Sterol synthesis. A timely look at the capabilities of conventional and silver ion high performance liquid chromatography for the separation of C_{27} sterols related to cholesterol biosynthesis

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Abstract Sterol intermediates in the biosynthesis of cholesterol have recently assumed a very prominent position in a number of important problems in medicine and biology. In studies of these matters, the separation and identification of the sterol intermediates present formidable challenges, a situation which does not appear to be generally appreciated. High performance liquid chromatography (HPLC) is a simple and rapid approach for the separation of the concerned compounds. Reversed phase HPLC is very commonly used for this purpose. In the present studies, we have evaluated the capabilities of reversed phase, normal phase, and silver ion HPLC for the separation of sterols. Using an extensive collection of authentic sterols, our studies indicate very limited capabilities of reversed phase and normal phase HPLC for the separation of C_{27} sterols differing in the number and location of olefinic double bonds. In contrast, silver ion HPLC provided remarkable separations of the same compounds, either as the free sterols or their acetate derivatives. These findings, coupled with the results of recent studies of the properties of the same compounds by gas chromatography and by nuclear magnetic resonance and mass spectroscopy, have important implications regarding current application of methodologies for the separation, identification, and quantitation of sterol intermediates in cholesterol biosynthesis as critical portions of investigations on a number of current and emerging problems in biology and medicine.—**Ruan, B., N. Gerst, G.T. Emmons, J. Shey, and G.J. Schroepfer, Jr.** Sterol synthesis. A timely look at the capabilities of conventional and silver ion high performance liquid chromatography for the separation of C_{27} sterols related to cholesterol biosynthesis. *J. Lipid Res.* 1997. **38:** 2615–2626.

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We have had a longstanding interest in the chemical nature of sterol intermediates in the biosynthesis of cholesterol (1–3). A number of recent developments have brought these compounds to a very high level of interest and prominence. One outstanding example is the Smith-Lemli-Opitz syndrome, a severe hereditary disorder of human development (4), that is associated with an accumulation of 7-dehydrocholesterol and other sterols in the blood and tissues of affected subjects (5–13). The biochemical defect in this disease has been ascribed to a deficient conversion of 7-dehydrocholesterol to cholesterol (5, 14–16), an enzymatic reaction demonstrated approximately 35 years ago (17, 18). Other sterols reported to accumulate in the Smith-Lemli-Opitz syndrome include cholesta-5,8-dien-3β-ol (7), 5α-cholesta-6,8-dien-3β-ol (8–10), 5α-cholesta-6,8(14)-dien-3β-ol (6), 5α-cholest-7-en-3β-ol (9, 10), 5α-cholest-8(14)-en-3β-ol (9), 5α-cholesta-5,7,9(11)-trien-

Abbreviations: Abbreviations: Ag^+, silver ion; GC, gas chromatography; HPLC, high performance liquid chromatography; lanosterol, lanosta-8,24-dien-3β-ol; MS, mass spectrometry; MTBE, methyl *tert*-butyl ether; NMR, nuclear magnetic resonance (spectroscopy); RRT, relative retention time (relative to cholesterol or cholesteryl acetate); TLC, thin-layer chromatography; t_R, retention time; UV, ultraviolet; Δ^0, 5α-cholestan-3β-ol; Δ^4, cholest-4-en-3β-ol; Δ^5, cholest-5-en-3β-ol; Δ^6, 5α-cholest-6-en-3β-ol; Δ^7, 5α-cholest-7-en-3β-ol; Δ^8, 5α-cholest-8-en-3β-ol; $\Delta^{8(14)}$, 5α-cholest-8(14)-en-3β-ol; Δ^{14}, 5α-cholest-14-en-3β-ol; $\Delta^{4,6}$, cholesta-4,6-dien-3β-ol; $\Delta^{5,7}$, cholesta-5,7-dien-3β-ol; $\Delta^{5,8}$, cholesta-5,8-dien-3β-ol; $\Delta^{5,8(14)}$, cholesta-5,8(14)-dien-3β-ol; $\Delta^{5,20(22)E}$, (20(22)*E*)-cholesta-5,20(22)-dien-3β-ol; $\Delta^{5,20(22)Z}$, (20(22)*Z*)-cholesta-5,20(22)-dien-3β-ol; $\Delta^{5,22F}$, (22*E*)cholesta-5,22-dien-3β-ol; $\Delta^{5,22Z}$, (22*Z*)-cholesta-5,22-dien-3β-ol; $\Delta^{5,24}$, cholesta-5,24-dien-3β-ol; $\Delta^{6,8}$, 5α-cholesta-6,8-dien-3β-ol; $\Delta^{6,8(14)}$, 5α-cholesta-6,8(14)-dien-3β-ol; $\Delta^{7,9(11)}$, 5α-cholesta-7, 9(11)-dien-3β-ol; $\Delta^{7,14}$, 5α-cholesta-7,14-dien-3β-ol; $\Delta^{7,24}$, 5α-cholesta-7,24-dien-3β-ol; $\Delta^{8,14}$, 5α-cholesta-8,14-dien-3β-ol; $\Delta^{8,24}$, 5α-cholesta-8,24-dien-3β-ol; $\Delta^{5,7,9(11)}$, cholesta-5,7,9(11)-trien-3β-ol; $\Delta^{6,8,14}$, 5α-cholesta-6,8,14-trien-3β-ol; 19-nor-$\Delta^{5,7,9}$, 19-norcholesta-5,7,9-trien-3β-ol; 24,25-dihydrolanosterol, lanost-8-en-3β-ol.

