) was transferred to tubes containing heptadecanoic acid (50 μg, dried under N_2) as internal standard. Aliquots were then lyophilized, transesterified with BF_3 in MeOH (20%) to the corresponding fatty acid methyl esters, and analyzed by GC-FID (30). Quantification of the individual fatty acids was performed by peak area comparison with the internal standard.

For oxysterol determination, 2.5-ml aliquots of the oxidation mixture were lyophilized, then resuspended in 200 μl water and hydrolyzed at 70°C for 60 min in the presence of 800 μl ethanol and 240 μl 50% KOH. After the addition of 1 ml water, lipids were extracted twice with 600 μl hexane. The organic layers were combined, dried under nitrogen, and converted to the corresponding *o*-trimethylsilyl ether-derivatives with N,O-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethyl chlorosilane (TMS) in acetone. Dried derivatives were redissolved in 75 μl toluene and 1 μl was injected into the GC system (Hewlett Packard, Palo Alto, CA). The lipids were separated on a 25-m Ultrasphere 2 crosslinked column (Chrompack, Middelburg, Netherlands) with an internal diameter of 0.25 mm. The injection port temperature was 280°C and the detector temperature was 300°C. The column temperature was held at 110°C for 2 min, increased to 290°C at a rate of 15°C/min where it was held constant for 21 min (31).

Plaque analysis

Atherosclerotic lesion tissue [Type IV, (32)] was obtained postmortem from the aorta thoracica or aorta abdominalis from nine patients (aged between 39 and 60 years). The plaque material was kept at −20°C under argon for 2 weeks. Lipid extraction was performed as described in ref. 33. The tissue was carefully blotted dry and weighed. Samples of about 0.7 g wet tissue weight were cut into small pieces under argon in 1 ml PBS buffer containing 0.2 mg/ml BHT. The chopped tissue was transferred into a screw-cap glass vial, suspended in 2.5 ml diethyl ether, and shaken under argon on ice for 15 h. After addition of 2 ml MeOH, the extraction was carried on for another 3 h. Samples were then extracted twice with 2 ml hexane. Organic extracts were combined, dried under vacuum, and weighed. The residues were redissolved in 500 μl of $CHCl_3$–MeOH 2:1 (v/v).

For α-tocopherol analysis, 20 μl of the lipid extract was evaporated under nitrogen and redissolved in 100 μl MeOH. Twenty μl of the supernatant was analyzed by HPLC with fluorescence detection (Ex = 292, Em = 335 nm). Separations were performed on an LC-18 column (Supelco, Bellefonte, PA, 25 × 0.46 cm) with methanol as the mobile phase (1 ml/min).

