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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF CHOLESTEROL AUTOXIDATION PRODUCTS

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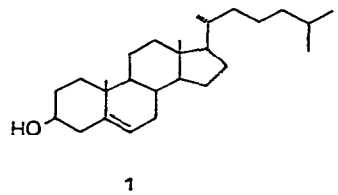
SUMMARY

High-performance liquid chromatography with microparticulate silica gel and octadecyl silica reversed-phase columns accords ready resolution of the common autoxidation products of cholesterol. Resolution of two pairs of isomeric sterols (5,6α-epoxy-5α-cholestan-3β-ol and 5,6β-epoxy-5β-cholestan-3β-ol, 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide and 3β-hydroxycholest-5-ene-7α-hydroperoxide) not previously resolved chromatographically has been achieved in both systems. Application of the procedures to the isolation of cholesta-4,6-dien-3-one from autoxidized cholesterol is described.

INTRODUCTION

Over fifty identified sterols have been variously implicated in the autoxidation of cholesterol (1). We have previously described thin-layer[1,2], gas[3,5] and Sephadex LH-20 liquid[6] chromatography for analytical studies of these oxidation products, but the resolution of two critical pairs of sterols, that of the isomeric cholesterol



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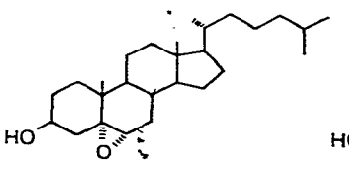

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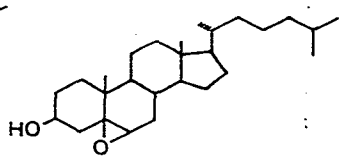

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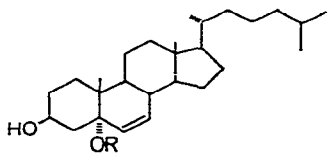

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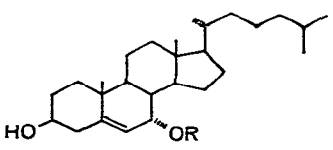

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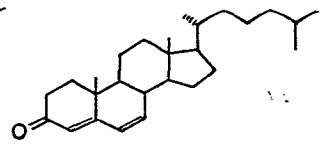

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5,6-epoxides 5,6α-epoxy-5α-cholestan-3β-ol (2) and 5,6β-epoxy-5β-cholestan-3β-ol (3) and that of the isomeric hydroperoxides 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide (4) and 3β-hydroxycholest-5-ene-7α-hydroperoxide (6), has not been achieved with these methods. The power of high-performance liquid chromatography (HPLC) for resolution of analogous oxidized ecdysterol[7] and calciferol[8,9] derivatives prompted us to investigate this additional means of analysis of oxidized cholesterol derivatives as well. We describe here our results in applying HPLC to studies of cholesterol autoxidation, including resolution of the heretofore unresolved sterol pairs and isolation of cholesta-4,6-dien-3-one (8) from air-aged cholesterol. HPLC has since been successfully applied also to studies of the common plant sterols[10] and to cholesterol biosynthesis precursors[11].

EXPERIMENTAL

Chromatography

Thin-layer chromatography (TLC) was conducted on 0.25-mm thick 20 × 20 cm chromatoplates of silica gel HF_{254} (E. Merck, Darmstadt, G.F.R.) using benzene–ethyl acetate (1:3 or 3:1), heptane–ethyl acetate (4:1) and other binary mixtures. Sterols were visualized by ultraviolet light (254 nm) absorption for detection of unsaturated ketones, N,N-dimethyl-*p*-phenylenediamine for detection of sterol hydroperoxides, and 50% aqueous sulfuric acid for detection of all steroids[1,2]. Commercially available HPTLC chromatoplates (10 × 10 cm) of silica gel 60F-245 (E. Merck) irrigated once with chloroform were used for sterol 3β-benzoate derivatives.

