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Silver ion high pressure liquid chromatography provides unprecedented separation of sterols: Application to the enzymatic formation of cholesta-5,8-dien-3β-ol

BENFANG RUAN, JUSTIN SHEY, NICOLAS GERST, WILLIAM K. WILSON, AND GEORGE J. SCHROEPFER, JR.*

Departments of Chemistry and of Biochemistry and Cell Biology, MS 140, Rice University, P.O. Box 1892, Houston, TX 77251-1892

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ABSTRACT **We report that silver ion HPLC provides remarkable separations of C_{27} sterols differing only in the number or location of olefinic double bonds. This technique has been extended to LC-MS, analysis of purified components by GC, GC-MS, and ^1H NMR, and to its use on a semipreparative scale. The application of this methodology for the demonstration of the catalysis, by rat liver microsomes, of the conversion of 7-dehydrocholesterol to cholesta-5,8-dien-3β-ol is also presented.**

The biosynthesis of cholesterol involves a large number of potential sterol intermediates (1–4). Apart from fundamental matters concerning the definition of the chemical nature of intermediates in the formation of cholesterol in mammalian cells, interest in this area has been markedly enhanced by reports of their potential importance in a wide variety of critical cellular processes. For example, the sterol composition of cell culture media and of cells (and subcellular fractions derived therefrom) is of considerable interest with regard to the intracellular transport (5–8) and efflux (9, 10) of sterols and cell morphology and function (6, 11). A number of mammalian cell lines have been described that are defective in one or more steps in the overall conversion of lanosterol to cholesterol (11–14). One of these cell lines (11), an HIV-susceptible T cell line showing essentially complete replacement of cellular cholesterol by lanosterol and 24,25-dihydrolanosterol, has been suggested to be a potentially valuable system for studies of the role of cholesterol in membrane fusion and in immunodeficiency virus-induced syncitia formation and pathological effects. Additional interest in sterol precursors of cholesterol arises from a recent paper (15) that reported that certain di- and triunsaturated sterol intermediates activate meiosis in mammalian oocytes.

Sterol intermediates in cholesterol biosynthesis are also of considerable importance in research in human subjects and animals. The steady-state levels of sterol precursors in blood and in most tissues are normally very low. However, important exceptions are found in the cases of skin (16), brain and spinal cord during the period of myelination (17, 18), spermatozoa (of some species) after maturation in the epididymis (19, 20), and human milk (21, 22). Changes in the low levels of sterol precursors of cholesterol in blood continue to be investigated as indirect indicators of hepatic 3-hydroxy-3-methylglutaryl-CoA reductase activity and of whole body cholesterol biosynthesis (23–25). Very low levels of a sterol believed to be cholesta-5,8-dien-3β-ol have been reported in blood from patients with cerebrotendinous xanthomatosis (26) and from normal subjects (27). 7-Dehydrocholesterol and 5α-cholesta-6,8-dien-3β-ol were also observed at low levels in blood from normal subjects and the levels of these sterols were elevated in patients with ileal resection or cholestyramine treatment (27). The $\Delta^{5,8}$ sterol has also been observed at substantial levels in liver from newborn rats of mothers treated with AY-9944 in the last 7–10 days of pregnancy (28).

Another major stimulus for research on sterol intermediates derives from important reports of the presence at substantial levels of noncholesterol sterols in blood and tissues of patients with the Smith–Lemli–Opitz syndrome, a severe developmental disorder affecting multiple organ systems (29, 30). 7-Dehydrocholesterol has been reported to accumulate in blood and tissues in this disorder (on the basis of GC and/or GC-MS studies; refs. 29–35) and, more definitively, by chromatographic, physical, and spectral (including NMR) studies of the sterol from feces (34). The biochemical defect in Smith–Lemli–Opitz syndrome has been ascribed to the conversion of 7-dehydrocholesterol to cholesterol (30, 36), a key enzymatic reaction in the biosynthesis of cholesterol as demonstrated ≈35 years ago (37, 38). Other reported structures for sterols accumulating in Smith–Lemli–Opitz syndrome (based on GC and/or GC-MS studies) include cholesta-5,8-dien-3β-ol (33–35), 5α-cholesta-6,8(14)-dien-3β-ol (31), 5α-cholesta-6,8-dien-3β-ol (29, 33), 5α-cholest-8(14)-en-3β-ol (33), and 19-norcholesta-5,7,9(10)-trien-3β-ol (35). The $\Delta^{5,8}$ sterol from feces has been characterized by chromatographic and spectral (including MS and NMR) studies (34), but without comparisons with an authentic sample.

