Free Rad. Res., Vol. 35, pp. 31–41
Reprints available directly from the publisher
Photocopying permitted by license only

Oxysterol Profiles of Normal Human Arteries, Fatty Streaks and Advanced Lesions

SANDRA GARCIA-CRUSET[a,b,*], KERI L.H. CARPENTER[a], FRANCESC GUARDIOLA[b], BRIDGET K. STEIN[c] and MALCOLM J. MITCHINSON[a]

[a]*University of Cambridge, Department of Pathology, Tennis Court Road, Cambridge CB2 1QP, UK;* [b]*Nutrition and Food Science Department-CeRTA, Faculty of Pharmacy, University of Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain;* [c]*EPSRC National Mass Spectrometry Service Centre, Department of Chemistry, University of Wales, Singleton Park, Swansea SA2 8PP, UK*

Accepted by Prof. Roland Stocker

(Received 1 June 1999; In revised form 28 October 2000)

Objective: Human atherosclerotic lesions of different stages have quantitative differences in cholesterol and oxysterol content, but information on the oxysterol profile in fatty streaks is limited. This study aims to provide more detailed oxysterol quantification in human fatty streaks, as well as normal aorta and advanced lesions.

Methods: A newly adapted method was used, including oxysterol purification by means of a silica cartridge; and it was ensured that artifactual oxysterol formation was kept to a minimum. Cholesterol and oxysterols were estimated by GC and identification confirmed by GC-MS in samples of normal human arterial intima, intima with near-confluent fatty streaks and advanced lesions, in necropsy samples.

Results: The oxysterols 7α-hydroxycholesterol, cholesterol-5β, 6β-epoxide, cholesterol-5α, 6α-epoxide, 7β-hydroxycholesterol, 7-ketocholesterol and 27-hydroxycholesterol (formerly known as 26-hydroxycholesterol) were found in all the lesions, but were at most very low in the normal aorta, both when related to wet weight and when related to cholesterol. Most components of the normal artery showed some cross-correlation on linear regression analysis, but cross-correlations were weaker in the fatty streaks and advanced lesions. However, in fatty streak there was a marked positive correlation between 27-hydroxycholesterol and cholesterol.

Conclusion: The findings confirm that oxysterols are present in fatty streaks and advanced lesions and may arise from different cholesterol oxidation mechanisms, including free radical-mediated oxidation and enzymatic oxidation.

Keywords: Atherosclerosis, normal artery, fatty streak, advanced lesion, cholesterol, oxysterols, (human)

1. INTRODUCTION

Fatty streaks (FS) represent an early stage in the formation of atherosclerotic lesions. Their chemical composition compared to that of advanced lesions is therefore of interest; the oxidation hypothesis of atherogenesis suggests that oxidation products of cholesterol may be particularly relevant.

*Corresponding author. Tel.: +44 1223 333723. Fax: +44 1223 333720.

Oxysterols are known to be present in foods,[1,2] notably cholesterol-rich foods (dairy, egg and meat products), especially those products which are heated in air during processing or stored for lengthy periods.[3] The most commonly detected oxysterols in foods are the major products of cholesterol autoxidation: 7-ketocholesterol (7-KC), 7α-hydroxycholesterol (7α-HC), 7β-hydroxycholesterol (7β-HC), cholesterol-5α,6α-epoxide (α-CE) and cholesterol-5β,6β-epoxide (β-CE).

Reported oxysterol data in biological samples include nine oxysterols measured in human plasma, namely 7-KC, 7α-HC, 7β-HC, α-CE, β-CE, cholestanetriol (CT), 24-hydroxycholesterol (24-HC), 25-hydroxycholesterol (25-HC) and 27-hydroxycholesterol (27-HC).[4] Some oxysterols, including 7β-HC and 27-HC (termed 26-hydroxycholesterol in some of the previous literature) as well as other lipid oxidation products, have been measured in human atherosclerotic lesions at several stages of development,[5–8] but other oxysterols may also be relevant.[9,10] Whether the oxysterols in lesions derive from the diet[3,11] or as a result of local oxidation in the lesion, or both, is not yet certain.

Oxysterols are cytotoxic to many cell types, including endothelial cells, macrophages, smooth muscle cells and lymphocytes.[12,13] Death of any of these cell types might affect atherogenesis and macrophage death due to oxysterols has been suggested to contribute to the formation of the acellular lipid core of advanced lesions.[8,14] Amongst all the oxysterols, 27-HC seems to be the most cytotoxic followed by the 7-oxysterols (7β-HC, 7-KC and 7α-HC) according to the *in vitro* studies with human monocyte-macrophages (HMM), where the epoxides were not tested.[12] However, it was also shown that the toxicity of 27-HC decreased with cholesterol co-addition in HMM culture.[12] Thus, it is possible that in the lesion 27-HC might not be toxic as cholesterol is present in high concentrations.

This study is the first attempt to compare the oxysterol profiles of normal artery, FS and advanced lesions, using a newly adapted technique providing data on their content of 7α-HC, β-CE, α-CE, 7-KC, 7β-HC and 27-HC, reported earlier to be present in human advanced lesions.[15,16]

2. MATERIALS AND METHODS

2.1. Sampling Procedures

Samples came from human common carotid arteries and aortas removed post mortem at Addenbrooke's Hospital, Cambridge. Fifteen specimens of normal artery, 15 specimens of FS and 15 specimens of advanced lesion were obtained from a total of 41 subjects. A scalpel was inserted into the inner media and the outer media and adventitia removed. Samples of normal intima ("normal artery") were taken from regions macroscopically free of lesions, but histology showed that some contained scanty isolated foam cells not apparent to the naked eye. Each FS sample consisted of inner media and intima with near-confluent fatty streaks constituting an estimated 30% of each sample. The samples were stored at $-70\,^{\circ}C$ under argon within an hour of dissection.

