Journal of Chromatography, 75 (1973) 108–113

Note

CHROM. 6308

Sterol metabolism

XXII. Gas chromatographic differentiation among cholesterol hydroperoxides

Hydroperoxides[1–4] formed in cholesterol autoxidation may be classified by structure into two groups. Those hydroperoxides substituted in the sterol side-chain (the cholesterol 20α-,24-,25-, and 26-hydroperoxides[1–3]) constitute a chromatographically more mobile group giving red or brown-gray colors with sulfuric sprays which may be resolved by thin-layer chromatography (TLC) as such or as their respective sodium borohydride reduction products (the cholest-5-ene-3β,20α-,3β,24-,3β,25-, and 3β,26-diols). They may also be differentiated by gas chromatography (GC) via their individually distinct pyrolysis patterns[2,3,5] in the manner previously demonstrated for ergosterol and cholesta-5,7-dien-3β-ol 5α,8α-peroxides[6].

The cholesterol B-ring hydroperoxides 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide, cholesterol 7α-hydroperoxide, and cholesterol 7β-hydroperoxide[4] constitute a poorly resolved more polar class characterized by blue colors with sulfuric acid on thin-layer chromatograms. Their differentiation is accomplished by prior sodium borohydride reduction to their respective reduction products 5α-cholest-6-ene-3β,5-diol, cholest-5-ene-3β,7α-diol and cholest-5-ene-3β,7β-diol which are readily resolved. This method cannot be employed in samples containing 3β-hydroxycholest-5-en-7-one which is also reduced by borohydride to the epimeric cholest-5-ene-3β,7-diols. Differentiation among the B-ring hydroperoxides by GC has not been achieved heretofore for lack of suitable liquid phases and chromatographic conditions which yield unique pyrolysis patterns for each hydroperoxide. Such failure results from thermal decomposition of the B-ring hydroperoxides to yield mixtures of the epimeric cholest-5-ene-3β,7-diols which are themselves unstable to GC[7–12].

We have now developed suitable GC systems using 2–3% OV-210 and 2–3% SP-2401 (fluoroalkyl silicone) liquid phases which serve acceptably for reliable differentiation between the epimeric cholest-5-ene-3β,7-diols and accordingly among their parent epimeric cholesterol 7-hydroperoxides and 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide.

Experimental

Samples of the sterol allylic alcohols 5α-cholest-6-ene-3β,5-diol (m.p. 147–148°), cholest-5-ene-3β,7α-diol (m.p. 182–183°), and cholest-5-ene-3β,7β-diol (m.p. 174–175°) and of the sterol hydroperoxides 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide, cholesterol 7α-hydroperoxide, and cholesterol 7β-hydroperoxide[4] pure by melting point and infrared absorption spectra criteria were all shown to be free of other detectable sterols by TLC using 20 × 20 cm chromatoplates, 0.25 mm thick, of Silica Gel HF_{254} (E. Merck, GmbH, Darmstadt) using solvent systems previously

described[1-4,13] and visualization procedures including sulfuric acid for sterols[13] and N,N-dimethyl-*p*-phenylenediamine for sterol hydroperoxides[14].

GC was conducted on 1.83-m long silanized glass U-tubes packed with 2 % or 3 % OV-210 and with 2 % or 3 % SP-2401 phases on 100–120 mesh Supelcoport (Supelco Inc., Bellafonte, Pa.) as well as on 3 % QF-1 and 3 % SE-30 for comparison purposes, as previously described[12]. Analyses were performed using 4-mm I.D. columns with sterol samples weighing no more than 5 μg. Preparative GC was conducted using 6-mm I.D. columns. A Hewlett-Packard Corp. F and M Model 400 or Model 402 gas chromatograph equipped with a hydrogen flame ionization detector system was used throughout, with nitrogen as carrier gas (20 ml/min) operated with injection port temperature 250 °, column temperature 230 °, and detector temperature 250 °. Sterol samples dissolved in chloroform–methanol (2 : 1) were injected on to the columns with a syringe in the usual manner. Elution curves for a given sample were reproducible in every case, but product proportions and absolute retention times varied with column operating conditions (aging and loading of liquid phase) and with the amount of sterol sample injected. Furthermore, the relative retention times of Table I, measured *versus* cholesterol as unit time, were subject to a greater variability than usually encountered for these same sterols on 3 % QF-1 or 3 % SE-30 liquid phases under similar operating conditions[12].