The isolation, characterization, and quantitation of potential sterol intermediates in the biosynthesis of cholesterol is critically dependent upon efficient methods for the chromatographic separation of the concerned compounds. These sterols differ in the number and location of double bonds in the sterol nucleus as well as in the presence or absence of a Δ^{24}-double bond in the side chain. Nuclear double bond locations of current biological interest include those at the Δ^8, $\Delta^{8(14)}$, Δ^7, Δ^5, $\Delta^{8,14}$, $\Delta^{7,14}$, $\Delta^{7,9(11)}$, $\Delta^{5,7}$, $\Delta^{5,8}$, $\Delta^{5,8(14)}$, $\Delta^{6,8}$, $\Delta^{6,8(14)}$, and $\Delta^{5,7,9(11)}$ positions (Fig. 1). Other sterol variants of interest in synthetic or natural product research include those with nuclear double bonds at the Δ^4, Δ^6, $\Delta^{9(11)}$, Δ^{14}, and $\Delta^{4,6}$ positions. Simple TLC on silica gel plates provides resolution of only a very few of C_{27} sterols from each other and from other sterol intermediates. Silicic acid-Super Cel column chromatography has been shown to provide useful separations of some C_{27} sterols [e.g., Δ^5 from Δ^6 (37), and Δ^5, $\Delta^{5,7}$, and Δ^7 from each other (39)]. However, this chromatography is very slow (several days) and resolution of many of the C_{27} sterols (e.g., Δ^8 and Δ^7) is not possible.

Chromatography on silica gel-Super Cel-$AgNO_3$ columns permitted the separation of certain monounsaturated C_{27} steryl acetates [Δ^5, $\Delta^{8(14)}$, Δ^8, and Δ^7] from certain diunsaturated C_{27} steryl acetates ($\Delta^{7,14}$, $\Delta^{8,14}$, and $\Delta^{5,7}$) and the resolution of these diunsaturated steryl acetates from each other (ref. 3 and references cited therein). Chromatography on alumina-Super Cel-$AgNO_3$ columns provided excellent separations of the $\Delta^{8(14)}$, Δ^8, Δ^7, and Δ^5 C_{27} steryl acetates (refs. 3 and 40 and references cited therein). While these approaches

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Abbreviations: MTBE, methyl *tert*-butyl ether; TMS, trimethylsilyl.

*To whom reprint requests should be addressed.

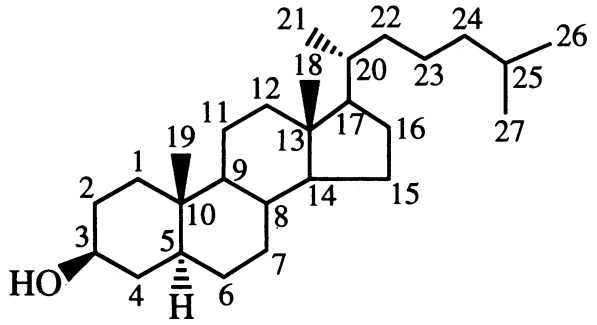

FIG. 1. Structure of 5α-cholestan-3β-ol with atom numbering to indicate the location of double bonds in unsaturated sterols described herein.

proved to be very valuable in the isolation and characterization of the various sterols, a significant limitation of these columns is the very long time required to complete the chromatographic analyses. Medium pressure liquid chromatography on columns of alumina impregnated with $AgNO_3$ permitted a more rapid analysis and provided useful separations (albeit not complete in some cases) of acetates of the $\Delta^{8(14)}$, Δ^8, Δ^7, Δ^5, $\Delta^{8,24}$, $\Delta^{5,24}$, $\Delta^{7,9(11)}$, $\Delta^{7,14}$, $\Delta^{8,14}$, and $\Delta^{5,7}$ C_{27} sterols (41). Under the conditions used, separation of the acetates of the $\Delta^{8,24}$ and $\Delta^{7,24}$ C_{27} sterols was not observed. However, in a subsequent study, this method was successfully used to isolate the $\Delta^{8,24}$-steryl acetate free of its $\Delta^{7,24}$ isomer (42). Despite the power and relatively high capacity of this method, it has not been widely applied outside of our laboratory, perhaps because of the need to prepare the columns and the length of time required for the chromatography (usually 16 to 20 h).

Reversed-phase HPLC, providing a much more rapid analysis, has been extensively used to separate C_{27} sterols and their derivatives. However, the results of a number of studies, most with only a limited number of sterol standards, have indicated difficulties in the separations of a number of C_{27} sterols or their derivatives. For example, the reported data from different laboratories with various reversed-phase systems indicated little or no separation of the $\Delta^{8,24}$ and $\Delta^{5,24}$ sterols and little or no resolution of the $\Delta^{8(14)}$, Δ^8, Δ^7, and Δ^5 sterols (42), no separations of the $\Delta^{8(14)}$ and Δ^7 C_{27} sterols (43), and only slight differences in the relative retention times of the Δ^8 and Δ^7 C_{27} sterols (44). Other reports indicated little or no differences in the retention times of the $\Delta^{5,7}$, $\Delta^{5,24}$, and $\Delta^{8(14)}$ sterols and little differences between the Δ^7 and Δ^5 sterols (45) and between $\Delta^{5,7,24}$, $\Delta^{7,24}$, $\Delta^{5,24}$, and Δ^8 C_{27} sterols (46). Morisaki and Ikekawa (47) reported no differences in the retention times of $\Delta^{7,14}$ and $\Delta^{8,14}$ C_{27} steryl benzoates and very little differences in the mobilities of other groups of C_{27} steryl benzoates.