The age of the patients from which normal artery was taken ranged from 29 to 83 years (mean 65.33 ± 16.01), while for fatty streaks the age range was 73 to 94 years (mean 82.73 ± 6.75) and for advanced lesions 49 to 99 years (mean 75.27 ± 14.97). More information on subject characteristics is given in Table I. The time-intervals after death ranged from 15 to 112 hours. The length of this time-interval does not affect results significantly.[5] The wet weight of the normal artery samples ranged from 195.3 to 327.6 mg, that of the FS samples from 139.9 to 401.8 mg and that of advanced lesions from 125.0 to 330.0 mg.

2.2. Extraction and Work-up of Lipids

The method used to extract and process lipids was adapted from that described previously,[5] with modifications to facilitate more detailed determination of oxysterols. Special care was

TABLE I Subject characteristics, including patient code, sex, age and artery from which sample was taken

Patient	Sex	Age	Lesion stage	Sample type (CC/TA/AA)
P99.020	F	63	N	CC, TA
P99.027	F	44	N	CC, TA
P99.033	M	60	N	TA
P99.080	M	51	N	CC, TA
P99.085	M	54	N	CC, TA
P99.157	M	83	N	TA
P99.158	M	60	N	TA
P99.159	M	75	N	TA
P99.184	M	70	N	TA
P99.185	F	82	N	TA
P99.199	M	81	N	TA
P99.201	M	79	N	TA
P99.208	F	82	N	TA
P99.209	F	29	N	TA
P99.228	M	67	N	TA
P98.425	M	81	FS	CC
P98.468	M	88	FS	CC
P98.504	M	89	FS	CC
P98.510	F	88	FS	CC
P98.513	F	76	FS	CC
P98.526	F	86	FS	CC, TA
P98.528	M	79	FS	CC
P98.535	F	73	FS	CC
P98.575	M	77	FS	CC
P98.577	M	77	FS	CC
P98.582	M	86	FS	CC, TA
P98.596	M	77	FS	CC
P98.622	M	77	FS	CC
P98.687	F	94	FS	CC
P98.688	F	93	FS	CC
P99.814	M	85	A	AA
P99.826	M	74	A	AA
P00.259	M	64	A	AA
P00.260	M	67	A	AA
P00.261	M	94	A	TA
P00.262	F	78	A	AA
P00.263	M	99	A	AA
P00.264	M	86	A	AA
P00.265	M	61	A	AA
P00.266	M	49	A	AA
P00.271	F	71	A	AA

CC: common carotid; TA: thoracic aorta; AA: abdominal aorta.

taken during sample processing to ensure artifactual formation of oxysterols was minimised, and this involved the following low oxidative conditions as previously:[6,17] dim light was used throughout the analysis, contact with air was minimised, cold saponification was used rather than hot; saponification, derivatisation, and storage of samples were all under argon.

The procedure involved weighing each sample (wet weight) and adding internal standards (300 μg 5α-cholestane and 60 μg 19-hydroxycholesterol (19-HC)). The choice of 19-HC as the internal standard for oxysterol quantitation is widespread since it has a high recovery factor through the oxysterol enrichment step with silica cartridges.[18,31,32] 50 μg BHT as a solution of 1 mg/ml chloroform were also added. The sample was then homogenised (at ca. 10,000 rpm for 5–10 min), taking care to rinse the dispersing element between samples in order to clean it. This was followed by Bligh and Dyer extraction and saponification at room temperature for 18 h,[6] under which saponification temperature successful recovery of 7-KC has been repeatedly demonstrated.[18,31,33–35] Sodium borohydride reduction was not performed. The extract was evaporated under nitrogen and dissolved in 3 ml hexane, from which an aliquot (500 μl) was taken, evaporated under nitrogen and derivatised with *N,O*-bis (trimethylsilyl)trifluoroacetamide +10% trimethylchlorosilane (BSTFA + 10% TMCS) for cholesterol GC determination. The remainder of the extract underwent further processing to achieve oxysterol purification.[17,18] After adjusting the total volume of extract to 5 ml, it was vortexed and eluted (elution speed 2 ml/min) through a Sep-Pak silica cartridge (previously pre-eluted with 5 ml hexane) by means of a vacuum manifold. The extract was washed with a further 5 ml hexane, vortexed and eluted. Sequential elution with 10 ml hexane:diethyl ether (95:5, v/v); 30 ml hexane:diethyl ether (90:10, v/v) and 10 ml hexane:diethyl ether (80:20, v/v) followed. After drying the cartridge by drawing air through it, the oxysterols were eluted and collected with 10 ml acetone:methanol (60:40, v/v), evaporated under nitrogen and derivatised with BSTFA +10% TMCS for GC determination. Processed samples were stored at −20 °C under argon until GC analysis, which was carried out within

a week of dissection. All extracts were analysed in duplicate. This oxysterol purification method was validated and the loss of oxysterols in the cartridge was proved to be negligible.[1]

The equipment used was as follows: The homogeniser was an Ika Labortechnik Ultra-Turrax T8 (S8N-5G, S8N-8G). The 24-position vacuum manifold was supplied by Waters, Millipore (Milford, MA, USA), as were the silica Sep-Pak cartridges (WAT051900). BSTFA +10% TMCS was supplied by Pierce and Warriner (Chester, UK) and 19-HC (Sigma, Poole, Dorset, UK). All other chemicals were as described previously, as was the cleaning of analytical glassware.[5]

Reagent blanks were run to check for contamination. Pure cholesterol (Sigma, Poole, Dorset, UK) was processed identically to test for artifactual oxidation. Artifactual production of oxysterols by this newly adapted method was 0.006 μg/100 μg cholesterol, out of which 7α-HC contributed 0.001 μg/100 μg cholesterol and α-CE contributed 0.005 μg/100 μg cholesterol. β-CE, 7β-HC, 7-KC and 27-HC were not produced artifactually.