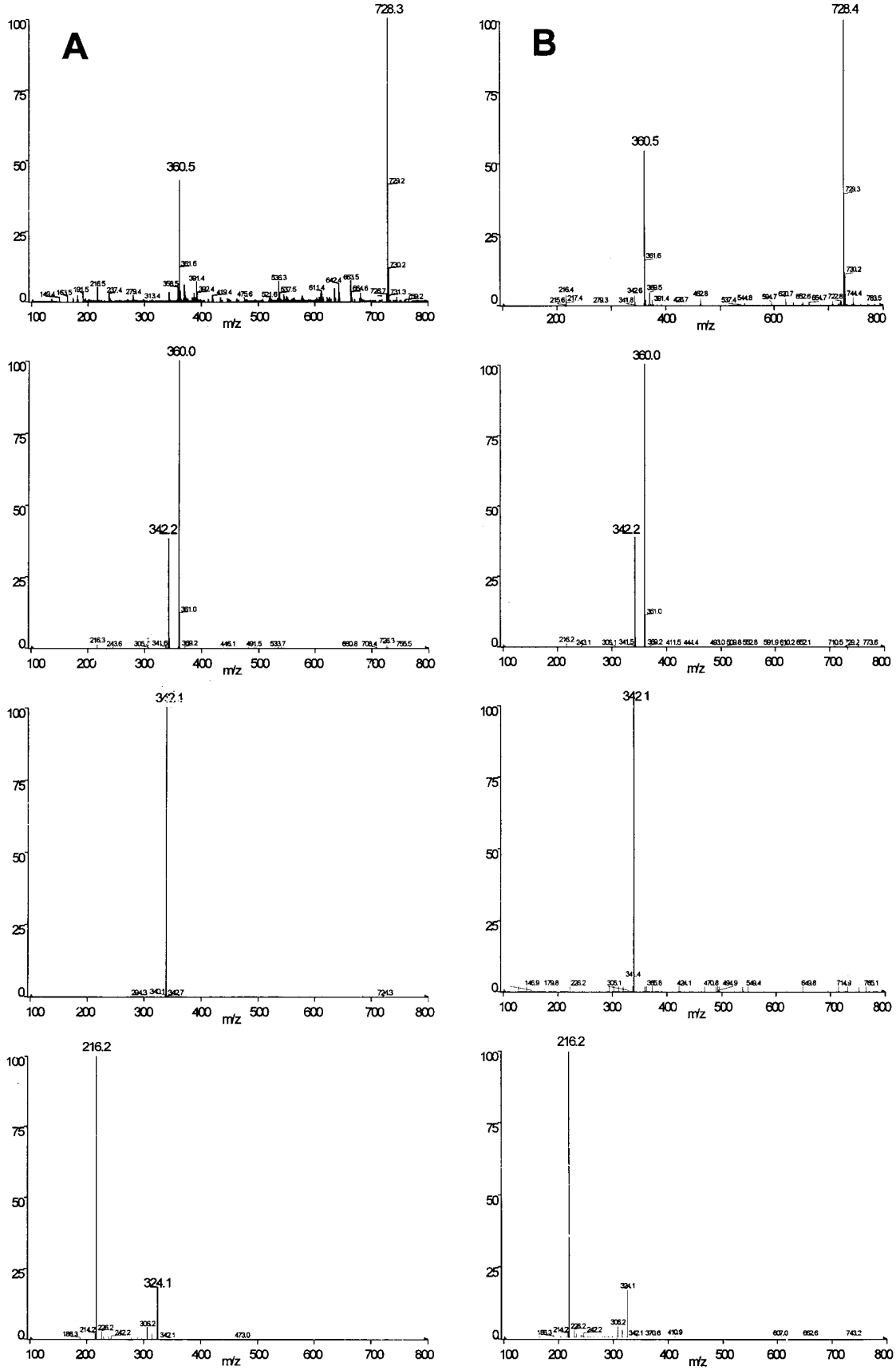


Fig. 2

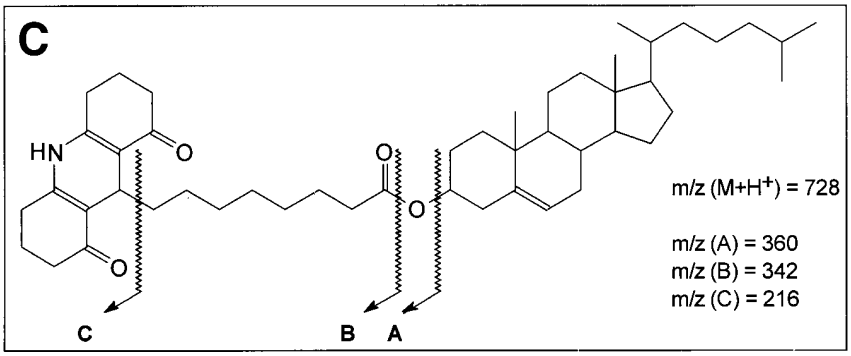


Fig. 2. Comparison of apCI-mass spectra and collision-induced daughter ion formation of 9-oxononanoyl cholesterol (A) and the coeluting peak isolated from the HPLC traces obtained from t-BuOOH/Fe^{2+} oxidized Ch18:2 (B). To identify the peak present in oxidized Ch18:2 coeluting with the 9-oxononanoyl cholesterol standard, app. 5 μg of the coeluting material was manually collected, dried under vacuum, and analyzed by direct flow injection and apCI-MS. C: Proposed fragmentation scheme of 9-oxononanoyl cholesterol during apCI-MS. Cleavage at the sites A–C yields fragments of m/z = 360, 342, and 216, respectively.

Cholesterol and cholesteryl esters in plaque lipid extracts were analyzed by RP-HPLC and UV detection at 210 nm. Twenty μl of the lipid extract was evaporated under nitrogen and redissolved in 10 μl $CHCl_3$–MeOH 2:1 (v/v) and 90 μl methanol–2-propanol 1:1. Twenty μl was separated over a Chromspher 5 C18, column (25 × 0.2 cm, Chrompack, Middelburg, Netherlands), using a solvent system of methanol–2-propanol 1:1, (v/v) at a flow rate of 0.2 ml/min. Quantitation of cholesterol and cholesteryl linoleate (Ch18:2) was performed by peak area comparison with external standards of known concentrations (34). 9-Oxononanoyl cholesterol was determined as described above by derivatizing 40 μl of the lipid extract with 20 μl CHD reagent followed by SPE purification.