Normal phase HPLC has been applied less extensively for the separation of C_{27} sterols and their derivatives. The separation of the acetate derivatives of the Δ^0, $\Delta^{8(14)}$, Δ^8, Δ^7, $\Delta^{5,7}$, $\Delta^{8,14}$, and $\Delta^{7,14}$ sterols has been reported (48). However, four columns of μPorasil (30 cm × 4 mm) were required. Morisaki and Ikekawa (47) reported data on a number of steryl benzoates on a Zorbax SIL column. They observed identical retention times for the benzoates of the Δ^8, $\Delta^{8(14)}$, and Δ^{14} C_{27} sterols. They also reported that the $\Delta^{6,8(14)}$ and $\Delta^{7,9(11)}$ steryl benzoates and the $\Delta^{7,14}$ and $\Delta^{8,14}$ steryl benzoates did not differ from each other.

GC has also been extensively used to separate C_{27} sterols and their derivatives. However, GC alone is limited by the close similarity of retention times of many sterols differing only in the location of olefinic double bonds (49–51).

Encouraged by our previous experience in the separation of sterols and their acetate derivatives by standard chromatography and medium pressure liquid chromatography on columns of silica gel or alumina impregnated with $AgNO_3$, we explored the possible application of silver ion HPLC. Herein, we report unprecedented separations of a wide variety of C_{27} sterols and steryl acetates differing in the number and location of olefinic double bonds. This methodology has been extended to LC-MS, analysis of purified components by GC, GC-MS, and NMR, and to its use on a semipreparative scale. We also describe the use of this methodology for the demonstration of the enzymatic conversion of 7-dehydrocholesterol to cholesta-5,8-dien-3β-ol.

MATERIALS AND METHODS

Sterols. The preparation and purities of 3β-acetoxy-5α-cholestane, unsaturated analogs with double bonds at the $\Delta^{8(14)}$, Δ^8, Δ^7, Δ^5, Δ^{14}, $\Delta^{5,24}$, $\Delta^{7,24}$, $\Delta^{8,24}$, $\Delta^{5,8}$, $\Delta^{5,8(14)}$, $\Delta^{6,8}$, $\Delta^{6,8(14)}$, $\Delta^{7,9(11)}$, $\Delta^{7,14}$, $\Delta^{8,14}$, $\Delta^{5,7}$, and $\Delta^{5,7,9(11)}$ positions, and the corresponding free sterols have been described previously (52). Trimethylsilyl (TMS) derivatives were prepared by treatment of the free sterols with a 1:1 mixture of bis(trimethylsilyl)trifluoroacetamide/pyridine at room temperature for 1 h. [4-^{14}C]7-Dehydrocholesterol (2.50 mCi per mmol; 1 Ci = 37 GBq) was prepared from [4-^{14}C]cholesterol (Amersham) by treatment of its acetate with 1,3-dibromo-5,5-dimethylhydantoin, followed by treatment with tetrabutylammonium bromide and tetrabutylammonium fluoride as described previously for the preparation of the unlabeled compound (53), followed by saponification of the $\Delta^{5,7}$-steryl acetate. The identity of the product was based upon UV, GC-MS (TMS derivative), and HPLC (reversed-phase and Ag^+ ion) analyses. The radiopurity was judged to be in excess of 99% on the basis of radio-HPLC (reversed-phase and Ag^+ ion). 25,26,26,26,27,27,27-Heptafluorocholest-5-en-3β-ol (F_7-cholesterol) was prepared as described (54).

General Methods. Reversed-phase HPLC was carried out with a 5-μm Customsil ODS column (250 mm × 4.6 mm; Custom LC; Houston) using 2% water in methanol as the solvent (1 ml/min). Sterols were injected in hexane (1–50 μl) using a Rheodyne (Cotati, CA) model 7125 injector. Semipreparative normal phase HPLC was done on a 5-μm Adsorbosphere XL silica column (250 mm × 10 mm; 90 Å pore size; Alltech Associates) using 4% acetone in hexane as the eluting solvent at 3 ml/min. With the exception of LC-MS (see below), the elution of the sterols and steryl acetates from the various HPLC columns was detected by their absorbance at 210 nm. Capillary GC analyses were made with split injection on a Shimadzu GC-9A unit with a 30 m DB-5 column using the TMS derivative of F_7-cholesterol as an internal standard. The injector and column temperatures were both 250°C, and nitrogen was used as the carrier gas with a head pressure of 1.1 kg/cm^2. Capillary GC-MS was carried out on a VG Analytical (Manchester, U.K.) ZAB-HF mass spectrometer with a Hewlett–Packard model HP-5890A GC unit containing a 60-m DB-5 column. The temperatures of the injector and GC-MS interface were 290°C, and the column temperature was maintained at 250°C. Helium was used as the carrier gas with a head pressure of 1.4 kg/cm^2. The ion source was maintained at 200°C and the mass range scanned was from m/z 50 to 750. The ionizing voltage was 70 eV. As described previously (52), COSYDEC (f_1-decoupled ^{1}H-^{1}H correlation spectroscopy) and ^{1}H NMR spectra were acquired on a Bruker (Billerica, MA) model AMX500 spectrometer (500.1 MHz for ^{1}H) at 25°C in $CDCl_3$ solution.