2.3. Gas Chomatography and Gas Chromatography – Mass Spectrometry

After workup, GC analysis (using a Carlo Erba 8000 GC) was performed for all the samples. A cold on-column injector and a 30 m DB-1 capillary column (film thickness 0.1 μm and internal diameter 0.32 mm) was used as described previously[14] and the oven programmed at 50–210 °C at 10 °C/min and held at 210 °C for 1 min, 210–264 °C at 2 °C/min, 264–290 °C at 3.5 °C/min and held at 290 °C for 10 min. As before, detection was by a flame ionisation detector.[14] Quantitation was by peak areas, measured electronically using an integrator, relative to internal standards. Cholesterol was quantified relative to 5α-cholestane internal standard, whilst the oxysterols were quantified relative to 19-HC. The response factors (RF) and linearity of response (r^2) relative to the relevant internal standard were as follows: cholesterol (RF = 1.089, $r^2 = .9923$), 7α-HC (RF = 0.905, $r^2 = .9938$), β-CE (RF = 1.083, $r^2 = .9996$), α-CE (RF = 0.997, $r^2 = .9997$), 7β-HC (RF = 0.970, $r^2 = .994$), 7KC (RF = 0.969, $r^2 = .9962$) and 27-HC (RF = 0.847, $r^2 = .9307$). GC-MS was performed on selected samples, using a similar capillary column, and confirmed identities of all components. The GC-MS instrumentation was as described previously.[14]

2.4. Statistical Analysis of Data

Student's unpaired *t*-tests were carried out using SigmaPlot 3.0™ software. Significance as judged by Student's unpaired *t*-test was taken as $p \leqslant .05$. Linear regression analysis (r^2) and their levels of significance were computed using StatView 4.5® software.

3. RESULTS

3.1. Cholesterol and its Oxidation Products

The compounds measured in all samples were cholesterol, 7α-HC, β-CE, α-CE, 7β-HC, 7-KC and 27-HC, as can be seen in Figure 1, a typical chromatogram of the purified oxysterols in FS (also containing residual cholesterol that elutes through the silica cartridge). The identity of the peak labelled as CT was confirmed by GC-MS and, although not quantifiable by our method as it is incompletely silylated under our conditions, it is included in Figure 1. The unlabelled peaks in Figure 1 are unknowns; their mass spectra did not correspond to any recognisable oxysterols. The oxysterol profiles of normal artery, FS and advanced lesion were found to be qualitatively the same but quantitatively distinct. No information on esterification state was sought.

The results of the GC analysis were expressed in two ways as previously:[14] (i) micrograms of each component per milligram wet weight of tissue; (ii) micrograms of each component per 100 micrograms of cholesterol. The main features of the results are described below and in Table II.

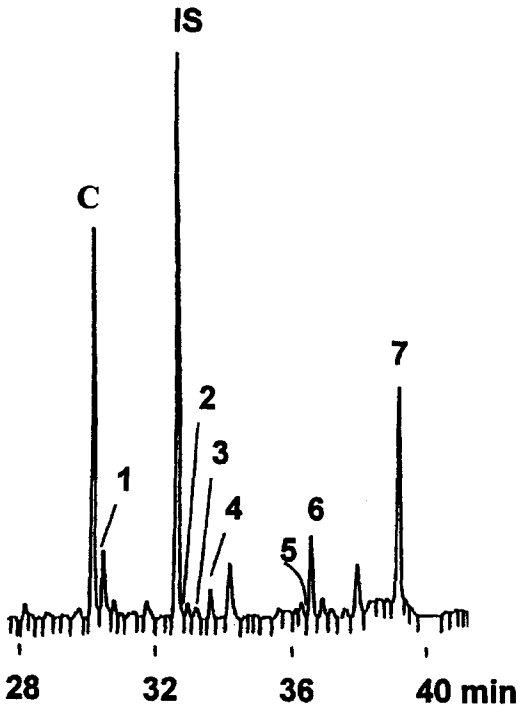

FIGURE 1 Representative GC/FID trace of the oxysterol fraction of a human fatty streak after oxysterol enrichment. Peaks are labelled as follows: **C**, residual cholesterol; **1**, 7α-HC; **IS**, 19-HC; **2**, β-CE; **3**, α-CE; **4**, 7β-HC; **5**, CT; **6**, 7-KC; **7**, 27-HC.

3.2. Cholesterol

Cholesterol levels were significantly higher in FS than in normal artery when expressed as μg/mg wet weight and, in turn, higher in advanced lesion than in FS when expressed as μg/mg wet weight. The means, standard deviations and p values for cholesterol are shown in Table II.

3.3. Oxysterols

7α-HC, β-CE, α-CE, 7β-HC, 7-KC and 27-HC levels were all significantly higher in FS than in normal artery when expressed as μg/mg wet weight and when expressed as μg per 100 μg cholesterol. 7α-HC, β-CE, α-CE, 7β-HC, 7-KC and 27-HC levels were all significantly higher in advanced lesion than in FS when expressed as μg/mg wet. When expressed as μg per 100 μg cholesterol, none of the oxysterols analysed were significantly different in advanced lesion than in FS. The means, standard deviations and p values for the oxysterols are shown in Table II.

