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⁂High Performance Liquid Chromatography of Oxygenated Cholesterols and Related Compounds

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ABSTRACT

Twenty-four oxygenated cholesterols and structurally related compounds were analyzed by high performance liquid chromatography with silicic acid column and various mobile phases. Hexane/2-propanol was superior to hexane/tetrahydrofuran and hexane/ethyl acetate for the separation of oxygenated cholesterols. The retention volumes of oxygenated cholesterols depended on the characteristics of the substituting group, its position of substitution, as well as its orientation. The effect of various functional groups at different positions on cholesterol molecules, in general order of decreasing retention volumes, were: hydroxy on the ring, carbonyl on the ring, epoxy on the ring, hydroxy on the side chain, and carbonyl on the side chain. Synergistic effect of multiple hydroxyl substitutions on cholesterol was observed.

INTRODUCTION

In developing a quantitative method for the determination of isomeric 5α-cholestan-5,6α-epoxy-3β-ol and 5β-cholestan-5,6β-epoxy-3β-ol (1) in dehydrated eggs, we observed the superior resolution of high performance liquid chromatography (HPLC) over thin layer (TLC) and gas liquid chromatographies (GLC) for the complex mixture of cholesterol autoxidation products. HPLC simplified greatly the quantitation procedure and introduced fewer artifacts.

Among the 50 or more reported cholesterol autoxidation products (2-4), the primary stable derivatives are oxygenated cholesterols derived from substituting hydroxy, carbonyl, and/or epoxy groups on cholesterol. Recently, Ansari and Smith (5) reported the HPLC of a selected number of the cholesterol autoxidation products and demonstrated the powerful capability of HPLC for resolving isomers. In this paper, we report the HPLC of some cholesterol autoxidation products along with some structurally analogous compounds which are not necessarily found among the autoxidation products of cholesterol. The results illustrate the effect of oxygenated functional groups on retention volumes. This knowledge is useful for predicting the HPLC characteristics of related compounds and for identifying unknown compounds in the oxidation mixtures.

EXPERIMENTAL PROCEDURES

Materials

Table I lists the compounds studied by HPLC. All compounds were found chromatographically pure except 5α-cholestan-5,6α-epoxy-3β-ol which contained ca. 5% 5β-cholestan-5,6β-epoxy-3β-ol. No further purification was attempted. The solvents were glass-distilled, HPLC grade, purchased either from Burdick and Jackson, Muskegan, MI, or Fisher Scientific Co., Pittsburgh, PA.

HPLC

HPLC was carried out with a Waters Associates' instrument (Model ALC/GPC 244, Milford, MA) using μPorasil column (3.9 mm × 30 cm). Elution was monitored by an absorption detector (variable wavelength Model 450, Waters Associates) set at 210 nm and a differential refractometer (R-401, Waters Associates) connected in series. Chromatograms were recorded with a dual pen strip chart recorder (Linear Instrument Co., Irvin, CA). Refractometer signals were integrated using Auto-lab System I (Perkin Elmer, Norwalk, CN).

TABLE I

Compounds Studied by HPLC

	mp (C)	Source[a]	Remark[b]
5-Cholesten-3β-ol (Cholesterol)	147-149	1 & 2	
5α-Cholestane	78-80	1	(N)
5-Cholestene	89-90.5	1	(N)
3,5-Cholestadiene	77-79	1	(N)
5α-Cholestan-3β-ol	142-144	2	(N)
5β-Cholestan-3β-ol	101-103	2	(N)
5-Cholesten-3α-ol	142.5-143	1	(N)
5,7-Cholestadien-3β-ol	148-150	3	(N)
5,24-Cholestadien-3β-ol	118-120	1	(N)
5α-Cholestane-3β,6β-diol	–	1	(N)
5-Cholestene-3β,7α-diol	183.5-184.5	1	(O)
5-Cholestene-3β,7β-diol	178-180	1	(O)
5-Cholestene-3β,20-diol	127-128	1	(O)
5-Cholestene-3β,25-diol	178-179	1	(O)
5α-Cholestane-3β,5,6β-triol	230-234	1	(O)
5α-Cholestan-3-one	129-130	2	(O)
5-Cholesten-3-one	127.5-130	1	(O)
5,3-Cholestadien-7-one	111-113	1	(O)
5α-Cholestan-3β-ol-6-one	145-147	3	(N)
5α-Cholestan-3β-ol-7-one	161-164	1	(N)
5-Cholesten-3β-ol-7-one	174-177	1	(O)
5-Cholesten-3β-ol-22-one	–	3	(N)
5α-Cholestan-5,6α-epoxy-3β-ol	137-139	1	(O)
5β-Cholestan-5,6β-epoxy-3β-ol	132-132.5	4	(O)

[a]Sources of supply were: 1) Steraloids Inc., Wilton, NH 03086; 2) Supelco, Inc., Bellefonte, PA 16823; 3) Sigma Chemical Co., St. Louis, MO 63178; 4) synthesized according to the procedure reported by Chicaye, Powrie and Fennema (6).