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3β-ol (11), and 19-norcholesta-5,7,9,-trien-3β-ol (11–13). Another genetic disorder of human development has been suggested to be due to a defect in the enzyme catalyzing the reduction of the Δ^{24} double bond of sterol precursors of cholesterol, leading to the accumulation of cholesta-5,24-dien-3β-ol (19). Another very recent report (20) indicated the direct involvement of a sterol in the processing of the hedgehog protein, a species known to be intimately involved in pattern development in early differentiation, and included speculation as to the potential importance of these findings in the pathogenesis of the Smith-Lemli-Opitz syndrome. In another recent study (21), certain di- and triunsaturated sterol precursors of cholesterol were reported to be activators of meiosis in mammalian eggs. One of these sterols, 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol, was also recently reported (22) to activate gene transcription through the orphan nuclear receptor LXRα. Other prominent problems focusing on the sterol composition of cell culture media and of cells and their subcellular fractions include the role of sterols in cell morphology and growth (23, 24), the intracellular transport and efflux of sterols (25–29), and the isolation of mammalian cell mutants deficient in the overall conversion of lanosterol to cholesterol (24, 30–32). The latter includes an HIV-susceptible T cell line that showed essentially complete replacement of cellular cholesterol by lanosterol and 24,25-dihydrolanosterol (24). The use of these cell mutants, as well as the use of cultured mammalian cells grown under conditions under which very major changes in their sterol composition can be effected by appropriate inhibitors or by the addition of sterols (23), will permit a variety of studies of the functional roles of sterols in mammalian cellular physiology.

The steady state concentrations of the sterol intermediates in blood and tissues are normally very low relative to that of cholesterol. Exceptions exist in the cases of brain and spinal cord during myelination (33–35), skin (36), human milk (37, 38), and spermatozoa (in some species) after their maturation in the epididymis (39–41). Very low blood levels of a sterol believed to be cholesta-5,8-dien-3β-ol have been reported for normal subjects (42) and patients with cerebrotendinous xanthomatosis (43). Changes in the low concentrations of sterol intermediates continue to be of interest in medicine as indirect indicators of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and of whole body cholesterol synthesis (44-47), although this approach is not without some uncertainty (46). Changes in sterol composition of blood and tissues induced by drugs inhibiting various reactions in the overall conversion of lanosterol to cholesterol continue to be of interest, and especially in the case of animals treated with inhibitors of the conversion of 7-dehydrocholesterol to cholesterol as potential animal models of the Smith-Lemli-Opitz syndrome (13, 48–51). Increases in the levels of 5α-cholest-8-en-3β-ol in blood of women undergoing treatment with tamoxifen or toremifene for breast cancer have recently been reported (52), and increases in blood levels of lanosterol and 24,25-dihydrolanosterol have been noted in patients and animals undergoing treatment with ketoconazole (53, 54). Low levels of certain C_{27} sterol precursors of cholesterol (7-dehydrocholesterol, cholesta-5,24-dien-3β-ol, and cholesta-5,7,24-trien-3β-ol) have been noted to be present in brain and sciatic nerve of 60-day-old rats and mice (55), and the levels of the former two sterols were reported to be lower in brains of mutant dysmyelinating mice. Dysmyelinating mutant mice (trembler, shiverer, and quaking) were reported to show lower levels of 7-dehydrocholesterol in sciatic nerve. Lower levels of cholesta-5,7,24-trien-3β-ol were noted for quaking mice, and lower levels of the $\Delta^{5,24}$ sterol were reported for trembler mice. Application of carcinogenic polycyclic aromatic hydrocarbons to skin of rats has been reported to lower the levels of 5α-cholest-7-en-3β-ol without affecting cholesterol concentrations (56 and references cited therein). This enumeration of current and expanding problems, hardly comprehensive, indicates, many exciting areas involving sterol intermediates, in cholesterol biosynthesis in biomedical research.

The overall conversion of lanosterol to cholesterol involves a very large number of potential intermediates, a subject which has been reviewed previously (1–3). Considerable complexity is reduced by consideration of C_{27} 3β-hydroxysterols alone. However, even with this limited class of intermediates, isomers shown to be convertible to cholesterol and/or shown to be formed or to have been isolated from tissues include the following: Δ^{7}, Δ^{8}, $\Delta^{8(14)}$, $\Delta^{5,7}$, $\Delta^{5,24}$, $\Delta^{7,24}$, $\Delta^{8,24}$, $\Delta^{7,9,(11)}$, $\Delta^{7,14}$, $\Delta^{8,14}$, $\Delta^{5,7,24}$, and $\Delta^{8,14,24}$ (2, 3 and references cited therein). Recent reports have suggested the possible occurrence and/or formation of other C_{27} sterols, including $\Delta^{6,8}$ (8–10, 42), $\Delta^{6,8,(14)}$ (6), and $\Delta^{5,7,9(11)}$ (11). In addition, other sterols are also deserving of consideration, i.e., $\Delta^{5,8(14)}$, $\Delta^{8(14),24}$, $\Delta^{7,9(11),24}$, $\Delta^{7,14,24}$, $\Delta^{6,8,24}$, $\Delta^{6,8(14),24}$, $\Delta^{5,8(14),24}$, and $\Delta^{5,7,9(11),24}$. Thus, these combined considerations lead, for C_{27} 3β-hydroxysterols alone, to a total of at least 26 sterols of this type (including cholesterol and 5α-cholestan-3β-ol) which could be contemplated as existing in a sample derived from mammalian cells. The separation and identification of these sterols, differing only in the number and location of olefinic double bonds, are obviously nontrivial matters. Samples of ^{3}H- or ^{14}C-labeled sterols formed from appropriate precursors (e.g., labeled acetate or mevalonate) present an even more formidable problem as almost invariably insufficient material is available for

the use of physical methods (nuclear magnetic resonance (NMR) or mass spectrometry (MS)) to assist in the characterization of the sterols. Thus, knowledge of the capabilities of existing chromatographic methodologies for the separation of the various sterols is extremely important. Currently, it appears that a real problem exists with respect to the recognition and/or consideration of the potential complexity of sterols present in mammalian samples as well as the capabilities and limitations of existing methodologies for their separation. For example, even in highly respected journals, "characterization" or "identification" of a sterol is frequently limited to determination of its chromatographic behavior on simple silica gel thin-layer chromatography (TLC) or reversed phase high performance liquid chromatography (HPLC), commonly in the absence of a comprehensive set of authentic standards.

