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CHROMATOGRAPHY OF SOME CHOLESTEROL AUTOXIDATION PRODUCTS ON SEPHADEX LH-20

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SUMMARY

The resolution on Sephadex LH-20 lipophylic gel of several C_{27}, C_{24}, C_{21}, and C_{19} steroids of interest in our studies of cholesterol autoxidation has been achieved. Relative mobilities of twenty steroids on adsorption thin-layer chromatograms are compared with relative mobilities on Sephadex LH-20, and a marked retardation effect on Sephadex LH-20 of four sterol hydroperoxides was noted.

INTRODUCTION

Gel permeation chromatography of steroids on the lipophylic gel Sephadex LH-20 include resolution of C_{19} steroid conjugates[1–4], C_{21} steroid conjugates[4,5], C_{24} bile acid conjugates[2], and estrogens[6,7], as well as of a variety of neutral C_{19}–C_{30} steroids[7–9]. In our continued examination of the air oxidation of cholesterol[10,11] we have demonstrated the presence of several previously undescribed cholesterol hydroperoxide derivatives[12,13]. These hydroperoxides were readily resolved from their closely related hydroxysterol analogs by chromatography on Sephadex LH-20 using organic solvents for development, whereas the usual adsorption chromatography technique failed to resolve these sterols satisfactorily.

On examination of the relative position of elution of these several hydroperoxides relative to their respective (reduced) alcohols it was clear that a marked retardation on Sephadex LH-20 was obtained for the hydroperoxides, whereas their affinity in adsorption systems was diminished. Thus, 3β-hydroxycholest-5-ene-25-hydroperoxide (No. 13) and 3β-hydroxycholest-5-ene-20α-hydroperoxide (No. 12) were eluted at about twice the volumes required to elute cholest-5-ene-3β,25-diol (No. 8) and cholest-5-ene-3β,20α-diol (No. 7) respectively. This selective retardation of cholesterol hydroperoxides prompted our present report on the potential use of Sephadex LH-20 for resolution of oxygenated steroids.

EXPERIMENTAL

Materials

All sterols and sterol hydroperoxides were purified by column and thin-layer

chromatography and by recrystallization. All solvents used were manufacturers' analytical reagent quality and were redistilled shortly before use. Column fractions were analyzed by thin-layer chromatography and in certain cases also by gas chromatography[11]. Appropriate fractions were pooled and evaporated under vacuum on a rotary evaporator or under a stream of nitrogen and stored at 0° pending further disposition.

Thin-layer chromatography

Thin-layer chromatography was conducted on 20 × 20 cm chromatoplates of Silica Gel HF_{254} (E. Merck, Darmstadt), 0.5 mm in thickness using benzene–ethyl acetate (3:2) and other related solvent systems previously described[10] for irrigation. Solvent rise was allowed to proceed to 17.5 cm. The chromatoplates were dried, and the resolved steroids visualized by initially viewing the chromatoplate under ultraviolet light (254 and 365 nm), followed by lightly spraying with 50 % aqueous sulfuric acid and heating at 110° until characteristic colors were developed. Sterol hydroperoxides were detected on chromatoplates not sprayed with sulfuric acid by means of the ammonium thiocyanate-ferrous sulfate reagent or the potassium iodide–starch reagent listed by WALDI (No. 5 and 85 respectively)[14], the potassium iodide–starch test being preferred.

Sterol hydroperoxides were recognized on thin-layer chromatograms on the basis of double criteria: the peroxide color tests and chromatographic behavior of the sterol after sodium borohydride reduction directly on the chromatoplate. After a test sterol sample (2–10 µg) was spotted in the usual manner on the start line of the Silica Gel HF_{254} chromatoplate (spotted in chloroform or diethyl ether to minimize spreading of the sterol on the chromatoplate), 5 µl of a freshly prepared 1 % solution of sodium borohydride in methanol was carefully spotted directly onto the sterol spot. After the methanol had evaporated the chromatoplate was irrigated in the usual fashion, dried, and visualized. For those samples containing sterol hydroperoxides a characteristic shift in position occurred in the borohydride reduced test sample, the reduced sterol alcohol being more polar in every instance than the parent hydroperoxide. By comparison of sterol sample "as is" and as reduced *in situ* by borohydride it was possible to recognize the presence of the relevant sterol hydroperoxides.

Necessarily, the positive peroxide color responses obtained on the reduced hydroperoxides were not obtained on the borohydride reduced components.