[b]N: not a reported autoxidation product of cholesterol; O: a reported autoxidation product of cholesterol.

RESULTS

A study to determine the optimal mobile phase was carried out initially. Cholesterol was eluted from a μPorasil column with 3 different binary mobile systems containing hexane. Hexane alone was unable to elute cholesterol from a silicic acid column. Polar additives, 2-propanol, tetrahydrofuran, and ethyl acetate, were selected from 3 different chemical groups but have virtually identical polarity indexes: 4.3, 4.2, and 4.3, respectively (7).

Figure 1 shows the effect of various mobile phases on the retention of cholesterol expressed as the ratio of net retention volume to the void volume of the column, K′. Initially, K′ declined sharply with the addition of polar solvents. The rate of decline decreased as the level of polar additives increased. While 2-propanol was, by far, the most effective additive, tetrahydrofuran was more effective than ethyl acetate. Cholesterol was eluted with K′ of 1.0 by hexane/2-propanol (100:3), hexane/tetrahydrofuran (100:30) or hexane/ethyl acetate (100:40). Hexane/2-propanol (100:3) was the only mobile phase which resolved the isomers of 5α-cholestan-5,6α-epoxy-3β-ol and 5β-cholestan-5,6β-epoxy-3β-ol. Furthermore, hexane/2-propanol systems absorbed the least amount of ultraviolet light so that column flow could be monitored by the absorption detector.

Cholesterol, 3,5-cholestadiene and 8 major cholesterol autoxidation products were resolved isocratically with hexane/2-propanol (100:3) as the mobile phase from a μPorasil column (Fig. 2). Each addition of oxygenated functional group on the cholesterol molecule increased retention volume and dehydroxylated compounds were eluted prior to cholesterol. The sensitivities of the differential refractometer for peaks 1 to 8 was about 10 μg, and 20 μg for peaks 9 and 10. The absorption detector at 210 nm showed higher sensitivity than the differential refractometer except for peaks 6 and 7 which did not respond at all. This mobile phase failed to elute 5α-cholestane-3β,5,6β-triol, a major autoxidation product of cholesterol.

5α-Cholestane-3β,5,6β-triol was eluted successfully by hexane/2-propanol (100:10) (Fig. 3). The increase in 2-propanol content improved the resolution between 5-cholesten-3β,7β-diol and its 7α-epimer, and also the detecting sensitivities of these compounds by both absorption and refractive detectors. However, hexane/2-propanol (100:10) appeared to be too polar for the other compounds included in Figure 2. It failed to resolve 5-cholestene-3β,20-diol and cholesterol, or 5α-cholestan-5,6α-epoxy-3β-ol and its 5,6β-epoxy isomer.

Table II summarizes the relative retention volumes of 24

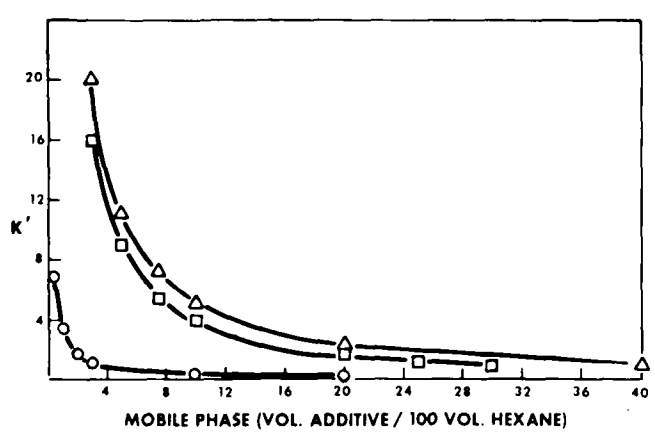

FIG. 1. Relative retention volume, K′, of cholesterol in μPorasil column (3.9 mm D X 30 cm L) eluted with hexane/2-propanol (○), hexane/tetrahydrofuran (□) and hexane/ethyl acetate (△).