The increasing recognition of the potential importance of sterol intermediates in cholesterol biosynthesis in biology and medicine (see above) stimulated a comprehensive evaluation of existing methodologies for the separation and characterization of C_{27} 3β-hydroxysterols potentially present in mammalian samples. Exploration of this matter required the availability of a significant number of authentic sterol samples of known structure and purity. Unfortunately, very few of the concerned sterols are available commercially. We have devoted considerable effort towards the chemical synthesis of the appropriate sterol standards (57), an endeavor which permitted detailed studies of their NMR (57) and GC and GC–MS (58) properties. In the present study, we have determined the capabilities and limitations of conventional reversed phase and normal phase HPLC in the separation of these sterols. In addition, we present extensions of our experience with silver ion HPLC (Ag^+-HPLC) (59), an approach which has recently been shown to provide unprecedented resolution of various C_{27} sterols. The structures of the C_{27} sterols studied herein are presented in **Fig 1.**

MATERIALS AND METHODS

Solvents for thin-layer chromatography (TLC) were of HPLC grade and were obtained from Burdick and Jackson (Muskegon, MI). Hexane, acetone, methanol, and methyl *tert*-butyl ether (MTBE) for HPLC (Omnisolve grade) were products of EM Science (Gibbstown, NJ). The preparation and characterization of C_{27} sterol standards and their acetate derivatives have been described previously (57 and references cited therein). 19-Norcholesta-5,7,9-trien-3β-ol and its acetate derivative were prepared as described previously (60). (20(22)*E*)-Cholesta-5,20(22)-dien-3β-ol was prepared essentially as described previously (61), and its $\Delta^{5,20(22)Z}$ isomer was obtained as a minor byproduct. Acetates of the $\Delta^{5,20(22)}$ dienes were prepared by treatment of the free sterols with acetic anhydride–pyridine 1:1 overnight at room temperature. 4,4-Dimethylcholest-5-en-3β-ol, 4,4-dimethylcholesta-5,7-dien-3β-ol, 4,4-dimethyl-5α-cholesta-7,14-dien-3β-ol, and 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol were prepared as described previously (62). 4α-Methylcholest-5-en-3β-ol and 4β-methylcholest-5-en-3β-ol were prepared as described previously (63). 4α-Methyl-5α-cholest-7-en-3β-ol, 4β-methyl-5α-cholest-8-en-3β-ol, and 4β-methyl-5α-cholesta-8,14-dien-3β-ol have been described previously (64). 4α-Methyl-5α-cholest-8-en-3β-ol, prepared by chemical synthesis (65), was a gift from Dr. A. A. Kandutsch (Bar Harbor, ME). The preparation and characterization of lanosterol, 24,25-dihydrolanosterol, and their acetate derivatives have been described previously (66). Samples of squalene, geraniol, *trans,trans*-farnesol, and geranylgeraniol were products of Sigma Chemical Co. (St. Louis, MO).

TLC was carried out on plates (20 cm × 20 cm) of silica gel G (250 μm in thickness) which were purchased from Analtech, Inc. (Newark, DE). Components on the plates were visualized after spraying with molybdic acid (67). HPLC was carried out using a Rheodyne 7125 or Waters U6K injector and Waters 510 pump with ultraviolet (UV) detection at 210 nm. Reversed phase HPLC was performed with a 5-μm Customsil ODS column (250 mm × 4.6 mm i.d.; Custom LC; Houston, TX), and normal phase separations were done on a 5-μm Adsorbosphere silica column (250 mm × 4.6 mm i.d.; Alltech Associates; Deerfield, IL) or an 8-μm Dynamax silica column (250 mm × 10 mm i.d.; Rainin Instrument Co.; Woburn, MA). Ag^+-HPLC was carried out as described previously (59). In the present study, 5-μm Nucleosil SA cation exchange (100 Å pore size) columns (250 mm × 4.6 mm i.d. and 300 mm × 3.2 mm i.d.) were obtained form Alltech Associates. The ion exchange columns each showed approximately 80,000 theoretical plates per meter (as tested by Alltech). The columns were charged with silver ion and prepared for use as described previously (59).

RESULTS

As anticipated, simple TLC on silica gel plates provided little capability to separate C_{27} sterols from each other. Using MTBE-hexane 1:1 as the developing solvent, the following R_f values were observed: Δ^5, 0.42; $\Delta^{5,7}$, 0.41; $\Delta^{5,24}$, 0.41; Δ^8, 0.41; $\Delta^{8(14)}$, 0.38; $\Delta^{8,24}$, 0.38;