Silver Ion HPLC Columns. The silver ion HPLC columns used herein were prepared by minor modifications of the method of Christie (55) using commercial columns containing 5 μm Nucleosil SA (100 Å pore size), an ion exchange material consisting of phenylsulfonic acid groups bonded to a silica backbone. The Nucleosil SA columns were connected to a Rheodyne injector and Waters pump, with column effluent flowing directly to waste. Two Ag^+ HPLC columns (250 mm ×

4.6 mm; Alltech Associates) were prepared as described by Christie (55) except that methyl *tert*-butyl ether (MTBE) was used to rinse the column as described below. One Ag^+ HPLC column (300 mm × 3.2 mm; Alltech Associates) was prepared by flushing at 0.5 ml/min with aqueous ammonium acetate (1%) for 1.5 h and with water for 2 h. During continued flushing with water, $AgNO_3$ solution (0.2 g dissolved in 1 ml water) was injected in 20-μl portions every minute for a total of 50 injections. The column was then rinsed at 0.5 ml/min with water for 1 h, methanol for 4 h, and MTBE for ≈10 h. Before use, the column was rinsed with hexane (1 ml/min) for 2 h. A semipreparative Ag^+ HPLC column (300 mm × 10 mm; Column Engineering, Ontario, CA) was prepared similarly by flushing with 1% aqueous ammonium acetate (1.5 ml/min for 2 h) and water (3 ml/min for 2 h), injecting an $AgNO_3$ solution (1.2 g in 7 ml water) in 250-μl portions every minute for a total of 28 injections using a flow rate of 1.5 ml/min, followed by rinsing at 3 ml/min with water for 20 min, methanol for 1 h, MTBE for 3 h, and hexane for 1 h. The longevity of the columns varied markedly depending on conditions of use. One column operated as described herein and subjected only to samples purified on silica gel showed little change in retention times and resolving power during 6 months of use (about 1000 injections). When required, columns were regenerated by washing with methanol, water, and aqueous ammonium acetate, followed by conversion to the silver ion form as described above.

LC-MS was done with atmospheric pressure chemical ionization using an Ag^+ HPLC column (300 mm × 3.2 mm) interfaced to a Finnigan (San Jose, CA) model MAT 95 spectrometer: eluting solvent, acetone-hexane (3:97) at 0.5 ml/min; vaporizer temperature, 300°C; capillary temperature, 230°C; corona voltage, 5 kV; auxiliary nitrogen gas flow, ≈35 ml/min; and nitrogen sheath gas pressure of ≈3.6 kg/cm^2. Steryl acetate samples (≈20-50 μg per component) were injected in the mobile phase (50 μl).

Incubations of [4-^{14}C]7-Dehydrocholesterol with Rat Liver Microsomes. Washed rat liver microsomes, in potassium phosphate buffer (100 mM) containing $MgCl_2$ (5 mM), were obtained from female Sprague Dawley rats (body weight ≈125 g) as described previously (56). [4-^{14}C]7-Dehydrocholesterol (27.9 μg; 3.94 × 10^5 dpm) in propylene glycol (50 μl) was mixed with a suspension of washed microsomes (10 ml; 7 mg protein per ml) in a modified Warburg flask (125 ml) and incubated in the dark for 2 h at 37°C under argon with shaking (80 cycles per min). The incubation mixture was transferred to an anaerobic glove box (Vacuum Atmospheres, Hawthorne, CA) using an atmosphere of nitrogen, and it was heated with 15% ethanolic KOH (10 ml) for 2 h at 70°C. The nonsaponifiable lipids were obtained by extraction (4×) with degassed hexane (40 ml portions) that was washed once with a saturated NaCl solution. The resulting nonsaponifiable lipids were removed from the anaerobic chamber, dried over anhydrous Na_2SO_4, evaporated to dryness under argon, and passed through a short (5 cm × 0.5 cm) column of silica gel (230–400 mesh) using 5% acetone in hexane as the eluting solvent prior to HPLC. A portion of this material was applied to a normal phase HPLC column (250 mm × 10 mm) using 4% acetone in hexane as the eluting solvent at a rate of 3 ml per min. Fractions 3 ml in volume were collected. The material in fractions 40–55, corresponding to the mobility of C_{27} sterols, was pooled and, after evaporation of the solvent under nitrogen, was treated with a mixture of acetic anhydride (500 μl) and pyridine (500 μl) for 24 h at room temperature in the dark. Water (1 ml) was added and the resulting mixture was extracted (4×) with MTBE (4-ml portions). The combined extracts were washed and dried as described above, evaporated to dryness under nitrogen, and passed through a short silica gel (230–400 mesh) column (5 cm × 0.5 cm) using 3% acetone in hexane as the eluting solvent. The resulting steryl acetates were dissolved in hexane (200 μl) and an aliquot (10 μl) was injected onto an Ag^+ HPLC column (250 mm × 4.6 mm) along with authentic samples of unlabeled cholesteryl acetate, 7-dehydrocholesteryl acetate, 3β-acetoxycholesta-5,8-diene, and 3β-acetoxycholesta-5,7,9(11)-triene. Using 3% acetone in hexane as the eluting solvent (1 ml per min), fractions 1 ml in volume were collected for assay of ^{14}C. An identical incubation was carried out with heat-treated microsomes (≈10 min in boiling water).