3.9. Correlations between Components

Each of the above components was assessed for correlations with others in the samples, using linear regression analysis. Each of the three samples (normal artery, FS and advanced lesion) was tested separately. Most components listed in Table III showed significant cross-correlation in the normal artery when expressed as μg/mg wet weight, but they showed less correlation in the FS and in the advanced lesion. This suggests that

TABLE II Mean levels and standard deviations for selected components of normal artery, fatty streak and advanced lesion expressed as μg/mg wet weight and as μg/100 μg cholesterol

	Normal artery				Fatty streak				Advanced lesion			
	(μg/mg wet wt.)		(μg/100 μg chol)		(μg/mg wet wt.)		(μg/100 μg chol)		(μg/mg wet wt.)		(μg/100 μg chol)	
	mean	S.D.	mean	S.D.	mean	S.D.	mean	S.D.	mean	S.D.	mean	S.D.
Cholesterol	3.71	1.54	–	–	25.74[a]	15.63	–	–	38.57[f]	11.59	–	–
7α-HC	0.00091	0.00087	0.036	0.032	0.045[a]	0.026	0.28[b]	0.25	0.13[e]	0.11	0.37[g]	0.29
β-CE	0.00069	0.0014	0.016	0.034	0.017[a]	0.0073	0.097[c]	0.069	0.034[e]	0.016	0.10[g]	0.045
α-CE	0.00099	0.0016	0.028	0.048	0.029[a]	0.011	0.16[a]	0.10	0.060[e]	0.038	0.17[g]	0.080
7β-HC	0.00051	0.00070	0.016	0.019	0.020[a]	0.011	0.12[d]	0.11	0.057[e]	0.040	0.16[g]	0.090
7-KC	0.00049	0.00098	0.011	0.019	0.045[a]	0.019	0.22[a]	0.13	0.10[e]	0.063	0.28[g]	0.14
27-HC	0.0016	0.0046	0.032	0.068	0.093[a]	0.078	0.43[a]	0.25	0.22[f]	0.25	0.75[g]	0.82

The superscripts denote the level of significance for the difference between the mean values for normal artery and fatty streak ([a]$p < .0001$; [b]$p = .0003$; [c]$p = .0007$; [d]$p = .001$) and between fatty streak and advanced lesion ([e]$p \leq .006$ [f]$p = .03$ [g]$p > .10$). The difference between the mean values for normal artery and advanced lesion was significant for all the components analysed.

TABLE III Cross correlations of components of normal artery, fatty streak and advanced lesion using least squares linear regression analysis

Components correlated		Normal artery		Fatty streak		Advanced lesion	
		(r^2)	(p)	(r^2)	(p)	(r^2)	(p)
Cholesterol vs.	7α-HC	.31	.03	.10	.25	.14	.17
	7β-HC	.13	.18	.12	.20	.34	.02
	7KC	.38	.01	.24	.06	.46	.004
	27-HC	.43	.007	.42	.008	.02	.64
7α-HC vs.	β-CE	.41	.009	.01	.73	.02	.59
	α-CE	.57	.0007	.03	.57	.60	.0004
β-CE vs.	α-CE	.75	< .0001	.17	.12	.03	.53
	7β-HC	.26	.05	.28	.04	.48	.003
	7-KC	.09	.27	.30	.03	.18	.12
	27-HC	.26	.05	.17	.13	.62	.0002
7β-HC vs.	7-KC	.43	.006	.08	.33	.39	.01
	27-HC	.26	.05	.006	.79	.30	.03
7-KC vs.	27-HC	.82	< .0001	.16	.14	.001	.93

For linear regression analysis, all components were expressed as μg/mg wet weight. The components not listed in the table showed very weak cross-correlation or no correlation at all, both in the normal artery and in the fatty streak ($p \geq .06$).

normal artery samples are similar in the abundance of the various components. Notably, in FS there was a marked positive correlation between 27-HC and cholesterol (Figure 2).

Using the same linear regression analysis, each component was assessed for the correlation of its abundance in the normal artery versus the FS and in the FS versus advanced lesion. All components correlated very weakly or not at all, giving values of $r^2 \leq .21$; $p > .09$ for the comparison of normal artery versus the FS and $r^2 \leq .11$; $p > .24$ for the comparison of FS versus advanced lesion (all the components expressed as μg/mg wet weight).

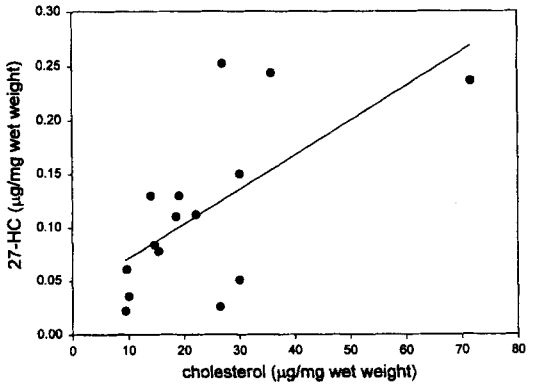

FIGURE 2 Graph of 27-HC vs. cholesterol (both expressed as μg/mg wet weight) for fatty streaks. Linear regression $r^2 = .42$, $p = .008$.

4. DISCUSSION

4.1. Cholesterol

Cholesterol levels were of course much higher than in normal artery in lesions, especially advanced lesions, as shown previously.[6]

4.2. Oxysterols

All the oxysterols were more abundant in FS than in normal artery, whether related to wet weight or to cholesterol. All were also more abundant in the advanced lesion than in the FS when related to wet weight, but there was no difference between the two when related to cholesterol.

7β-HC and 27-HC were both significantly higher in FS than in normal artery when related either to wet weight or to cholesterol in previous reports.[6,7] However, while in previous reports[6]

7β-HC was found to be higher in FS than in advanced lesion when related to cholesterol, in this study there was no significant difference between the two lesion categories. The levels of 27-HC were higher in advanced lesion than in FS in previous reports[6] but no significant difference was found in this study between the levels of 27-HC in the two lesion categories when related to cholesterol.