Chromatography on Sephadex LH-20

A sufficient amount of Sephadex LH-20 (Pharmacia Fine Chemicals Inc., Uppsala) was slurried with 3–4 volumes of methylene chloride so as to provide enough packing for the column to be used. Rapid swelling of the Sephadex LH-20 occurred, to 220 % of the initial dry volume. Additional methylene chloride was added to permit pouring the slurry readily into the column. The slurry was carefully poured into the column and was packed with slight air pressure. When the desired column height was attained, any remaining slurry was pipetted from the column, the glass walls rinsed with methylene chloride, and slight air pressure again applied to give a firm packing. When the surface of the Sephadex LH-20 just became free of wetting solvent the solvent flow was stopped and 0.5 cm of sand was added to the surface of the packing to prevent floating of the upper layer of the Sephadex LH-20. Methylene chloride was

added to wet just the sand, and the column was then ready for the application of the sample. For preparative chromatography involving very close separations of sterol components a 1 cm layer of alumina (activity I) was placed on the top of the prepared Sephadex LH-20 column packing, with an additional 0.5 cm of sand atop the alumina layer. The sample in methylene chloride was washed onto the column with as much methylene chloride as needed. The sample adsorbed on the alumina layer was then eluted by frontal elution using a few milliliters of methylene chloride-methanol (4:1) directly onto the Sephadex LH-20 bed, and elution was continued with methylene chloride.

Glass columns 1 cm in diameter and 30 or 60 cm long were used for analytical and small scale preparative work, with 20–200 mg of sterol sample dissolved in 0.5 ml of methylene chloride being chromatographed on 18 g of Sephadex LH-20 on the 1 × 60 cm columns, 3 ml fractions being taken on an automatic fraction collector. For larger preparative separations (0.1–2 g of sample) 2.5 × 60 cm columns (110 g of Sephadex LH-20) were used, with 20 ml fractions being collected. For sterol samples not completely dissolved by methylene chloride a few drops of methanol were added to complete solution.

Analytical and most preparative work was done with neat methylene chloride as irrigating solvent. However, methylene chloride–10% acetone (by volume) was used in gross group separations and in initial separations of larger amounts of material (up to 20 g), with 5 × 40 cm columns. Methylene chloride–10% acetone caused the Sephadex LH-20 to swell less and also eluted components at greatly reduced elution volumes (approximately 30–50% of elution volumes with neat methylene chloride).

Solvent flow rates of 0.5–1.0 ml/min were optimum for 1 cm diameter columns, 2–4 ml/min for the 2.5 cm diameter columns. The desired flow rate was obtained by application of slight pressure to the top of the column or by adjustment of the height of the solvent reservoir.

Mobility data were accumulated in terms of elution volumes—the volume of irrigating solvent required to elute the component at maximum concentration from the Sephadex LH-20 column under fixed, uniform conditions. Data of Table I were obtained on 1 × 60 cm columns as previously described. A given sterol was completely eluted in a volume of solvent equal to 10–15% of the peak elution volume, the exact total volume of solvent needed to elute all of the component depending on the weight of sample applied and on the volume of solvent used to bring the sample onto the column.

DISCUSSION

Relative mobility data on thin-layer adsorption chromatograms and elution volumes on Sephadex LH-20 columns are presented in Table I. From these data a decrease in elution volume on Sephadex LH-20 is apparent for cholesteryl acetate (No. 2) and for cholesta-3,5-dien-7-one (No. 3), two sterol derivatives regularly considered to be less polar than cholesterol on adsorption chromatography[10].

Similarly, an increase in elution volume is obtained for sterols with additional ketone and hydroxyl groups, in the approximate order anticipated from prior adsorption chromatography experience. Thus 3β-hydroxycholest-5-en-7-one (No. 4) is retarded relative to cholesterol (No. 1), and its reduction products, the epimeric

