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Sterol synthesis. High-pressure liquid chromatography of C_{27} sterol precursors of cholesterol

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Summary Conditions have been described which permit the rapid (~3.5 hr) column chromatographic separation of the acetate derivatives of a number of C_{27} sterol precursors of cholesterol differing only in the number and position of double bonds in the sterol nucleus. On columns containing μPorasil with hexane–benzene 9:1 as the eluting solvent, acetate derivatives of the Δ^5, $\Delta^{8(14)}$, Δ^8, Δ^7, $\Delta^{5,7}$, $\Delta^{8,14}$, and $\Delta^{7,14}$ sterols were separated.—**Thowsen, J. R., and G. J. Schroepfer, Jr.** Sterol synthesis. High-pressure liquid chromatography of C_{27} sterol precursors of cholesterol. *J. Lipid Res.* 1979. **20:** 681–685.

Supplementary key words sterol precursors of cholesterol

The enzymatic formation of cholesterol from lanosterol (4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol) involves a very large number of potential intermediates (1–4). A number of these potential intermediates include sterols that differ only in the number and position of nuclear double bonds. These variants include sterols with nuclear double bonds in the following positions: Δ^8, $\Delta^{8(14)}$, Δ^7, Δ^5, $\Delta^{8,14}$, $\Delta^{7,14}$ and $\Delta^{5,7}$. The separation of these compounds from one another is a less than trivial matter. In the C_{27} series, the separation of cholesterol, cholesta-5,7-dien-3β-ol, and 5α-cholest-7-en-3β-ol has been achieved by chromatography on silicic acid–Super Cel columns (5–10). However, the resolution of these sterols was frequently less than complete and very long development times (2–3 days) were required using these columns. Complete separations of the above

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Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; HPLC, high-pressure liquid chromatography.

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sterols can be achieved by chromatography on alumina–silver nitrate thin-layer plates (11) or on alumina–Super Cel–silver nitrate columns (12). The separation of 5α-cholest-8-en-3β-ol and 5α-cholest-7-en-3β-ol could not be effected on the silicic acid columns (8). However, the latter sterols could be separated from each other by gas–liquid chromatography of suitable derivatives (8, 13, 14) or by alumina–Super Cel–silver nitrate column chromatography of the free sterols or their acetate derivatives (14–16). The results of research from several laboratories have indicated the possible intermediary role of $\Delta^{8(14)}$, $\Delta^{8,14}$, and $\Delta^{7,14}$-sterols in the biosynthesis of cholesterol (4, 16–27). Exploration of the metabolism of those sterols required the development of chromatographic methods to allow the separation of these compounds from one another and from the sterols noted above. To this end we have described methodology that permits the resolution of these compounds from one another by a combination of column chromatographic techniques. Silicic acid–Super Cel column chromatography can be used to isolate the C_{27} monohydroxysterols (4, 8). Chromatography of the acetylated C_{27} sterols on columns of silica gel G–Super Cel–silver nitrate permitted the separation of the monounsaturated steryl acetates from diunsaturated steryl acetates and, in addition, permitted separation of the $\Delta^{7,14}$, $\Delta^{8,14}$ and $\Delta^{5,7}$-steryl acetates from one another (4, 25, 27, 28). Subsequent chromatography of the monounsaturated steryl acetate fraction (from the silica gel G–Super Cel–silver nitrate column) on columns of alumina–Super Cel–silver nitrate permitted separation of the acetates of 5α-cholest-8(14)-en-3β-ol, 5α-cholest-8-en-3β-ol, 5α-cholest-7-en-3β-ol, and cholesterol (4, 14, 16, 24, 25, 28). While this methodology has proved to be extremely valuable in the purification, isolation, and identification of various C_{27} sterols, a very serious defect of this approach has been the extraordinarily long time required to complete the three separate column chromatographic analyses.

Several laboratories have explored the use of reversed-phase high-pressure liquid chromatography for the separation of sterols through their derivatives (29–32). Rees, Donnahey, and Goodwin (29) have reported on the chromatographic behavior of the acetate derivatives of a number of C_{27}, C_{28}, and C_{29} sterols upon reversed-phase high-pressure liquid chromatography. While 3β-acetoxy-5α-cholestane could be resolved from the acetate derivatives of cholesterol and 5α-cholest-7-en-3β-ol, effective separations of the latter compounds from one another was not achieved. 3β-Acetoxy-5α-cholest-7-ene was cleanly separated from 3β-acetoxy-cholesta-5,24-diene and 3β-acetoxy-cholesta-5,7-diene while only partial separation of the latter two compounds was achieved. Trocha, Jasne, and Sprinson (30) recently reported the results of the study of the behavior of a number of yeast sterols upon reversed-phase high-pressure liquid chromatography. A number of 4- and 14-methylated sterols and ergosterol derivatives were studied. Separation of ergost-8-en-3β-ol from ergost-7-en-3β-ol, but not of ergosta-8,22,24(28)-trien-3β-ol from ergosta-7,22,24(28)-trien-3β-ol, was achieved in the chromatographic system employed. Hunter, Walden, and Heftmann (31) recently reported the resolution of a mixture of 5α-cholest-7-en-3β-ol, cholesterol, and 7-dehydrocholesterol by reversed-phase high-pressure chromatography. Hansbury and Scallen (32) have also recently reported the separations of desmosterol from cholesterol, lanosterol from dihydrolanosterol, and 7-dehydrocholesterol from cholesterol using reversed-phase high-pressure liquid chromatography. The utility of this form of chromatography to separate sterols differing only in the presence or absence of a Δ^{24}-double bond had also been found previously by Trocha et al. (30).