HPLC was conducted using two 3.9 mm × 30 cm μPorasil microparticulate (10 μm) silica gel columns in tandem and with 3.9 mm × 30 cm μBondapak C_{18} reversed-phase columns and sample injector, solvent delivery pumps, and solvent programming accessory, all from Waters Assoc. (Milford, Mass., U.S.A.). Column effluent was monitored with a Perkin-Elmer model LC-55 variable wavelength ultraviolet spectrophotometer (set at 212 nm for general use, at 230 nm for detection of cholesterol 5,6-epoxide 3β-benzoates, or at 284 nm for detection of cholesta-4,6-dien-3-one) and then with a Waters Assoc. differential refractometer model R401 in series. Samples were dissolved (1 μg/μl) in the solvent to be used or in chloroform, and sample volumes of 1–50 μl injected for analysis.

The adsorption mode μPorasil columns were used under isocratic conditions with simple binary solvent mixtures of hexane–isopropyl alcohol (99:1, 24:1 or 9:1), hexane–butanol (99:1) or isooctane–isopropyl alcohol (499:1). Programmed solvent delivery was also useful, with initial 90% hexane–isopropyl alcohol (99:1) and 10% hexane–isopropyl alcohol (9:1) mixtures changing linearly to 80% hexane–isopropyl alcohol (99:1) and 20% hexane–isopropyl alcohol (9:1). Reversed-phase μBondapak C_{18} columns were irrigated with acetonitrile–water (19:1, 9:1, 17:3 and 4:1) and with methanol–water (9:1). All solvents were from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.).

Melting points were taken with a Kofler block used under a microscope. Ultraviolet absorption spectra were made on a Cary Model 14 spectrophotometer. Mass spectra were obtained with a Finnigan Model 3200 quadrupole mass spectrometer equipped for electron-impact (70 e/V) and chemical-ionization operations, with methane as reagent gas. Samples were introduced as solids via the direct inlet probe.

Special samples

Reference sterol samples obtained by purchase, gift, or synthesis in our laboratory were identified by chromatographic and spectral properties. Chromatographically homogenous cholesterol 5,6-epoxides (2) and (3) and several sterol benzoate esters prepared by standard methods were characterized: (2), m.p. 139–141°; (2) 3β-benzoate, m.p. 172–173°; (3), m.p. 130–132°; (3) 3β-benzoate, m.p. 176–178°; 5α-cholestane-3β,5,6β-triol 3β,6β-dibenzoate, m.p. 110–111°.

Cholesterol autoxidation products (20.9 g) were recovered by evaporation under vacuum of mother liquor from the recrystallization of 200 g air-aged USP cholesterol (stored in the laboratory for approximately eight years) from methanol. This mixture of autoxidation products gave TLC patterns characteristic of those previously described[1,2] and was used as a reference preparation for our present study.

Preliminary fractionation of the mixture necessary prior to HPLC was conducted using 200 g silica gel in a 4 × 35 cm column. Elution with 500 ml hexane gave 234 mg nonpolar material which was rechromatographed on 40 g silica gel in a 2.5 × 19 cm column using hexane, hexane–chloroform and chloroform for elution. From chloroform eluates was recovered 107 mg solids which were subjected to preparative TLC with heptane–ethyl acetate (4:1) in three ascending irrigations. Elution with chloroform of the cholesta-4,6-dien-3-one (8) region gave 3.5 mg solids which were then chromatographed on the μPorasil column using hexane–isopropyl alcohol (99:1) at 1.0 ml/min. Eluate monitoring at 212 and 284 nm established that cholesta-4,6-dien-3-one and cholest-4-en-3-one were unresolved. Rechromatography of eluted material absorbing at 284 nm on the μBondapak C_{18} column using acetonitrile–water (9:1) at 2.0 ml/min and effluent monitoring at 212 and 284 nm resolved cholesta-4,6-dien-3-one from contaminating cholest-4-en-3-one. Evaporation of eluates under vacuum gave 8 μg of chromatographically homogenous cholesta-4,6-dien-3-one identified by ultraviolet light absorption ($\lambda_{max.}^{EtOH} = 285$ nm) and mass spectra in the electron-impact (*m/z* 382(M), 269 (M − C_8H_{17}), 247, 227, 173, 158, 136) and chemical-ionization (with methane: *m/z* 423 (3.9%, M+41), 411 (21.4%, M+29), 383 (100%, M+1), 382 (20.7%, M), 371 (15%), 367 (10.7%)) modes, in comparison with spectra of authentic cholesta-4,6-dien-3-one.