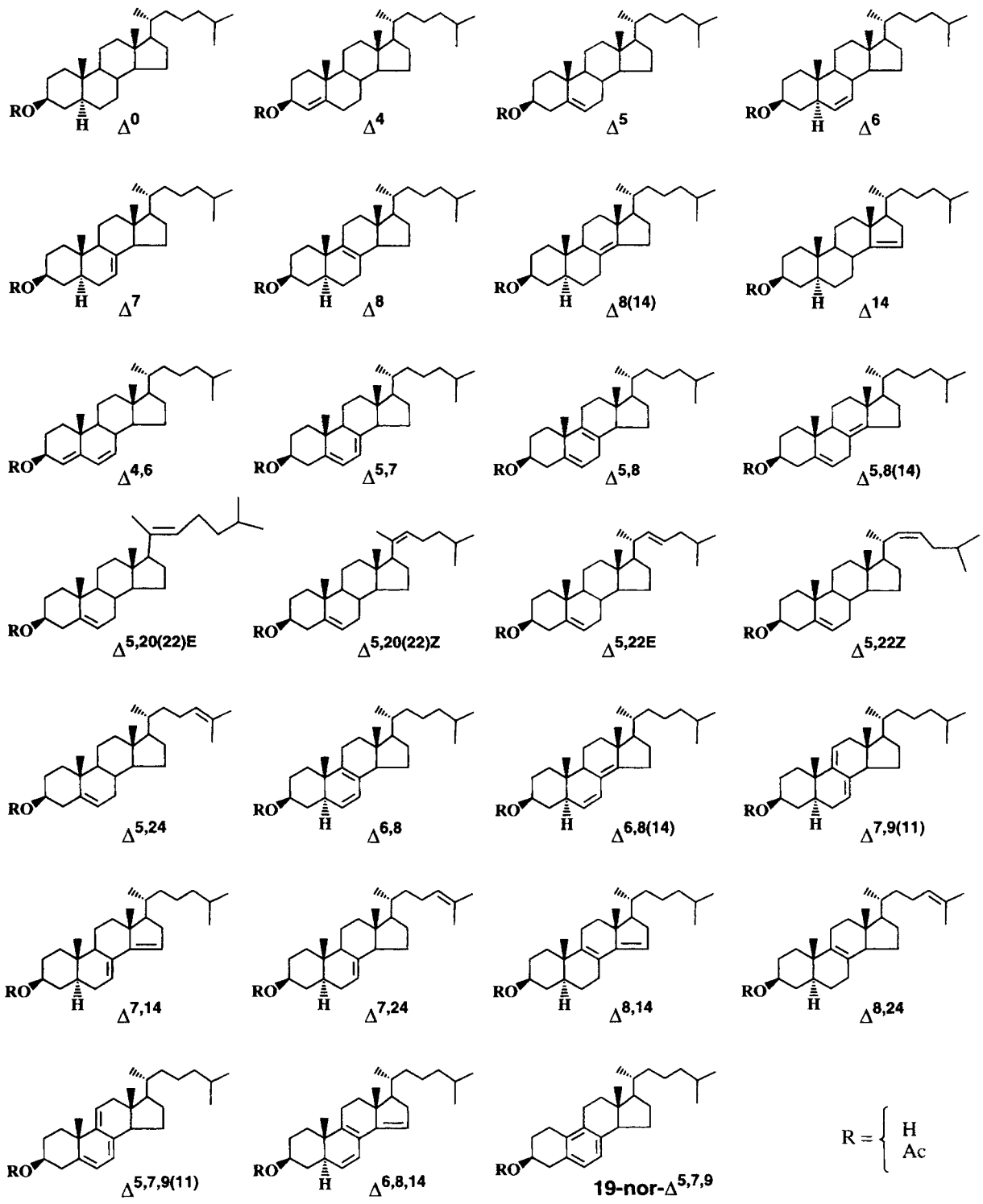

Fig. 1. Structures of unsaturated C_{27} 3β-hydroxysterols and related sterols described therein.

Δ^7, 0.38; $\Delta^{7,9(11)}$, 0.38; $\Delta^{7,14}$, 0.37; and $\Delta^{8,14}$, 0.37. Under the same conditions, 24,25-dihydrolanosterol, lanosterol, and 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol showed R_f values of 0.66, 0.63, and 0.54, respectively.

The chromatographic behavior of a substantial number of unsaturated C_{27} sterols and their acetate derivatives on reversed phase HPLC on a Customsil ODS column are presented in **Table 1** along with previously

TABLE 1. Reversed phase HPLC retention data for C_{27} free sterols, acetates, and benzoates

	Retention Times Relative to Cholesterol (or Its Esters)									
	Free sterol							Acetate		Benzoate
Sterol	This work[a]	Ref. 73[b]	Ref. 68[c]	Ref. 72[d]	Ref. 70[e]	Ref. 71[f]	Ref.69[g]	This work[a]	Ref. 74[h]	Ref. 75[i]
$\Delta^{5,17(20)Z}$		0.61				0.63				
$\Delta^{5,17(20)E}$		0.64				0.67				
$\Delta^{5,20(22)Z}$	0.65									0.68
$\Delta^{5,20(22)E}$	0.71	0.69				0.67				0.75
$\Delta^{5,23E}$										0.75
$\Delta^{5,23Z}$										0.75
$\Delta^{7,14}$	0.73									0.66
$\Delta^{5,7,22}$						0.58				
$\Delta^{5,7,24}$			0.71		0.53					
$\Delta^{8,14}$	0.73	0.76	0.76							0.66
$\Delta^{8,24}$	0.74	0.67	0.78		0.69		0.67	0.71		
$\Delta^{6,8(14)}$	0.76							0.76		0.75
$\Delta^{7,24}$	0.78		0.77					0.75		
$\Delta^{5,24}$	0.79	0.72	0.87	0.73	0.68	0.68	0.74		0.79	0.78
$\Delta^{5,22Z}$		0.74				0.70			0.72	0.71
$\Delta^{5,25}$		0.78								0.76
$\Delta^{5,22E}$		0.81		0.78		0.78			0.80	0.79
$\Delta^{7,9(11)}$	0.80							0.75		0.73
$\Delta^{5,8}$	0.80							0.81		
$\Delta^{4,6}$	0.81					0.66		0.78		0.75
$\Delta^{6,8}$	0.82							0.79		
$\Delta^{5,8(14)}$	0.82							0.81		
$\Delta^{9(11)}$										0.86
$\Delta^{8(14)}$	0.86	0.88	0.88		0.96	1.06	0.87	0.88		0.89
$\Delta^{5,7}$	0.88	0.86	0.87		0.75	0.74		0.82	0.83	0.84
$\Delta^{20(22)E}$										0.81
Δ^{1}										0.90
Δ^{14}	0.88	0.91						0.87		0.95
Δ^{4}	0.90	0.93				0.90				0.83
Δ^{8}	0.94	0.90	0.92		1.00		0.93	0.92		0.93
Δ^{6}	0.96									0.97
Δ^{7}	1.00	0.97	0.98	1.04	1.00	1.06	0.96	1.00	0.98	0.97
Δ^{5}	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Δ^{0}		1.10		0.89	1.29	1.63			1.14	1.12

[a] 5-μm Customsil ODS column (250 mm × 4.6 mm i.d.); isocratic elution with methanol–water 98:2 at 1 ml/min; t_R 22.6 min for cholesterol, t_R 49.0 min for cholesteryl acetate. 19-Norcholesta-5,7,9-trien-3β-ol showed a relative retention time of 0.60.