The purpose of the present report is to summarize the results of preliminary investigations of the behavior of the acetate derivatives of a number of C_{27} sterols on both reversed-phase and absorption high-pressure liquid chromatography. Described herein is a system that permits the resolution of the acetates of C_{27} sterols with double bonds in the Δ^5, Δ^7, Δ^8, $\Delta^{8(14)}$, $\Delta^{8,14}$, $\Delta^{7,14}$, and $\Delta^{5,7}$ positions from one another in a relatively short period of time. A preliminary account of this work has been presented (33).

MATERIALS AND METHODS

Thin-layer chromatography was performed on plates (250 μm in thickness) of silica gel G (Analtech, Inc., Newark, DE; solvent, benzene–ether 1:1) or 15% silver nitrate–silica gel G (34), (solvents, ether–hexane 9:1 or chloroform). Components on the plate were visualized after spraying with a molybdic acid solution (35) followed by heating at 100–120°C for 10 min. Gas–liquid chromatography was performed using a Hewlett-Packard Model 402 unit equipped with flame ionization detector. A silanized glass column (6 ft × 4 mm) of 3% QF-1 on Gas Chrom Q (100–120 mesh) was used with a column temperature of 240°C. Gas–liquid chromatography–mass spectral analyses were made using an LKB 9000S unit equipped with a 3% OV-17 on Gas Chrom Q

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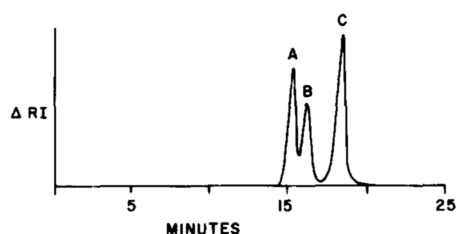

Fig. 1. Reversed-phase chromatography of 3β-acetoxy-5α-cholesta-8,14-diene (*A*), 3β-acetoxy-cholesta-5,7-diene (*B*), and cholesteryl acetate (*C*) on three columns (30 cm × 4 mm each) of μBondapak/C_{18}. The eluting solvent was methanol–acetonitrile 4:1 and the flow rate was 2.0 ml per min. Approximately 100–200 μg of the steryl acetates was injected.

(60–80 mesh) column. Operating conditions for the mass spectrometer were as described previously (36).

Solvents for high-pressure liquid chromatography were of analytical grade "Distilled in Glass" (Burdick and Jackson, Inc., Muskegon, MI), and all except acetonitrile were filtered prior to use using a Millipore filtration apparatus (FHLP 047 filters). The chloroform used in this work did not contain stabilizers. Hexane was UV grade. HPLC analyses were carried out using a Waters liquid chromatograph ALC 201 equipped with a differential refractometer and a U6K injector. Prepacked columns (30 cm × 4 mm) of μBondapak/C_{18} and μPorasil were obtained from Waters Associates (Milford, MA). Eluting solvents were elevated above the instrument and gently stirred; the eluting solvent vessels were positioned slightly above the stirring motor to prevent heating of the solvents. Eluted fractions were collected (using an automatic fraction collector via a Teflon tubing connected to the outlet of the detector unit) and the identity of each of the compounds eluted from the various HPLC columns was confirmed by the results of TLC, GLC, and GLC–MS analyses. The preparation of 3β-acetoxy-5α-cholestane, 3β-acetoxy-5α-cholest-8(14)-ene, 3β-acetoxy-5α-cholest-8-ene, 3β-acetoxy-5α-cholest-7-ene, 3β-acetoxy-5α-cholesta-8,14-diene, 3β-acetoxy-5α-cholesta-7,14-diene, 3β-acetoxy-cholesta-5,7-diene, and cholesteryl acetate have been described previously (12, 14, 16, 24, 25).