Two other special samples were also analyzed for their cholesta-4,6-dien-3-one content using μBondapak C_{18} columns and acetonitrile–water (9:1). A ten-year old, air-aged mixture of epimeric cholest-5-ene-3β,7-diols derived by sodium borohydride reduction of 3β-hydroxycholest-5-ene-7-one and a pure 3β,7α-diol (7) sample subjected to air oxidation induced by ^{60}Co γ-radiation (2.4 krad) both exhibited a minor component with retention time and 284 nm absorption of the dienone (8).

RESULTS

In Fig. 1 are presented elution curves from μPorasil and μBondapak C_{18} column resolutions of the standard preparation of cholesterol autoxidation products. Resolution of over thirty components indicated is reminiscent of our prior resolutions of over twenty components by gas chromatography[3] and of over thirty components by TLC[1]. The identities of individually identified components has been determined by isolation of components and by comparison of retention times with those of

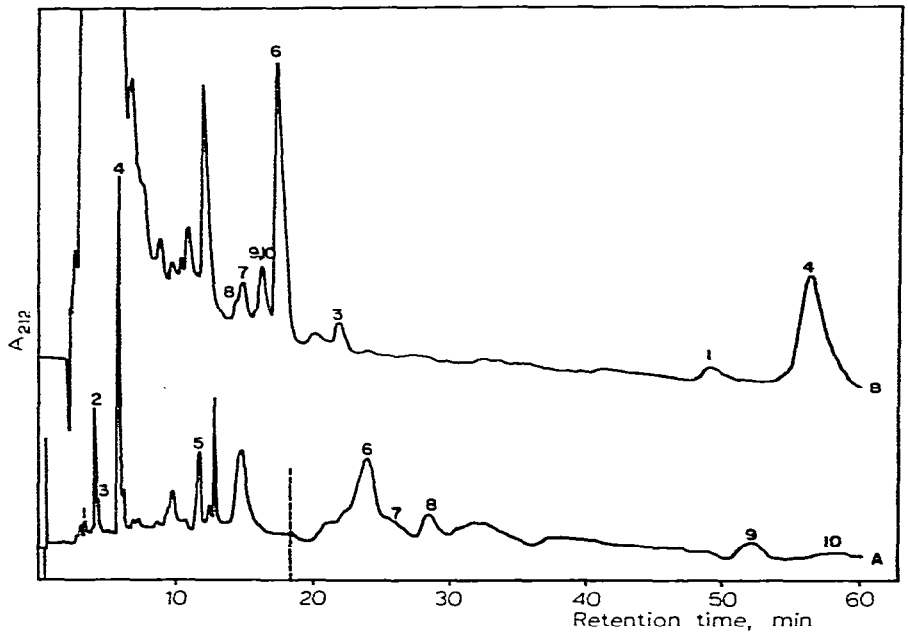

Fig. 1. HPLC of cholesterol autoxidation products, detected by 212 nm light absorption. A, 1.0 mg concentrated autoxidation products mixture, μPorasil, hexane–isopropyl alcohol (24:1), 2.0 ml/min; attenuation 10× up to 9.2 min (dotted line), thereafter attenuation 1×. B, 100 μg concentrated autoxidation products mixture, μBondapak C_{18}, acetonitrile–water (9:1), 1.0 ml/min. Components recognized include: 1 = cholesta-3,5-dien-7-one; 2 = cholest-4-en-3-one; 3 = cholest-4-ene-3,6-dione; 4 = cholesterol; 5 = cholest-5-ene-3β,25-diol; 6 = 3β-hydroxycholest-5-en-7-one; 7 = 3β-hydroxycholest-5-ene-7β-hydroperoxide; 8 = 3β-hydroxycholest-5-ene-7α-hydroperoxide; 9 = cholest-5-ene-3β,7β-diol; 10 = cholest-5-ene-3β,7α-diol.

authentic steroids previously recognized to be cholesterol autoxidation products, retention data for which are recorded in Table I.

Detection of oxidized sterols by absorption at 212 nm is effective for most. Where a specific chromophore is sought, the wavelength of effluent monitoring can be set at other values for optimum sensitivity, such as 284 nm for cholesta-4,6-dienone. Detection by light absorption fails for the isomeric 5,6-epoxides (2) and (3) as these sterols do not absorb well even at 212 nm, and reliance on detection with the differential refractometer must be had. Data of Table II and Fig. 2 were obtained for the 5,6-epoxides (2) and (3) in this manner.