The other oxysterols determined in this study, namely 7α-HC, β-CE, α-CE, and 7-KC, were also found to be significantly higher in FS than in normal artery when related to wet weight or to cholesterol. This is the first attempt to compare the levels of these oxysterols in normal artery and FS, although some data exist on advanced lesions[15] and in normal artery versus advanced lesions.[9] In FS, the means of the oxysterol contents were ranked as follows: 27-HC > 7α-HC = 7-KC > α-CE > 7β -HC > β-CE, when related to wet weight, and 27-HC > 7α-HC > 7-KC > α-CE > 7β-HC > β-CE, when related to cholesterol. In advanced lesions, the means of the oxysterol contents were ranked as follows: 27-HC > 7α-HC > 7-KC > α-CE > 7β-HC > β-CE, whether related to wet weight or to cholesterol. Some reports suggested a different order for the means of oxysterol contents in advanced lesions, namely 27-HC > 7-KC > 7β-HC > 7α-HC,[9] when an HPLC method that does not detect epoxides was used, and 27-HC > 7-KC > 7β-HC > 7α-HC > α-CE = β-CE when isotope-dilution mass spectrometry with individual deuterated standards was used.[15]

Although information on oxysterol production mechanisms cannot be directly obtained from the present study, some interpretation is possible. The greater abundance of 27-OH-CHOL related to cholesterol in FS than in normal artery indicates a greater contribution of cytochrome P450 sterol 27-hydroxylase-mediated oxidation of cholesterol in FS. This enzyme is present in various mammalian tissues and cell types.[15,19,20,21] 27-HC concentrations correlated positively with cholesterol concentration in FS ($r^2 = .42$) as in previous reports.[6,7] This suggests that this oxysterol, produced by sterol 27-hydroxylase in cells of the lesion in response to cholesterol build-up, might theoretically slow down this cholesterol increase by down-regulating HMGCoA reductase and LDL receptors.[22] However, these processes already appear to be down-regulated in foam cells which accumulate cholesterol; the LDL receptors are probably not important for cholesterol accumulation, since in these cells cholesterol is mainly taken up by scavenger receptors. Which cell type is responsible for 27-hydroxylation is unknown, but because the main cell-type that is increased in numbers in fatty streaks is the macrophage foam cell, it raises the possibility that they might be the main source. The co-localisation of sterol 27-hydroxylase with macrophages in advanced lesions is consistent with this.[16]

27-hydroxylase may constitute a cell cholesterol removal mechanism.[15] 27-OH-CHOL can be oxidised further to 3β-hydroxy-5-cholestenoic acid by 27-hydroxylase, which has the capacity to hydroxylate the same methyl group three times.[15] 27-HC and 3β-hydroxy-5-cholestenoic acid are more polar than cholesterol and thus are more easily transported out of the tissue into the plasma and ultimately converted to bile acids by the liver.[15,23,24] The product of the mitochondrial cytochrome P450 sterol 27-hydroxylase is 25R-cholest-5-en-3β, 26-diol, termed 26-hydroxycholesterol formerly and in some of the recent literature. However, the term 27-hydroxycholesterol is now increasingly used for the same molecule.

Cholesterol autoxidation is initiated by hydrogen abstraction at C-7 forming a C-7 allylic radical, which then reacts with oxygen to produce a peroxyl radical. This peroxyl radical then abstracts hydrogen and results in 7α- and 7β-hydroperoxycholesterol (7α- and 7β-OOH). 7α- and 7β-OOH are thermally unstable, leading to 7α-HC, 7β-HC and 7-KC as secondary products,[2] which is in agreement with the trace amounts of 7α- and 7β-OOH found in the atherosclerotic lesion.[9] Peroxidases can also convert 7α- and 7β-OOH to 7α-HC, 7β-HC.[9]

The mean level of 7α-HC relative to the other oxysterols found in advanced lesions is higher than reported previously;[9,15] the explanation is unknown. The original GC estimations of all the oxysterols, including 7α-HC, were obtained using a DB-1 column (Figure 1 and Table II) as stated in the Materials and Methods section. A DB-5 column was used to re-analyse 7α-HC by GC and the peak for this compound was observed to shift from eluting just after cholesterol (on DB-1) to a retention time just before cholesterol (on DB-5), in accordance with authentic standard 7α-HC run under the same conditions, and with other reports.[36,37] The measured levels of 7α-HC calculated from GC peak areas (relative to the 19-HC internal standard, as before) were the same on DB-5 (data not shown) as on DB-1. The identity of 7α-HC was also verified in the present study by its mass spectrum on GC-MS, in comparison with that of authentic standard 7α-HC (Figure 3).

7α-HC can be produced by free radical oxidation of cholesterol,[26] and has been reported previously in atherosclerotic lesions.[26] 7α-HC can also be produced enzymically from cholesterol, by cholesterol 7α-hydroxylase.[25] This enzyme is reputedly liver-specific,[25] although recently it has been reported in rat pancreatic hepatocyte-like cells.[38] It is possible that the enzyme might exist in other tissues, by analogy with 27-hydroxylase, which was originally identified in liver,[15] and later found in other tissues, including atherosclerotic lesions.[16,20] Cholesterol 7α-hydroxylase and 27-hydroxylase are both key enzymes in the early steps of the bile acid biosynthetic pathway in the liver.[25] We have alluded earlier to the likelihood that 27-hydroxylase constitutes a cholesterol removal mechanism from tissues including atherosclerotic lesions. Cholesterol 7α-hydroxylase might have an analogous role, albeit on a much smaller scale than its activity in liver.