[b] Whatman 5 μm 110 mm × 4.7 mm i.d. column operated at 40°C, elution with methanol–water 94:6.

[c] Brownlee Spheri-5RP-18 column (100 mm × 4.6 mm i.d.), elution with methanol.

[d] Altex Ultrasphere-ODS column (250 mm × 4.6 mm i.d.), linear gradient elution over 15 min from methanol–acetonitrile 60:40 to methanol–acetonitrile 22.5:81.5. Relative retention was calculated as ratios of capacity factors (k'). The reported retention time for the Δ^0 sterol in ref. 72 appears to be anomalous and may represent a misprint.

[e] Waters μBondepak-C_{18} column (300mm × 3.9 mm i.d.), elution with acetonitrile–water 88:12.

[f] Waters μBondepak-C_{18} column (300 mm × 3.9 mm i.d.), elution with acetonitrile. Relative retention was calculated as ratios of capacity factors (k').

[g] Altex 5 μm Ultrasphere ODS column (250 mm × 4.6 mm i.d.), elution with methanol–water 96:4.

[h] Waters μBondepak-C_{18} column (300 mm × 3.9 mm i.d.), elution with methanol–chloroform–water 71:16:13. Relative retention was calculated as ratios of capacity factors (k').

[i] Zorbax ODS column (250 mm × 4.6 mm i.d.), elution with methanol.

published reversed phase HPLC data for free sterols (68–73) and their acetate (74) and benzoate derivatives (75). The combined results indicate very substantial limitations of reversed phase HPLC to provide separations of many of the sterols (or their acetate or benzoate derivatives). In fact, little or no separations of numerous sterols were observed.

Table 2 presents retention time data for C_{27} sterols and their acetate derivatives on normal phase HPLC on an Adsorbosphere silica column along with previously published normal phase HPLC data for acetate (70, 76) and benzoate (75) derivatives. As in the case of reversed phase HPLC, normal phase HPLC provided little or no separation of numerous sterols or their acetate derivatives. In general, the order of elution of the acetate derivatives followed that of the free sterols.

TABLE 2. Normal phase HPLC retention data for C_{27} free sterols, acetates, and benzoates

Sterol	Retention Times Relative to Cholesterol (or Its Esters)					
	This Work[a] Free Sterol	This Work[b] Free Sterol	This Work[c] Acetate	Ref.76[d] Acetate	Ref.70[e] Acetate	Ref.75[f] Benzoate
Δ^{4}	0.87					1.07
Δ^{1}						0.91
Δ^{0}				0.94	1.02	0.95
Δ^{5}	1.00	1.00	1.00	1.00	1.00	1.00
Δ^{6}			1.00			1.16
$\Delta^{4,6}$	1.03		1.18			1.10
$\Delta^{5,8(14)}$	1.03		1.10			
$\Delta^{5,22E}$						1.17
$\Delta^{9(11)}$						1.27
$\Delta^{5,23E}$						1.29
$\Delta^{5,22Z}$						1.32
$\Delta^{20(22)E}$						1.34
$\Delta^{5,23Z}$						1.34
$\Delta^{5,20(22)E}$	1.04					1.54
$\Delta^{5,24}$	1.04	1.02	1.12		1.16	1.45
$\Delta^{8(14)}$	1.05	1.16		1.04	1.11	1.39
Δ^{8}	1.07	1.18	1.04	1.09	1.20	1.39
$\Delta^{5,8}$	1.10		1.16			
Δ^{14}	1.11		1.10			1.39
$\Delta^{5,25}$						1.40
$\Delta^{8,24}$	1.11	1.19	1.24		1.42	
Δ^{7}	1.12	1.22	1.09		1.27	1.48
$\Delta^{7,24}$	1.17		1.25			
$\Delta^{5,7}$	1.19	1.12	1.28		1.28	1.55
$\Delta^{7,9(11)}$	1.19					1.75
$\Delta^{6,8(14)}$	1.22		1.28			1.75
$\Delta^{6,8}$	1.23					
$\Delta^{8,14}$	1.26	1.36	1.33	1.18		2.30
$\Delta^{7,14}$	1.27	1.38	1.37	1.24		2.34
$\Delta^{5,7,9(11)}$	1.30		1.33			
$\Delta^{5,20(22)Z}$			1.35			1.17
$\Delta^{5,7,24}$					1.54	
$\Delta^{6,8,14}$	1.52					

[a]Five-μm Adsorbosphere silica column (250 mm × 4.6 mm i.d.); isocratic elution with acetone–hexane 3:97 at 1 ml/min; t_R 29.6 min for cholesterol. 19-Norcholesta-5,7,9-trien-3β-ol showed a relative retention time of 1.47.

[b]Eight-μm Dynamax silica column (250 mm × 10 mm i.d.): isocratic elution with MTBE–hexane 1:4 at 4ml/min; t_R 31.1 min for cholesterol.

[c]Five-μm Adsorbosphere silica column (250 mm × 4.6 mm i.d.); isocratic elution with acetone–hexane 0.25:100 at 1 ml/min; t_R 12.2 min for cholesteryl acetate.