The μPorasil columns are effective for the oxidized sterols listed, but the very long retention time for more polar sterols such as 5α-cholestane-3β,5,6β-triol limits use of the adsorption columns for general analyses. Although binary mixtures of hexane–isopropyl alcohol are very useful for these oxidized sterols, inadequacies do occur, for instance in the resolution of cholest-4-en-3-one from cholesta-4,6-dien-3-one (*cf.* Table I). In this case, the two are resolved using hexane–butanol (99:1), 1.0 ml/min, the enone being more mobile than the dienone. Other nonpolar hydrocarbons are also of use, an isooctane–isopropyl alcohol mixture giving base line resolution of the 3β-benzoate esters of the isomeric 5,6-epoxides (2) and (3) (*cf.* Table II).

Reversed-phase operations using μBondapak C_{18} columns accord more ver-

TABLE I

RELATIVE RETENTION DATA FOR COMMON CHOLESTEROL OXIDATION PRODUCTS

Systems: I, μPorasil, hexane–isopropyl alcohol (24:1), isocratic, 2.0 ml/min; II, μPorasil, linear programming from 90% hexane–isopropyl alcohol (99:1)–10% hexane–isopropyl alcohol (9:1) to 80% hexane–isopropyl alcohol (99:1)–20% hexane–isopropyl alcohol (9:1) over 30 min, 2.0 ml/min; III, μBondapak C_{18}, acetonitrile–water (9:1) isocratic, 1.0 ml/min; IV, μBondapakC_{18}, acetonitrile–water (17:3) isocratic, 1.0 ml/min; V, μBondapak C_{18}, acetonitrile–water (4:1), isocratic 1.0 ml/min. Absolute retention time for cholesterol: I, 5.9 min; II, 12.0 min; III, 57.8 min.

Sterol	*Relative retention time**				
	I	*II*	*III*	*IV*	*V*
Cholesterol	1.00	1.00	1.00	—	—
Cholesta-3,5-dien-7-one	0.13	0.06	0.77	—	—
Cholesta-4,6-dien-3-one	0.44	—	0.75	—	—
Cholest-5-en-3-one	0.12	0.11	1.02	—	—
Cholest-4-en-3-one	0.44	0.39	0.91	—	—
Cholest-4-ene-3,6-dione	0.54	0.47	0.34	—	—
Cholest-5-ene-3β,25-diol	2.01	—	—	—	—
3β-Hydroxycholest-5-en-7-one	5.93	2.80	0.28	1.21	1.21
3β-Hydroxy-5α-cholest-6-ene-5-hydroperoxide	5.35	—	0.24	1.12	1.13
3β-Hydroxycholest-5-ene-7α-hydroperoxide	7.82	3.05	0.21	1.00	1.00
3β-Hydroxycholest-5-ene-7β-hydroperoxide	7.28	2.86	0.23	1.04	1.04
5α-Cholest-6-ene-3β,5-diol	8.51	3.21	0.30	—	—
Cholest-5-ene-3β,7α-diol	19.4	4.27	0.24	—	—
Cholest-5-ene-3β,7β-diol	16.3	3.95	0.25	—	—

* Retention data relative to cholesterol as unity for systems I–III, relative to the 7α-hydroperoxide (6) as unity for systems IV and V.

satility with the polar cholesterol autoxidation products of interest. Here aqueous acetonitrile or methanol mixtures are useful. Acetonitrile–water (9:1) is particularly effective, and increase in the amount of water to as high as a 4:1 v/v proportion retains good resolving power for the isomeric sterol hydroperoxides (*cf.* Table I).

TABLE II

RETENTION DATA FOR ISOMERIC CHOLESTEROL 5,6-EPOXIDE DERIVATIVES

HPLC in system I, μPorasil, hexane–isopropyl alcohol (24:1), 2.0 ml/min; II, μPorasil, isooctane–isopropyl alcohol (499:1), 1.0 ml/min; III, μBondapak C_{18}, acetonitrile–water (9:1), 1.0 ml/min; IV, μBondapak C_{18}, acetonitrile–water (19:1), 2.0 ml/min; V, μBondapak C_{18}, methanol–water (9:1), 1.0 ml/min. Data in parenthesis were obtained with the same system but at a flow-rate of 2.0 ml/min.