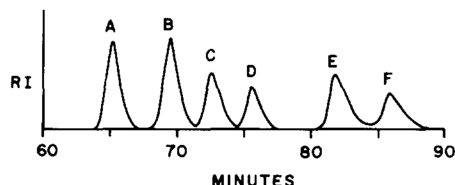

Fig. 2. Chromatographic separation of 3β-acetoxy-5α-cholestane (*A*), 3β-acetoxy-cholest-5-ene (*B*), 3β-acetoxy-5α-cholest-8(14)-ene (*C*), 3β-acetoxy-5α-cholest-8-ene (*D*), 3β-acetoxy-5α-cholesta-8,14-diene (*E*), and 3β-acetoxy-5α-cholesta-7,14-diene (*F*) on four columns of μPorasil (30 cm × 4 mm each) using chloroform–hexane 4:6 as the eluting solvent at a flow rate of 1.0 ml per min. The amounts of the steryl acetates applied to the column varied from ~75 to 170 μg.

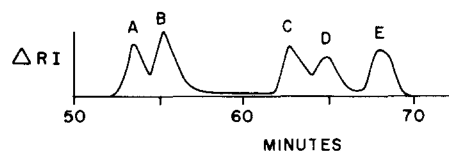

Fig. 3. Chromatographic separation of 3β-acetoxy-5α-cholestane (*A*), cholesteryl acetate (*B*), 3β-acetoxy-5α-cholest-8(14)-ene (*C*), 3β-acetoxy-5α-cholest-8-ene (*D*), and 3β-acetoxy-5α-cholest-7-ene (*E*) on four columns of μPorasil (30 cm × 4 mm each) using hexane–benzene 8:2 as the eluting solvent at a flow rate of 2.5 ml per min. The amount of each steryl acetate injected was ~180–240 μg.

RESULTS

Reversed-phase chromatography

Our initial efforts involved reversed-phase chromatography on columns of μBondapak/C_{18}. Using a mixture of methanol and acetonitrile (4:1) as the solvent, resolution of the acetate derivatives of cholesterol, cholesta-5,7-dien-3β-ol, and 5α-cholesta-8,14-dien-3β-ol was achieved (**Fig. 1**). Further studies indicated that the acetate derivatives of 5α-cholesta-7,14-dien-3β-ol and 5α-cholesta-8,14-dien-3β-ol were not separable on these columns even on prolonged recycling or using a variety of other eluting solvent mixtures.

Adsorption chromatography

Fig. 2 illustrates the chromatographic separation of the acetate derivatives of 5α-cholestan-3β-ol, cholesterol, 5α-cholest-8(14)-en-3β-ol, 5α-cholest-8-en-3β-ol, 5α-cholesta-8,14-dien-3β-ol, and 5α-cholesta-7,14-dien-3β-ol on columns of μPorasil using chloroform–hexane 4:6 as the eluting solvent. While excellent resolution of the above compounds was achieved in only 90 min, useful separations of the

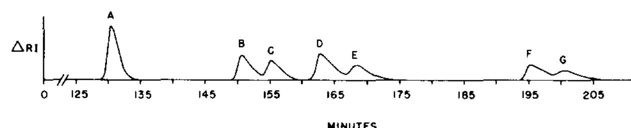

Fig. 4. Chromatography of cholesteryl acetate (*A*), 3β-acetoxy-5α-cholest-8(14)-ene (*B*), 3β-acetoxy-5α-cholest-8-ene (*C*), 3β-acetoxy-5α-cholest-7-ene (*D*), 3β-acetoxy-cholesta-5,7-diene (*E*), 3β-acetoxy-5α-cholesta-8,14-diene (*F*), and 3β-acetoxy-5α-cholesta-7,14-diene (*G*) on four columns of μPorasil (30 cm × 4 mm each) using hexane–benzene 9:1 as the eluting solvent at a flow rate of 1.0 ml per min. The loads of the steryl acetates varied from ~170 μg to 470 μg.

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acetates of cholest-5,7-dien-3β-ol and 5α-cholesta-8,14-dien-3β-ol were not achieved under the same conditions. In view of the latter situation, we systematically explored other solvent mixtures for the elution of the steryl acetate from the μPorasil columns. Hexane–benzene 8:2 gave encouraging results. **Fig. 3** shows the partial resolution of the acetate derivatives of 5α-cholestan-3β-ol, cholesterol, 5α-cholest-8(14)-en-3β-ol, 5α-cholest-8-en-3β-ol, and 5α-cholest-7-en-3β-ol. Decreasing the polarity of the eluting solvent mixture (hexane–benzene 9:1) permitted the separation of the acetate derivatives of the Δ^5, $\Delta^{8(14)}$, Δ^8, Δ^7, $\Delta^{5,7}$, $\Delta^{8,14}$, and $\Delta^{7,14}$ sterols as shown in **Fig. 4.**

DISCUSSION

The results presented herein indicate that HPLC provides very rapid and useful separations of a number of steryl acetates differing only in the number and position of double bonds in the sterol nucleus. While we have not as yet systematically investigated such important matters as the column capacity and detector response for each of the concerned steryl acetates, a presentation of our results at this state is prompted by our determination of conditions permitting the very rapid (~3.5 hr) separation of steryl acetates which can be achieved only in a minimum of 7–10 days using previously described methodology.

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