A larger scale incubation of the [4-^{14}C]7-dehydrocholesterol (2.53 mg; 3.94 × 10^5 dpm) in propylene glycol (5.05 ml) with microsomes (50 ml; 7 mg protein per ml) was carried out and the nonsaponifiable lipids were processed as described above to give, after normal phase HPLC (250 mm × 10 mm) and then Ag^+ HPLC (300 mm × 10 mm) using 9.1% acetone in hexane as the eluting solvent (3 ml per min), the $\Delta^{5,8}$ sterol (3.32 × 10^4 dpm; 8.4% of incubated ^{14}C). This material was studied by NMR and by GC and GC-MS (TMS derivative). In addition, a portion of the purified material was subjected to analytical Ag^+ HPLC (300 mm × 3.2 mm) along with authentic samples of the unlabeled Δ^5, $\Delta^{5,8}$, and $\Delta^{5,7}$ sterols using 9.1% acetone in hexane as the solvent. A single radioactive component was observed that had the same chromatographic behavior as the authentic $\Delta^{5,8}$ sterol (t_R, ≈31.3 min) and clearly different from those of the Δ^5 sterol (t_R, ≈9 min) and the $\Delta^{5,7}$ sterol (t_R, ≈76 min). In addition, the purified ^{14}C-labeled $\Delta^{5,8}$ sterol recovered after NMR analysis was acetylated as described above, and the resulting acetate was subjected to Ag^+ HPLC (250 mm × 4.6 mm), along with an authentic sample of unlabeled 3β-acetoxycholesta-5,8-diene, using 3% acetone in hexane (1 ml per min, 1-min fractions). Approximately 96% of the ^{14}C showed the same chromatographic mobility as the authentic $\Delta^{5,8}$ acetate (t_R, 26.7 min). Approximately 4% of the ^{14}C eluted from the column at 44–45 min. The nature of this minor component was not studied further.

RESULTS

Silver Ion HPLC of Sterols and Steryl Acetates. With 3% acetone in hexane as the eluting solvent, the acetate derivatives of the $\Delta^{8(14)}$, Δ^8, Δ^7, Δ^5, $\Delta^{8,24}$, $\Delta^{7,24}$, $\Delta^{5,7,9(11)}$, $\Delta^{5,8(14)}$, $\Delta^{7,9(11)}$, $\Delta^{6,8}$, $\Delta^{5,8}$, $\Delta^{6,8(14)}$, $\Delta^{8,14}$, $\Delta^{7,14}$, $\Delta^{5,7}$, and Δ^{14} sterols (≈0.2-4 μg each) were cleanly resolved on Ag^+ HPLC (250 mm × 4.6 mm) (Fig. 2). In a separate run on the same column, the $\Delta^{5,24}$ steryl acetate eluted between the $\Delta^{7,24}$ and $\Delta^{5,7,9(11)}$ steryl acetates [with a t_R (relative to cholesteryl acetate) of 2.58]. Chromatographic peaks were identified by individual injection of authentic standards and, in separate experiments, by collection of the eluent followed by GC-MS and, in many cases, ^{1}H NMR analysis. Changing the elution solvent to 1% acetone in hexane resulted in improved separations of the acetate derivatives of the $\Delta^{8(14)}$, Δ^8, Δ^7, and Δ^5 sterols (Fig. 3). Ag^+ HPLC (same column as above) also provided useful separations of a number of C_{27} mono- and diunsaturated free sterols (solvent, 10% acetone in hexane). However, the separations of the acetate derivatives were superior to those observed with the free sterols. Moreover, it is very important to note that the order of elution of some of the diunsaturated free sterols differed from that observed with the acetate derivatives. In addition, no separation of the $\Delta^{5,8(14)}$ and $\Delta^{7,14}$ free sterols was observed.

Ag^+ HPLC (300 mm × 3.2 mm; solvent, 3% acetone in hexane, 0.5 ml per min) was also extended to LC-MS of the acetate derivatives of the Δ^0 (t_R, 6.4 min), Δ^8 (t_R, 7.8 min), Δ^5 (t_R, 10.2 min), $\Delta^{8,24}$ (t_R, 17.0 min), $\Delta^{5,7,9(11)}$ (t_R, 26.7 min), $\Delta^{5,8}$ (t_R, 37.4 min), and $\Delta^{5,7}$ (t_R, 97.1 min) sterols with atmospheric pressure chemical ionization. Total ion current monitoring provided for detection of the elution of cholestanyl acetate (not detected by UV absorbance at 210 nm). The MS of each acetate showed a base peak corresponding to M+H−CH_3COOH, an overly large (≈60% relative intensity) isotope peak (corresponding in part to M+2H − CH_3COOH),