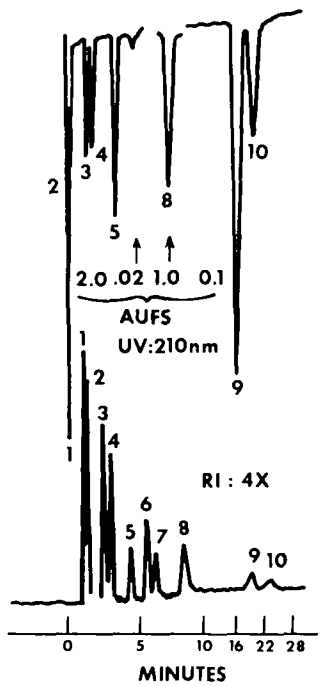

FIG. 2. HPLC of 1) 3,5-cholestadiene; 2) 3,5-cholestadien-7-one; 3) cholesterol; 4) 5-cholestene-3β,20-diol; 5) 5-cholestene-3β,25-diol; 6) 5α-cholestan-5,6α-epoxy-3β-ol; 7) 5β-cholestan-5,6β-epoxy-3β-ol; 8) 5-cholesten-3β-ol-7-one; 9) 5-cholestene-3β,7β-diol; 10) 5-cholestene-3β,7α-diol. Sample dissolved in 100 μL mobile phase was injected onto a μPorasil column (3.9 mm X 30 cm) and eluted isocratically with hexane/2-propanol (100:3) at 3.0 mL/min. Elution was monitored at 210 nm and with a differential refractometer in series. The chart speed was initially at 1/2 cm/min and changed to 1/6 cm/min after 10 min. AUFS = Absorbance Units Full Scale.

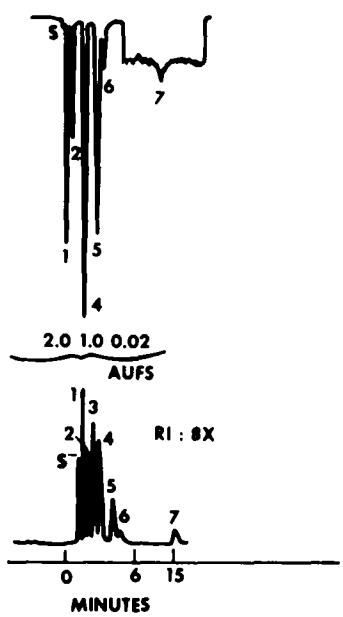

FIG. 3. HPLC of 1) cholesterol; 2) 5-cholestene-3β,25-diol; 3) 5α-cholestan-5,6α-epoxy-3β-ol and 5β-cholestan-5,6β-epoxy-3β-ol; 4) 5-cholesten-3β-ol-7-one; 5) 5-cholestene-3β,7β-diol; 6) 5-cholestene-3β,7α-diol; 7) 5α-cholestane-3β,5,6β-triol. Sample dissolved in 50 μL of mobile phase was injected onto a μPorasil column (3.9 mm X 30 cm) and eluted isocratically with hexane/2-propanol (100:10) at 3.0 mL/min. Elution was monitored at 210 nm and with a differential refractometer in series. Chart speed was initially at 1/2 cm/min and changed to 1/6 cm/min after 6 min. AUFS = Absorbance Units Full Scale.

TABLE II

Relative Retention Volumes, K'*, of Cholesterol and Related Compounds on a μPorasil Column