RESULTS

Separation and identification of 9-oxononanoyl cholesterol in oxidized cholesteryl linoleate and LDL

Cholesteryl ester core-aldehydes were converted to fluorescent decahydroacridine derivatives by reaction with 1,3-cyclohexanedione in the presence of ammonium acetate and analyzed by RP-HPLC with fluorescence detection as outlined in Scheme 1. **Figure 1A** shows the HPLC trace of the CHD-derivative of a 9-oxononanoyl cholesterol standard. Figures 1B–D show the HPLC traces of oxidized cholesteryl linoleate (t-BuOOH/$FeSO_4$), Cu^{2+}-oxidized LDL and postmortem plaque lipid extracts. To verify the nature of the peaks coeluting with the 9-oxononanoyl cholesterol standard, 5 μg of the coeluting material was manually collected and subjected to apCI–MS analysis. The results of these LC–MS analyses are shown in **Fig. 2A** (9-oxononanoyl cholesterol) and Fig. 2B (oxCh18:2). The structure of the corresponding derivative of 9-oxononanoyl cholesterol and the proposed fragmentation scheme during MS^n are shown in Fig. 2C. apCI analysis of 9-oxononanoyl cholesterol resulted in spectra with an intense $[M+H]^+$ ion at m/z 728 with a minor fragment at m/z 360 (Fig. 2A, B, and C). Collision-induced decomposition of the molecular ion led to the formation of two daughter ions with m/z of 360 and 342. These fragments correspond to decahydroacridine derivatives arising from cleavage of the ester-bond to cholesterol. Collision-induced fragmentation of the ion at m/z 360 resulted in daughter ion formation at m/z 342; subsequent fragmentation of this ion gave rise to an ion at m/z 216. Irrespective of whether the authentic 9-oxononanoyl cholesterol standard or the coeluting peak from the oxCh18:2 lipid extract was analyzed by apCI-MS, we have observed the same fragmentation pattern of the $[M+H]^+$ mother ion m/z 728, indicating that the peaks contain identical analytes,

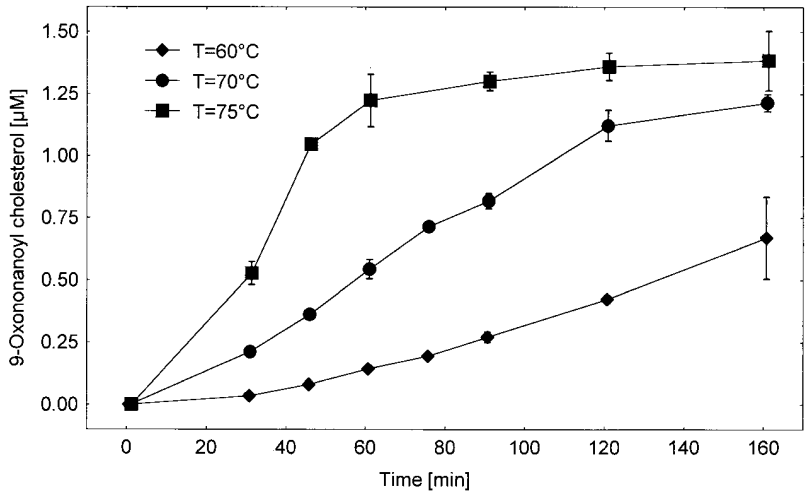


Fig. 3. Effect of derivatization temperature on the formation of decahydroacridine derivatives. LDL lipid extracts (1 mg/ml of total lipid) were spiked with 9-oxononanoyl cholesterol (final concentration 1 μm) and derivatized with CHD at 60°, 70°, and 75°C for the indicated time. Each point represents mean ± SD of triplicate analyses under the conditions described in Fig. 1.

i.e., 9-oxononanoyl cholesterol. Identical spectra were obtained when the coeluting material from oxLDL or plaque lipid extracts was analyzed by LC–apCI–MS (data not shown).

Additional fluorescent compounds with retention times shorter than 9-oxononanoyl cholesterol (Fig. 1) are most probably due to the formation of other, more polar, cholesteryl ester core-aldehydes e.g., 5-oxovaleroyl cholesterol, 8-oxooctonoate cholesterol, or core aldehydes derived from oxysterols. The presence of these compounds in oxLDL or oxidized Ch18:2 has already been described (10, 13). During the present study, identification of these compounds has not yet been attempted.