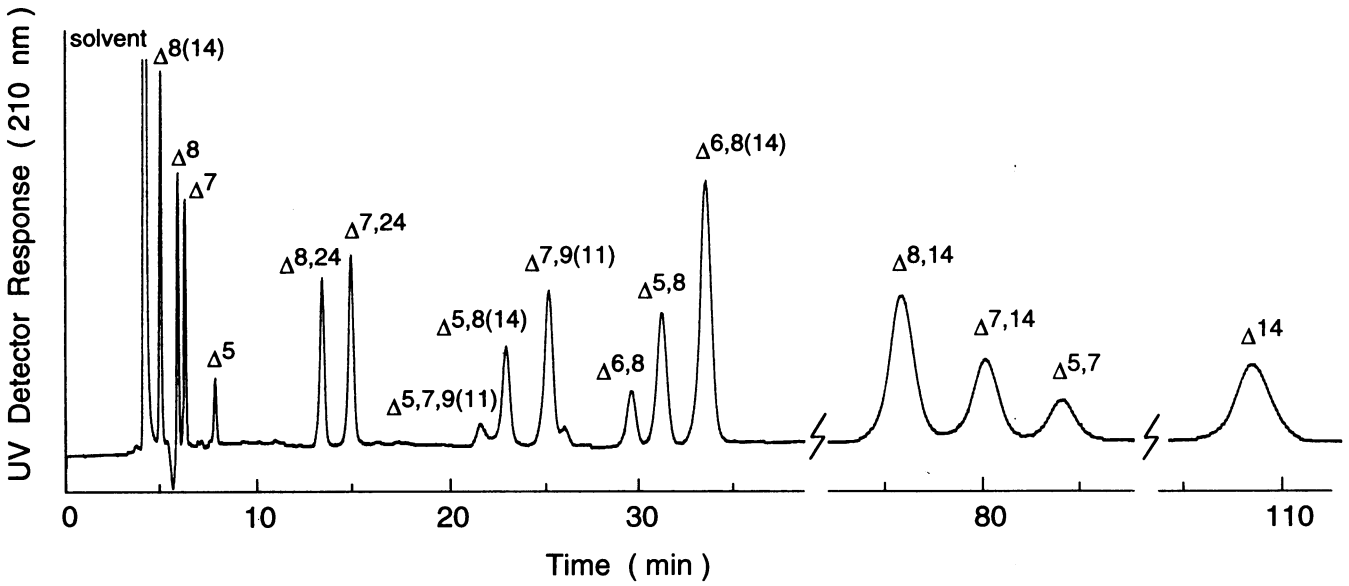

FIG. 2. Silver ion HPLC analysis of unsaturated C_{27} steryl acetates (250 × 4.6 mm column; elution with 3:97 acetone-hexane).

and, in most cases, minor ions at M+H (up to 5% relative intensity) and M−61 (up to 20% relative intensity). Under the conditions studied, little other structural information was observed.

Ag^+ HPLC was also used to separate C_{27} sterols on a semipreparative scale. Semipreparative Ag^+ HPLC (300 mm × 10 mm; solvent, 16% acetone in hexane; 3 ml per min) provided complete resolution of cholesterol (7 mg; t_R, 16.1 min), cholesta-5,8-dien-3β-ol (3 mg; t_R, 34.7 min), and 7-dehydrocholesterol (5 mg; t_R, 69 min).

Enzymatic Conversion of 7-Dehydrocholesterol to Cholesta-5,8-dien-3β-ol. The analytical Ag^+ HPLC of the steryl acetates recovered after incubation of the ^{14}C $\Delta^{5,7}$ sterol with washed liver microsomes under anaerobic conditions is shown in Fig. 4. Approximately 11% had the same mobility as the authentic $\Delta^{5,8}$ acetate. The remainder of the radioactivity (≈89%) corresponded to the mobility of the acetate of the authentic $\Delta^{5,7}$ sterol. Little or no ^{14}C was associated with cholesteryl acetate, a finding compatible with requirement for NADPH in the conversion of 7-dehydrocholesterol to cholesterol (38). The Ag^+ HPLC of the steryl acetates recovered after incubation of the ^{14}C $\Delta^{5,7}$ sterol with heat-treated microsomes showed a single peak of radioactivity with the chromatographic behavior of the acetate of the incubated substrate.

A larger scale incubation was carried out to provide sufficient $\Delta^{5,8}$ sterol for its further characterization. The purified labeled product showed a single labeled component (≈99%) with the chromatographic behavior of the $\Delta^{5,8}$ sterol on Ag^+ HPLC. Its TMS ether derivative showed a single component (≈99%) upon GC analysis on a 30 m DB-5 column, with essentially the same t_R relative to that of the TMS derivative of F_7 cholesterol (1.892) as that of the TMS of the authentic $\Delta^{5,8}$ sterol (1.893). GC-MS analysis of the TMS derivatives of the enzymatic product on a 60 m DB-5 column also showed that 98% of the material had essentially the same t_R (41.35 min) as that of an authentic sample (41.42 min). The mass

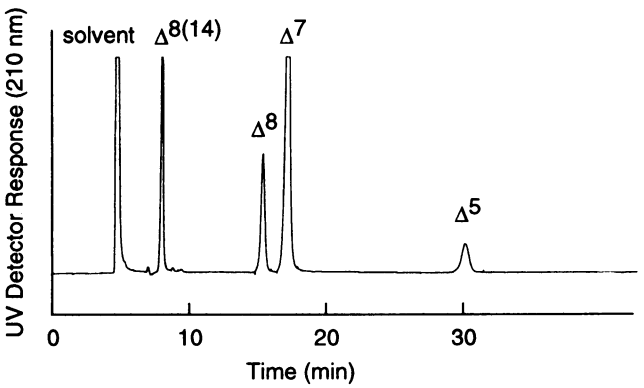

FIG. 3. Silver ion HPLC analysis of monounsaturated C_{27} steryl acetates (≈1 μg each; 250 × 4.6 mm column; elution with 1:99 acetone-hexane).