Sterol	*TLC**	*Retention time (min)*				
		I	*II*	*III*	*IV*	*V*
Cholesterol (1)	—	—	—	—	61.8	57.0
Cholesterol 3β-benzoate	1.00	—	8.3	—	—	—
5,6α-Epoxy-5α-cholestan-3β-ol (2)	—	14.4	—	29.5 (14.0)	—	25.9
5,6α-Epoxy-5α-cholestan-3β-ol 3β-benzoate	0.68	—	20.4	—	26.4	88.6 (43.3)
5,6β-Epoxy-5β-cholestan-3β-ol (3)	—	16.4	—	27.2 (12.0)	—	23.4
5,6β-Epoxy-5β-cholestan-3β-ol 3β-benzoate	0.87	—	18.8	—	23.0	83.6 (41.1)
5α-Cholestane-3β,5,6β-triol	—	—	—	11.9 (6.4)	—	12.4
5α-Cholestane-3β,5,6β-triol 3β,6β-dibenzoate	0.27	—	66.9	—	25.3	—

* TLC on high-performance chromatoplates irrigated with chloroform; mobilities relative to cholesterol 3β-benzoate as unity.

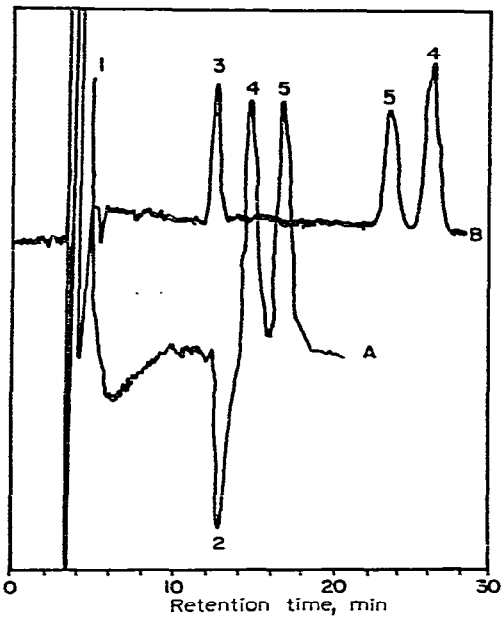

Fig. 2. Resolution of 50 μg each of the isomeric cholesterol 5,6-epoxides and 5α-cholestane-3β,5,6β-triol. A, μPorasil, hexane–isopropyl alcohol (24:1), 2.0 ml/min. B, μBondapak C_{18}, acetonitrile–water (9:1), 1.0 ml/min. Component identities: 1 = chloroform; 2 = impurity in chloroform; 3 = 5α-cholestane-3β,5,6β-triol; 4 = 5,6α-epoxy-5α-cholestan-3β-ol (2); 5 = 5,6β-epoxy-5β-cholestan-3β-ol (3).

A decrease in water proportion accommodates less polar sterols such as the 5,6-epoxide 3β-benzoates (*cf.* Table II).

In Fig. 2 and Table II resolution of the isomeric 5,6-epoxides (2) and (3) is shown using both μPorasil and μBondapak C_{18} columns. Base-line resolutions of the free sterols were obtained in all cases and of the 3β-benzoate esters in all systems save that of aqueous methanol. The 5α,6α-epoxide (2) is the more mobile on adsorption columns, the isomeric 5β,6β-epoxide (3) being more mobile on reversed-phase columns, as anticipated. The 3β-benzoate esters of (2) and (3) are also resolved in both systems; however, the 5β,6β-epoxide 3β-benzoate is the more mobile in both systems, and no inversion of elution order is observed in going from μPorasil to μBondapak C_{18} columns.

The polar sterol 5α-cholestane-3β,5,6β-triol, the common hydration product of both 5,6-epoxides (2) and (3), is handily resolved from the 5,6-epoxides on the μBondapak C_{18} columns, as is also 5α-cholestane-3β,5,6β-triol 3β,6β-dibenzoate from the 5,6-epoxide 3β-benzoates (*cf.* Table II).