[d]Four Waters μPorasil columns (300 mm × 4.0 mm i.d.) in series; isocratic elution with chloroform–hexane 4:6.

[e]Waters μPorasil column (300 mm × 3.9 mm i.d.), elution with isooctane–cyclohexane–toluene 50:30:20.

[f]Zorbax SIL column (150 mm × 4.6 mm i.d.); isocratic elution with dichloromethane–hexane 2:98.

However, a number of exceptions were observed (e.g., $\Delta^{4,6}$, Δ^{8}, Δ^{14}, Δ^{7}, and $\Delta^{5,7,9(11)}$). Additional data were obtained with C_{30}, C_{29}, and C_{28} sterols and prenyl alcohols and squalene, all of which eluted prior to cholesterol, indicating the utility of normal phase HPLC for rapid separation of the C_{27} free sterols from C_{30}, C_{29}, and C_{28} known or potential intermediates in cholesterol biosynthesis and from squalene and prenyl alcohols. Retention times (relative to cholesterol) were as follows: squalene, 0.11; geranylgeraniol, 0.79; farnesol, 0.86; geraniol, 0.94; 24,25-dihydrolanosterol, 0.48; lanosterol, 0.50; 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol, 0.54: 4,4-dimethyl-5α-cholest-7,14-dien-3β-ol, 0.55; 4α-methyl-5α-cholest-8-en-3β-ol, 0.61; and 4α-methyl-5α-cholest-7-en-3β-ol, 0.64.[3]

As described previously (59), Ag^{+}-HPLC provides unprecedented separations of the acetate derivatives of C_{27} sterols differing in the location and number of olefinic

[3]Additional relative retention time data were observed for the following sterols, which, on the basis of current knowledge (3), do not appear to represent intermediates in the biosynthesis of cholesterol: 4,4-dimethylcholest-5-en-3β-ol, 0.44; 4,4-dimethylcholesta-5,7-dien-3β-ol, 0.49; 4α-methylcholest-5-en-3β-ol, 0.54; 4β-methylcholest-5-en-3β-ol, 0.70; 4β-methyl-5α-cholest-8-en-3β-ol, 0.79; and 4β-methyl-5α-cholesta-8,14-dien-3β-ol, 0.92.

TABLE 3. Ag^+-HPLC retention data for C_{27} free sterols and acetates[a]

Sterol	Retention Times Relative to Cholesterol (or Its Acetate Derivative)				
	Free Sterol	Acetate			
	A	B	C	D	E
$\Delta^{8(14)}$	0.69	0.73	0.63	0.65	0.60
Δ^{8}	0.73	0.80	0.75	0.73	0.71
Δ^{7}	0.76	0.84	0.79	0.79	0.75
Δ^{4}	1.04	0.87			
Δ^{5}	1.00	1.00	1.00	1.00	1.00
$\Delta^{5,22Z}$		1.44			
$\Delta^{8,24}$	1.06	1.52	1.73	1.57	1.41
$\Delta^{5,20(22)Z}$	1.10				
$\Delta^{7,24}$	1.11	1.66	1.92	1.68	1.57
$\Delta^{5,22E}$		1.74		1.85	
$\Delta^{5,24}$	1.49	2.05		2.44	
$\Delta^{5,7,9(11)}$	1.96	2.37	2.79	2.69	
$\Delta^{5,22,24}$		2.50			
$\Delta^{5,8(14)}$	2.65	2.53	2.97	2.93	3.13
$\Delta^{7,9(11)}$	1.54	2.67	3.27	3.15	2.86
$\Delta^{5,20(22)E}$	1.64	2.82			
$\Delta^{6,8}$	2.21	3.16	3.83	3.67	3.54
$\Delta^{5,8}$	3.12	3.30	4.03	3.98	4.11
$\Delta^{4,6}$	2.46	3.52			4.12
$\Delta^{6,8(14)}$	2.31	3.53	4.34	4.16	3.96
$\Delta^{8,14}$	2.56	7.31	9.81	9.62	8.20
$\Delta^{7,14}$	2.71	7.68	10.38	10.10	8.69
Δ^{6}		8.00			
$\Delta^{5,7}$	7.16	8.24	10.87	10.58	10.79
Δ^{14}	3.54	10.47	14.11	14.16	11.60

[a]Data obtained on Nucleosil SA strong cation exchange columns in the Ag^+ form with isocratic elution at 1 ml/min. A, 300 mm × 3.2 mm i.d. column; elution with acetone–hexane 1:10, t_R 11.4 min for cholesterol; B, 300 mm × 4.6 mm i.d. column; elution with acetone–hexane 4:96, t_R 8.7 min for cholesteryl acetate; C, 250 mm × 4.6 mm i.d. column, elution with acetone–hexane 3:97 (data corresponding to those in ref. 59), t_R 7.7 min for cholesteryl acetate; D, 300 mm × 4.6 mm i.d. column, elution with acetone–hexane 3:97, t_R 10.4 min for cholesteryl acetate; E, 300 mm × 3.2 mm id. column, elution with MTBE–hexane 1:2, t_R 4.0 min for cholesteryl acetate. The following relative retention times were observed for the 19-nor-$\Delta^{5,7,9}$ sterol: 1.45 (free sterol, system A), 1.44 (acetate, system B), 1.50 (acetate, system D).

double bonds. In the present study, we have extended our investigations to include a larger number of C_{27} steryl acetates and also the corresponding free sterols (**Table 3**). Significant differences in retention times were observed for each of the C_{27} steryl acetates, with the minor exceptions of the $\Delta^{4,6}$ and $\Delta^{6,8(14)}$ compounds with the acetone–hexane mixture (condition B) and the $\Delta^{4,6}$ and $\Delta^{5,8}$ compounds with the MTBE-hexane mixture (condition E, Table 3). **Figure 2** shows a typical chromatogram obtained using 3% acetone in hexane as the eluting solvent.[4] As noted previously (59), changing the elution solvent to 1% acetone in hexane provides a clearer separation of the monounsaturated C_{27} steryl acetates from each other and, as noted in **Fig. 3**, provided resolution of the $\Delta^{8(14)}$, Δ^{8}, Δ^{7}, Δ^{4} and Δ^{5} compounds. Notable separations of a substantial number of C_{27} steryl acetates were also achieved using a mixture of MTBE and hexane (1:2) as the eluting solvent (Table 3). The order of elution of the steryl acetates with this solvent system showed some differences relative to that observed with acetone–hexane mixtures. Ag^+-HPLC also provided notable separations of the C_{27} free sterols (Table 3). It is important to note that the order of elution of the free sterols differed from that of the acetate derivatives.