In the next set of experiments we have optimized the derivatization conditions with respect to time, temperature and reagent concentrations. As can be seen from **Fig. 3**, derivatization yields were highly dependent on the time and temperature at which 9-oxononanoyl cholesterol was converted to the corresponding decahydroacridine derivative. For these experiments, LDL lipid extracts (1mg/ml total lipid) were spiked with 9-oxononanoyl cholesterol (1 μm) and derivatized at 60°, 70°, and 75°C. At the time points indicated, samples were loaded on SPE cartridges and analyzed by HPLC as described above. At 60°C, recovery of the analyte was app. 40% after 160 min of derivatization. At 70°C, recovery was nearly quantitative after 160 min and quantitative after a 70-min incubation at 75°C. These conditions (75°C, 70 min) were thus routinely used for the preparation of the decahydroacridine-derivatives of 9-oxononanoyl cholesterol.

In light of the applicability of the method for core-aldehyde analysis in more complex biological samples (e.g., oxLDL or atherosclerotic plaques), the CHD concentration necessary for optimum derivatization yields in oxLDL lipid extracts was estimated. During these experiments, LDL was oxidized for 3 h at 37°C as described in Materials and Methods, lipids were extracted at the indicated time points, converted to the fluorescent derivatives, and analyzed by HPLC. Forty μl of oxLDL lipid extracts was mixed with 20 μl of CHD reagent (containing 20–200 mg CHD/ml) and kept for 70 min at 75°C. From the results shown in **Fig. 4**, it became evident that CHD concentrations >100 mg/ml did not significantly increase derivatization yields. Therefore, a concentration of 100 mg CHD/ml was routinely used for the derivatization of standards, oxidized cholesteryl esters, oxLDL, and plaque lipid extracts.

Quantitative validation of the methodology

Due to the fact that injection of the crude derivatization mixtures led to huge injection peaks with heavily tailing base lines, a direct comparison of peak areas prior to and after solid-phase extraction was not possible. We have therefore compared the peak areas of standards purified on C-18 cartridges and standards extracted from the derivatization mixture with $CHCl_3$–MeOH 2:1 (v/v). Under these experimental conditions, recovery of the fluorescent decahydroacridine derivative after solid phase extraction was between 98 and 105% at 9-oxononanoyl cholesterol concentrations between 10 nm and 10 μm.

Calibration curves of 9-oxononanoyl cholesterol standard (purified by solid-phase extraction) were established in a concentration range of 0.05 to 12.5 pmol aldehyde injected. As can be seen from **Fig. 5**, the detector response was linear over a concentration range of nearly three orders of magnitude. The upper limit of linearity was not a result of incomplete derivatization but rather caused by the linear range of the detector. Dilution of the samples outside the linear range with mobile phase returned the detector signals back into the linear range of the calibration curve. To further test the recovery of 9-oxononanoyl cholesterol from LDL, increasing amounts of the standard were added to an LDL solution (1.3 mg/ml in PBS), extracted after a 20-min incubation (25°C), derivatized, and analyzed by HPLC. In a separate set of experiments, equivalent concentrations of the standard were added to PBS, derivatized, and analyzed. The areas of the 9-oxononanoyl cholesterol peak obtained from the PBS or LDL matrix are compared in **Fig. 6**. The linear fit of the data showed a slope of 0.97 for the fitted line, indicating quantitative recovery of the aldehyde from the LDL matrix under these conditions.

The limit of quantification at the lowest point of the calibration curve was estimated as 50 fmol injected CHD-de-

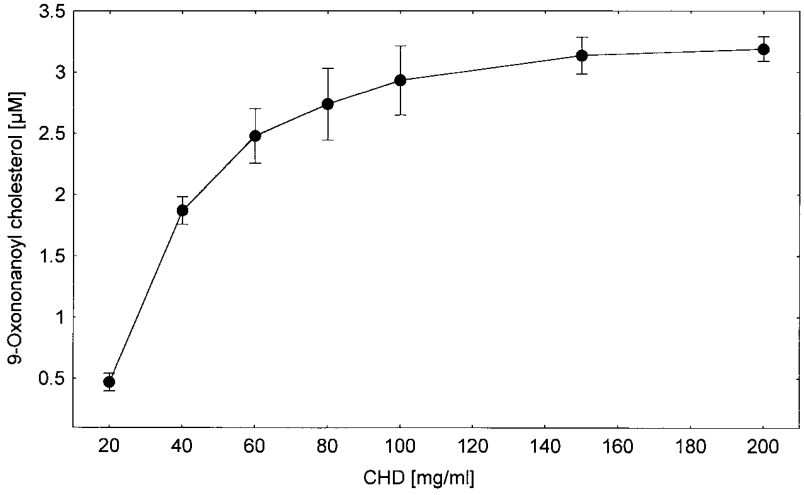


Fig. 4. Effect of CHD concentration on the formation of decahydroacridine derivatives. Lipid extracts (0.2 mg/ml of total lipid, 40 μl) obtained from 3 h Cu^{2+}-oxidized LDL were derivatized with CHD reagent at 75°C for 70 min. Solid-phase extraction and chromatographic conditions as described in Materials and Methods. Each point represents mean ± SD of triplicate analyses under the conditions described in Fig. 1.