In Fig. 3 resolution of the isomeric sterol hydroperoxides 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide (4), 3β-hydroxycholest-5-ene-7α-hydroperoxide (6) and 3β-hydroxycholest-5-ene-7β-hydroperoxide is recorded, again with both μPorasil and μBondapak C_{18} column operations. On μPorasil columns the 5α-hydroperoxide (4) is the most mobile, with the quasiequatorial 3β-hydroxycholest-5-ene-7β-hydroperoxide following (4) but preceding the quasiaxial 7α-hydroperoxide (6). On μ-Bondapak C_{18} columns the order of elution is the reverse. Although 3β-hydroxy-

cholest-5-en-7-one so often present in sterol hydroperoxide preparations lies very close to 3β-hydroxycholest-5-ene-7β-hydroperoxide on the μPorasil column, the 7-ketone is resolved from all three sterol hydroperoxides on the μBondapak C_{18} column.

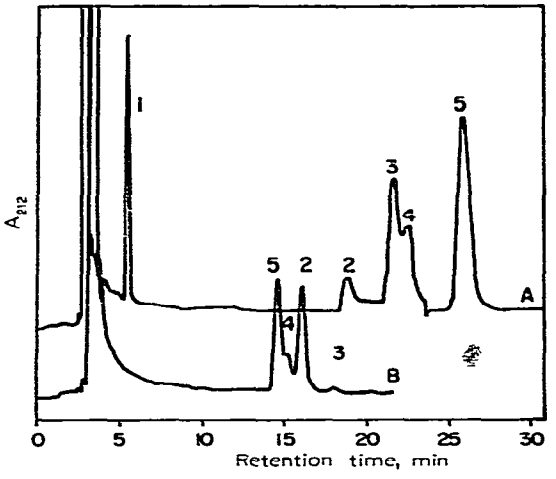

Fig. 3. Resolution of three sterol hydroperoxides and 3β-hydroxycholest-5-en-7-one, detected by 212 nm light absorption. A, μPorasil, hexane–isopropyl alcohol (24:1), 2.0 ml/min. B, μBondapak C_{18}, acetonitrile–water (9:1), 1.0 ml/min. Component identities: 1 = cholesterol; 2 = 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide (4); 3 = 3β-hydroxycholest-5-en-7-one; 4 = 3β-hydroxycholest-5-ene-7β-hydroperoxide; 5 = 3β-hydroxycholest-5-ene-7α-hydroperoxide (6).

HPLC procedures are very useful for isolation of resolved components, as evinced in our recovery of cholesta-4,6-dien-3-one (8) present in autoxidized cholesterol at approximately 40 ng/g levels! Cholesta-4,6-dien-3-one was also found similarly in samples of air-aged cholest-5-ene-3β,7-diol epimers and of cholest-5-ene-3β,7α-diol (7) oxidized by air induced by ionizing radiation.

Attempts to detect 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide (4) and 5α-cholest-6-ene-3β,5-diol (5) were also conducted. Material eluted from regions of thin-layer chromatograms where these components would be found were subjected to HPLC on μPorasil columns using hexane–isopropyl alcohol (24:1) at 2.0 ml/min with component detection by 212 nm light absorption. A small pen excursion on the elution curve at the retention time of the 5α-hydroperoxide (4) was observed but none was seen at the retention time of the 3β,5α-diol (5). Recovery of material from the putative 5α-hydroperoxide (4) fraction and rechromatography on thin-layer chromatoplates using N,N-dimethyl-*p*-phenylenediamine for specific detection of sterol hydroperoxides failed to confirm the presence of the 5α-hydroperoxide (4). Reduction of the material with sodium borohydride in methanol and rechromatography on μPorasil using hexane–isopropyl alcohol (24:1) revealed the presence of the same component at the retention time of the 5α-hydroperoxide (4), thus unaltered by borohydride reduction. Moreover, the expected product 5α-cholest-6-ene-3β,5-diol (5) was not detected in the reduced fraction. Repeated attempts to detect (4) and (5) in different autoxidized cholesterol preparations were equally negative.

DISCUSSION

HPLC is a powerful adjunct to other chromatographic techniques for analysis of mixtures of cholesterol autoxidation products and for recovery of individual components, as evinced by our present results. The procedures are particularly useful for resolution of oxidation products not heretofore resolved chromatographically.