Retention times given above and in Tables 1–3 were determined by injecting each sterol individually and in mixtures and were quite reproducible. For example, 15 unsaturated sterols analyzed 4–6 times over 2 months on a Ag^+-HPLC column showed retention times having coefficients of variation of 1.2–3.4%. The same data expressed as relative retention times gave coefficients of variation of 0.4–1.1%. Although some Ag^+-HPLC columns exhibited nearly constant retention times and efficiencies over a year of regular use, other Ag^+-HPLC columns suffered loss of resolving power. When these columns were regenerated as described previously (59), the resolving power decreased slightly relative to that observed initially.

DISCUSSION

In view of the very high prominence of sterol intermediates in biology and medicine, a brief consideration of some aspects of the current status of methodologies for the separation and identification of the concerned compounds appears to be in order. This is especially important in view of the apparently widespread lack of appreciation of the capabilities and, more importantly, the limitations of existing methodologies.

Our results indicate that simple TLC on silica gel plates has little capability for the separation of C_{27} 3β-hydroxysterols differing in the number and location of olefinic double bonds, findings in agreement with the results of Xu et al. (73) and, we believe, general experi-

[4]Using UV detection at 210 nm to monitor the elution of the various unsaturated sterols and steryl acetates, the choice of elution solvents was somewhat limited. The use of dichloroethane or dichloromethane, solvents used extensively for Ag^+-HPLC work with unsaturated fatty acid methyl esters and triacylglycerols (77), was precluded by their high absorbance at 210 nm. MTBE-hexane mixtures provided good UV transparency and appropriate solvent strength for Ag^+-HPLC. However, separations were better with the acetone–hexane mixtures. Although acetone shows an absorbance maximum at 270 nm (ϵ = 16), it shows low absorbance at 210 nm (~3.5% of that observed at 270 nm).

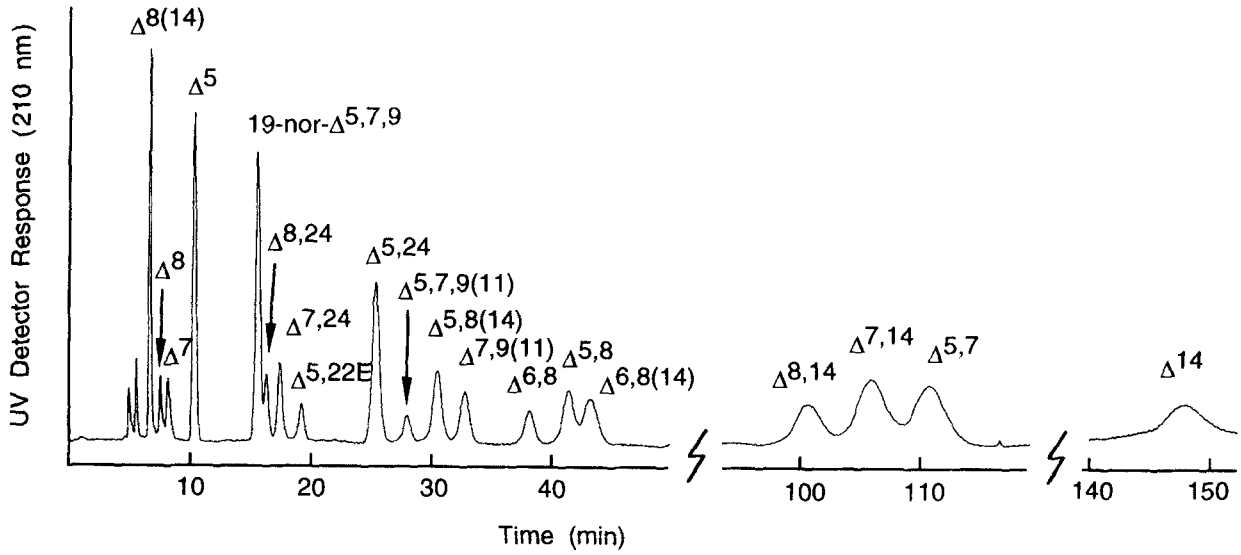

Fig. 2. Silver ion HPLC analysis of unsaturated C_{27} steryl acetates and 3β-acetoxy-19-norcholesta-5,7,9-triene: 300 mm × 4.6 mm i.d. column; elution with acetone–hexane 3:97.

ence. Impregnation of silica gel (73) or alumina (78) with $AgNO_3$ provides for the TLC separation of some of the concerned compounds from each other. However, a significant fraction of the limited number of C_{27} sterols evaluated are not resolved by these simple, rapid methods (73, 78).

Chromatography on silicic acid-Super Cel columns allows the separation of some C_{27} sterols from each other. For example, the separations of the Δ^5-, $\Delta^{5,7}$-, and Δ^7-sterols (17, 79), the separation of the Δ^5- and Δ^6-sterols (17), and the separation of the Δ^5 and $\Delta^{5,24}$ sterols (80) have been effected by this methodology. However, the separation of other sterols (e.g., Δ^8 from Δ^7) was not observed, and this methodology could hardly be expected to resolve a significant number of the C_{27} diunsaturated sterols noted previously. Moreover, this chromatography is very labor intensive and excruciatingly slow (3–5 days).