7-KC is a cholesterol autoxidation product, initiated by hydrogen abstraction at C-7 forming a C-7 allylic carbon-centred radical. The mechanism proceeds as described above for the production of 7β-HC and it also applies to the production of 7α-HC.[2] While it is generally considered that 7-KC and 7β-HC are not formed enzymically in mammals, there is some evidence to the contrary, such as the conversion of cholesterol to 7-KC in rat liver microsomes (perhaps via the intermediate formation of 7β-HC) and conversion of 7α-HC to 7-KC by an $NADP^+$-dependent dehydrogenase in liver microsomes of hamster, human, and cow.[26] The enzymic conversion of 7-KC to 7β-HC (particularly in rat liver microsomes) is well documented. However, the extent to which these pathways contribute to total 7-KC and 7β-HC *in vivo* remains to be determined.[26]

Little has been reported about the origin of α-CE and β-CE. It has been suggested that oxidative action of H_2O_2 and cholesterol hydroperoxides on cholesterol can lead to the formation of these two epoxides.[27] α-CE and β-CE might be of dietary origin since they have been detected in foods[2] and there is no solid evidence for their formation *in vivo*.[26] α-CE has been suggested to stimulate cellular sterol accumulation in J774 macrophages, and thus possibly enhance atherosclerosis.[28] Although not major oxysterols in oxidised LDL[29,30] or in human atherosclerotic lesions,[15] α-CE and β-CE contribute to the total oxysterol level in human atherosclerosis.

It was not possible to quantify cholestanetriol in the present study as, unlike the other oxysterols, it was incompletely silylated under the conditions used. However, a peak eluting just in front of 7-KC was seen in all but one of the fatty streaks and in all the advanced lesions and its identity was confirmed to be CT by mass spectrometry. Moreover, the presence of CT cannot be ignored since it was not a result of artifactual oxidation, as shown by a test carried out with pure cholesterol put through the experimental method. CT has been found in foods, such as heated beef tallow,[33] egg and milk powders[32] and freeze-dried pork.[39] It has been suggested

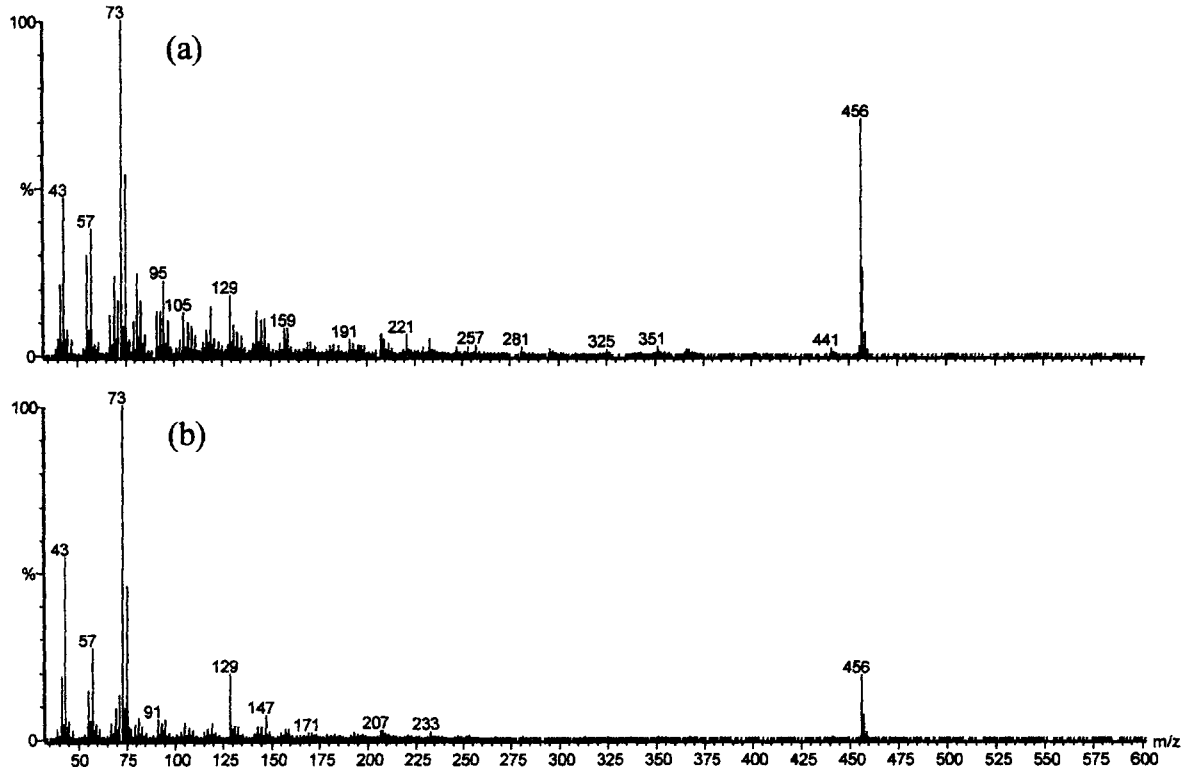

FIGURE 3 Mass spectra of standard 7α-HC (a) and 7α-HC from an advanced atherosclerotic lesion (b).

that cholestanetriol may result from the hydration of α-CE.[40]

4.3. Overall Considerations

Accumulation of cholesterol and oxysterols within the fatty streak is in general consistent with increased oxidative activity taking place in this first stage of atherosclerotic lesion formation as compared to that in the normal artery. Cytochrome P-450 enzymatically-mediated oxidation of cholesterol is higher in FS and so is free radical-mediated oxidation, according to the oxysterol levels found in this study. Since only about 30% of the mass of the FS samples was actually FS (see section 2.1), all the lipid and oxidised lipid contents found here are certainly underestimates.

A factor often overlooked in chemical studies of tissues is that a given specimen, whether removed in life or at necropsy, provides only the data at that specific time. Too little is known about the factors that might cause even quite rapid changes in chemistry. Results may for instance be different if diurnal or seasonal changes are considered, such as variations in lipid and antioxidant uptake in the diet.

Another major factor affecting results from different laboratories is the choice of quantification technique. Gas chromatography (GC) with flame ionisation detection is the commonest method of oxysterol determination in foods and biological samples and was preferred to high performance liquid chromatography (HPLC) for the present study. HPLC has low separation temperatures that avoid thermal decomposition of certain oxysterols (e.g. 7α- and 7β-OOH) which have not been reported to be in relevant amounts in the human artery, but it does not detect the α- and β-epoxides. Moreover, GC

provides a relatively inexpensive, quantitative and sensitive means to determine oxysterols and it is more easily linked to a GC-MS system for the confirmation of compound identities. It is also relevant that the method used here requires quite large arterial samples (~200 mg), especially for normal artery, so that minor components such as the six oxysterols can be quantifiable by GC.