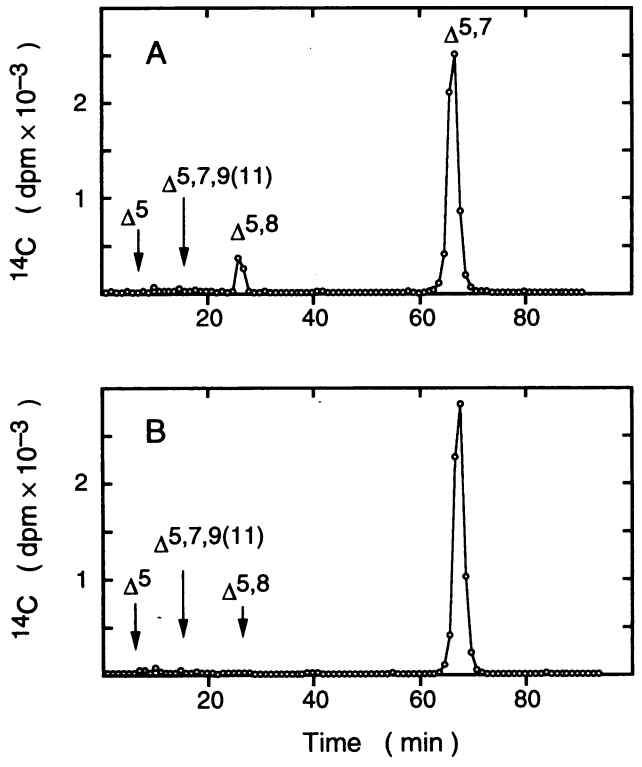

FIG. 4. Comparison of silver ion HPLC analyses of steryl acetates recovered from incubations of [4-^{14}C]7-dehydrocholesterol with washed rat liver microsomes under anaerobic conditions in the absence of added cofactors. (*A*) Untreated microsomes. (*B*) Heat-treated microsomes.

spectrum of the TMS derivative of the enzymatic product was essentially the same as that of the TMS ether of an authentic $\Delta^{5,8}$ sample, and the ^{1}H NMR spectrum was essentially identical with that of an authentic sample (52) of cholesta-5,8-dien-3β-ol (Table 1). Ag^+ HPLC analysis of the acetate derivative of the enzymatic product recovered after NMR analysis showed that ≈96% of the ^{14}C had the same chromatographic behavior as the authentic $\Delta^{5,8}$ acetate.

DISCUSSION

Our results demonstrate that silver ion HPLC provides unprecedented separations of a variety of C_{27} sterols differing only in the number and location of olefinic double bonds. Whereas Ag^+ HPLC has been successfully applied for the separation of other lipids [most notably fatty acids and triglycerides (57) and cholesterol esters (58)], the present work represents, to our knowledge, the first application of this methodology for the separation of sterols. This appears to be very timely in view of a large number of recent reports indicating the importance of sterol intermediates in cholesterol formation in a variety of critical cellular processes. We have presented an application of Ag^+ HPLC to one problem of current medical importance, i.e., the origin of cholesta-5,8-dien-3β-ol, a sterol reported to accumulate in blood and tissues of patients with a human hereditary disorder of development.

Table 1. Comparison of ^{1}H NMR chemical shifts reported for cholesta-5,8-dien-3β-ol with NMR data for the purified $\Delta^{5,8}$ sterol obtained after incubation of [4-^{14}C]7-dehydrocholesterol with rat liver microsomes

Hydrogen atom	Incubation product	$\Delta^{5,8}$ reported
H-1α	1.362*	1.361
H-1β	1.87	1.874*
H-2α	1.89	1.886*
H-2β	1.559*	1.560
H-3α	3.549	3.549
H-4α	2.348	2.347
H-4β	2.309	2.309
H-6	5.435	5.435
H-7α	2.513	2.513
H-7β	2.562	2.562
H-11α	2.11	2.111*
H-11β	2.17	2.166*
H-12α	1.440*	1.439
H-12β	2.003*	2.002
H-14α	2.103*	2.104*
H-15α	1.619*	1.620
H-15β	1.289*	1.289*
H-16α	1.916*	1.915
H-16β	1.333*	1.335*
H-17α	1.169*	1.168
H-18	0.652	0.652
H-19	1.189	1.189
H-20	1.398*	1.398
H-21	0.934	0.934
H-22R	1.35	1.35
H-22S	0.993*	0.992*
H-23R	—	1.35
H-23S	—	1.15
H-24	—	1.11
H-24	—	1.14
H-25	1.521*	1.521
H-26	0.865	0.865
H-27	0.870	0.870

Data obtained at 500 MHz in $CDCl_3$ solution at 25°C. Chemical shifts given to two and three decimal places are generally accurate to ±0.01 and ±0.001 ppm, respectively, except that values marked by an asterisk are accurate to about ±0.003 ppm. R and S denote pro-R and pro-S hydrogens, respectively. Multiplicities and coupling constants of the ^{1}H NMR signals of the incubation product were essentially identical to those described in ref. 52. Dashes indicate that chemical shifts could not be estimated reliably from the available spectral data.