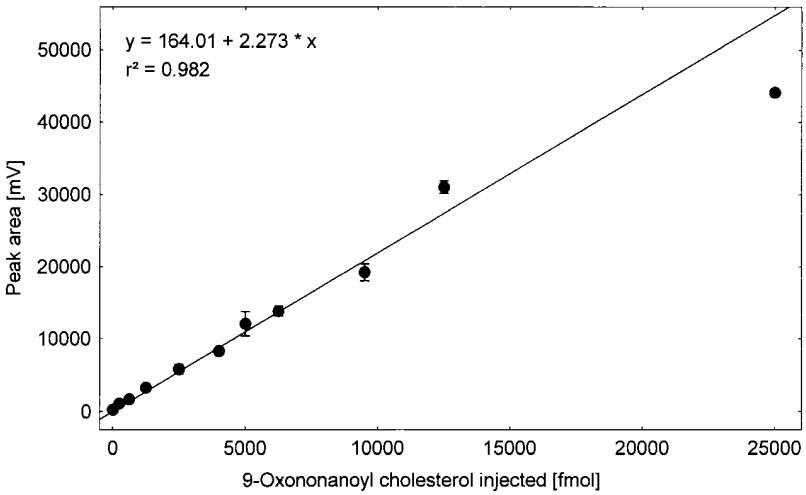


Fig. 5. Linearity of response. 9-Oxononanoyl cholesterol was derivatized at 75°C for 70 min. Excess derivatization reagent was removed by solid-phase extraction and the fluorescent derivative was analyzed as described in Fig. 1.

rivative, permitting analysis of samples containing 9-oxononanoyl cholesterol concentrations as low as 10 nm. The absolute limit of detection at a signal-to-noise ratio of 6:1 was estimated to be 15 fmol decahydroacridine derivative injected. The intra-assay variation was ≈4–5%, while inter-assay variation was about 5–15%, depending on the concentration of the analyte.

In order to test the selectivity of the method presented, epoxides, hydroxides, hydroperoxides, ketones, and native cholesteryl esters as well as native LDL were tested for reaction with the derivatization reagent. Under these experimental conditions no formation of fluorescent compounds was observed. In contrast to these compounds, alkanals, hydroxyalkanals, and other aliphatic aldehydes formed during lipid peroxidation reacted with CHD to form fluorescent derivatives. The latter are, however, removed during the solid-phase extraction procedure in the acetonitrile fraction. This offers the possibility to analyze this aldehydic fraction under separate chromatographic conditions.

To test the stability of the decahydroacridine derivatives of 9-oxononanoyl cholesterol, derivatized samples were kept at room temperature in the dark and analyzed at the time points indicated. The CHD-derivative proved to be stable for as long as 160 h, providing sufficient stability for even extended time-dependent studies (data not shown).

Association of 9-oxononanoyl cholesterol with LDL

In order to determine the reactivity of the cholesteryl ester core aldehyde towards LDL, mixtures of LDL and 9-oxononanoyl cholesterol were incubated at 37°C for up to 24 h. Free aldehyde was quantified after lipid extraction. In the absence of LDL, ≈10% of the aldehyde was lost after 24 h. In the presence of LDL, 9-oxononanoyl cholesterol recovery decreased by 25% after a 2-h incubation, indicating that about 5–6 mol core-aldehyde was trapped per mol LDL. After 24 h, about 50% of the aldehyde was associated with LDL, corresponding to approximately 15–16 mol core-aldehyde per mol LDL (data not shown). ApoB contains 356 mol lysine and 115 mol histidine (2), so that only about 3.4% of potentially reactive amino groups was modified. This relatively small modification rate of apoB did not lead to an altered electrophoretic mobility in agarose gels or to a measurable decrease in reactive amino groups as estimated by the photometric TNBS assay (35) (data not shown).