The complex interactions between oxysterols, cholesterol and antioxidants in the fatty streaks need further study. Not all the oxysterols have the same origin. Some may derive from the diet,[2] some being produced or scavenged by different factors in the lesion. It is certainly no longer possible to consider oxysterols as a class that behaves uniformly in atherogenesis. To elucidate the lipid oxidation hypothesis, it would be helpful to estimate oxysterols in human plasma and/or in arterial samples in patients receiving antioxidant supplementation. Such results would also help to explain how much of the oxysterols in lesions are of dietary origin and how much due to local oxidation in the arterial wall. These studies are in progress.

Acknowledgements

We gratefully acknowledge the British Heart Foundation (KLHC) and Universitat de Barcelona-Beques de Formació en la Recerca i la Docència 1998–1999 (Spain) (SGC) for financial support. We thank Prof. D.E. Games and Dr. J.A. Ballantine (EPSRC National Mass Spectrometry Service Centre, Chemistry Department, University of Wales, Swansea) for providing GC-MS facilities and Mr. G. Llewelyn for assisting with GC-MS analysis.

References

[1] F. Guardiola, R. Codony, M. Rafecas and J. Boatella (1994) Metodología analítica para la determinación de oxisteroles. *Grasas y Aceites*, **45**, 164–191.

[2] P.B. Addis, P.W. Park, F. Guardiola and R. Codony (1996) Analysis and health effects of cholesterol oxides. In *Food Lipids and Health* (eds. R.E. McDonald and D. Min), Marcel Dekker, New York, pp. 199–240.

[3] M.S. Jacobson (1987) Cholesterol oxides in Indian ghee: possible cause of unexplained high risk of atherosclerosis in Indian immigrant populations. *Lancet*, **2**, 656–658.

[4] S. Dzeletovic, O. Breuer, E. Lund and U. Diczfalusy (1995) Determination of cholesterol oxidation products in human plasma by isotope dilution-mass spectrometry. *Analytical Biochemistry*, **225**, 73–80.

[5] K.L.H. Carpenter, S.E. Taylor, J.A. Ballantine, B. Fussell, B. Halliwell and M.J. Mitchinson (1993) Lipids and oxidised lipids in human atheroma and normal aorta. *Biochimica et Biophysica Acta*, **1167**, 121–130.

[6] K.L.H. Carpenter, S.E. Taylor, C. van der Veen, B.K. Williamson, J.A. Ballantine and M.J. Mitchinson (1995) Lipids and oxidised lipids in human atherosclerotic lesions at different stages of development. *Biochimica et Biophysica Acta*, **1256**, 141–150.

[7] K.L.H. Carpenter, S.E. Taylor, C. van der Veen and M.J. Mitchinson (1995) Evidence of lipid oxidation in pulmonary artery atherosclerosis. *Atherosclerosis*, **118**, 169–172.

[8] K.L.H. Carpenter, C. van der Veen, S.E. Taylor, S.J. Hardwick, K. Clare, L. Hegyi and M.J. Mitchinson (1995) Macrophages, lipid oxidation, ceroid accumulation and alpha-tocopherol depletion in human atherosclerotic lesions. *Gerontology*, **41**, 53–67.

[9] A.J. Brown, S. Leong, R.T. Dean and W. Jessup (1997) 7-Hydroperoxycholesterol and its products in oxidized low density lipoprotein and human atherosclerotic plaque. *Journal of Lipid Research*, **38**, 1730–1745.

[10] F. Guardiola, R. Codony, P.B. Addis, M. Rafecas and J. Boatella (1996) Biological effects of oxysterols: current status. *Food Chemistry and Toxicology*, **34**, 193–211.

[11] P. van de Bovenkamp, T.G. Kosmeijer-Schuil and M.B. Katan (1988) Quantification of oxysterols in Dutch foods: egg products and mixed diets. *Lipids*, **23**, 1079–1085.

[12] K. Clare, S.J. Hardwick, K.L.H. Carpenter, N. Weeratunge and M.J. Mitchinson (1995) Toxicity of oxysterols to human monocyte-macrophages. *Atherosclerosis*, **118**, 67–75.

[13] H. Hughes, B. Mathews, M.L. Lenz and J.R. Guyton (1994) Cytotoxicity of oxidized LDL to porcine aortic smooth muscle cells in association with the oxysterols 7-ketocholesterol and 7-hydroxycholesterol. *Arteriosclerosis, Thrombosis and Vascular Biology*, **14**, 1177–1185.

[14] S. Garcia-Cruset, K.L.H. Carpenter, F. Guardiola and M.J. Mitchinson (1999) Oxysterols in cap and core of advanced atherosclerotic lesions. *Free Radical Research*, **30**, 341–350.

[15] I. Björkhem, O. Andersson, U. Diczfalusy, B. Sevastik, R.J. Xiu, C. Duan and E. Lund (1994) Atherosclerosis and sterol 27-hydroxylase: Evidence for a role of this enzyme in elimination of cholesterol from human macrophages. *Proceedings of the National Academy of Sciences of the USA*, **91**, 8592–8596.

[16] M. Crisby, J. Nilsson, V. Kostulas, I. Bjorkhem and U. Diczfalusy (1997) Localization of sterol 27-hydroxylase immuno-reactivity in human atherosclerotic plaques. *Biochimica et Biophysica Acta*, **1344**, 278–285.

[17] F. Guardiola, A. Jordán, A. Grau, S. Garcia-Cruset, J. Boatella, M. Rafecas and R. Codony (1998) Recent methodological advances in oxysterol determination. In *Recent Research Developments in Oil Chemistry* (ed. S.G. Pandalai), Transworld Research Network, Trivandrum, pp. 77–88.