The cholesterol 5,6-epoxides (2) and (3) are cholesterol autoxidation products formed by the attack of initially formed cholesterol 7-hydroperoxides on cholesterol (where the 5β,6β-epoxide (3) predominates[12]) and by oxidation of cholesterol by hydroxyl radical (where the 5α,6α-epoxide (2) predominates[13]). However, direct chromatographic analysis of mixtures of the isomeric 5,6-epoxides has not heretofore been achieved for adequate resolution[12–17], although chromatography of 3β-acetate[16,17] and 3β-benzoate[18] esters and of 3β-trimethylsilyl ethers[16] resolves the isomers. Other analysis means include lithium aluminium hydride reduction of the 5,6-epoxides and chromatography of the resulting product alcohols[12] or proton nuclear magnetic resonance spectroscopy wherein the doublet signal of the 6β-hydrogen of the 5α,6α-epoxide (2) is distinguished from that of the isomeric 5β,6β-epoxide (3)[19–23].

HPLC analysis of 5,6-epoxides mixtures must be conducted with care, whether free sterol, ester or trimethylsilyl ether be used, for the order of elution of the isomeric derivatives varies from system to system. Thus, the 5β,6β-epoxide (3) is the more mobile on thin-layer chromatograms and on gas chromatography on 3% SE-30 and 1% QF-1[12,14] and on μBondapak C_{18} reversed-phase columns of our present study, whereas the isomeric 5α,6α-epoxide (2) is the more mobile in gas chromatography on 1% NGS[14] and on μPorasil columns described herein. Furthermore, the 3β-acetate of (3) is the more mobile on TLC and gas chromatography[16]. Although the 5β,6β-epoxide (3) 3β-trimethylsilyl ether is the more mobile on gas chromatography[14,16], the isomeric 5α,6α-epoxide (2) 3β-trimethylsilyl ether is the more mobile on thin-layer chromatograms[16]. Moreover, the 5β,6β-epoxide (3) 3β-benzoate is the more mobile on thin-layer chromatograms and on μPorasil columns but also on μBondapak C_{18} reversed-phase columns! These data thus evince an inversion of elution order for 3β-benzoates on the reversed-phase system. The use of appropriate reference sterols is obviously necessary for reliance on these means of analysis.

Direct chromatographic analysis of oxidized cholesterol preparations for the 5α-hydroperoxide (4), the major product of attack of singlet molecular oxygen on cholesterol[24] and for the 7α-hydroperoxide (6), formed by free radical autoxidation of cholesterol[25], has also not been possible heretofore for want of adequate resolution. Pyrolysis patterns obtained on gas chromatography distinguish the isomeric hydroperoxides[5], but for analysis of their mixtures recourse to sodium borohydride reduction to the corresponding alcohols 5α-cholest-6-ene-3β,5-diol (5) and cholest-5-ene-3β,7α-diol (7) has been necessary[26]. HPLC on μPorasil or on μBondapak C_{18} columns now accords ready analysis of oxidized cholesterol samples for the 5α-hydroperoxide (4), the 7α-hydroperoxide (6) and for 3β-hydroxycholest-5-ene-7β-hydroperoxide also formed by free radical autoxidation of cholesterol[25].

We have utilized HPLC to search naturally air-aged samples of cholesterol for possible presence of the 5α-hydroperoxide (4). In this regard, we had previously recorded inconclusive data suggesting that the 5α-hydroperoxide (4) be present in

air-aged cholesterol samples[2,26]. Our present HPLC studies now make it clear that the 5α-hydroperoxide (4) is not present in air-aged cholesterol. Moreover, its reduction product 5α-cholest-6-ene-3β,5-diol (5) is also not present.

However, we have detected cholesta-4,6-dien-3-one (8) at 40 ng/g levels in air-aged cholesterol using HPLC. The dienone (8) though a major thermal degradation product of the 5α-hydroperoxide (4)[27] and also a product of singlet molecular oxidation of cholest-5-en-3-one[28] is also a minor thermal degradation product of the epimeric cholest-5-ene-3β,7-diols[27]. Moreover, our present detection of the dienone (8) in oxidized samples containing the 3β,7α-diol (7) supports formation of the dienone (8) from these secondary cholesterol autoxidation products.

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