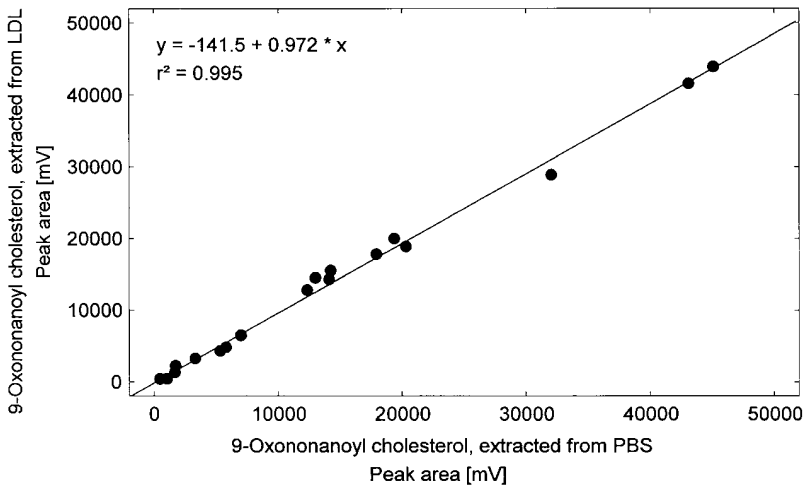


Fig. 6. Recovery of 9-oxononanoyl cholesterol from LDL and PBS. LDL (1.3 mg/ml) was spiked with increasing amounts of 9-oxononanoyl cholesterol, incubated at 25°C for 20 min, extracted with $CHCl_3$–MeOH 2:1, (v/v, 3 times), dried and derivatized (75°C, 70 min). Peak areas obtained from the spiked LDL sample were plotted versus the peak areas obtained from a 9-oxononanoyl cholesterol standard added to PBS at identical concentrations.

Time course of 9-oxononanoyl cholesterol formation during Cu^{2+}-initiated LDL oxidation

In the next set of experiments we analyzed the time-dependent formation of 9-oxononanoyl cholesterol during Cu^{2+}-initiated LDL-oxidation. During this experiment we measured the formation of conjugated dienes, consumption of linoleic acid (18:2), formation of 7-ketocholesterol and 9-oxononanoyl cholesterol (**Fig. 7**). The concentration of linoleic acid was determined after transmethylation of total LDL-lipids, 7-ketocholesterol was analyzed after KOH-hydrolysis of total LDL-lipids (for details, see Materials and Methods). After an apparent lag phase of about 60 min, the formation of conjugated dienes and the consumption of linoleic acid were closely accompanied by the formation of 9-oxononanoyl cholesterol. It is, however, important to note that the maximum rates of 9-oxononanoyl cholesterol formation were delayed by app. 20 min in comparison to diene formation. Maximum diene concentrations were obtained after 2 h, whereas 9-oxononanoyl cholesterol concentrations reached a plateau after 3 h of oxidation, with app. 30 mol core-aldehyde formed per mol LDL. Assuming an initial concentration of 304 nmol Ch18:2/mg LDL (30), ≈3% of Ch18:2 would be converted into 9-oxononanoyl cholesterol. After 5 h of oxidation, the core-aldehyde concentrations started to decrease. Whether this is a result of covalent binding to apoB-100 or oxidation of the cholesterol core of the aldehyde (and thus different chromatographic properties) is presently not clear.

The concentration of 7-ketocholesterol increased throughout the whole period of oxidation without reaching a plateau value. The differences in formation of the compounds are best reflected by a comparison of the maximal rates of reaction (in $mol \cdot min^{-1} \cdot mol\ LDL^{-1}$): $\triangle c/\triangle t_{PUFA} = -4.9$; $\triangle c/\triangle t_{dienes} = 6.0$; $\triangle c/\triangle t_{9\text{-oxononchol}} = 0.30$; $\triangle c/\triangle t_{ketochol} = 0.05$.

Analysis of postmortem lipoid plaque material

In order to test the applicability of the method, we also analyzed lipid extracts obtained from advanced human atherosclerotic lesions for their 9-oxononanoyl cholesterol content. Plaque material was characterized by the amount of extractable lipids, the content of 9-oxononanoyl cholesterol, α-tocopherol, cholesteryl esters, and cholesterol (**Table 1**). The percentage of extractable lipids from these samples ranged from 1 to 10% (w/w). Cholesterol was the most abundant of all lipids with concentrations varying in a range from 0.05 to 0.70 μmol per mg lipid extract. The most abundant cholesteryl ester in the plaque lipids was Ch18:2 with mean concentrations of 0.52 ± 0.18 mol/mol cholesterol. Ch18:1 and Ch20:4 contents were somewhat lower with concentrations of 0.26 ± 0.10 and 0.13 ± 0.04 mol/mol cholesterol, respectively. Despite the presence of α-tocopherol, cholesterol core-aldehydes were present in each of the plaque samples analyzed. The mean concentration of 9-oxononanoyl cholesterol was 28.7 ± 14.2 μmol per mol cholesterol corresponding to 59.3 ± 36.2 μmol per mol cholesteryl linoleate. Mean α-tocopherol concentrations in plaque lipid were 4.0 ± 1.2 mmol per mol cholesterol corresponding to 7.7 ± 4.1 mmol per mol Ch18:2. The α-tocopherol and cholesteryl ester concentrations obtained during our plaque analysis are in relatively good agreement with data reported by Suarna et al. (36).