[18] F. Guardiola, R. Codony, M. Rafecas and J. Boatella (1995) Comparison of three methods for the determination of oxysterols in spray-dried egg. *Journal of Chromatography A*, **705**, 289–304.

[19] R. Fumagalli, G. Galli and G. Urna (1971) Cholestanol and 26-hydroxycholesterol in normal and atherosclerotic human aorta. *Life Sciences*, **10**, 25–33.

[20] S. Andersson, D.L. Davis, H. Dahlback, H. Jornvall and D.W. Russell (1989) Cloning, structure, and expression of the mitochondrial cytochome P-450 sterol 26-hydroxylase, a bile-acid biosynthetic enzyme. *Journal of Biological Chemistry*, **264**, 8222–8229.

[21] A.B. Reiss, K.O. Martin, N.B. Javitt, D.W. Martin, E.A. Grossi and A.C. Galloway (1994) Sterol 27-hydroxylase. High levels of activity in vascular endothelium. *Journal of Lipid Research*, **35**, 1026–1030.

[22] N.B. Javitt (1990) 26-hydroxycholesterol: synthesis, metabolism, and biologic activities. *Journal of Lipid Research*, **31**, 1527–1533.

[23] E. Lund, O. Andersson, J. Zhang, A. Babiker, G. Ahlborg, U. Diczfalusy, K. Einarsson, J. Sjövall and I. Björkhem (1996) Importance of a novel oxidative mechanism for elimination of intracellular cholesterol in humans. *Arteriosclerosis, Thrombosis and Vascular Biology*, **16**, 208–212.

[24] I. Björkhem (1992) Mechanism of degradation of the steroid side chain in the formation of bile acids. *Journal of Lipid Research*, **33**, 455–471.

[25] M. Schwarz, E.G. Lund and D.W. Russell (1998) Two 7α-hydroxylase enzymes in bile acid biosynthesis. *Current Opinion in Lipidology*, **9**, 113–118.

[26] A.J. Brown and W. Jessup (1999) Oxysterols and atherosclerosis. *Atherosclerosis*, **142**, 1–28.

[27] L.L. Smith, J.M. Kulig, D. Miller and G.A.S. Ansari (1978) Oxidation of cholesterol by dioxygen species. *Journal of the American Chemical Society*, **100**, 6206–6211.

[28] J. Cao, H.M. Fales and C.P. Schaffner (1995) Cellular sterol accumulation stimulated by cholesterol-5β,6β-epoxide in J774 macrophages. *Proceedings of the Society for Experimental Biology and Medicine*, **209**, 195–204.

[29] A.J. Brown, R.T. Dean and W. Jessup (1996) Free and esterified oxysterol: formation during copper-oxidation of low density lipoprotein and uptake by macrophages. *Journal of Lipid Research*, **37**, 320–335.

[30] S. Dzeletovic, A. Babiker, E. Lund and U. Diczfalusy (1995) Time course of oxysterol formation during *in vitro* oxidation of low density lipoprotein. *Chemistry and Physics of Lipids*, **78**, 119–128.

[31] J.E. Pie, K. Spahis and C. Seillan (1990) Evaluation of oxidative degradation of cholesterol in food and food ingredients: identification and quantification of cholesterol oxides. *Journal of Agricultural and Food Chemistry*, **38**, 973–979.

[32] P.C. Dutta, M.F. Caboni, U. Diczfalusy, F. Dionisi, S. Dzeletovic, A. Grandgirard, F. Guardiola, J. Kumpulainen, V.K. Levovics, J.-M. Pihlava, M.T. Rodriguez-Estrada and F. Ulberth (1999) Measurements of cholesterol oxides in foods: results of an interlaboratory comparison study. In *Natural Antioxidants and Anticarcinogens in Nutrition, Health and Disease* (eds. J.T. Kumpulainen and J.T. Salonen), The Royal Society of Chemistry, Cambridge, pp. 309–315.

[33] S.W. Park and P.B. Addis (1986) Identification and quantitative estimation of oxidized cholesterol derivatives in heated tallow. *Journal of Agricultural and Food Chemistry*, **34**, 653–659.

[34] P.W. Park (1995) Toxic compounds derived from lipids. In *Analyzing Food for Nutrition Labelling and Hazardous Contaminants* (eds. I.J. Jeon and W.G. Ikins), Marcel Dekker, New York, pp. 363–434.

[35] P.W. Park (1996) Kinetic evaluation of 3β-hydroxycholest-5-en-7-one (ketocholesterol) stability during saponification. *Journal of the American Oil Chemists Society*, **5**, 623–629.

[36] P.W. Park and P.B. Addis (1985) Capillary column gas–liquid chromatographic resolution of oxidized cholesterol derivatives. *Analytical Biochemistry*, **149**, 275–283.

[37] P.W. Park and P.B. Addis (1992) Methods of analysis of cholesterol oxides. In *Biological effects of cholesterol oxides* (eds. S.-K. Peng and R.J. Morin), CRC Press, Florida, pp. 33–70.

[38] Y. Ando, H. Ide, S. Kosai, R. Kamimura, Y. Maeda, S. Higashi and T. Setoguchi (1999) Expression of cholesterol 7α-hydroxylase and Δ^4-3-ketosteroid 5β-reductase genes in rat pancreatic hepatocyte-like cells. *Journal of Lipid Research*, **40**, 1793–1798.

[39] P.W. Park and P.B. Addis (1987) Cholesterol oxidation products in some muscle foods. *Journal of Food Science*, **52**, 1500–1503.

[40] S.W. Park and P.B. Addis (1989) Derivatization of 5α-cholestane-3β,5,6β-triol into trimethylsilyl ether sterol for GC analysis. *Journal of the American Oil Chemists Society*, **66**, 1632–1634.