Compound	Mobile phases		
	Hexane/2-propanol (100:3)	Hexane/2-propanol (100:10)	Hexane/ethyl acetate (100:5)
5α-cholestane	0.05		0.05
5-cholestene	0.05		0.05
3,5-cholestadiene	0.05		0.05
3,5-cholestadien-7-one	0.14		0.70
5α-cholestan-3-one	0.22		1.4
5-cholesten-3-one	0.30		1.6
5-cholesten-3α-ol	0.46		5.1
5β-cholestan-3β-ol	0.70		6.5
5-cholesten-3β-ol (cholesterol)	1.0	0.38	10.4
5α-cholestan-3β-ol	1.1		
5,24-cholestadien-3β-ol	1.2		
5,7-cholestadien-3β-ol	1.2		
5-cholesten-3β-ol-22-one	1.4		
5-cholestene-3β,20-diol	1.5	0.38	
5-cholestene-3β,25-diol	2.9	0.70	
5α-cholestan-5,6α-epoxy-3β-ol	4.4	1.0	
5β-cholestan-5,6β-epoxy-3β-ol	5.0		
5α-cholestan-3β-ol-6-one	5.8		
5α-cholestan-3β-ol-7-one	7.3		
5-cholesten-3β-ol-7-one	7.6	1.4	
5-cholestene-3β,7β-diol	18.5	2.2	
5-cholestene-3β,7α-diol	21.7	2.7	
5α-cholestane-3β,6β-diol	21.7	3.1	
5α-cholestane-3β,5,6β-triol	>50.0	12.2	

*K' = V-Vo/Vo, where V is the retention volume of the compound and Vo the void volume of column.

compounds on a μPorasil column eluted with different mobile phases. Using hexane/2-propanol (100:3) and (100:10) all compounds were eluted within K' of 15, which was the optimal range for our study. Most of the compounds were well resolved except those which differed by only 1 or 2 unsaturated carbons such as 5α-cholestane, 5-cholestene, and 3,5-cholestadiene; 5α-cholestan-3-one and 5-cholesten-3-one; 5α-cholestan-3β-ol, 5-cholesten-3β-ol, 5,7-cholestadien-3β-ol and 5,24-cholestadien-3β-ol; 5α-cholestan-3β-ol-7-one and 5-cholesten-3β-ol-7-one.

The K' of cholesterol in hexane/ethyl acetate (100:5) was about 10 times that in hexane/2-propanol (100:3). Hexane/ethyl acetate (100:5) was found to be an efficient mobile system for the separation of those compounds eluted prior to cholesterol. These compounds included primarily the reduced or dehydroxylated products of cholesterol. Hexane/ethyl acetate (100:5) was also unable to resolve those compounds which differed only in 1 or 2 unsaturated carbons.

The effectiveness of HPLC was demonstrated by resolving epimers such as those listed in Table III. The high resolution factors (>0.85) indicated that K' were affected significantly by the orientation of hydroxy and epoxy substituents.

DISCUSSION

Silicic acid chromatography of lipids has been extensively reviewed (8-11). The work by Hirsch and Ahrens, Jr. (12) and the comprehensive review by Wren (8) are frequently

TABLE III

Effect of Position and Orientation of Hydroxyl Substituent on the Retention of Cholestane and Cholestene Derivatives on Silicic Acid

Compound	Substituent		K'	R_S*
	Position	Orientation	Hexane/2-propanol (100:3)	
5-Cholesten-3α-ol	3-OH	Axial	0.46	1.71
5-Cholesten-3β-ol	3-OH	Equatorial	1.0	
5β-Cholestan-3β-ol	3-OH	Axial	0.70	1.25
5α-Cholestan-3β-ol	3-OH	Equatorial	1.1	
5-Cholestene-3β,7β-diol	3-OH	Equatorial	18.5	1.24
	7-OH	Equatorial		
5-Cholestene-3β,7α-diol	3-OH	Equatorial	21.7	
	7-OH	Axial		
5α-Cholestane-3β,6β-diol	3-OH	Equatorial	21.7	
	6-OH	Axial		
5α-Cholestan-5,6α-epoxy-3β-ol	3-OH	Equatorial	4.35	0.85
	5,6-Epoxy			
5β-Cholestan-5,6β-epoxy-3β-ol	3-OH	Axial	5.0	
	5,6-Epoxy			

*R_S represents resolution factor which is the ratio of the distance between 2 peaks to 1/2 of the sum of the peaks' width on the baseline.

cited literature. Silicic acid absorbs compounds through the free electron pair of the oxygen bridges and the hydrogen bonding of the hydroxyl group. It was not surprising to find that the relative elution power of the three polar additives, 2-propanol, tetrahydrofuran, and ethyl acetate, correlated with their hydrogen bonding capabilities (13).

The retention volumes of oxygenated cholesterols should also correlate to the hydrogen bonding capabilities of their functional groups. However, the K′ as shown in Tables II and III were dependent not only on the nature of the functional groups, but also on their position and orientation. The K′ were more profoundly affected by hydroxyl substituents on carbon-7, such as in 5-cholestene-3β,7α-diol and 5-cholestene-3β,7β-diol, than by those on the side chain, such as in 5-cholestene-3β,20-diol and 5-cholestene-3β,25-diol. Functional groups at various positions of the cholesterol molecule, in general order of decreasing effect on K′, were as follows: hydroxy on the ring, carbonyl on the ring, epoxy on the ring, hydroxy on the side chain, and carbonyl on the side chain.