DISCUSSION

A variety of different derivatization reactions have been described for the analysis of unesterified aldehydes, among them formation of hydrazone or oxime derivatives (26, 37). HPLC analysis of core-aldehydes as DNPH derivatives has been reported by Kamido et al. (9), which, however, seems to be a tedious and time-consuming procedure. Here we report the analysis of 9-oxononanoyl cholesterol (the main cholesterol core-aldehyde present in oxLDL and in atherosclerotic plaques; compare Fig. 1 C and D) as the corresponding decahydroacridine derivative. The procedure described is simple, yields fluorescent derivatives with excellent chromatographic properties and stability, and could also be successfully applied to the analysis of aliphatic aldehydes (21–24, 38, 39). Instead of a thin-layer chromatographic preseparation step (as in case of DNPH-derivatives), we have used solid-phase extraction

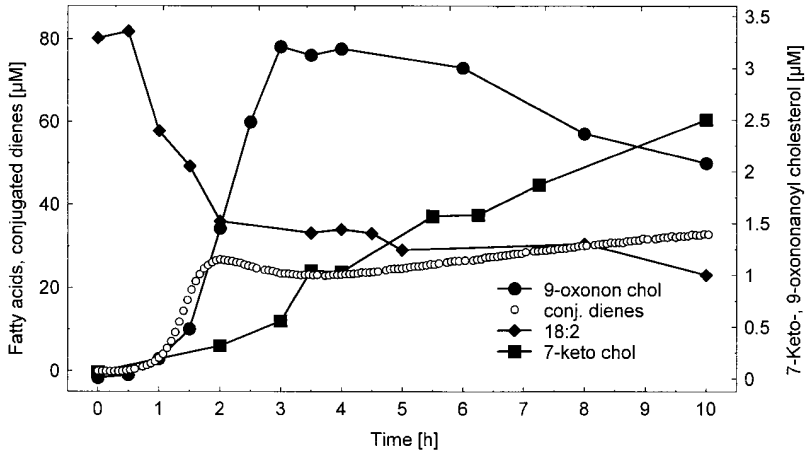


Fig. 7. Kinetics of 9-oxononanoyl cholesterol formation during Cu^{2+}-initiated oxidation of LDL (0.25 mg/ml; ≈ 0.1 μm in PBS) was oxidized by 1.6 μm $CuSO_4$. Aliquots of the reaction mixture were taken at the time points indicated and assessed for their fatty acid, oxysterol, and 9-oxononanoyl cholesterol content as described in Materials and Methods.

TABLE 1. Lipid content of postmortem atherosclerotic plaques

Sample	9-Oxononanoyl-cholesterol	α-TocH	Ch20:4	Ch18:2	Ch18:1	Cholesterol
	μmol/mol cholesterol	*mmol/mol cholesterol*		*mol/mol cholesterol*		*μmol/100 mg lipid extract*
1	27.5	2.1	0.06	0.34	0.13	69.8
2	45.8	2.8	0.07	0.34	0.19	42.1
3	15.8	3.6	0.14	0.46	0.27	22.1
4	6.2	4.9	0.10	0.28	0.11	4.7
5	25.8	5.0	0.16	0.55	0.26	55.7
6	44.8	5.0	0.17	0.58	0.29	25.9
7	16.7	3.6	0.15	0.79	0.38	21.3
8	43.3	3.1	0.15	0.68	0.38	38.0
9	32.2	2.5	0.13	0.64	0.33	20.7
Mean ± SD	28.7 ± 14.18	4.0 ± 1.1	0.13 ± 0.041	0.52 ± 0.176	0.26 ± 0.098	33.4 ± 20.13

Nine samples of aortic tissue from three different donors aged between 39 and 60 years were extracted as described in Materials and Methods. Lipid extracts were assessed for cholesterol, cholesteryl esters, α-tocopherol and free 9-oxononanoyl cholesterol. Values for cholesteryl esters are given in mol/mol cholesterol, α-tocopherol in mmol/mol cholesterol, 9-oxononanoyl cholesterol in μmol/mol cholesterol and the cholesterol content as μmol/100 mg lipid extract.