TABLE I

GC RETENTION DATA FOR SELECT STEROLS

Sterol	*Relative retention time*[a]		
	3% OV-210	*3% SP-2401*	*3% QF-1*[b]
Cholesterol (cholest-5-en-3β-ol)	1.00	1.00	1.00
Cholesta-2,4,6-triene	0.41	0.41	0.47
Cholesta-3,5-dien-7-one	2.16	2.00	2.14
Cholest-5-ene-3β,7α-diol	2.27	2.16	2.18
Cholest-5-ene-3β,7β-diol	2.46	2.31	2.34
Cholesta-4,6-dien-3-one	3.46	3.31	3.24
3β-Hydroxycholest-5-en-7-one	5.01	4.73	4.70

[a] Determined on suitably aged columns. Relative retention times decrease with an increase in column aging.

[b] Data from VAN LIER AND SMITH[12].

Adequate thermal stability and suitable resolution of the epimeric cholest-5-ene-3β,7-diols is the key to successful gas chromatographic differentiation among the three B-ring hydroperoxides. Both criteria are met by either OV-210 or SP-2401 liquid phases using usual operating conditions. Typical elution curves for the allylic alcohols 5α-cholest-6-ene-3β,5-diol, cholest-5-ene-3β,7α-diol, and cholest-5-ene-3β,7β-diol on analytical columns of 3 % SP-2401 liquid phase are presented in Fig. 1. Very similar behavior was obtained on 2 % SP-2401 and on 2–3 % OV-210 phases. The quasi-axial 3β,7α-diol was eluted ahead of the quasi-equatorial 3β,7β-diol as is the case for this epimeric pair on 3 % QF-1 (ref. 12) and in other liquid partition chromatographic systems[13,15-17]. The epimerization of one 3β,7-diol to the other with concomitant dehydration to a common cholestatriene which compromised previous attempted GC of these allylic alcohols[7-12] was absent under our present

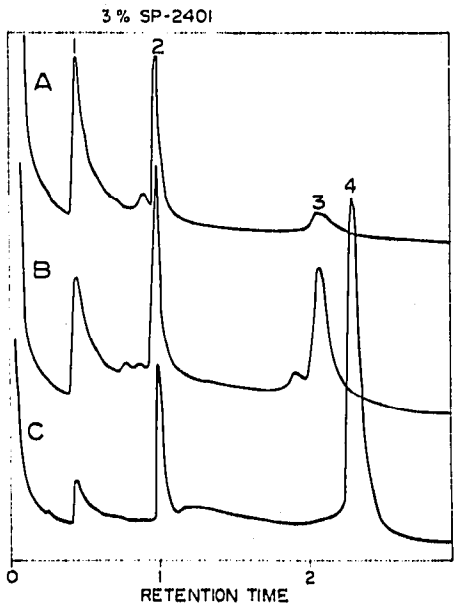

Fig. 1. GC elution curve of sterol allylic alcohols on 3% SP-2401 aged column. (A) 5α-cholest-6-ene-3β,5-diol. (B) cholest-5-ene-3β,7α-diol. (C) cholest-5-ene-3β,7β-diol. Component identities: No. 1, cholesta-2,4,6-triene; No. 2, cholesterol added for precise retention time measurement; No. 3, cholest-5-ene-3β,7α-diol; No. 4, cholest-5-ene-3β,7β-diol.

operating conditions. Dehydration of ether 3β,7-diol to the common cholestatriene, most probably cholesta-2,4,6-triene, was the only major thermal decomposition reaction encountered on analytical columns of either OV-210 or SP-2401. The full proof of structure for this product and the several other thermal decomposition products identified herein will be reported elsewhere.

These results were dependent on the amounts of sterols injected and on the aging properties of the liquid phases on the columns used. Analysis conditions were best achieved with sterol samples no larger than 5 μg. Larger sterol samples on analytical columns or on larger preparative columns regularly yielded additional thermal decomposition products. Newly prepared columns of either phase were less suitable for analysis of these unstable sterols, and in our hands an aging through use of several weeks was required for optimal performance. We have regularly used these OV-210 and SP-2401 columns for analysis of allylic alcohols and hydroperoxides only after such aging. Retention times of cholesterol and other sterols become shorter as aging proceeds, apparently from loss of fluoroalkyl silicone liquid phase. A 2% OV-210 or 2% SP-2401 loading is superior for these studies than is a 3% loading, although both 2% and 3% loadings afford the stability and resolution required for these purposes.

In contrast to the behavior of the epimeric cholest-5-ene-3β,7-diols the isomeric 5α-cholest-6-ene-3β,5-diol on either OV-210 or on SP-2401 did not survive GC as such, dehydration to cholesta-2,4,6-triene and isomerization to cholest-5-ene-3β,7α-diol being the major thermal processes involved (Fig. 1A).