The combined effects of position and the nature of functional groups on retention volume might be illustrated with hydroxy derivatives. Comparing 5α-cholestane (0.05) to 5α-cholestan-3β-ol (1.1) and 5-cholestene (0.05) to 5-cholesten-3α-ol (0.46) and 5-cholesten-3β-ol (1.0), the introduction of a hydroxy to carbon-3 increased K′ about 1. But when a second hydroxy was introduced, the magnitude of increase of K′ was far greater than the sum of simple addition. For example, the K′ of 5-cholestene-3β,7α-diol, 5-cholestene-3β,7β-diol, and 5-cholestene-3β,25-diol were 21.7, 18.5, and 2.9, respectively. This synergistic effect was apparently the result of increasing accessibility of the second hydroxy once the molecule was absorbed through hydrogen bonding of the other hydroxy. Therefore, the effect would be more profound for a compound whose 2 hydroxyl groups were in close proximity such as 5-cholestene-3β,7α-diol and 5-cholestene-3β,7β-diol (14), than for a compound with 2 hydroxyl groups far apart, such as 5-cholestene-3β,25-diol. Theoretically, if 2 hydroxyl groups were infinitely apart and completely free from each other, the synergism would diminish and the theoretical K′ of the compound would approach a magnitude of 2. The unusually low K′ of 5-cholestene-3β,20-diol (1.5) may be attributed to the steric hindrance by the bulky 4-member ring, the hexanyl, and the methyl groups which prevented the tertiary hydroxy from hydrogen bonding.

Steric effect was apparently the predominant determining factor for the resolution of epimers and isomers (Table III). The greater K′ of 5-cholesten-3β-ol (1.0) than 5-cholesten-3α-ol (0.46) was due to lesser steric hindrance of 3β-hydroxy for hydrogen bonding. The 3β-hydroxy was equatorial and extended horizontally away from the multi-ring plane whereas the 3β-hydroxy was axial and perpendicular to the multi-ring plane. On the other hand, at carbon-7 an equatorial hydroxy rendered less effect on retention time than an axial because of the interference of the hydrogens on carbon-15. Consequently, the K′ of 5-cholestene-3β,7β-diol (18.5) was less than that of 5-cholestene-3β,7α-diol (21.7). The axial 6β-hydroxy of 5α-cholestane-3β,6β-diol rendered an identical elution effect to the axial 7α-hydroxy of 5-cholestene-3β,7α-diol, although the 6β- and 7α-hydroxies were located on the opposite sides of the multi-ring planes of the corresponding molecules. It was likely that most of the axial hydroxies had similar elution effect except the 8β- and 11β-hydroxies, which might have interference from the 2 angular methyl groups of carbons-18 and -19.

The difference in K′ between 5α-cholestan-3β-ol (1.1) and 5β-cholestan-3β-ol (0.7) was due to their difference in molecular configuration. 5α-Cholesten-3β-ol, similar to 5-cholesten-3β-ol, had a rather flat multi-ring plane with all *trans*-configurations. In 5β-cholestan-3β-ol, rings A and B were fused together in *cis*-configuration. Consequently, ring A was at an angle with the flat plane of rings B, C and D, and resulted in a bulkier 3-dimensional molecule with respect to its 3β-hydroxy than the all *trans* 5α-cholestan-3β-ol.

Configurations of α- and β-epoxides were similar to those of 5α- and 5β-cholestan-3β-ol, respectively, except that in cholestanol, carbon-5 and 6 were in staggered conformation, whereas in epoxides, the 2 carbons were eclipsed in order to accomodate the epoxy bond formation. The conformational change was achieved by flipping carbons-6 and -7, which transformed B-ring into boat form. In α-epoxide, the epoxy group was oriented downward from the multi-ring plane and opposite to the equatorial 3β-hydroxy. On the other hand, the epoxy and 3β-hydroxy groups of the β-epoxide were on the same side of the molecule and neighboring to each other. A higher synergistic effect of the 2 neighboring groups in the β-isomer was expected and accounted for the greater K′ for the compound.

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