to separate cholesterol core-aldehydes from most of the other lipid classes. The solid-phase extraction procedure allows analysis of complex lipid extracts obtained from biological samples without further work-up because phospholipids as well as native cholesteryl esters are separated from the derivatized cholesterol core-aldehydes. Derivatives of unesterified aldehydes are removed in the acetonitrile fraction, permitting separate quantitation of this fraction. However, even after pre-purification, the HPLC chromatograms of oxLDL or atherosclerotic plaque extracts revealed the presence of a number of fluorescent compounds distinct from 9-oxononanoyl cholesterol. Identification of these analytes is currently in progress.

It is well established that unesterified aldehydes formed during LDL oxidation can modify lysine or histidine residues of apolipoproteins by Schiff-base or Michael adduct formation (40, 41). LDL modified in this way is no longer recognized by the LDL receptor but internalized via hepatic- or macrophage scavenger receptor-mediated pathways (42). Recently, it has been reported that in analogy to short-chain aldehydes, cholesterol core-aldehydes are also capable of Schiff-base adduct formation with ϵ-amino groups of lysine (13). In addition, Schiff-base adducts of phospholipid core-aldehydes with lysine residues of myoglobin (43) and thyroglobin (44) have been identified. Under our experimental conditions we have obtained two lines of evidence that the reactivity of 9-oxononanoyl cholesterol towards apoB-100 is relatively low: First, after a 24-h coincubation with LDL, about 50% of the aldehyde was recovered. Second, amino acid analysis of LDL incubated in the presence of a 2000 molar excess of 9-oxononanoyl cholesterol revealed only minor lysine modification (≈15%) and unchanged histidine and arginine content (B. Karten and W. Sattler, unpublished data). However, the feasibility of these studies is somewhat limited due to the poor solubility of the core aldehyde in the aqueous LDL solution if drastic conditions, e.g., sonication of the incubation mixture, are avoided. Another explanation for the relatively high recovery of 9-oxononanoyl cholesterol by lipid extraction after coincubation with LDL is binding of 9-oxononanoyl cholesterol to amino head groups of phosphatidylethanolamine constituting ≈4.5% of total PLs in LDL. This observation is in line with a recent report demonstrating the formation of Schiff-base adducts between phospholipid core-aldehydes and amino phospholipids (43). Although we have no direct evidence, one could expect extraction of these phospholipid–cholesterol core-aldehyde adducts into the organic solvent and cleavage of the Schiff-base adducts under the slightly acidic conditions used for derivatization. It is important to note that under our experimental conditions protein-bound aldehydes are not detected as only the extractable lipids are derivatized.

During copper-initiated oxidation of LDL, we observed the formation of 9-oxononanoyl cholesterol as the main cholesterol core-aldehyde. The formation of 9-oxononanoyl cholesterol closely accompanied the increase in conjugated dienes but reached its maximal rate of formation later than the conjugated dienes. This reflects the fact that aldehydes are formed by decomposition of hydroperoxides constituting the majority of conjugated dienes (5, 7). As lipid hydroperoxides decompose to a variety of secondary degradation products (12) we were not able to establish a stoichiometric precursor–product relationship. Another point worth mentioning is the fact that the consumption of linoleic acid preceded the formation of conjugated dienes and that the loss in linoleic acid cannot be fully accounted for by the formation of conjugated dienes. This might be a result of concomitant formation of secondary degradation products. The decrease in 9-oxononanoyl cholesterol concentrations observed after 5 h of copper oxidation is most probably due to further oxidation of the cholesterol moiety of the molecule resulting in a shift of retention time under the chromatographic conditions described.

To test the practical applicability of the method presented, we have quantitatively analyzed the 9-oxononanoyl cholesterol content of human atherosclerotic lesions. The

presence of core-aldehydes in plaque lipids has been shown by Hoppe and coworkers (13), but the amount of cholesterol core-aldehydes was not quantitated. We have identified 9-oxononanoyl cholesterol in each of the plaque samples analyzed at mean concentrations of 11 nmol/g lipid extract (28 μmol/mol cholesterol). It is interesting to note that the relative amounts of the different cholesterol core-aldehyde peaks eluting from the HPLC system differ markedly from those found in oxLDL. How and where the core-aldehydes are formed in the arterial wall is presently not clear.

Taken together, the selectivity, sensitivity, and broad applicability of the presented method should make it potentially useful for the diagnostic assessment of lipid peroxidation in various diseases and mechanistic studies of lipid/lipoprotein peroxidation.

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