Injection of larger amounts (10–20 μg) of the allylic alcohols gave more complex curves which contained, in addition to the major thermal degradation products

mentioned, low variable amounts of several other components, of which cholesta-3,5-dien-7-one, cholesta-4,6-dien-3-one, and 3β-hydroxycholest-5-en-7-one have been identified. Although these are minor products obtained only at the higher sample injection levels, one must recognize that these B-ring allylic alcohols are subject to dehydrogenation reactions as well as allylic rearrangement and dehydration reactions. However, even at the higher sample charges epimerization of either cholest-5-ene-3β,7-diol one to the other was not detected. We conclude that where either the 3β,7α-diol or the 3β,7β-diol be detected in these analyses that neither arose via epimerization from the other.

GC of the B-ring hydroperoxides on either OV-210 or SP-2401 phases afforded unique elution curves of the pyrolysis products such that recognition and identification of the cholesterol hydroperoxide injected could be determined. In Fig. 2 the elution curves for the Δ^6-5α-hydroperoxide, the Δ^6-7α-hydroperoxide, and the Δ^6-7β-hydroperoxide are presented. Differentiation between the epimeric cholesterol may be had by inspection, the 7α-hydroperoxide decomposing thermally to cholest-5-ene-3β,7α-diol as a major product, the 7β-hydroperoxide decomposing similarly to cholest-5-ene-3β,7β-diol as a major product. Mixtures of the epimeric cholesterol 7-hydroperoxides afford GC elution curves containing both epimeric

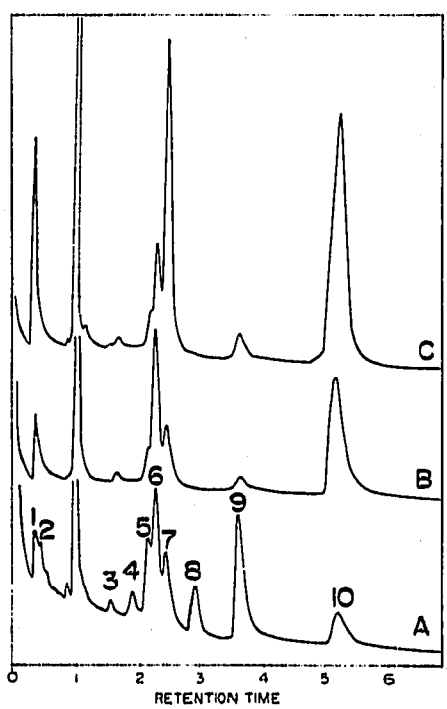

Fig. 2. GC elution curve of sterol hydroperoxides on 3% OV-210 aged column. (A) 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide. (B) cholesterol 7α-hydroperoxide. (C) cholesterol 7β-hydroperoxide. Component retention times and identities are: No. 1, 0.43, cholesta-2,4,6-triene; No. 2, 0.51, unidentified; No. 3, 1.56, unidentified; No. 4, 1.90, unidentified; No. 5, 2.17, cholesta-3,5-dien-7-one; No. 6, 2.25, cholest-5-ene-3β,7α-diol; No. 7, 2.45, cholest-5-ene-3β,7β-diol; No. 8, 2.87, unidentified; No. 9, 3.57, cholesta-4,6-dien-3-one; No. 10, 5.04, 3β-hydroxycholest-5-en-7-one. Cholesterol (retention time 1.00) was added for precise retention time measurement but is not numbered as a component.

cholest-5-ene-3β,7-diols. It must be noted that minor amounts of epimerization of the 7α-hydroperoxide prior to thermal degradation must occur, as small amounts of cholest-5-ene-3β,7β-diol appear in the elution curve from the 7α-hydroperoxide. Similarly the 7β-hydroperoxide must also epimerize to a minor extent, as small amounts of the 3β,7α-diol are present among its pyrolysis products. The other major thermal decomposition product from the cholesterol 7-hydroperoxides is the dehydration product cholesta-2,4,6-triene. Minor products identified include 3β-hydroxycholest-5-en-7-one, its dehydration product cholesta-3,5-dien-7-one, and cholesta-4,6-dien-3-one (Figs. 2B and 2C).

The Δ^6-5α-hydroperoxide decomposes along similar pathways but in different proportions so as to afford a unique pyrolysis pattern (Fig. 2A). The chief pyrolysis product is cholesta-4,6-dien-3-one, the epimeric cholest-5-ene-3β,7-diols, 3β-hydroxycholest-5-en-7-one, and cholesta-3,5-dien-7-one being minor products. Furthermore, the dehydration product cholesta-2,4,6-triene formed uniformly by all of these allylic alcohols and hydroperoxides is accompanied by a second putative cholestatriene of undetermined structure. The combination of two rapidly eluted cholestatriene components with major amounts of cholesta-4,6-dien-3-one (and minor amounts of the other decomposition components) serves adequately to identify the Δ^6-5α-hydroperoxide and to differentiate it from either epimeric cholesterol 7-hydroperoxide.