Very useful separations of a number of C_{27} sterols can be achieved by chromatography on columns of silica gel or alumina impregnated with $AgNO_3$. For example, chromatography on alumina-Super Cel-$AgNO_3$ columns permitted the separation of the Δ^8, Δ^7, Δ^5, $\Delta^{8,24}$, $\Delta^{7,24}$, and $\Delta^{5,7}$ sterols (81). Chromatography of the acetate derivatives of the C_{27} sterols on silica gel-Super Cel-$AgNO_3$ columns allows the separation of a number of important monounsaturated steryl acetates (e.g., $\Delta^{8(14)}$, Δ^8, Δ^7, and Δ^5) from diunsaturated steryl acetates and, in addition, provides for the separation of the $\Delta^{8,14}$-, $\Delta^{7,14}$-, and $\Delta^{5,7}$-steryl acetates form each other (2, 82). Chromatography of the C_{27} monounsaturated steryl acetates on alumina-Super Cel-$AgNO_3$ columns permitted the resolution of the $\Delta^{8(14)}$, Δ^8, Δ^7, and Δ^5 compounds from each other (2, 83). These chromatographic methods are limited by the relatively long times required to complete an analysis and the need to repeatedly prepare and pack the adsorbent. In 1980, Pascal, Farris, and Schroepfer (84) described an improved method involving medium pressure liquid chromatography of C_{27} steryl acetates on columns of alumina-$AgNO_3$. With this method, useful separations (albeit not complete in some cases) 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 were achieved. Advantages of this methodology included

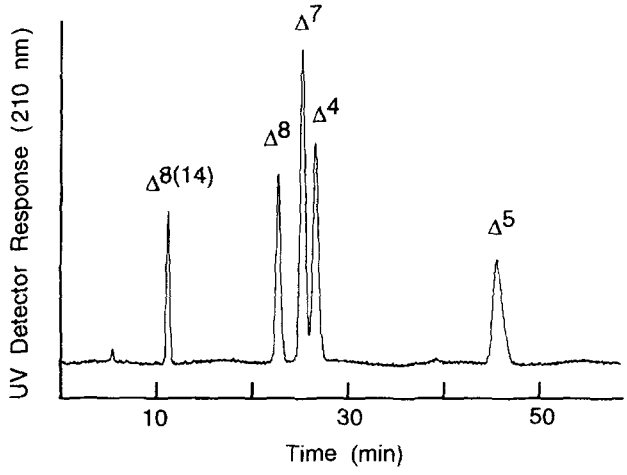

Fig. 3. Silver ion HPLC analysis of monounsaturated C_{27} steryl acetates: 300 mm × 4.6 mm i.d. column; elution with acetone–hexane 1:99.

shorter times to complete analyses, extended use of the columns, and high loading capacity.

Gas chromatography (GC) and GC–MS, methods providing high sensitivity, are in common use in studies of C_{27} sterols. GC on packed or capillary columns provides separations of a number of C_{27} sterols or their trimethylsiloxy, methoxy, acetate, or benzoate derivatives. However, in studies involving a significant number of authentic reference compounds (58, 73, 85–87), numerous sterols were inseparable on polar or nonpolar columns. Increasing the level of sophistication to GC–MS has been complicated by the fact that a number of the C_{27} sterols showed very similar and, in some cases, essentially identical mass spectra (42, 58).

HPLC also provides a relatively simple and rapid separation method. Reversed phase HPLC is currently extensively used for sterol analyses. However, simple inspection of the results of the present study and those of others (Table 1) indicates that, with a significant collection of standards, reversed phase HPLC actually provides relatively little capability to separate numerous individual C_{27} sterols (or their acetate or benzoate derivatives). Similarly, normal phase HPLC provides little or no separations of numerous C_{27} sterols (or their acetate or benzoate derivatives) (Table 2). However, our results indicate that normal phase HPLC provides a simple, rapid approach for the separation of C_{27} sterols from C_{30}, C_{29}, and C_{28} sterol precursors of cholesterol.

As noted previously (59), Ag^+-HPLC provides unprecedented separations of the acetate derivatives of C_{27} sterols differing in the number and location of olefinic double bonds. In the present study, we have reported extensions of our experience with this method to include additional C_{27} steryl acetates and its use for the separation of free sterols. Our results demonstrate that Ag^+-HPLC represents a unique and extremely powerful method for the separation of these closely related compounds. Moreover, the chromatographic mobility of an individual unknown C_{27} sterol (or its acetate derivative) on Ag^+-HPLC can provide important information regarding its tentative structure, assuming the use of a column that is carefully calibrated with a significant number of appropriate authentic standards. The level of certainty in the assignment of structure can be further raised by supplementation with GC, GC–MS, or LC–MS (59) studies and appropriate chemical modification. ^1H NMR analysis is extraordinarily powerful in this regard, providing essentially unambiguous structural information. It should be noted that ^1H NMR, with careful attention to experimental conditions, can distinguish between each of the C_{27} sterols shown in Fig. 1 (57). Studies of the nature of ^3H- or ^{14}C-labeled sterols derived from incubations of cells (or subcellular fractions therefrom) with commonly studied precursors (acetate, mevalonate, etc.) are considerably more difficult and are critically dependent upon chromatography supplemented with chemical modification and cocrystallization studies.

Numerous exciting current and emerging problems in biology and medicine involve sterol intermediates in the biosynthesis of cholesterol (see above). However, failure to recognize the potential complexity of sterol mixtures obtained from mammalian cells and/or failure to carefully apply state of the art methodologies for the separation and identification of the concerned compounds will provide, at best, a very weak foundation for understanding of these important matters.

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