TABLE I

RELATIVE MOBILITIES OF STEROIDS ON SEPHADEX LH-20

No.	*Steroid*	*Relative mobilities*	
		TLC[a]	*LH-20*[b]
1	Cholest-5-en-3β-ol (cholesterol)	1.00	1.00
2	Cholest-5-en-3β-yl (cholesteryl) acetate	1.52	0.67[c]
3	Cholesta-3,5-dien-7-one	1.48	0.67
4	3β-Hydroxycholest-5-en-7-one	0.48	1.17[c]
5	Cholest-5-ene-3β,7α-diol	0.22	1.50
6	Cholest-5-ene-3β,7β-diol	0.27	1.57
7	Cholest-5-ene-3β,20α-diol	0.83	1.17
8	Cholest-5-ene-3β,25-diol	0.52	1.47
9	5α-Cholestane-3β,5,6β-triol	0.05	5.33
10	Cholest-5-ene-3β,20α,22R-triol	0.56	2.36
11	3β-Hydroxycholest-5-ene-17α-hydroperoxide	0.91	2.80
12	3β-Hydroxycholest-5-ene-20α-hydroperoxide	0.93	2.13
13	3β-Hydroxycholest-5-ene-25-hydroperoxide	0.84	2.90
14	3β-Hydroxy-5α-cholest-6-ene 5-hydroperoxide	0.59	3.60
15	3β-Hydroxychol-5-en-24-ic acid	0.10	>6.0
16	3β-Hydroxypregn-5-en-20-one	0.74	1.17
17	Pregn-5-ene-3β,20α-diol	0.41	1.90
18	Pregn-5-ene-3β,20β-diol	0.51	1.73
19	Pregn-5-en-3β-ol	0.94	1.23
20	3β-Hydroxyandrost-5-en-17-one	0.65	1.23
21	Androst-5-ene-3β,17β-diol	0.41	2.33

[a] Relative mobility on thin-layer chromatograms irrigated with benzene–ethyl acetate (3:2), with cholesterol (No. 1) as unity. The absolute R_F value of cholesterol was 0.50.
[b] Relative elution volumes on Sephadex LH-20 columns irrigated with methylene chloride, with the elution volume of cholesterol as unity. The absolute elution volume of cholesterol was 30 ml.
[c] Comparable data from ENEROTH AND NYSTRÖM[7] included 0.68 for No. 2, 1.11 for No. 4.

7-hydroxycholesterols cholest-5-ene-3β,7α-diol (No. 5) and cholest-5-ene-3β,7β-diol (No. 6) are still more retarded on Sephadex LH-20. Notably, however, the elution volume of the 7α-epimer (No. 5) is slightly less than that of the 7β-epimer (No. 6) and thereby is the reverse of the order of elution obtained on adsorption chromatography[10]. Thus, elution volumes of the 7-hydroxycholesterol epimers more nearly correspond with the order obtained on partition columns and on partition paper chromatographic systems[10,15].

The several hydroxycholesterol derivatives cholest-5-ene-3β,7α-diol (No. 5), cholest-5-ene-3β,7β-diol (No. 6), cholest-5-ene-3β,20α-diol (No. 7), and cholest-5-ene-3β,25-diol (No. 8) exhibit differential retardation of Sephadex LH-20, such that resolution of their mixtures may be possible, and the order of elution from Sephadex LH-20 roughly parallels their R_F values on adsorption chromatography with the exception of the 7-hydroxycholesterols already noted. It is noteworthy that the 20α-hydroxyl group of cholest-5-ene-3β,20α-diol (No. 7) has exactly the same retardation effect on Sephadex LH-20 as does the 7-ketone group of 3β-hydroxycholest-5-en-7-one (No. 4); however, the polarity of the 7-ketone group on adsorption thin-layer chromatography is marked in comparision with that of the 20α-hydroxyl group.

It is of interest that several ketones have about the same elution volume on Sephadex LH-20, without regard to molecular size. Thus 3β-hydroxycholest-5-en-7-

one (No. 4), 3β-hydroxypregn-5-en-20-one (No. 16), and 3β-hydroxyandrost-5-en-17-one (No. 20) have about the same elution volume. As previously noted for the cholestene derivatives, the alcohols are much more retarded on Sephadex LH-20 than are the corresponding ketones. Thus, both epimeric pregn-5-ene-3β,20-diols (No. 17 and No. 18) are substantially retarded in comparison with the parent 20-ketone (No. 16), and androst-5-ene-3β,17β-diol (No. 21) is retarded *versus* the 17-ketone (No. 20).

The mobility behavior of the 20-ketone 3β-hydroxypregn-5-en-20-one (No. 16) constitutes another clear case of the "ketone exclusion" effect described by ENEROTH AND NYSTRÖM[7], since the related 20-deoxosteroid pregn-5-en-3β-ol (No. 19) is eluted after the 20-ketone. In regard to the "ketone exclusion" effect, we also confirm that 3β-hydroxycholest-5-en-7-one does not appear to follow this generality[7].