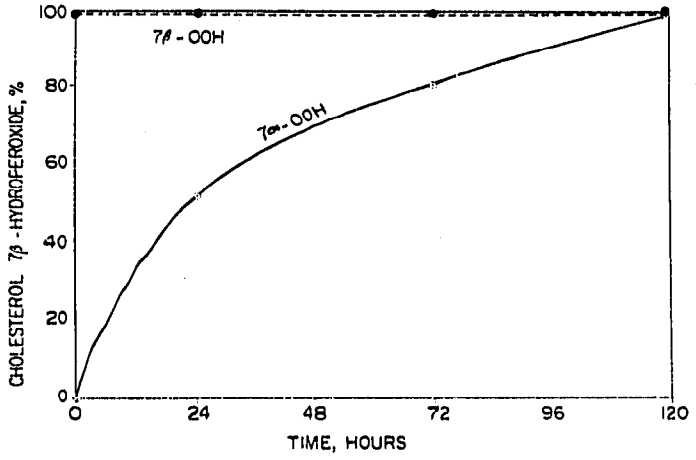

Fig. 3. Rate of epimerization of cholesterol 7α-hydroperoxide to cholesterol 7β-hydroperoxide in acetone at 50°, as determined by GC measurement of the proportion of cholest-5-ene-3β, 7α-diol and cholest-5-ene-3β,7β-diol formed on GC analysis.

The GC procedures permit detection and identification of minute amounts of the cholesterol B-ring hydroperoxides in a variety of circumstances known to form sterol hydroperoxides, including enzymic reactions of cholesterol such as soybean lipoxygenase[18] and a number of autoxidation conditions[14]. The utility of the method is further exemplified in following the time course of the epimerization of cholesterol 7α-hydroperoxide to cholesterol 7β-hydroperoxide, as presented in Fig. 3. These data were obtained with the present means at a considerable saving of time and effort over the demanding and tedious TLC methods previously used for this purpose[4].

This work was supported financially by research grants from the U.S. Public Health Service (AM-13520 and NS-08106).

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1 J. E. VAN LIER AND L. L. SMITH, *J. Org. Chem.*, 35 (1970) 2627.
2 J. E. VAN LIER AND L. L. SMITH, *J. Org. Chem.*, 36 (1971) 1007.
3 J. E. VAN LIER AND G. KAN, *J. Org. Chem.*, 37 (1972) 145.
4 J. I. TENG, M. J. KULIG, L. L. SMITH, G. KAN AND J. E. VAN LIER, *J. Org. Chem.*, in press.
5 J. E. VAN LIER AND L. L. SMITH, *Steroids*, 15 (1970) 485.
6 G. A. BLONDIN, B. D. KULKARNI, J. P. JOHN, R. T. VAN ALLER, P. T. RUSSELL AND W. R. NES, *Anal. Chem.*, 39 (1967) 36.
7 J. R. CLAUDE AND J. L. BEAUMONT, *C. R. Acad. Sci., Paris*, 260 (1965) 3204.
8 J. R. CLAUDE AND J. L. BEAUMONT, *J. Chromatogr.*, 21 (1966) 189.
9 J. R. CLAUDE, *J. Chromatogr.*, 23 (1966) 267.
10 A. FIORITI AND R. J. SIMS, *J. Amer. Oil. Chem. Soc.*, 44 (1967) 221.
11 E. CHICOYE, W. D. POWRIE AND O. FENNEMA, *Lipids*, 3 (1968) 551.
12 J. E. VAN LIER AND L. L. SMITH, *Anal. Biochem.*, 24 (1968) 419.
13 L. L. SMITH, W. S. MATTHEWS, J. C. PRICE, R. C. BACHMANN AND B. REYNOLDS, *J. Chromatogr.*, 27 (1967) 187.
14 L. L. SMITH AND F. L. HILL, *J. Chromatogr.*, 66 (1972) 101.
15 E. H. MOSBACH, M. NIERENBERG AND F. E. KENDALL, *J. Amer. Chem. Soc.*, 75 (1953) 2358.
16 L. L. SMITH, *J. Amer. Chem. Soc.*, 76 (1954) 3232.
17 K. SCHUBERT, G. ROSE, AND M. BÜRGER, *Hoppe Seyler's Z. Physiol. Chem.*, 326 (1961) 235.
18 J. I. TENG AND L. L. SMITH, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 31 (1972) 913.

Received August 1st, 1972