To explore the possible enzymatic conversion of 7-dehydrocholesterol to cholesta-5,8-dien-3β-ol or other metabolites, we have incubated the ^{14}C-labeled $\Delta^{5,7}$-sterol with rat liver microsomes under anaerobic conditions in the absence of added cofactors. Rat liver microsomes have been reported to catalyze the conversion of 5α-cholest-8-en-3β-ol and 5α-cholesta-8,24-dien-3β-ol to the corresponding Δ^7 sterols (40, 59, 60). The reaction occurs with the uptake of solvent hydrogen at C-9 (40, 60) and proceeds under anaerobic conditions in the absence of added cofactors (59, 61). The enzyme from rat liver microsomes has been solubilized and partially purified (61), and a highly purified enzyme has been obtained from rats treated with cholestyramine and lovastatin (62). Reversal of the reaction, i.e., the conversion of the 5α-cholest-7-en-3β-ol to 5α-cholest-8-en-3β-ol, has been reported for incubations with rat liver microsomes (63) or with the partially purified enzyme (61). The combined results of the studies cited above indicate that, for the C_{27} $\Delta^{8,24}$ and Δ^8 sterols, the predominant product at equilibrium is the corresponding Δ^7-sterol (≈95%). With other substrates, the ratio of the Δ^8 and Δ^7 sterols at equilibrium may be different. For example, whereas no conversion of the C_{27} $\Delta^{8,14}$ sterol to the corresponding $\Delta^{7,14}$ diene was detected with the partially purified enzyme (61), the catalysis by rat liver microsomes of the conversion of the $\Delta^{7,14}$ sterol to its $\Delta^{8,14}$ isomer has been reported (64). Further, whereas no conversion of 14α-methyl substituted Δ^8 sterols (14α-methyl-5α-cholest-8-en-3β-ol, 24,25-dihydrolanosterol, and lanosterol) to the corresponding Δ^7 sterols was observed with rat liver microsomes (59), conversion of 14α-methyl-5α-cholest-7-en-3β-ol and 14α-hydroxymethyl-5α-cholest-7-en-3β-ol to the corresponding Δ^8 sterols has been reported (56). Thus, the position of equilibrium for the $\Delta^8 \rightarrow \Delta^7$ isomerization may vary with different substrates. In addition, the microsomal enzyme may catalyze unanticipated reactions. For example, anaerobic incubation of 5α-cholesta-7,9(11)-dien-3β-ol with rat liver microsomes has been reported to give the corresponding $\Delta^{8,14}$-sterol in 30% yield (65).

In the present study, the Ag^+ HPLC methodology has been used in studies demonstrating the conversion of 7-dehydrocholesterol to cholesta-5,8-dien-3β-ol (≈11% yield). No other products were detected. The reaction did not require molecular oxygen or added cofactors. The product was characterized as the $\Delta^{5,8}$ sterol by the chromatographic behavior of the free sterol and its acetate derivative on Ag^+ HPLC and the chromatographic behavior of the TMS derivative on GC. The observation that the MS of the TMS derivative was essentially the same as that of the TMS derivative of an authentic sample of the $\Delta^{5,8}$ sterol is consistent with the assigned structure. However, these MS results, taken alone, are not definitive inasmuch as the MS of the TMS derivatives of the $\Delta^{5,7}$ sterol and a number of its isomers (including the $\Delta^{5,8}$ sterol) are essentially indistinguishable (ref. 27 and unpublished data). In contrast, the essentially identical ^{1}H NMR spectra of the enzymatic product and the chemically synthesized $\Delta^{5,8}$ sterol provide the basis for a definitive structure assignment since we have shown that each of a large number of C_{27} diunsaturated sterols [$\Delta^{5,7}$, $\Delta^{5,8}$, $\Delta^{5,8(14)}$, $\Delta^{6,8}$, $\Delta^{6,8(14)}$, $\Delta^{7,9(11)}$, $\Delta^{8,14}$, $\Delta^{7,14}$, $\Delta^{4,6}$, $\Delta^{8,24}$, and $\Delta^{7,24}$] can be differentiated by ^{1}H NMR (52). Our combined results, representing the first demonstration of the enzymatic conversion of a $\Delta^{5,7}$ sterol to a $\Delta^{5,8}$ sterol, are of importance in considerations of the origin of the $\Delta^{5,8}$ sterol reported to be present in blood of normal human subjects (27) and to accumulate, along with 7-dehydrocholesterol, in the blood, tissues, and feces of patients with the Smith–Lemli–Opitz syndrome (33–35).

In conclusion, Ag^+ HPLC is a simple, nondestructive, and rapid method providing unprecedented separations of closely

related sterols. The elution of the sterols can be monitored by UV or by LC-MS and isolated components can be studied, as illustrated above, by GC, GC-MS, and NMR. This methodology should be of considerable value for the isolation and characterization of sterols in a number of current and emerging problems in biology, chemistry, and medicine.

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