Although liquid-gel partition and molecular sieving appear to be major processes involved in chromatography of neutral steroids on Sephadex LH-20[7] other factors may obtain in the case of the sterol hydroperoxides of our interest. A plot of the relative mobility of fourteen C_{27} steroids on adsorption-type thin-layer chromatograms *versus* relative mobility on Sephadex LH-20 columns in Fig. 1 yields linear relationships with both methylene chloride and with methylene chloride–10 % acetone as irrigating solvents in the Sephadex LH-20 chromatography (use of methylene chloride–10% acetone diminishes the slopes of the lines but does not destroy the separate linear relationships). This plot indicates that steroids bearing three oxygen atoms (the four sterol hydroperoxides Nos. 11–14 and the two triols Nos. 9 and 10) behave differently from related steroids bearing only one or two oxygen atoms per molecule.

These several differences in mobilities on Sephadex LH-20 and silica gel adsorption systems may be put to good use in resolution of complex mixtures of cholesterol autoxidation products, where reliance on one of the systems alone may not permit satisfactory resolution of the particularly complex samples and where gas chromatography cannot be applied[11].

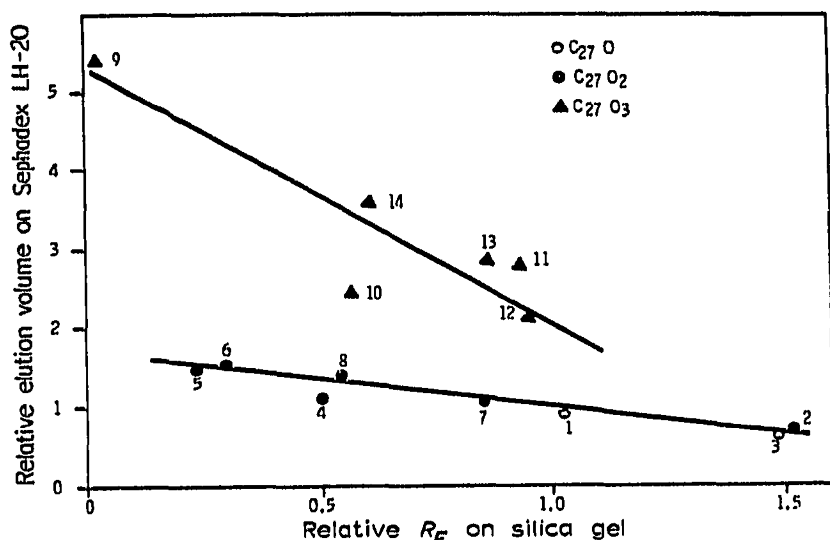

Fig. 1. Differential behavior of $C_{27}O_3$ sterol derivatives on adsorption chromatography and on Sephadex LH-20 *versus* $C_{27}O$ and $C_{27}O_2$ derivatives.

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REFERENCES

1 R. VIHKO, *Acta Endocrinol.*, 52, Supplement 109 (1966) 15, 21.
2 J. SJÖVALL AND R. VIHKO, *Acta Chem. Scand.*, 20 (1966) 1419.
3 J. SJÖVALL, K. SJÖVALL AND R. VIHKO, *Steroids*, 11 (1968) 703.
4 J. SJÖVALL AND R. VIHKO, *Acta Endocrinol.*, 57 (1968) 247.
5 J. SJÖVALL AND K. SJÖVALL, *Steroids*, 12 (1968) 359.
6 H. VAN BAELEN, W. HEYNS AND P. DE MOOR, *J. Chromatog.*, 30 (1967) 226.
7 P. ENEROTH AND E. NYSTRÖM, *Biochim. Biophys. Acta*, 144 (1967) 149.
8 T. SEKI, *J. Chromatog.*, 29 (1967) 246.
9 P. ENEROTH AND E. NYSTRÖM, *Steroids*, 11 (1968) 187.
10 L. L. SMITH, W. S. MATTHEWS, J. C. PRICE, R. C. BACHMANN AND B. REYNOLDS, *J. Chromatog.*, 27 (1967) 187.
11 J. E. VAN LIER AND L. L. SMITH, *Anal. Biochem.*, 24 (1968) 419.
12 J. E. VAN LIER AND L. L. SMITH, *5th Intern. Symp. on the Chemistry of Natural Products, London, July 8–13, 1968, Abstracts*, p. 360.
13 J. E. VAN LIER AND L. L. SMITH, *156th Natl. Meeting Am. Chem. Soc., Atlantic City, Sept. 8–13, 1968, Abstracts of Papers*, ORGN-58.
14 D. WALDI, in E. STAHL (Editor), *Thin Layer Chromatography, A Laboratory Handbook*, Springer Verlag, Berlin, 1965, pp. 483–502.
15 L. L. SMITH, *J. Am. Chem. Soc.*, 76 (1954) 3232.